

## Vapor as a carrier of toxicity in a health troubled building

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### SUMMARY

*Penicillium expansum* was identified as a major contaminant in indoor air, settled dust and materials of several buildings connected to indoor air related health complaints. This fungus emitted large quantities of exudates when cultivated on laboratory media. The exudates proved toxic towards four different mammalian test cells up to 10000 fold dilution. Toxins identified by LC-MS/MS were communesins and chaetoglobosin. Air dispersal of the toxic exudates was investigated with an experimental set-up where natural convection was generated by temperature gradient. It was found that the exudate with the contained toxins became airborne transported from the warmer surface to the colder surface. The results thus demonstrate transportation of microbially produced toxic substances across the air space. The role of liquid emissions from indoor molds represents a novel mechanism for human exposure in mold contaminated buildings. In this paper we report that vapor condensed from the indoor air of building affected with molds *Aspergillus versicolor*, *Aspergillus calidoustus* and *Penicillium expansum* contained substances that were acutely toxic when exposed to mammalian cells *in vitro*. The results encourage further study of condensed indoor water vapor as a tool to assess the presence of airborne substances with possible adverse health effects.

### INTRODUCTION

Building materials and house dust have been shown to contain microbially produced substances that are toxic to higher organisms, including man. These substances, referred in this paper as microbial toxins, have been found in indoor samples at low concentration levels (Täubel et al., 2011). Therefore, the potential health risk they may pose in indoor air is still under debates. Microbial toxins are assumed to be carried in the air via the spores and microbial cell fragments. However, intervention studies where buildings were remediated to reduce viable spores failed to show

reduction of building related ill health complaints (Sauni et al., 2011). Microbial and building material emitted air contaminants (VOC, MVOC) have been measured in a large number of studies but no proof for correlations with indoor health has been reported (Pasanen et al., 1998) Thus indoor air contaminating particulates and gaseous compounds has been extensively studied but indoor air water vapor as a carrier of adverse agents appears to have escaped research attention so far. Thus the true nature of a causative agent of adverse indoor health continues to be poorly understood. In the present study we offer evidence suggesting that condensed indoor water vapor may be useful as a tool to detect the presence of building contained undesirable substances.

## METHODOLOGIES

Buildings where the occupants' complaints were suspected to depend on indoor air quality were sampled by surface wiping (with autoclaved microfiber fabric, 16 cm<sup>2</sup>), settled dust collection, 6-stage Andersen impactor, deposition plates and specimens of construction material. The samples were cultured on 2% malt extract agar plates (35 ml per plate, Ø 9 cm, 63 cm<sup>2</sup>) sealed with adhesive tape immediately after the exposure and cultured under ambient light and 20 - 22°C. Biocidal resistance of the indoor fungi was tested using malt extract agar plates supplemented with boric acid (CAS 10043-35-3), borax (CAS 1303-96-4), arsenic pentoxide (CAS 1303-28-2) or arsenic trioxide (CAS 1327-53-3), PHMG (CAS 57028-96-3) and PHMG (CAS 91403-50-8) up to 500 - 2000 ppm. Fungal identification was by microscopic inspection (Samson et al., 1995) and verification by ITS or calmodulin sequences as described elsewhere (Kredics et al., 2007). Water vapor in indoor air was condensed into a stainless steel reservoir, with a surface area of 1380 cm<sup>2</sup> cooled to 3°C. The harvested condensate was concentrated by evaporation at 62 °C and the residue dissolved in ethanol.

The individual colonies emerging on the plates within 4 weeks of incubation, as well as their liquid exudates, were assessed for toxicity using cell toxicological methods described elsewhere (Hoornstra et al., 2013; Rasimus et al., 2012) and for fluorescent emission using black light (360 nm, UVA Finland, Kauniainen). The target cells for toxicological assays were porcine spermatozoa from healthy live donors (delivered by an artificial insemination station, Figen OY, Tuomikylä, Finland) diluted in MR-A extender to 27 × 10<sup>6</sup> sperms/ml and used within 3 days of delivery. Cell lines were cultured and exposed in tissue culture cabinet (5 vol % CO<sub>2</sub>, 19 % O<sub>2</sub> in N<sub>2</sub>, 95 % RH): kidney tubular epithelial cells (PK-15), feline fetal lung cells (FL) and murine neuroblastoma cells (MNA) were retrieved from EVIRA (The Finnish Food Safety Authority, Helsinki, Finland). Chemical identification of *P. expansum* mycotoxins was performed by LC-MS-methods described by Mikkola et al. (2012).

## RESULTS AND DISCUSSION

We studied a Finnish university building with reported indoor related human illness, constructed in 1959 - 1967, renovations made in 1997 - 2001 (Table 1). The building has a concrete frame, mineral wool and cork board isolation and a tile façade. The offices were sampled with four different methods. Several different toxin producing fungi were obtained by each method and from more than one office space. The most prevalent toxin producers are displayed in Figure 1.

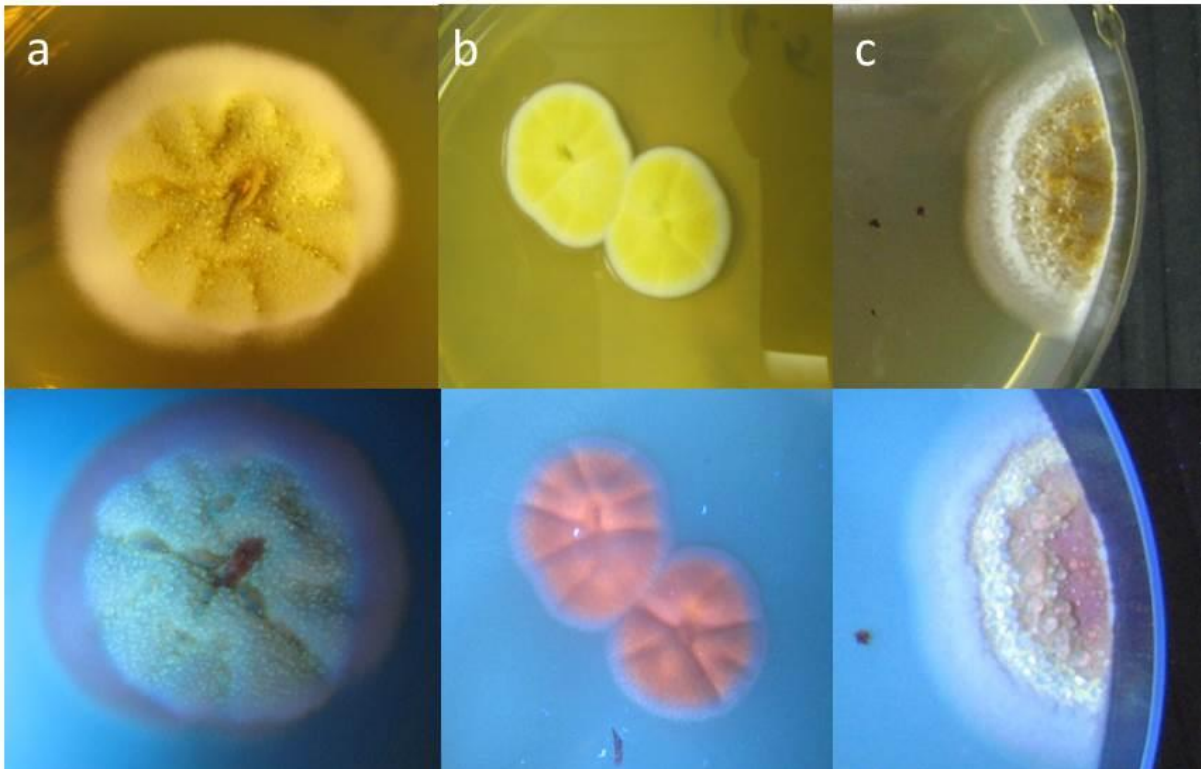


Figure 1. Colonies grown on malt extract agar plate from indoor samples in a health troubled office building (#1, Table 1). A) *Aspergillus calidoustus*, b) *Aspergillus versicolor* and c) *Penicillium expansum*. Top row: photographed under ambient light; bottom row: illuminated at 360 nm.

The most prevalent indoor fungus in offices in the building (#1, Table 1) was *Penicillium expansum* (Figures 1, 2). This fungus was found in settled dust, building materials and on deposition plates (1 h exposed). All colonies identified as *P. expansum* were toxic in *in vitro* tests. *P. expansum* was prevalent also in several other buildings investigated because of building related health complaints (Table 1).

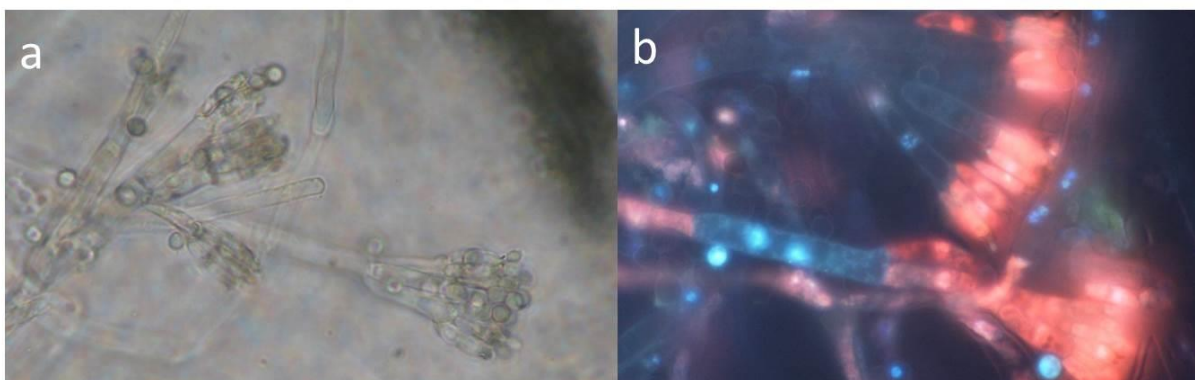


Figure 2. *Penicillium expansum* from a primary colony on fall out plate (#3, Table 1). A) phase contrast microscopic image and b) fluorescence (ex. 450 - 490 nm) microscopic image double stained with propidium iodide (dyes single stranded DNA and RNA pink) and dsDNA dye Hoechst 33342 (blue).

Table 1. Public buildings in southern Finland colonized by *Penicillium expansum*.

Number	Buildings where <i>P. expansum</i> was found as a major colonizer	Year of construction	Sampling	Year investigated
1	Several offices of a university institute (Espoo)	1958 - 1963	Indoor Air (Andersen 6-stage impactor), settled dust, deposition plate, building material: cork	2013, 2014
2	Offices in an education institute (Helsinki)	In the 1950s	Wipe sample from horizontal surface	2014
3	Elementary school (Helsinki)	2009	Deposition plate	2010
4	Elementary school (Lahti)	In the 1960s	Deposition plate + settled dust	2013
5	Ice rink (Nivala)	1995	Settled dust	2014
Material	Unused gypsum board in warehouse		Building material: gypsum board	2013

Adan et al. (2011) and Samson (2011) indicated *P. expansum* as a common contaminant in indoor air. Our findings indicate that toxin producing *P. expansum* is an important colonizer of health troubled buildings in Finland and was also found as contaminant in unused building materials (Table 1). So far, *P. expansum* received little attention as a harmful indoor space colonizer of buildings in Finland (Nevalainen et al., 2015, Pietarinen et al., 2008).

An explanation for not observing *P. expansum* may be found in the official method of the Finnish Ministry of Social Affairs and Health (STM, 2003) that limits the sampling season to the months when the soil is frozen and the cultivation time to 7 days,  $25 \pm 3$  °C with no sealing of plates. In order to be able to cultivate for 28 d enabling slow growing fungi to form colonies, the agar plates were sealed to reduce excessive drying in the laboratory air, usually < RH 30 %. In the winter months the outdoor monthly average water vapor content in Finland varies between 1.17–3.28 g/m<sup>3</sup> (RIL, 2000), equivalent to relative humidities of 5 - 14 %, when warmed to 25 °C and RH 4 - 12 % at 28 °C.

Buildings in Finland contain large amounts of antifungal chemicals aimed to increase the fungal resistance of building materials. According to statistics of the Finnish Ministry of Environment, the summed usage in years 1994 - 2006 for building materials was 3652 000 kg of arsenic pentoxide and 432 000 kg of boron compounds (Repo, 2009). In addition, biocidal polyguanides PHMG and PHMB were used at a rate of 10000 kg per year since 1995, with the aim to protect indoor space against molds. The Finnish building stock contains about 434 million square meters, of which 63 % is in residential buildings and 37 % in other buildings (Reijula et al., 2012).

We investigated the efficacy of these building antifungals against *P. expansum* cultivated from health troubled buildings and found that *P. expansum* was not

prevented by the presence of arsenic, boron or polyguanide compounds at concentrations equal or higher than those expected in buildings (Figure 3).

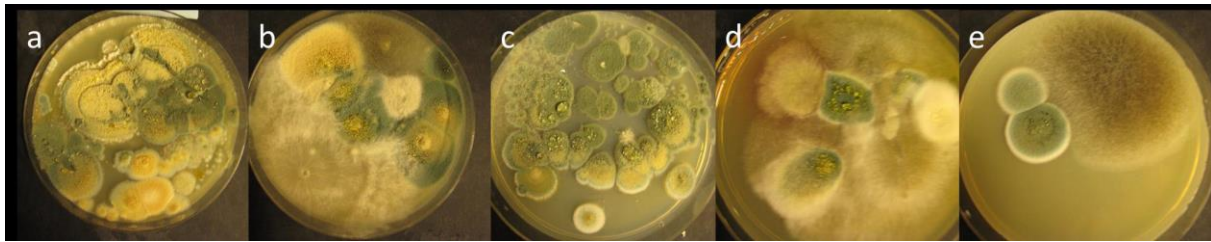


Figure 3. Fall out plates of indoor air microbes from a school building (#4, Table 1). Malt extract agar plate supplemented with a) Arsenic  $As_2O_3$  200 ppm, b) Borax 500 ppm, c) PHMB 500 ppm, d) Boric acid  $H_3BO_3$  2000ppm or e) PHMG 500 ppm.

The results thus indicate that the current and the near past practice in using biocidal antimicrobials may offer selective advantage for *P. expansum* and is likely to be ineffective in remediating buildings already colonized by this fungus.

Indoor fungi are well known to produce highly toxic metabolites called mycotoxins. Nevertheless, investigations targeted to finding these toxins in indoor air at toxicologically relevant concentrations failed for many years. In 2008, Gottschalk et al. reported toxic concentrations of satratoxins G and H in indoor air. Later they reported that *Stachybotrys spp.* emitted toxins as exudate droplets rather than spores as was assumed before (Gareis and Gottschalk, 2014). Interestingly, indoor isolates of *P. expansum* also appear to extrude droplets (Figures 3, 4).

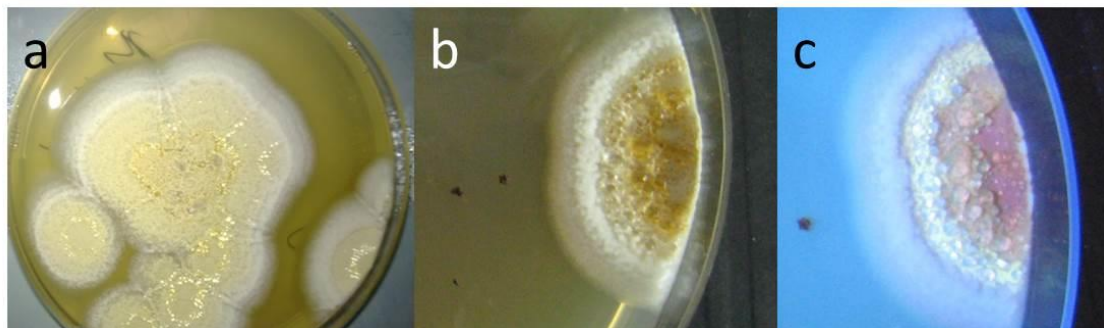


Figure 4. Droplets produced by a) the indoor isolate *Penicillium expansum* P61 growing on malt extract agar, b) cork board sample from an exterior wall of university office building (#1, Table 1) growing on malt extract agar and c) the same plate photographed after illumination at 360 nm. On both plates exudate droplets produced by the fungus are visible.

Exudates produced by *P. expansum* P61 were collected by micropipetting and tested for toxicity. It was found that exudates were toxic towards two porcine cell types (kidney epithelium and spermatozoa), feline lung cells (FL) and murine neuroblastoma cells (MNA). The exudates proved toxic toward these test cells at dilutions up to 1000–10000 fold. Communesins A, B and D and chaetoglobosin were identified from these exudates by LC-MS/MS. The present findings are, to our knowledge, the first report of highly concentrated, toxicologically significant quantities of mycotoxins emitted in liquid form by *P. expansum*. The same mycotoxins of *P. expansum* were earlier reported by Andersen et al. (2004) from plate cultured hyphal biomass of food isolates and two indoor air isolates from Denmark.

The question whether toxic exudates of *P. expansum* could disperse into air space was tested with an experimental setup using a Petri dish where natural convection was generated by temperature gradient. The dish temperature was 15.7 °C and the lid cooled to 12.1 °C. We found that exudate droplets with the contained toxins, became airborne transported from the warmer surface to the colder surface, and condensed as droplets visible on the lid (Figure 5). The droplets were harvested by using a capillary pipette and analyzed for toxicity and by LC-MS methods described earlier (Andersson et al. 2010, Mikkola et al. 2012). The droplets proved toxic and mass spectrometry showed that the droplets contained communesins and chaetoglobosin.

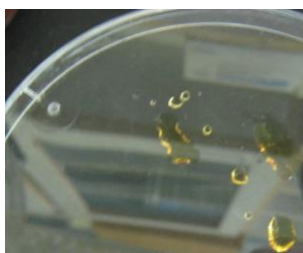


Figure 5. Droplets produced by *Penicillium expansum* P61 airborne transported from the agar surface of the petri dish (Ø 90 mm) to the lid of the dish.

Indoor moisture has, so far, been considered as a promoter of mold growth, but as shown in Figure 5, it also may function as a carrier of toxic compounds. Next we asked a question whether airborne transport of indoor toxicants with water vapor could also operate in a large space like an office room. To this end we condensed indoor vapor as water and assayed its toxicity with *in vitro* methods as described above for *P. expansum* exudate droplets. The obtained yields were 7 - 68 ml of condensate containing up to 1 mg of evaporation residual.

Table 2. Toxicity of water vapor condensed from indoor air and the relevant average parameters during the harvest period. Harvested quantity of water corresponded to water vapor contained in 17.5 - 21 cubic meters of indoor air in that space and time.

Indoor space from where the water vapor condensed was harvested	Volume of the room [m <sup>3</sup> ]	Length of the collection period [d]	Average parameters during the collection period			Toxic *
			RH	T	v <sub>s</sub>	
			[%]	[°C]	[g/m <sup>3</sup> ]	
Negative control (RO-water)		1				-
Office room 1	84	3	40.8	26.1	9.9	+
Office room 2	45	4.5	25.8	26.6	6.5	+
Class room in elementary school	210	5	26	23.0	5.3	-
Positive reference toxicant (Triclosan)						+

v<sub>s</sub> = water vapor content [g/m<sup>3</sup>], \*Toxic response by one or several of the target cells (n = 3) used.

The results show that toxic condensates were obtained within 3 - 5 days from two offices where the occupants reported building related ill health symptoms. This shows that harvesting airborne vapor was more effective for obtaining samples applicable for toxicity assays than sampling of airborne dust where a minimum of 10 mg dust and  $\geq 30$  d of harvesting was required for samples sufficient for toxicity assays (Andersson et al. 2010). The class room not involved with health complaints yielded condensates with no detectable cell toxicity. These results encourage to further study condensed indoor water vapor as a tool to assess the presence of airborne substances with possible adverse health effects. Condensing airborne moisture enables repeated and quantitative sampling and furthermore, was sufficient for assaying harmful air contaminants by cell toxicological methods.

## CONCLUSIONS

1) Toxin producing *P. expansum* was found as a major contaminant in several health troubled public buildings. 2) This fungus appeared to be tolerant to high concentrations (500 - 2000 ppm) of wood preserving chemicals (arsenic, boric acid, borax) and polyguanide antimicrobials (PHMG, PHMB). 3) a *P. expansum* indoor isolate was shown to emit its toxins communines A, B, D and chaetoglobosin as exudate droplets from which the toxins mobilized into the air. 4) We demonstrated that airborne toxic substances could be harvested by condensing indoor air water vapor. 5) Our results indicate that water condensed from the indoor vapor is a useful tool for assessing toxic contaminants in inhaled air.

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