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*Published in:*
Sisäilmastoseminaari 2019

Published: 01/01/2019

**Document Version**
Publisher's PDF, also known as Version of record

*Please cite the original version:*
OLD DRY MOLD GROWTH SEEMS TO EMIT LESS BIOACTIVE METABOLITES AND SURFACTANTS THAN ACTIVELY GROWING MICROBES

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ABSTRACT
We characterized the toxicity and surfactant properties of old and fresh biomasses of indoor isolates of Trichoderma atroviride, Aspergillus versicolor, Chaetomium sp., Stachybotrys sp, and Rhizopus sp. Toxicity of cell dispersals from old dry biomasses (> 12 months old) and actively growing molds (< 3 weeks old) was tested as inhibition of proliferation with somatic mammalian cell lines (PK-15 and MNA). Fresh biomasses were up to 100 times more toxic than old dry biomasses. When calculated per conidial particle, 100 times more toxicity was associated with the fresh conidia than the old ones. Surfactant activity was detected both in biomass dispersals and exudates. We hypothesized that the metabolic state of the fungal growth may influence fungal metabolite emission into indoor air. This information may be important when mouldy buildings are renovated.

INTRODUCTION
Respiratory illness is associated with exposure to dampness and molds /1/. Mold odor has been connected to unhealthy indoor air and is a possible indicator for active fungal growth /2/. Mold growth indoors depends on moisture enabling biological activities as cell division, mycelial growth, sporulation, formation of membrane-surrounded liquid-containing organelles (vesicles, vacuoles and peroxisomes), synthesis of secondary metabolites and emission of volatile organic compounds (VOCs /3/4/5/). Fresh, hydrated fungal hyphae containing conidiophores, vesicles and vacuoles indicate ongoing, fungal growth, whereas desiccated conidia and dry hyphal fragments may be less relevant indicators /1/2/3/4/.

Available microbiological methods may not measure relevant indicators for hazardous mold exposure and no guidelines for unhealthy levels of indoor mold exposure have been defined /5/6/. Biologically active fungal secondary metabolites may be toxic to the producer organism and transported to the cell surface and liberated to the exterior by membrane-surrounded organelles like vacuoles and vesicles /2/3/. Compared to conidia and hyphal fragments, membrane-surrounded organelles of indoor fungi have gained little attention and their impact on indoor air quality is not yet understood. The aim of our study was to compare toxicity and surfactant activity (ability to reduce surface tension in water) in dry old and moist fresh fungal biomasses.
METHODS

Isolation and toxicity testing of fungal biomass dispersals and exudates

Settled dust, indoor building materials and inlet air filters were collected from urban buildings where severe health problems/7/ were observed. The samples were cultivated on malt extract agar /8/. Isolation of the indoor microbial isolates, rapid toxicity screening and toxicity testing of biomass dispersals and exudates of pure cultures with the somatic cell lines PK-15 (a continuous cell line of porcine kidney epithelial cells) and MNA (a malignant murine neuroblastoma cell line) were performed as described previously /8/.

Identification of indoor strains and measurement of reduction of surface tension of water

Isolates were identified to the genus level based on the colony morphology on MEA, conidiophore morphology, and the size of conidia. Aspergillus versicolor strain SL/3 was identified (in the year 2008) to the species level in DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). DNA barcoding (www.isth.info) was used to identify the indoor Trichoderma atroviride strains. Measurement of reduction of surface tension, i.e. the increase of the surface of a drop of pure water of 20-500 µl after addition of 5-50 µl of exudates or conidial dispersals is described in the paper of Castagnoli et al. 2018 b /9/.

RESULTS

Old resting and fresh active fungal growth - Liquid exudate droplets were not detected in old dry biomasses.

Figure 1. Stachybotrys biomass on a water damaged paper board (A) and on malt extract agar (B). The biomasses were >20 years (A) and 3 weeks old (B), respectively. Panel A shows conidiophores and conidia, no exudates or vesicles are visible. Panel B shows large liquid-containing membrane-surrounded organelles and black conidiophores.
A piece of paperboard (10 cm×10 cm), was sampled 1996 and stored in a sealed glass jar at room temp until 2017 (Figure 1A). Biomass scraped from the paper board from 3 different locations were a) cultivated on malt extract agar, b) dispersed in ethanol (60 mg ml⁻¹). The inoculated malt extract agar exhibited dominant growth of *Stachybotrys* sp. like colonies. Three colonies were pure cultured, identified by microscopy as *Stachybotrys* sp. One strain was stored in our culture collection as HJ5 and will be identified by molecular methods. Biomass from the pure cultures incubated at room temperature for 3 weeks were dispersed in ethanol (60 mg ml⁻¹). Dispersals from the paperboard (n=3) and pure cultures (n=3) were tested for toxicity with PK-15 and MNA cells. The EC₅₀ concentrations for the old dry biomasses in exposed PK-15 and MNA cells were >5000 µg ml⁻¹ and 1200 µg ml⁻¹, respectively. The EC₅₀ concentrations for the fresh biomasses from the three pure cultures were 16 µg ml⁻¹. Difference between the triplicate measurements were within one dilution step (two fold dilution).

Old and fresh fungal biomass growing on the old paper board and malt extract agar, respectively were inspected by stereo microscopy. Figure 1 shows the difference between old, dry biomass of *Stachybotrys* sp. on paper board and actively growing, fresh biomass on malt extract agar. The picture shows that the old, dry biomass exhibited no membrane surrounded organelles. The fresh biomass contained hyphae, conidiophores, conidia and large exudate-containing vesicles. Microscopic evaluation of biomasses from the fresh actively growing *Stachybotrys* sp. revealed that the volumes of membrane-surrounded organelles filled with liquid exudates exceeded the volumes of the solid particles (Figure 1B).

**In vitro toxicity of old, dry and wet, fresh fungal biomass**

Table 1. Toxic responses in vitro assays with somatic mammalian cell lines PK-15 and MNA of actively growing, fresh biomass and dry, old fungal biomass grown on malt extract agar.

<table>
<thead>
<tr>
<th>Age of biomass</th>
<th>EC₅₀ µg fungal biomass (wet wt) ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 weeks</td>
</tr>
<tr>
<td></td>
<td>PK-15</td>
</tr>
<tr>
<td><em>T. atroviride</em></td>
<td></td>
</tr>
<tr>
<td>T1/SKK</td>
<td>100</td>
</tr>
<tr>
<td>T7/SKK</td>
<td>130</td>
</tr>
<tr>
<td><em>Asp. versicolor</em></td>
<td></td>
</tr>
<tr>
<td>SL/3</td>
<td>100</td>
</tr>
<tr>
<td><em>Chaetomium sp</em></td>
<td></td>
</tr>
<tr>
<td>9/MØ</td>
<td>30</td>
</tr>
<tr>
<td>2b/26</td>
<td>40</td>
</tr>
<tr>
<td>2c/26</td>
<td>60</td>
</tr>
<tr>
<td>HAS5</td>
<td>50</td>
</tr>
</tbody>
</table>

Dispersals of old and fresh fungal biomass growing on malt extract agar were tested *In vitro* for toxicity as inhibition of proliferation with the PK-15 and MNA cell lines. The biomass dispersals contained 60 mg fungal biomass per ml. We found reduced toxicities, i.e. higher EC₅₀ values in the old biomasses compared to fresh ones representing 5 genera.
of common indoor molds; *Stachybotrys*, *Trichoderma*, *Chaetomium* and *Aspergillus* (Table 1). The toxicity endpoints of old biomass dispersals were >3 to 10 times higher than for the fresh biomass dispersals (Table 1). When calculating density of conidia per microscopic fields in dispersals of 60 mg (wt wt) ml⁻¹, the old, less toxic dispersals were found to contain 6 to 10 times more conidia than the more toxic, fresh dispersals. Thus, 100 times more toxicity was associated with the fresh conidia than with the old ones (Figure 2).

**Figure 2.** Phase contrast micrographs showing density of conidia in microscopic fields in dispersals of plate-grown biomasses (60 mg wet wt per ml) of *Aspergillus versicolor* SL3. The dispersal of >1-year-old biomass in Panel A visualizes 120 conidia. Panel B shows the dispersal from a 2-weeks-old biomass containing 20 conidia. The bar is 6 µm. The panels A and B are representative for 6 microscopic fields, respectively.

<table>
<thead>
<tr>
<th>Toxic response</th>
<th>Reduction of surface tension of water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exudates</td>
<td>Conidia</td>
</tr>
<tr>
<td><em>Rizopus sp. MK1</em></td>
<td>-</td>
</tr>
<tr>
<td><em>Asp. versicolor SL3</em></td>
<td>-</td>
</tr>
<tr>
<td><em>T. atroviride T1/SKK</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Stachybotrys sp HJ5</em></td>
<td>+</td>
</tr>
</tbody>
</table>

The results in Table 2 and Figure 3 show that both conidia and exudates from the indoor isolates reduced the surface tension of water. These results, although the number of tests is limited, also indicated that fungal exudates from different species differed in toxic responses in the applied bioassays, and that different substances are emitted in exudates than the ones bound to the conidia.

In conclusion, our results suggests that the metabolic state and vesicle formation influences the content of toxic metabolites and surfactants of indoor fungi and possibly influence the emission into indoor air.
DISCUSSION

We describe two biologically measurable activities in biomass and exudates emitted by indoor molds, i.e. toxicity against mammalian cells *in vitro*, and reduction of water surface tension. Our main result is that old, dry plate-grown fungal biomass seemed to contain less toxicity and less exudate-containing membrane-surrounded organelles than actively growing fungal biomass. Our results, eventhough based on a very low number of experiments, tempted us to hypotize that actively growing fungi may have a greater influence on indoor air quality than old, dry fungal growth, irrespective of the amount of liberated conidia. However, more experiments including biomasses collected after several different incubation times, i.e. 2, 6, 8, 12 and 24 months are needed to test our hypothesis.

The low toxicity recorded for the dry *Stachybotrys* sp. biomass scraped from the old paperboard could be explained by degradation of toxins, or inability to produce toxins on paperboard. This indicated that a finding of *Stachybotrys* sp. growth in a building is not necessarily connected to extreme toxicity, an information important for renovation work.

Recent studies suggest that mycotoxins are synthesized from precursors in toxisomes, i.e. extracellular trafficking vesicles that house secondary metabolite synthesis /2/3/. This explains why different substances may be found in toxisomes and in conidia, preliminary illustrated by the differences between the four indoor fungal strains in Table 2. Reduction of the surface tension of water may be caused by anthropogenic chemicals and/or be microbially produced, but is very likely absent in outdoor air /11. Tensidometrical measurement of surfactant activity in air condensates directly from indoor air, as described by Salonen et al. /12/ could be a measure of indoor air pollution.
AKNOWLEDGEMENTS

The authors thank the Finnish Work Environment Fund (Työsuojelurahasto, SIBI 117101) and the Academy of Finland (Suomen Akatemia, TOXICPM 289161). LK is grantee of the János Bolyai Research Scholarship (Hungarian Academy of Sciences). Maria Hautaniem at the Finnish Food Safety Authority for providing the somatic cells.

REFERENCES