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Assessment of Fungal Growth on Sodium Polyborate-Treated Cellulose Insulation

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Cellulose insulation has rapidly gained a large market share among general contractors and homeowners. Recent interest regarding health effects of high concentrations of fungi within indoor environments (building-related illnesses or sick building syndrome) has promoted concern about susceptibility of building materials, including wood products (in general) and cellulose insulation (specifically), to fungal attack. This study reports an assessment of fungal growth on cellulose insulation made from recycled paper and treated with varying concentrations of sodium polyborate within half-scale wall units exposed to variable and high ambient temperatures and relative humidities throughout the summer. Boron-treated and untreated (control) cellulose insulation within the wall units were challenged with a suspension containing high concentrations of spores of five fungal species commonly found in indoor environments. Our results suggest that cellulose insulation treated with sodium polyborate (a) precludes the growth of the five common fungal species; (b) harbors fewer fungal species before and after being challenged with the fungal spore suspension; and (c) is likely having a cytotoxic or sporocidal effect on many, if not all, fungal species. These results suggest that cellulose insulation treated with sodium polyborate, when properly applied and installed, precludes fungal growth for at least 124 days at high temperatures and relative humidities.

Keywords boron, cellulose insulation, fungi, indoor air quality, mold, sick building syndrome

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Cellulose insulation made from recycled paper products and treated with boron compounds has been used for several decades and has provided the home construction industry an environmentally friendly alternative to retard fire and to insulate with high R-values and low thermal conductance, while providing high sound proofing at moderate cost.[1,2]

Recent concerns about building-related illnesses and symptoms, including those mediated by fungi, have led to increased interest regarding the susceptibility of construction materials to fungal growth.[3] In some instances, published reports that cellulose insulation materials are particularly susceptible to fungal attack (e.g., Hyvarinen et al.)[4] have been generalized by the public and occupational health workers to mean that treated cellulose insulation also is susceptible to attack by fungi. The confusion about what types of cellulose insulation materials are susceptible to fungal attack comes at a time when the public and the construction industry are particularly sensitive to negative information about fungal growth on construction materials (in general) and cellulose insulation (specifically).

I was approached by a consortium of cellulose insulation manufacturers and borate supplier and asked to assess the ability of four different formulations of boron-treated cellulose insulation to prevent or retard fungal growth in a realistic setting using half-scale mock wall units that were prepared based on industry standards and insulated with cellulose insulation impregnated with different concentrations of sodium polyborate (Boron 10; CAS #183290-63-3), one of the most common borates used in the preparation of boron-treated cellulose insulation. To date, and to our knowledge, the efficacy of boron-treated cellulose insulation to retard or prevent fungal growth has rarely been empirically tested (but see Amburgey).[5]

We hypothesized that the cellulose insulation treated with different formulations of sodium polyborate would contain fewer viable fungal spores and less actively growing fungal mycelia than an untreated control assessed in a similar fashion.

MATERIALS AND METHODS

Products to Be Tested

Five cellulose insulation-based insulation formulations were tested: four prepared with different concentrations of sodium polyborate (1) Fiber-lite, Fiberlite Technologies, Inc., Joplin, Mo.; (2) Wallseal, Nu-Wool Inc., Jenison, Mich.; (3) Thermolok, Hamilton Mfg. Inc., Twin Falls, Idaho; and (4) Pest Control Insulation, InCide Technologies, Inc., Phoenix, Ariz.; and a control that had not been chemically treated. During 2002, manufacturing facilities at InCide Technologies randomly selected 5-kg batches of insulation from each of the four manufacturing facilities (plus one untreated control). These batches were shipped to InCide, where the bags were opened to select random subsamples for independent fungal assessment

(Fiberquant Analytical Services, Phoenix, Ariz.). Subsamples of each of the five batches also were shipped to U.S. Borax Corp. (Valencia, Calif.) to determine percentage boron and sodium (by weight).

Assessment by Fiberquant involved filling standard sterile petri dishes with autoclaved (sterilized) subsamples of borate-treated insulation. Subsamples of two formulations (Nu-Wool and InCide) were soaked and mixed separately with 780 μL of sterile distilled water/g of insulation. However, instead of using untreated cellulose insulation as a control, Fiberquant used a sterilized (autoclaved) wood tongue depressor mounted on a piece of clay and enclosed in a standard petri dish containing and just above 3 mL of sterile distilled water. A total of 10 μL of a suspension containing *Alternaria alternata* spores was deposited on the center of all subsamples, including the tongue depressor. Spore suspensions from four additional fungal species (*Aspergillus flavus*, *Aspergillus niger*, *Stachybotrys chartarum*, or *Cladosporium sphaerospermum*) were used separately to challenge four additional replicate sets of 3 plates (for a total of 15 plates). All plates were parafilmed and incubated at 30°C for 28 days. After incubation, each plate was removed and observed under a dissecting microscope using 30× magnification. Any emerging colonies were measured and the identity of the fungus confirmed using 1000× light microscope. The experiment was repreated three times during 2003.

After the subsamples were shipped to Fiberquant and U.S. Borax, the bags were resealed and forwarded to FiberLite. These five formulations were independently spray-applied into five separate half-scale wall units per industry specifications (Figure 1).[6] The formulation containing the lowest concentration of sodium polyborate was spray-applied first and the one containing the highest concentration was applied last (sequence: control, Nu-Wool, Fiberlite, Hamilton and InCide).

Construction of Wall Units

Construction materials and construction personnel were supplied by Fiberlite Technologies, Inc., and the construction of the wall units followed, as much as possible, industry standards. A total of five wall units (1.22 m × 1.22 m) were partially constructed in Joplin, Mo., and shipped to Truman State University (Kirksville, Mo.), completed, and set up on a concrete slab in the northwest corner of the new outdoor Agricultural Science Laboratory (ASL) building at the University Farm (Adair County, Mo.) on May 25, 2004. The portion of the ASL where the experiment took place was roofed and walled on only three sides, thus protecting the wall units from rain and sun but exposing them to ambient temperature and relative humidity throughout the study period.

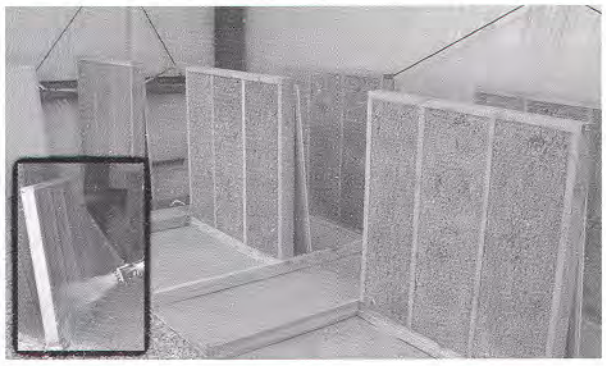

FIGURE 1. The nearly completed wall units set up at Truman State University's Agricultural Science Laboratory building. Cellulose insulation was wet spray applied in all three panels (inset) but only the two right-most panels were challenged with a fungal suspension containing five species of microfungi.

Each wall unit consisted of 1.3 cm thick oriented strand board (OSB) framed with standard 2″ × 4″ pine boards into three 40.6-cm sections (Figure 1). Prior to the addition of the cellulose insulation, a small (1 cm diameter) hole was drilled in the center portion of the middle panel of the OSB of each of the wall units to allow access for a temperature and relative humidity (RH) probe during the study. While not in use, this access hole was covered with a taped cork. Cellulose insulation was spray-applied with tap water in all three panels per manufacturer's protocol[6] to an average depth of about 7.6 cm at an average density of 48 kg/m^3. The leftmost section was sealed in plastic vapor block and left untreated to conduct additional long-term studies.

The sprayed cellulose insulation was allowed to dry and cure in all units for approximately 24 hours before they were sealed with 1.3-cm, industry-grade gypsum boards and secured with four C-clamps placed at the corners of each wall unit. On May 31, 2004, temperature (T) and relative humidity (RH) were measured within all wall units (through the drilled hole) and 1 m above the floor outside the wall units (ambient). The gypsum board covering the rightmost two sections was removed, and eight moisture readings (Delmhorst BD-10 moisture meter, Towaco, N.J.) were taken on the rightmost two panels (four readings on each panel). Then, before the wall units were challenged with a fungal spore suspension, we collected "prechallenge" (pretest) samples of cellulose insulation from each wall unit.

Collection of Samples

A grid (made of 10 cm × 10 cm quadrants) marked on the margins of the wall units was used to randomly select six quadrants within the two rightmost panels of each unit. Random coordinates consisted of two numbers selected by a computer-based random number generator (*STATISTICA* 5.5; StatSoft, Inc., 1999). The same random coordinates (locations) on each grid were used for sampling all wall units. The coordinates were re-randomized for each of the seven sampling bouts (sampling dates).

During each sampling bout, ambient T and RH (1 m above floor) and T and RH readings within each unit were taken through the drilled hole in the OSB. Then, the clamps and attending gypsum board were removed to measure moisture (eight readings per wall) and collect insulation samples before the gypsum board and clamps were replaced. Assessment of

moisture and sampling of the cellulose insulation on each unit generally took less than 5 min.

Collection of insulation samples consisted of taking approximately 0.25 g of insulation material from each of the six randomly selected quadrants with sterile forceps; placing the material into separate, sterile, resealable plastic bags; putting the bags in an iced cooler; transporting them back to Truman State University; and processing the samples within 6 hours.

Fungal Challenge

On May 31, 2004, after the pretest samples were collected, the cellulose insulation in the two rightmost panels of all wall units was challenged with 100 mL of an atomized (aerosol size approximately 10 μL) spore suspension consisting of about 3000 spores/mL of sterilized distilled water amended with the surfactant, Triton X (approximately 3×10^5 fungal spores in a 1% Triton X solution). The following fungal species and approximate spore concentrations (in spores/ml) were used: *Alternaria alternata* (39 or 1.3% of the total number of spores), *Aspergillus niger* (714 or 23.8%), *Cladosporium cladosporioides* (330 or 11%), *Penicillium chrysogenum* (1737 or 57.9%) and *Stachybotrys chartarum* (72 or 2.4%). All fungal species used in this study were cultured out from the Truman State University mold herbarium.

The identity of the fungal species and the proportion of spores used in this study are modeled after the mean number of airborne mold spores isolated from indoor airspora in apartments from 1998–2002.[7] Spore stock solutions of individual fungal species were prepared separately and mixed together to make a mixed-species solution that was vortexed and immediately atomized directly onto the surface of the insulation on the two rightmost panels of each wall unit, giving a total spore concentration of 30.27 spores/cm^2 of cellulose insulation (for comparison, there are usually less than 0.3 spores/cm^3 in most indoor environments).[7]

The spore suspension in each wall was allowed to dry for 1 hour before baseline samples were taken (Day 1 of study; sampling protocol described above). Then, each wall unit was resealed with the gypsum board and secured at the margins with the C-clamps. In total, cellulose insulation samples were collected from each of the five wall units on seven occasions: May 31 (pretest), May 31 (Day 1 of study), June 16 (Day 17), June 30 (Day 31), July 30 (Day 61), August 14 (Day 76), and October 2 (Day 124). Sampling was conducted when differences in T and RH between ambient and internal readings within wall units were at their minimum (usually around 11:00 a.m.). In total, we collected 210 samples throughout the study (six samples/formulation/sampling date $\times$ five formulations $\times$ seven sampling dates).

Processing of Samples

Washings

To obtain a more accurate understanding of both the diversity and frequency of fungal species inhabiting the insulation samples, a variation of the washing scheme described by Warcup[8] was used. This procedure involves washing away extraneous fungal spores and other propagules from the samples and isolating microfungi from cellulose insulation fragments in which those fungi are actively growing.

This process is labor intensive but reduces the possibility of overestimating the number of microfungi that specialize in investing most of their energy in producing many or long-lived spores.[9] Washing also would increase species richness estimates by increasing the probability of culturing out potentially important nonsporulating or slowly sporulating species actively growing in the insulation. About 0.05 g of each sample was placed separately into a sterile wire mesh cup and washed with a stream of pressurized distilled water and approximately 20 mL of sterile 1% Triton X solution (a detergent designed to wash out extraneous spores) for 5 min. Preliminary experiments revealed that this technique was effective at washing out almost all the extraneous spores while leaving actively growing fungi within cellulose insulation fibers.

Microfungal Assay

After washing the samples, small (approximately 0.5 mm $\times$ 0.5 mm) particles of insulation material were selected using watchman's forceps and imbedded into petri dishes filled with malt extract agar (MEA) containing antibiotics (0.4 g of streptomycin sulfate and 0.2 g of chlortetracycline/L of media) and MEA amended with 8 g of powdered cellulose/L of media (MEA-C; 5 sections/plate). A total of 10 pieces of insulation (5 on each of two plates containing MEA and MEA-C) was assessed per sample. All told, 2100 pieces of insulation were assessed for active fungal growth: 10 pieces/sample/sampling date $\times$ six samples/wall (formulation) $\times$ five walls $\times$ seven sampling dates; constituting a total of 480 plates. Plates containing embedded pieces of insulation material were incubated at 30°C and observed daily for fungal growth for a total of 10 days. I then isolated, identified, and enumerated any microbe arising from the plates using standard, morphological taxonomic techniques.

Microscopic Assessment

To determine if there were any nonculturable microbial growth on the insulation samples, we assessed a total of 420 pieces of cellulose insulation microscopically (two pieces/sample/sampling date $\times$ six samples $\times$ five walls (formulations) $\times$ seven sampling dates). Individual slides containing a drop of lactophenol acid fuchsin, covered with a coverslip were assessed for the presence of microbial cells or mycelia using an epifluorescence, phase-contrast microscope at 100–1000$\times$ magnification. Presence of bacteria and fungi were confirmed with a high-resolution digital imaging system (CIAS; CID Imaging, Inc., Camas, Wash.).

Statistical Analyses

Overall fungal growth rates for each of the five formulations were arc-sine transformed and compared with each other using an analysis of variance (ANOVA) with formulation as the independent variable, transformed growth rate as the dependent variable, and sampling date as the covariate (Ho: growth rate

for boron-treated samples = growth rate for control samples). When necessary, a Tukey's Honest Significance Difference (HSD) multiple comparison test was used to determine differences among the formulations.[10]

RESULTS

Elemental analyses of the cellulose insulation samples revealed that the highest concentration of boron existed in the Hamilton samples (2.45%), followed by Nu-Wool (2.35%), Fiberlite (2.18%), InCide (1.69%), and the untreated control (<0.01). Sodium levels also were highest in Hamilton (2.34%) but were followed by Fiberlite (2.11%), InCide (1.14%), Nu-Wool (0.17%), and control (0.05%). Neither percentage of boron nor sodium in the formulations appeared to be obviously related to fungal growth rates on the cellulose insulation.

Independent assessment of fungal growth on the cellulose insulation or wooden tongue depressors by Fiberquant indicated that none of the 30 boron-treated subsamples (five fungal species × two formulations × three replicate runs) exhibited fungal colonies. Conversely, all but 5 of the 15 (five fungal species × three replicate runs) tongue depressors (control) showed growth of fungal colonies with which they were challenged. Only those tongue depressors challenged with *Alternaria alternata* spores did not exhibit growth on the wood on any of the three replicate runs.

Cellulose insulation within the control wall unit (untreated) was yellowish after the 17th day of the study (June 16) and remained discolored through the rest of the study. No other obvious visual differences existed among the five different formulations.

Cellulose insulation particles embedded on MEA and MEA-C plates during the first three sampling bouts did not exhibit any obvious differences with respect to number of fungi or composition of the fungal community. Consequently, to simplify the comparisons and to increase our sampling size, we opted to pool data obtained from MEA and MEAC plates.

Moisture Measurements

Moisture readings and RH within the walls significantly decreased over time (univariate regression analysis, $F = 214.4$, $df = 278$, $p < 0.0001$; $F = 153.6$, $df = 33$, $p < 0.0001$, respectively; Figure 2).

Fungal Measurements

A total of 1959 cellulose insulation particles were assessed for fungal growth. Of those, 258 (or 13.2%) harbored fungi; most of these (197 or 76%) were isolated from untreated (control) cellulose insulation. Nu-Wool had 21/258 (8.1%), Fiberlite had 20/258 (7.8%); Hamilton had 11/258 (4.3%), and InCide had 9/258 (3.5%). Almost all the boron-treated samples harboring fungi, however, were observed during the first sample less than 12 hours after the insulation had been challenged with fungal spores (Day 1; Figure 3).

Although the fungal challenge increased the percentage growth rate of both control and boron-treated insulation, it was apparent that the untreated samples (control) had significantly higher growth rates during the pretest (Day 0) than the boron-treated samples (Figure 3). Furthermore, control samples were more likely to harbor more than one fungal species. Of the 258 cellulose insulation particles that were exhibiting fungal growth, 36 of them had more than one fungal species. All 36 of these particles originated from control wall units.

In addition, although we sprayed the insulation in our units with only five fungal species, we recovered a total of 23 species of microfungi, almost all of them from the control wall (21 of 23 species found on untreated cellulose insulation). Boron-treated samples contained an average of only 4.0 species ($N = 4$; $SE = 0.71$), but of those four species, an average of 2.5 species (or 63%) were one of the five original (core) species sprayed on the cellulose insulation. Conversely, only 4 of the 21 species found in control particles (19%) were core species. The remaining 17 species accounted for 44% (98/224) of the instances of fungal growth on control particles. The appearance of most of these "non-core" species growing on the particles ocurred

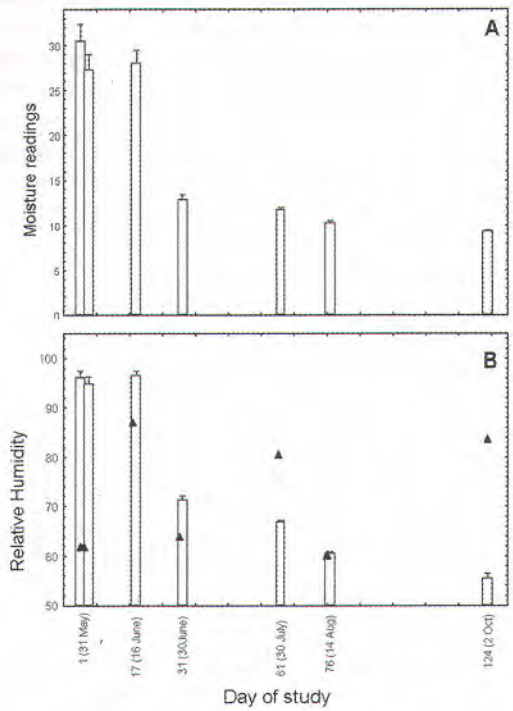

FIGURE 2. Mean (± SE) moisture (A, measured with a Delmhorst moisture meter) and relative humidity (B, measured with a hygrometer) readings measured within the five wall units. A total of 40 moisture measurements (five wall units × eight replicates, four measurements on each of the two rightmost panels) were used to calculate the mean moisture in the wall units for each sampling date. Similarly, five relative humidity measurements (one for every wall unit) were used to calculate the mean relative humidity for each sampling date. Note that the small SEs suggest little variation in moisture and relative humidity among wall units within sampling date. Each individual triangles represents a single ambient RH measurement one meter above the floor next to the wall units during each of the seven sampling dates.

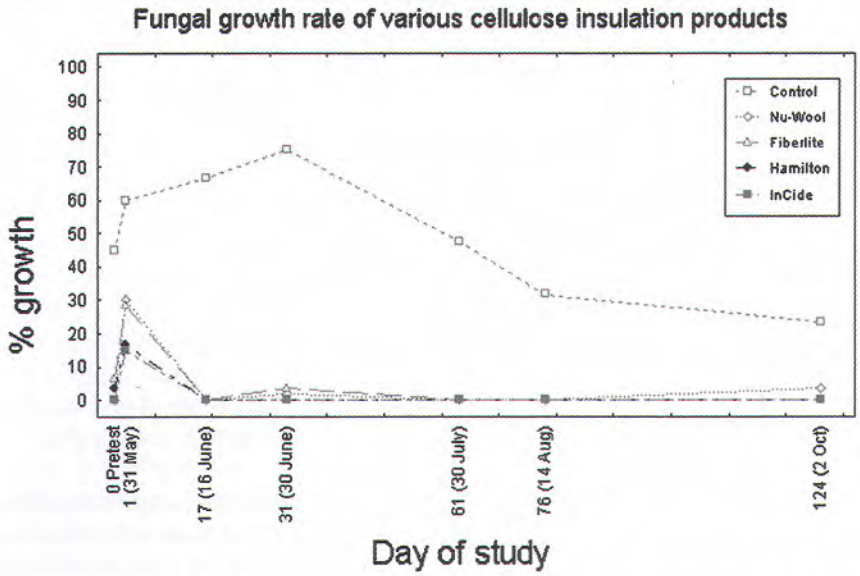

FIGURE 3. Percentage fungal growth on the five formulations of cellulose insulation tested throughout 2004; four of the products were treated with sodium polyborate and one was left untreated (control).

after Day 15 of the study (88.8% or 87/98). Only one of these non-core species (*Fusarium semitectum* complex) was isolated from one cellulose insulation particle on Day 0, and three non-core species (*Epicoccum nigrum*, *Chaetomium globosum*, and *Geotrichum* sp.) isolated from a total of 10 particles on Day 1.

Because there were so few instances of fungal growth on the boron-treated cellulose insulation, we opted to pool all dates of the study and test differences in growth rates among the formulations with a more conservative one-way ANOVA. The ANOVA and a subsequent post hoc Tukey's HSD test revealed that untreated cellulose insulation (control) samples were much more likely to harbor fungi (ANOVA $F = 33.80$, $df = 4$, $p < 0.0001$), and that all boron-treated groups differed from the control group ($p = 0.00013$) but not from each other ($p > 0.90$).

Although there were too few instances of fungal growth on which to examine the dynamics of the growth process on boron-treated samples, the control samples show a strong relationship between growth rate, moisture content, and RH (Figure 4). That is, as relative humidity and moisture content decreased, growth rate also decreases.

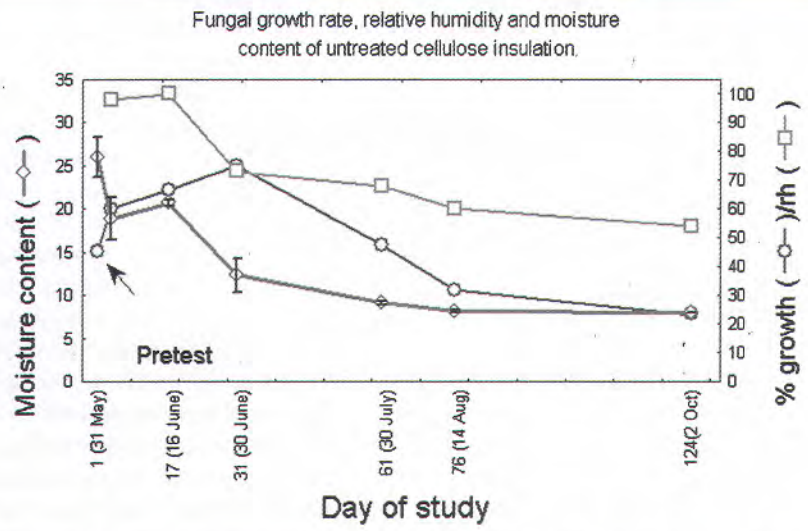

FIGURE 4. Comparison of mean (± SE) moisture content (diamonds; measured with a Delmhost moisture meter; n = eight measurements per sampling date), relative humidity (squares; measured with a hygrometer; one measurement per sampling date) and percentage fungal growth (circles) measured on untreated (control) cellulose insulation during 2004.

Microscopic Assessment

The microscopic work suggested that a large number of microbes exist as commensals on all cellulose insulation formulations studied. Almost all (141/210 or 67%) cellulose insulation samples contained viable microbes. Although we were unable to determine conclusively the identity of the viable cells, post hoc analyses with two general types of standard media (tryptic soy and nutrient agar) suggested that these organisms are primarily Gram positive bacteria or inactive microbial propagules that normally inhabit many types of insulation, including fiberglass.[11,12] Many of these cells in our study appear to be unculturable on standard media, since only a small number of particles yielded colonies within 7 days on standard media (tryptic soy and nutrient agar). The presence of actively growing mycelia discernable under the microscope was rare: only 5 of the 420 samples assessed (1.2%) were observed to contain actively growing mycelia. All of these observations of mycelia were made from control samples, spread sporadically throughout the sampling dates (2 on Day 17; 1 each on Day 0, 31, and 61 of the study).

DISCUSSION AND CONCLUSIONS

Our results clearly indicate that cellulose insulation made from recycled paper and treated with sodium polyborate (CAS #183290-63-3) is unlikely to exhibit fungal growth even when challenged with artificially high concentrations of viable fungal spores. In addition, although the growth rate was statistically higher in the untreated wall unit and we observed some yellowing of the cellulose insulation, we saw no additional obvious visual evidence of fungal growth on the untreated cellulose insulation through Day 124 (last day of study) of sampling. These observations were bolstered by a concurrent (and unpublished) experiment in the laboratory that placed small samples of untreated and boron-treated cellulose insulation in partitioned petri dishes with one section of the dish filled with distilled water (at 100% RH). These samples did not show any obvious visual evidence of fungal growth even after 150 days.

Although there were few apparent visual differences among the cellulose insulation samples sprayed into the wall units, several microbiological differences existed at the start and continued throughout our study:

- Our assessment revealed that untreated cellulose insulation contained about 9–10 times more viable fungi than the boron-treated samples prior to being challenged with our fungal spore suspension (Figure 3). It is likely that the fungal inhibiting properties of the treated cellulose insulation were functioning even before our study began.
- Control samples contained a disproportionate amount of "non-core" fungal species. These species occurred rarely during Days 0 and 1 of the study and were isolated more frequently toward the end of the study. This suggests that (a) non-core species of microfungi likely were introduced and grew after the start of our study (Day 1), and (b) because almost all fungi isolated during the pretest (Day 0) were members of the core group of five fungal species sprayed on Day 1, we propose that the five core species used in this study accurately model the fungal community that can grow on cellulose insulation.
- Control samples contained more fungal species per sample. That is, cellulose insulation particles were observed to harbor multiple species of fungi on 36 occasions; all were collected from control samples.
- Although, the percentage of fungal growth jumped in both control and treated samples after being challenged with our spore suspension, this increase was transient in the treated samples and remained high (and even increased for the first 30 days) in the control samples.

These differences suggest that although there were no apparent differences obvious to the naked eye with respect to fungal growth between the control and treated cellulose insulation, sodium polyborate appears to have a cytotoxic effect on fungal mycelia and/or has an inhibiting or sporocidal effect on asexual fungal spores (conidia).

Assessment of cellulose insulation samples also showed that as the summer progressed, both moisture and RH readings within all wall units decreased (Figure 2), and this decrease correlated well with a concomitant decrease in the percentage of untreated cellulose insulation particles exhibiting fungal growth (Figure 4) but not ambient RH measurements (Figure 2b). This suggests that water availability has a strong influence on the likelihood that spores and/or mycelia will remain viable and actively grow on the cellulose insulation. Since we did not see any significant fungal growth in the treated cellulose insulation after Day 1 of the study, we can only draw conclusions about untreated cellulose insulation.

Data from direct observations of the treated and untreated cellulose insulation on slides imply that most fungal growth observed on our slides was likely caused by viable and slow growing or dormant fungal spores. This is not surprising since cellulose insulation provides a recalcitrant substrate that is consumed only by a subset of specialized microbial species. Many fungal species observed on our plate samples are likely feeding (and slowly growing) on small amounts of organics trapped within the cellulose insulation (see Ref. 12 for a similar finding with fiberglass). Furthermore, we suspect that since the control cellulose insulation was not treated with sodium polyborate, these fungal propagules were able to remain viable until water activity dropped below some critical value. Had the water activity (indirectly measured by RH and moisture content) remained high, we suspect we would have observed high growth rates (as measured by the plate samples) in our controls. The fact that growth rates decreased in control samples, and the lack of obvious visual fungal growth on the control wall units, confirms that cellulose insulation is a difficult substrate to breakdown and consequently requires high water activities *and* specialized fungal species adapted to break down and grow on this substrate.

Nevertheless, although our study was not designed to address this, we admit that had the water activities remained high for a longer time, we may have seen more actively growing mycelia on our slides or visible signs of fungal growth on our samples. Moreover, our study would have benefited from: (a) additional and contemporaneous laboratory studies that addressed the degree to which microfungal populations grow on boron-treated or untreated (but sterilized) cellulose insulation in a standardized laboratory growth chamber environment; (b) the addition of more replicate wall units, which would have increased the likelihood to observe within-treatment variation; (c) more frequent or more accurate readings of water activity within the cellulose insulation, which may have given a more accurate or better insight about the fungal community dynamics and the threshold values for fungal growth on the different cellulose insulation formulations; and (d) although most fungi associated with indoor environments grow well in MEA at 30°C,[13] it is possible that our estimates of fungal diversity may have changed had we grown our samples at different temperatures or in different media.

Because it is likely that sodium polyborate does inhibit the growth or kills fungal cells, the exact biochemical mechanism remains unknown. Based on this study and other post hoc studies carried out in our laboratory, it appears that sodium polyborate may prevent the growth of mycelia, the actively growing and cellulose insulation-decomposing portion of the fungal life cycle. More studies on the nature of the effect of boron compounds on fungal growth should yield additional information on the mechanism(s) that protect boron-treated cellulose insulation (in particular) and wood products (in general).[14]

Finally, although small differences in fungal growth rates existed among the four formulations containing sodium polyborate, the post hoc Tukey's test suggested that these differences were not significantly different, and that differences in boron concentrations present in the four treatment formulations may not statistically change the likelihood of fungal growth.

In conclusion, our results suggest that at least over the span of more than 124 days and at high ambient relative humidities and temperatures (in the Midwest), cellulose insulation treated with sodium polyborate restricts the growth of five common species of indoor molds. Based on the diversity of different fungal species introduced on control samples after the study started, it is also likely to inhibit growth of most (if not all) species of mold. Studies have confirmed this finding on wood or wood products using sodium polyborate and other boron compounds.[14,15]

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The sporocidal and sporostatic effect of sodium polyborate and boron-treated cellulose insulation on common indoor fungal species

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ABSTRACT

Continuing interest in fungi and fungal-related health problems within indoor environments has spurred the building industry to develop ecologically-friendly, cost-effective, safe and useful antifungal additives for building materials. Treated cellulose insulation, made from recycled newsprint and amended with a variety of chemical compounds, has gathered attention and interest from a wide variety of sources including the building industry, environmentalists, and industrial mycologists. This study reports an assessment of antifungal properties of treated cellulose insulation (as a whole) and one of the most common principal active ingredients, sodium polyborate (CAS # 183290-63-3). Boron-treated cellulose and untreated paper homologs (controls) were challenged with a suspension containing a high concentration of fungal spores of six species of common fungi (*Alternaria alternata*, *Aspergillus niger*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Stachybotrys chartarum*). Results suggest that (a) paper entering processing facilities does not harbor large concentrations of fungi; (b) treated cellulose insulation is sporocidal to the six species of fungi used in this study, and possibly many other fungal species; and (c) unilateral exposure to sodium polyborate, the principal active ingredient in the samples of treated cellulose, is sufficient to preclude spore germination of these same species (actually killing spores of some).

Key words: Anti-fungal compounds, cellulose insulation, indoor air quality, mold, sick building syndrome, sodium polyborate.

INTRODUCTION

In light of persisting interest in the quality of indoor air and in the aftermath of water and fungal damage caused by hurricane flooding, many mycologists continue to work with various constituencies to reassess, test, develop, and modify building materials that are functional, ecologically friendly and cost effective. The focus of much of the interest has been laid on wood-based building materials. For example, cellulose insulation, made from recycled newsprint and amended with flame retardants[11] has garnered much attention by the building industry, environmentalists, and industrial mycologists interested in biodeterioration of building materials[7,13]. Although cellulose insulation has been used for several decades, a common concern voiced by some consumers and certain sections of the building industry has suggested that because cellulose insulation is made of paper, it is susceptible to fungal growth[5]. Recent evidence, however, suggests that a flame retardant, sodium polyborate (CAS # 183290-63-3), commonly added to some cellulose building products can reduce fungal growth within cellulose insulation at high humidity and temperature[4]. Although this finding provided mycologists, consumers and the building industry with necessary information, the results failed to disclose how boron-treated cellulose insulation manifests its antifungal properties. We were approached by a consortium of cellulose insulation manufacturers to systematically test whether sodium polyborate-treated cellulose insulation is sporocidal, sporostatic or hyphal toxic. We assessed sporocidal, sporostatic or hyphal toxic properties on five species of common indoor microfungi: *Alternaria alternata* (Fr.)Kiessl., *Aspergillus niger* Tiegh., *Cladosporium cladosporioides* (Fresen.)G. A. de Vries, *Penicillium chrysogenum* Thom, and *Stachybotrys chartarum* (Ehrenb.) S. Hughes and a species specialized to hydrolyze cellulose: *Chaetomium globosum* Kunze[3,12].

MATERIALS AND METHODS

Part A. Assessing sporocidal activity on treated cellulose insulation

Processing of samples. A total of four formulations (**Table 1**) containing different concentrations of sodium polyborate from three U. S. manufacturing facilities (Nu-Wool Co., Inc., Jenison, MI; Fiberlite Technologies, Joplin, MO; and Hamilton Mfg., Twin Falls, ID) were assessed. Each company provided five samples of raw paper not in the production stream, by collecting at least 500 g of raw newsprint and/or cardboard from different source streams. Two of the three manufacturing plants (Hamilton and Nu-Wool) opted to sample paper from five different sampling streams coming into their manufacturing facility on the same day. The remaining plant (Fiberlite Technologies) collected samples on different days and from different source streams (**Table 1**).

Five hundred grams of the sodium polyborate-treated homolog product (using the same paper as the pretreated samples) was packaged separately and mailed overnight to our laboratory. Two of the manufacturing plants (Hamilton Mfg. and Fiberlite Technologies) opted to send five homologs that correspond to the five different sampling streams of raw paper. Nu-Wool pooled their sampling streams and sent two samples each consisting of 500 g of randomly selected product. In addition, since these manufacturing facilities are licensed to produce a fourth formulation

(Incide Pest Control®), we opted to have Hamilton Mfg. use this formulation to treat their raw (pretreated) paper samples. The post-treatment homologs of Hamilton's raw paper were collected and shipped separately to our laboratory. In sum, we collected a total of 15 raw paper (pre-treatment) samples and 17 boron-treated homologs (**Table 1**).

Samples were collected by third party officials and sent to our laboratory per our directions (originals available upon request). The officials were instructed to take the samples "...randomly or haphazardly within each lot." and, "...not [to] take the entire pound [~500 g] of the sample from a single newspaper on top of the paper stack. Instead, care should be taken to collect a representative composite sample from each lot." Each of the composite samples from each lot was placed in one sterile, resealable bag until the requisite pound of raw paper was collected. The bags were refrigerated at 5 C until a total of five lots were collected and then mailed to our laboratory.

Once at our laboratory, all samples were refrigerated until processed. On 9 June 2006, all raw paper samples were shredded separately to approximately 0.25 cm^2 pieces, using a sterile shredder. To determine the native community of fungi inhabiting raw paper arriving at the three different plants, we opted to assess smaller (1 x 1 mm) pieces of paper from each of the processing facilities.

Table 1. Origins and chain of custody of samples (S) tested in the U.S.A. and Canada.

Manufacturing facility	Formulation	S	Date collected (2006)	Location(s) of source stream paper	Date treated with sodium polyborate (2006)	Date shipped (2006)
Fiberlite Technologies	Fiberlite®	1	28 March	Rogers, AR	28 March	12 April
		2	5 April	Fordland, MO	5 April	
		3	6 April-10 am	Des Moines, IA	6 April	
		4	6 April-2 pm	Jefferson City, MO	6 April	
		5	11 April	St. Joseph, MO	11 April	
Hamilton Mfg.	Thermolok®	1	12 May	Hailey, ID (H); Salt Lake City, UT (SLC); Laramie, WY (L)	12 May	7 June
		2		H, SLC, Jackson, WY (J)		
		3		L, SLC		
		4		J, SLC		
		5		L, SLC		
InCide Technologies	InCide Pest Control®	Same as Hamilton			12 May	7 June
Nu-Wool Co., Inc.	Wallseal®	1	14 March	Grand Rapids, MI	14 March	14 March
		2		Portage, IN		
		3		Mixed bales-IN & TN		
		4		Saginaw, MI		
		5		Canada		

A total of 225 particles of paper (15 particles/sample x 5 samples/facility x 3 facilities) were randomly selected and embedded into Petri dishes containing Malt Extract Agar with antibiotics [MEA, ME broth (Difco, BD diagnostics, Inc.) containing 6 g ME base, 1.8 g of maltose and amended with 15 g of agar, 0.4 g of streptomycin sulfate and 0.2 g of chlortetracycline/L media] and incubated at 30 C. Assessment of 135 larger pieces (1 cm x 1 cm; 9 particles/sample x 5 samples/facility x 3 facilities) taken from frayed edges of the paper (where there is a higher probability of fungal growth) was conducted when it became obvious that smaller pieces did not contain many fungi.

Fungal challenge. On 6 July 2006, approximately 10 g of each untreated and boron-treated subsamples from each company were placed in separate, large (150 x 15 mm) sterile Petri dishes (total of 32 dishes: 15 pretreatment, 17 treated homologs). Each subsample was challenged with a mixture of six fungal species (**Table 2**) by atomizing 10 ml of a fungal spore suspension consisting of about 2000 spores/ml in 1% Triton X solution. *Alternaria alternata* was approximately 1.25% of the total number of spores, *Aspergillus niger* 17.5%, *Chaetomium globosum* 1.0%, *Cladosporium cladosporioides* 7.5%, *Penicillium chrysogenum* 71.0%, and *Stachybotrys chartarum* 1.75%. The identity and percentage of each of the fungal species is modeled after our previous study[4]. The species were acquired from the Truman State University microfungal herbarium and subcultured onto MEA. Spore stock suspensions of individual fungal species were prepared separately, vortexed together and immediately atomized onto the surface of the shredded paper. Any leftover stock spore suspensions were stored separately at 5 C for use in Part B of this study. The Petri dishes containing the shredded paper and spore suspension were then sealed in parafilm™ and incubated at 30 C for two weeks.

Table 2. Estimated concentration of spores of the six different fungal species used in this study.

Species	Estimated spore concentration (spores/ml)
Alternaria alternata	711
Aspergillus niger	18,187
Chaetomium globosum	60
Cladosporium cladosporioides	11,072
Penicillium chrysogenum	23,917
Stachybotrys chartarum	150

Processing subsamples for media-based assay. After incubation, each subsample was assessed before and after being washed as described previously[4]. To determine if any extraneous fungal spores exist on the surface of unwashed samples, and prior to washing, five small (approximately 1 x 1 mm) particles of paper or insulation were randomly selected from each subsample and imbedded into MEA (total = 160 particles, 85 treated and 75 untreated).

Each of the subsamples was then washed separately for 5 min with a stream of distilled water and approximately 2 ml of sterile 1% Triton X solution. The first 25 ml of the stream were dripped over each of the samples and collected in separate Falcon™ tubes to be used in a subsequent spore viability assay (**Fig. 1**). After washing, small particles of paper or insulation were randomly selected and imbedded into MEA. A total of 320 washed particles were assessed

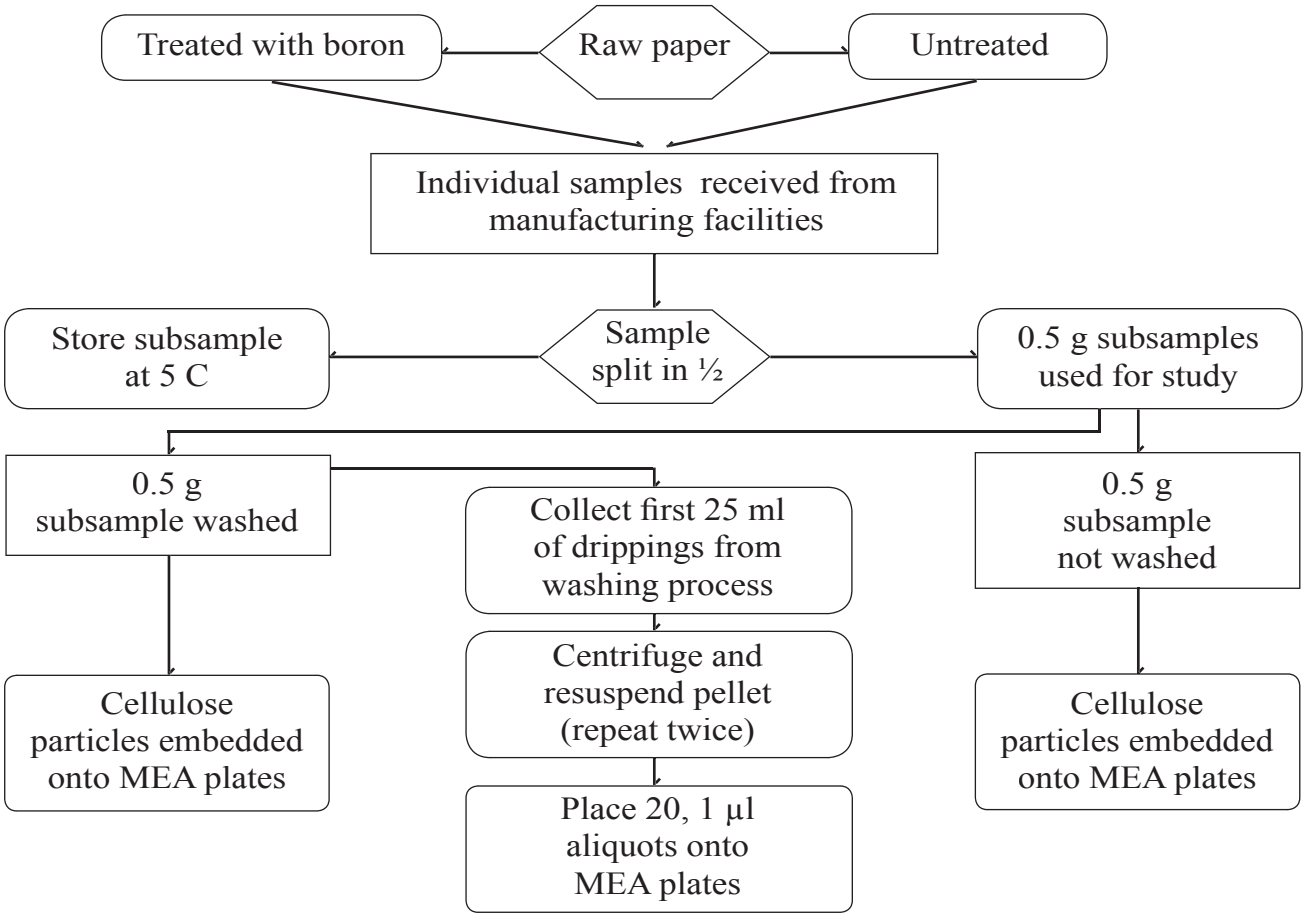

Fig. 1. Schematic diagram describing sampling and processing protocol for part A of study assessing sporocidal activity of sodium polyborate-treated insulation.

for active fungal growth. All plates were then incubated at 30 C for 7 days to 21 days. Any colonies emerging from the particles were enumerated and identified using standard, morphological taxonomic techniques.

Spore viability assay. The first 25 ml aliquots of water dripped over the subsamples were used to determine whether spores previously atomized onto the cellulose are still viable after interacting with boron-treated insulation. Residual borate left on the spores was rinsed off by centrifuging (4000 rpms for 10 min), resuspending the spores in 25 ml of sterile, distilled water and vortexing vigorously. After repeating the rinsing procedure, the spores were resuspended in 5 ml of sterile distilled water. Then 20, 1 µl aliquots of each spore suspension were placed onto plates containing MEA.

Collection of data and statistical comparisons. All MEA plates were incubated at 30 C and observed daily for fungal growth for a total of 10 days. Fungi arising from the plates were isolated, identified and enumerated. The proportion of boron-treated and untreated cellulose particles harboring fungi for each of the formulations were arcsine transformed and a paired-sample t-test used to compare the

effect of the addition of sodium polyborate (H_0: presence of actively growing fungi will not differ between treated and untreated samples). Similarly, the number of colonies emerging from aqueous drippings was compared between treated and untreated cellulose insulation (H_0: number of aliquots yielding colonies will not differ from treated and untreated samples). In addition, and using the same data, the number of colonies emerging from the spore viability assay for each formulation were compared to each other using an Analysis of Variance (ANOVA) with formulation as the independent variable, number of fungal colonies as the dependent variable and fungal species identity as the covariate (H_0: number of aliquots yielding fungal colonies should be equal among formulations). If necessary, a Tukey's multiple comparison test was used to determine differences among the formulations[14].

Part B. Assessing sporocidal and sporostatic activity of sodium polyborate

Processing of sodium polyborate. Sodium polyborate powder was supplied by Jim Blasius (InCide Technologies, Phoenix, AZ) in a resealable bag. This bag was refrigerated until used in the study, during the summer of 2006.

Sporocidal or sporostatic activity. Each of the six species of fungi from part A of this study were used again. However, to determine if there was an effect of spore concentrations on viability rates, the six spore suspensions were diluted to different concentrations (**Table 2**). The effect of exposure on viability was assessed using four standard Petri dishes containing media amended with 5% sodium polyborate (w/w; made by mixing powdered sodium polyborate into media previously autoclaved and molten, but cooled to 50 C). Previous preliminary studies showed that media containing 5% sodium polyborate was sufficient to reduce or eliminate fungal growth. Plates 1 and 2 contained MEA and water agar (WA), respectively. A sterile cellophane filter (Osmonics Inc., 0.45 micron pore size, Minnetonka, MN) was then placed on the surface of the media and 10, 1 µl aliquots of a fungal spore suspension were pipetted at the surface and margins of this filter. The filter is permeable to boron (and any nutrients), but does not allow fungal spores to pass through. Plates 3 and 4 were prepared in a similar fashion except that the spore suspensions contained Triton X. Because concurrent assessment of Triton X suggested that it did not influence spore viability, we opted to pool data describing spore viability with and without Triton X. An additional plate not containing boron was used as a positive control for each of the fungal species. Consequently, a total of 56 plates were used for this portion of the study (6 species of fungi x 4 plates + 12 control plates without boron, 6 with MEA and 6 with WA).

After the spores were exposed to the 5% sodium polyborate for 7 days, the filter was removed and cleaned free of any boron residue by rinsing the side exposed to the media with a gentle stream of sterile distilled water. Each filter then was placed on separate MEA plates (without boron) and incubated at 30 C for 10 days. The number and diameter of fungal colonies emerging from the second set of plates was assessed and compared to their corresponding control plates and among the six fungal species used.

Assessment of full submersion on sporocidal or sporostatic activity. In order to determine whether sodium polyborate can reduce/stop growth of (or kill) fungal spores by intimately interacting with them (rather

than coming into contact with them on just one side), the same spore suspensions were used to test the ability of sodium polyborate to preclude fungal growth in an aqueous solution.

Processing spore suspensions. One ml of each spore suspension was pipetted into each of two separate sterile 15 ml Falcon tubes. Then 9 ml of a 5% sodium polyborate stock solution was added to one and 9 ml of sterilized distilled water in the other to serve as the control. The 12 suspensions were placed at room temperature for 1 h. Then, 1 ml of each of the mixtures was dispensed into a 300 nm pore microfilterfuge apparatus (Spin X® centrifuge tubes; Corning, Inc., Corning, NY) and spun at low speed for 1 min. The sieved spores were then serially washed and rinsed to remove any residual boron (as described previously). After washing, the spores were resuspended in 1 ml of sterile distilled water and vortexed. Finally, 10, 1 µl aliquots were pipetted onto the surface of a Petri dish containing MEA (without boron). This procedure was repeated after the spores had been immersed in the boron solution for 1 day and again after 7 days.

Analysis of the data described above suggests that spores of some fungal species succumbed to the effects of sodium polyborate only after germinating. To determine if sodium polyborate was sporocidal, sporostatic, or hyphal toxic, we undertook the following protocol: 100 µl of the stock spore suspensions of three species (*A. niger*, *C. globosum*, and *P. chrysogenum*) were added to separate 1.5 ml microcentrifuge tubes containing Malt Extract Broth (MEB, Difco, BD diagnostics, Inc.) with or without (control) 5% sodium polyborate. The six tubes were incubated at 25 C and observed for turbidity after 1 h, 1 day and again after 7 days.

To determine if any of the spores were still viable, 200 µl of each suspension was dispensed into a 300 nm pore Spin X® tube and spun at low speed for 1 min. The sieved spores were serially washed and rinsed as described above and then pipetted on to the surface a Petri dish containing MEA, but no boron (a total of 10, 1 µl aliquots). This procedure was repeated after 1 day and again after 7 days.

Finally, to confirm that spore viability was not media-dependant, 150 µl of each of the washed and rinsed spore suspensions were placed in a 1% propidium iodine solution, warmed in a microwave for 30 s and allowed to sit for 24 h using a modified protocol described by Prigione *et al*.[9]. Approximately 20 spores in each of 6 mixtures were assessed for viability using a fluorescence microscope (Olympus BX40 with a 510-560nm filter).

RESULTS

Native community of fungi on paper. Only three of the 225 randomly selected particles of raw paper entering the three facilities harbored fungi or fungal spores. Nu-Wool samples 2, 3 and 4 harbored *Penicillium expansum* Link, *Paecilomyces variotii* Bainier, and *Penicillium chrysogenum*, respectively. Assessment of larger pieces taken from frayed edges of the incoming paper showed that 26% of the pieces harbored a variety of fungal species (**Table 3**).

Part A. Assessing sporocidal activity on treated cellulose insulation

Planned statistical comparisons were obviated since treated cellulose insulation harbored no viable fungi, even when extraneous spores were not washed away. Conversely, all particles of the untreated

Table 3. Number and identity of fungi isolated from non-random (from frayed edges) and larger (1 x 1 cm) pieces of paper entering the three different cellulose insulation manufacturing facilities.

Manufacturing facility	Sample	No. of particles containing fungi	Dominant species (number of isolates)
Fiberlite Technologies	1	2/9	*Penicillium purpurogenum* Stoll; *Aspergillus fumigatus* Fresen.
	2	2/9	*A. niger*
	3	1/9	*A. niger*
	4	7/9	*P. expansum* (1); *P. chrysogenum* (1); *P.* sp. (6); *P. purpurogenum* (1)
	5	1/9	*P. expansum*
	Total	13/45= 29%	
Hamilton Mfg.	1	4/9	*Cladosporium cladosporioides* (1); yeast (1); *P.* sp. (2)
	2	1/9	*Stachybotrys chartarum*; *P.* sp.
	3	2/9	*P. expansum*
	4	2/9	*Nigrospora oryza* (Berk. & Broome)Petch; *A. niger*
	5	2/9	*Mucor racemosus* Fresen.; *P.* sp.
	Total	11/45= 24%	
Incide Technologies	Same as Hamilton		
Nu-Wool, Inc.	1	1/9	*Alternaria alternata*; *P. expansum*
	2	0/9	
	3	3/9	*Paecilomyces variotii*; *P. chrysogenum*; *P. expansum* (2)
	4	3/9	*P. glabrum* (Wehmer)Westling; *P.* sp.
	5	3/9[a]	*P. purpurogenum*; *P. chrysogenum*; *P. variabile* Sopp; *P. expansum*; *P.* sp.
	Total	10/45= 22%	
Overall		34/135= 25%	

[a] Some particles harbored multiple species.

and unwashed paper homolog harbored fungi and 97% of the same samples that were washed free of fungal spores contained actively growing mycelia. The fungal community growing within these samples was dominated by *A. niger*, which inhabited 99% of the particles from unwashed and 75% of the washed samples.

Spore viability assay. After interacting with treated cellulose, spores lost viability. Only 2.5% of aliquots from collected drippings contained viable spores. Conversely, 95% of aliquots from similarly prepared untreated paper harbored fungi, with *A. niger* accounting 293/294 instances of growth.

Part B. Assessing sporocidal and sporostatic activity of sodium polyborate

No spores from any fungal species grew on filter paper on MEA or WA amended with 5% sodium polyborate. Once transferred to

MEA without boron, however, spores from the six species varied in their response. Aliquots containing spores of *A. alternata* and *Cladosporium cladosporioides* did not exhibit growth, while aliquots from *S. chartarum* and *P. chrysogenum* exhibited growth on MEA only when they were exposed to sodium polyborate in WA. In fact, spores exposed to sodium polyborate in WA were nearly twice as likely to grow compared to spores exposed to sodium polyborate in MEA. A Wilcoxon Signed Rank test exhibited marginal significance, suggesting that this was generally true ($Z = 1.63$, $P = 0.05$), and our data (**Table 4**) suggests that it was particularly true for *P. chrysogenum* and *S. chartarum*. Aliquots of *A. niger* and *Chaetomium globosum* exhibited growth regardless of whether they were exposed to sodium polyborate in MEA or WA (**Table 4**), though compared to controls, the maturation of colonies was protracted by about two to three days.

Effect of spore concentration. Spore concentration did not obviously influence viability rates of spores. For example, aliquots containing the spores of *C. globosum* (harboring the lowest spore concentration of the six species used) were most likely to exhibit growth (**Table 2**, **Table 4)**.

Assessment of full submersion on sporocidal or sporostatic activity. The sporocidal effects of aqueous sodium polyborate appeared to begin after 1 day but became fully evident with some fungal species on or before the seventh day (**Table 5**). After 7 days, spores from *A. alternata*, *Cladosporium cladosporioides*, and *S. chartarum* did not exhibit growth on MEB without sodium polyborate. Spores of *A. niger*, *Chaetomium globosum*, and *P. chrysogenum*, failed to germinate in the aqueous solution but continued to be viable even after 2 weeks (sporostatic effect).

Sporostatic and hyphal toxicity of sodium polyborate. The sporostatic effect of sodium polyborate was confirmed when spores from three species did not exhibit growth when placed in MEB amended with 5% sodium

Table 4. Percent of fungal spore inoculations exhibiting growth on Malt Extract Agar (MEA) without sodium polyborate after 7 days. Twenty, 1 µl aliquots of a suspension containing spores of six fungal species were placed on filter paper and unilaterally exposed to 5% sodium polyborate in MEA or Water Agar (WA) for seven days before being transferred to MEA without sodium polyborate.

Species	% inoculations growing on MEA after pre-exposure to sodium polyborate in MEA	% inoculations growing on MEA after pre-exposure to sodium polyborate in WA	% of inoculations growing on MEA after sodium polyborate pre-exposure (row totals)
Alternaria alternata	0%	0%	0% (0/40)
Aspergillus niger	90%	100%	95% (38/40)
Chaetomium globosum	100%	100%	100% (40/40)
Cladosporium cladosporioides	0%	0%	0% (0/40)
Penicillium chrysogenum	0%	100%	50% (20/40)
Stachybotrys chartarum	0%	90%	45% (18/40)
Totals	32% (38/120)	65% (78/120)	48% (116/240)

Table 5. Percent of fungal spore inoculations that exhibited growth on Malt Extract Agar (MEA) without sodium polyborate after 7 days. Ten, 1 µl aliquots of a suspension containing spores of six fungal species were exposed to 5% or 0% (control) sodium polyborate for 1 h, 1 day or 7 days prior to being washed and dispensed onto MEA without boron. Note: percentage of aliquots of controls shown in parentheses.

Species	% inoculations growing on MEA after 1 h pre-exposure to 5% aqueous sodium polyborate	% inoculations growing on MEA after 1day pre-exposure to 5% aqueous sodium polyborate	% inoculations growing on MEA after 7days pre-exposure to 5% aqueous sodium polyborate
Alternaria alternata	90% (100%)	20% (80%)	0% (100%)
Aspergillus niger	100% (100%)	100% (100%)	100 (100%)
Chaetomium globosum	70% (100%)	50% (90%)	80% (20%)
Cladosporium cladosporioides	90% (100%)	0% (100%)	0% (90%)
Penicillium chrysogenum	100% (100%)	100% (100%)	100% (100%)
Stachybotrys chartarum	100% (100%)	100% (100%)	0% (100%)
Totals	92% (55/60)	62% (37/60)	47% (28/60)
(Control)	100% (60/60)	95% (57/60)	85% (51/60)

polyborate (**Table 6**). Spores from these species, however, germinated and grew after they were removed from the 5% sodium polyborate solution, although the colonies emerging from these spores were delayed compared to controls (**Fig. 2**). Epifluorescence assessment of individual spores placed in MEB amended with 5% sodium polyborate for 7 days showed that, compared to controls, fewer spores from *A. niger* and *P. chrysogenum* were viable. Negative controls for *C. globosum* did not fluoresce and so were not assessed (**Fig. 2**, **Table 7)**.

DISCUSSION

Our results clearly indicate that cellulose insulation treated with formulations that include sodium polyborate are very effective at killing and preventing germination of high concentrations of six species of fungi commonly found on paper and indoor environments. Our initial assessment of paper entering the three manufacturing facilities suggests that raw newsprint entering the production stream does not contain many fungi. However, closer inspection of the frayed edges of larger pieces of the same samples indicates they were more likely to harbor fungi (about 25% of samples containing spores or mycelia). Furthermore, five out of the six species used to challenge the paper in part A of this study are also part of the native fungal community on paper that enters the manufacturing facilities (**Table 3**). Consequently, although fungal contamination of paper in the facilities is

Table 6. Percent of fungal spore inoculations that exhibited growth on Malt Extract Agar (MEA) without sodium polyborate for 7 days. Ten, 1 µl aliquots of a suspension containing spores of three fungal species were immersed with Malt Extract Broth (MEB) containing 5% or 0% (control) sodium polyborate for 1 h, 1 day or 7 days prior to being washed and dispensed onto MEA without sodium polyborate. Note: percentage of aliquots of controls exhibiting growth shown in parentheses.

Species	Turbidity on MEA broth containing 5% or 0% (control) sodium polyborate after 7 days	% inoculations growing on MEA after 1 h pre-exposure to MEB with 5% sodium polyborate	% inoculations growing on MEA after 1 day pre-exposure to MEB with 5% sodium polyborate	% inoculations growing on MEA after 7 days pre-exposure to MEB with 5% sodium polyborate
Aspergillus niger	-	100%	100%	100%
	(+)	(100%)	(100%)	(100%)
Chaetomium globosum	-	40%	30%	20%
	(+)	(30%)	(20%)	(30%)
Penicillium chrysogenum	-	100%	100%	70%
	(+)	(100%)	(100%)	(100%)

Table 7. Percent of viable fungal spores confirmed using a 1% propidium iodine solution. A total of 40 [20 exposed to 5% sodium polyborate in Malt Extract Agar (MEA) and 20 MEA control] spores were assessed/fungal species after 1 h, 1 day and 7 days prior to being washed and assessed for viability. Note: percentage of spores of controls shown in parentheses. MEB= Malt Extract Broth.

Species	% viable spores after 1 h pre-exposure to MEB with 5% sodium polyborate	% viable spores after 1 day pre-exposure to MEB with 5% sodium polyborate	% viable spores after 7 days pre-exposure to MEB with 5% sodium polyborate
Aspergillus niger	100%	90%	80%
	(100%)	(100%)	(100%[a])
Chaetomium globosum	Not assessed	Not assessed	Not assessed
Penicillium chrysogenum	100%	95%	10%
	(100%)	(100%)	(100%[a])

[a]Almost all spores observed had germinated and were a disappearing part of a mycelial network.

low, the results of this study suggest that any subsequent problems with fungal growth before treatment likely start on frayed and damaged edges of paper, where the integrity of the papers' fibers is likely to allow fungal growth to take a foothold.

Once paper is treated with borates, any viability or growth of fungi is nullified. That

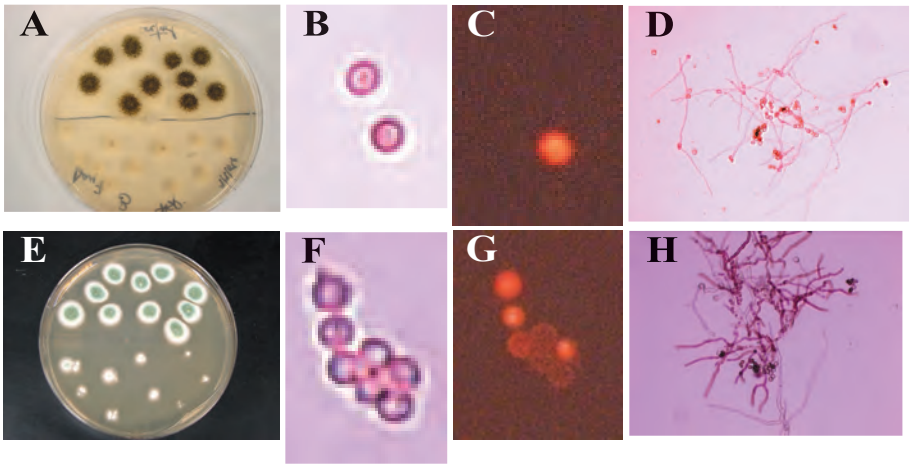

Fig. 2. Results of 7 days exposure of *Aspergillus niger* (A-D) and *Penicillium chrysogenum* (E-H) spores to 5% sodium polyborate in Malt Extract Broth (MEB). Colonies grew and matured on Malt Extract Agar (MEA) plates without sodium polyborate from controls after 3 days (top half of plates in panels A and E), while sodium polyborate-treated spores showed delayed maturation within 5 days (bottom half of plates in panels A and E). Spores placed in 1% propidium iodine solution showed, that compared to controls, some of the sodium polyborate-treated spores were inviable (fluorescing). Images B and F are of sodium polyborate-treated spores taken using light microscopy and images C and G are of the same spores taken with fluorescence wavelengths (400x). Images D and H are of control spores germinating after 3 days in MEB (200x).

was most evident in treated insulation (Part A). Treated cellulose insulation harbored no viable fungi regardless of whether the insulation had been washed. Even though the treated insulation was challenged with artificially high concentrations of fungal spores known to grow on paper, we found no viable spores on the treated cellulose particles. Conversely, nearly all of the equally treated washed and unwashed paper homolog samples harbored actively growing fungi or contained viable fungal spores. Nearly all of the growth observed was attributable to *A. niger*, which grew and sporulated readily on the control paper subsamples. This suggests that this species is comparatively numerous and/or adapted to growing on paper[2]; but see Siu[12].

Similarly, aqueous drippings containing spores previously atomized onto treated insulation harbored spores in less than 3% of aliquots examined. Although this is a low number, it represents a slight overestimate of the number of viable spores; since the drippings had high spore concentrations; each 1 µl aliquot contained an estimated 3 spores. Using this ratio, we would expect the probability of viability of an individual spore to be somewhere around 1%.

Part B. Sporocidal and sporostatic activity of sodium polyborate. Sodium polyborate, the principal component of all tested formulations was effective at preventing fungal growth even when fungal spores were exposed only on one side. Even when the spores were removed from contact with sodium polyborate and placed on media (MEA) containing no borates, only two of the six species grew, though this growth appeared to be protracted compared to their

controls. Interestingly, *P. chrysogenum* and *S. chartarum* exhibited growth if exposure to sodium polyborate occurred on WA, a media that does not provide carbohydrates and, consequently, does not encourage spore germination. Although sodium polyborate's sporostatic effect held for all fungal species, supplying a carbohydrate source may elicit germination of the spores and allow sodium polyborate to induce a hyphal toxic effect on *P. chrysogenum* and *S. chartarum*.

In sum, the results lead us to conclude that the presence of sodium polyborate within cellulose insulation prevents fungal growth.

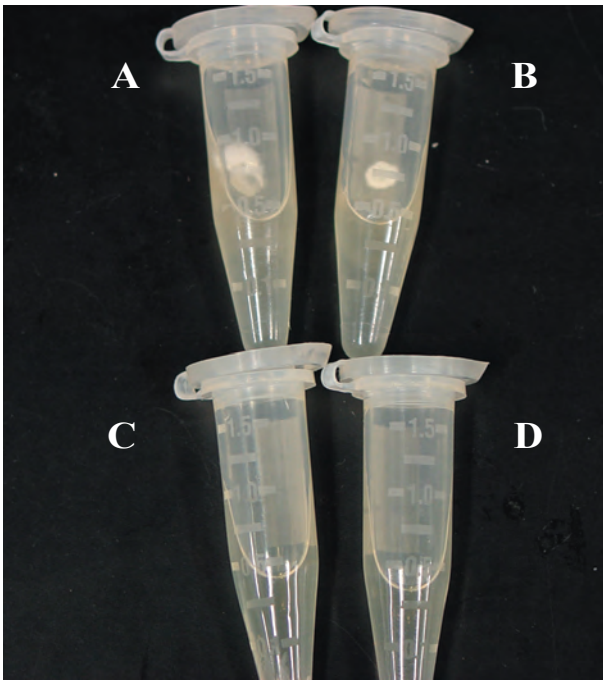

Fig. 3. Results of 35 days exposure of *Aspergillus niger* (tubes A and C) and *Penicillium chrysogenum* (tubes B and D) spores to 5% sodium polyborate in Malt Extract Broth (MEB). Colonies evident on MEB without boron after 7 days (tubes A and B), while no growth evident in MEB with sodium polyborate even after 35 days (tubes C and D).

At the concentration tested (5%), sodium polyborate seems to manifest this inhibition by preventing spore germination of all species (including *A. niger* and *C. globosum*; sporostatic), killing spores of some species (*A. alternata* and *Cladosporium cladosporioides*; sporocidal activity), and possibly killing the germinating (and metabolically active) spores of others (*e.g.*, *P. chrysogenum* and *S. chartarum*; hyphal toxic).

Allowing spores to interact with 5% sodium polyborate in an aqueous solution seems to confirm these conclusions and implies that the sporocidal effect for most fungal species occurs somewhere between one hour and seven days of interaction (**Table 5**). Though the sporocidal effects of sodium polyborate require at least a few hours of interaction, the sporostatic effects are immediate and long-lasting[4]: assessment of spores placed within MEB containing 5% sodium polyborate failed to show germination and growth (**Table 6**); continued assessment failed to show growth even after 5 weeks (**Fig. 3**).

Some of the discrepancy in sporocidal activity between the treated cellulose insulation and sodium polyborate, the principal ingredient, may include the following non-mutually exclusive possibilities:

1. Treated cellulose insulation has a much higher concentration of sodium polyborate. Although the formulations are proprietary, all have concentrations that exceed 10% sodium polyborate (by weight). These high concentrations are designed to retard fire and only coincidentally to retard fungal growth. Sporocidal activity in all of all fungal species would likely have been achieved in the *in vitro* study had we employed higher levels of sodium polyborate that more closely model those found in treated cellulose insulation.

2. The efficacy of the sporocidal properties exhibited by treated cellulose insulation may be, in part, accounted for by other additives. Many of these products contain other chemicals that, although making up a smaller proportion of the active compounds, may represent a measurable effect on the level of sporocidal activity exhibited by the product as a whole. If true, it is likely that the effectiveness of the treated insulation is potentiated or synergized by these compounds acting as complementary chemical units in an effective chemical mixture within the insulation. Indeed, Clausen and Yang[1] suggested as much in their work that showed anti-fungal effectiveness was improved when some borates were included in "multicomponent" mixtures. This complementation of antifungals is generally true for other antibiotics that kill a different suite of microbes via distinct mechanisms[10].

3. The spores did not interact with sodium polyborate sufficiently long enough. Spores sprayed onto treated insulation were allowed to interact with the product for 14 days while those placed on filter paper only interacted with sodium polyborate for 7 days. Although continued observation of the spores in aqueous suspensions past 7 days using epiflourescence viability staining suggests that at least some spores were still viable, none of the spores of *P. chrysogenum* and *A. niger* germinated after interacting with sodium polyborate for 7 days (sporostatic effect); and some of them were rendered inviable (**Fig. 2**). Perhaps, spores from these species are more resistant to the effects of sodium polyborate and take longer to kill.

The fact that spores of some species exposed to sodium polyborate on different media differed in their response is a bit perplexing. In particular, although germination and growth was inhibited by 5% sodium polyborate regardless of media and conditions, exposure on solid MEA plates appears to have a sporocidal effect while placing spores in MEB seemed to have a sporostatic effect or in the case of *Chaetomium globosum*, little to no affect at all. Perhaps the difference in response lies in the availability of water and its corresponding changes in osmolarity in aqueous solutions or in the differential effects of sodium polyborate's mechanism of action in solution. Although the chemical mechanism for sodium polyborate antifungal activity remains unknown, several investigators as early as 1971[8], have suggested that numerous borate compounds are effective as reversible inhibitors of serine proteases[6]. If true, inhibition of some members of this diverse family of enzymes may prevent the polymerization of chitin, an important fungal cell wall constituent and disturb some of the disparate metabolic pathways. The diverse targets and mechanisms of boron containing compounds may help explain why sodium polyborate is sporocidal to some species and sporostatic or hyphal toxic to others.

In conclusion, our results indicate that treated cellulose insulation is sporocidal for nearly all species of fungi tested and that the principal active ingredient, sodium polyborate, plays a predominant role in the product's antifungal properties. Boron's diverse mechanisms of activity seem to determine whether those antifungal properties are manifested as sporocidal, sporostatic or hyphal toxic effects on any one species of fungi.

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