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Production of mycotoxins on artificially and naturally infested building materials

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Abstract

In this study, the ability to produce mycotoxins during growth on artificially infested building materials was investigated for *Penicillium chrysogenum*, *Pen. polonicum*, *Pen. brevicompactum*, *Chaetomium* spp., *Aspergillus ustus*, *Asp. niger*, *Ulocladium* spp., *Alternaria* spp., and *Paecilomyces* spp., all isolated from water-damaged building materials. Spores from the different isolates of the above mentioned species were inoculated on gypsum board with and without wallpaper and on chipboard with and without wallpaper. Fungal material was scraped off the materials, extracted, and analyzed using high performance liquid chromatography-diode array detection and thin layer chromatography. All six isolates of *C. globosum* produced the toxic chaetoglobosins A and C, at levels of up to 50 and 7 μg/cm^2 respectively. The quantities of secondary metabolites produced by Penicillia were generally low, and no toxin production was detected from any of the five isolates of *Pen. chrysogenum*. Both isolates of *Pen. polonicum* produced 3-methoxy-viridicatin, verrucosidin, and verrucofortine. Two of five isolates of *Pen. brevicompactum* produced mycophenolic acid. From five out of six isolates of *Alternaria* spp., alternariol and alternariol monomethyl ether were detected. From *Ulocladium* spp., *Paecilomyces* spp., and *Asp. ustus* no known mycotoxins were detected, although the latter two are known mycotoxin producers. *Asp. niger* produced several naphtho-γ-pyrones and tetra-cyclic compounds. All investigated species, especially *Asp. ustus* and *Asp. niger* produced many unknown secondary metabolites on the building materials. Analyses of wallpaper and glass-fibre wallpaper naturally infested with *Asp. versicolor* revealed sterigmatocystin and 5-methoxysterigmatocystin. Analyses of naturally infested wallpaper showed that *C. globosum* produced the chaetoglobosins A and C, and *Pen. chrysogenum* produced the antibiotic meleagrin.

Keywords: alternariol, chaetoglobosin, indoor air, mycotoxin, sick building syndrome, sterigmatocystin

Abbreviations: a_w = water activity; a_w-min = minimal a_w for fungal growth; DAD = diode array detection; HPLC = high performance liquid chromotography; MVOC = microbial volatile organic compound; RH = relative humidity

Introduction

The correlation between dampness, mould growth, and symptoms such as allergy, airway problems, fatigue, irritation of the skin, headache, and mucous membrane symptoms e.g. itching eyes and stuffy nose, has been demonstrated during the last 5–10 years [1–6].

Fungal growth on building materials always occurs as a result of a high water activity (a_w) at the surface of the material. The minimal water activity for supporting fungal growth was examined by several authors indicating a_w-min. values around 0.67–0.75, but growth also depends on temperature, time and the material [7–9]. Many mycotoxigenic fungi have a demand for an a_w-min of 0.9 for growth, usually met by water ingress from leaking roofs or water pipes. It should be considered that, although $a_w \times 100$ = RH

(at equilibrium), the relative humidity (RH) changes much faster than a_w due to the faster diffusion of water in air than in materials.

Microfungi can be divided into three groups according to their a_w-min [9].

- **Primary colonizers** capable of growth below a_w 0.8, including species of the genera of *Wallemia*, *Penicillium*, *Aspergillus,* and *Eurotium* [9, 10].
- **Secondary colonizers,** with an a_w-min between 0.8 and 0.9, including species of *Cladosporium* [9].
- **Tertiary colonizers**, demanding an a_w of at least 0.9, including genera as: *Stachybotrys*, *Trichoderma*, *Fusarium*, *Chaetomium*, *Ulocladium* and *Alternaria* [9, 11, 12].

Only a very few species can grow on a specific building material. As fungal spores are ubiquitous, a given combination of water activity and material will support growth of one or a few species, named the associated funga [9, 13, 14].

Except for the specific fungal allergies, the causal agents for the symptoms mentioned above have not been found, but at least four fundamentally different types of fungal products could be involved:

- **Proteins** giving the well-known immediate allergic reactions within minutes of exposure (Type 1 allergy) [1, 2, 4] and also non IgE mediated histamine release [15].
- **β-(1,3)-D-glucanes** triggering inflammatory reactions very similar to symptoms observed on exposure to endotoxin [16, 17].
- **Microbial volatile organic compounds** (MVOCs) released from the fungi during growth [18–22].
- **Mycotoxins** including peptides released from fungal spores after inhalation. It has been shown that macrocyclic trichothecenes produced by *Stachybotrys chartarum* (synonym: *S. atra*) probably can induce mycotoxicosis in people living or working in buildings infested with this fungus [23–28].

Several authors have listed mycotoxins isolated in water-damaged building suggesting that these toxins might be produced in the actual buildings [1, 29, 30]. Unfortunately only few authors have confronted the problem of misidentified fungal strains and changes in taxonomy, which reduces the number of mycotoxins likely to be found in indoor environments shown in Table 1 [10, 13, 14, 31–34].

Many fungi found on contaminated building materials are well-known mycotoxin producers as demonstrated after growth on foodstuff or laboratory substrates [29, 33].

Several authors have screened fungal isolates from water-damaged building materials and indoor air for production of mycotoxins on laboratory substrates, giving more reliable suggestions of which mycotoxins might be found in water-damaged buildings [10, 28, 33, 35]. The production of mycotoxins depends on the substrate, a_w, temperature and other environmental factors [12, 14], meaning that determination of the mycotoxins actually produced in water-damaged buildings should preferable be done by analysing contaminated building materials. This has been performed for the following species:

- *Stachybotrys chartarum*, which produced the extremely toxic macrocyclic trichothecenes, satratoxins H and G, roridin E, and verrucarins J and B on building materials [12, 23, 36–38].
- *Aspergillus versicolor* which produced sterigmatocystin and 5-methoxysterigmatocystin on wallpapered gypsum boards [12].
- *Trichoderma* spp. which did not produce any detectable quantity of trichothecenes or other mycotoxins on several building materials [12].
- *Alternaria alternata* which produced alternariol and alternariol monomethyl ether on cellulosic acoustic ceiling tiles [39].

The scope of this work was to detect the mycotoxins de facto produced by common fungi growing on building materials. This was done through analyses of artificially mould infested building materials and material samples from a recently infested building.

Materials and methods

Sampling in a domestic residence

Fungal growth on the wall was caused by a leaking water pipe below the floor, leaking approximately 1–5 L/day for 3–6 months. Capillary forces facilitated water transport upwards in the walls, and visual growth was present to a height of 0.5–1 meter on each wall within a radius of 3 meters from the leaking pipe. One sampling was performed by collecting fungal material from heavily infested wallpaper onto a 0.45 μm filter by means of a sampling device attached to a vacuum cleaner [40]. The other samplings took place in four rooms, where infested wallpaper was removed with a scalpel and transported to the laboratory for mycological and chemical analyses. People collecting the

Table 1. Mycotoxin production reported from fungi found in water-damaged buildings[1]

Genus	Species	Secondary metabolites and **toxins**[2]
Alternaria	*tenuissima*	**Alternariol***, **alternariol mono ethyl ether***, **tenuazonic acid,*** **altertoxins I***
Alternaria	*infectoria*	No known metabolites[3]
Alternaria	*alternata*[5]	**Alternariol***, **alternariol mono ethyl ether***, **tenuazonic acid,*** **altertoxins I***
Alternaria	sp.	**Altenuene***, **alternariol***, **iso-altenuene**, **alternariol mono ethyl ether***, **tenuazonic acid,*** **altertoxins I*** & **II**, dihydrotentoxin, alterlosin II, tentoxin*
Aspergillus	*niger*	**Naphtho-t-pyrones**, **Malformins A***, **B***, **C***, tetra-cyclic compounds, aspergilin, asnipyrone, asperrubrol, asperenones, aurasperones, 4-hyroxymandelic acid, orlandin*, nigrazines, orobols, pyrophen, flavinines, tubingensin A and B, nigragillin*, kotanin*, **Ochratoxin A***[4]
Aspergillus	*ustus*	**Austamide*****, austdiol*** and **austocystins A*** **& B***
Aspergillus	*versicolor*[5]	**Sterigmatocystin, 5-methoxysterigmatocystin**
Chaetomium	*globosum*	**Chaetoglobosins A***, **B** and **C***
Paecilomyces	*variotii*	**Patulin***, **viriditoxin***, ferrirubin, fusigen, variotin, indole-3-acetic acid
Paecilomyces	*lilacinus*	
Penicillium	*expansum*	**Chaetoglobosins A*** and **C*****, communosins A*** and **B***, expansolide*, **roquefortine C***, **citrinin***, **patulin***
Penicillium	*chrysogenum*	**Roquefortine C***, meleagrin*, chrysogine*, penicillin g*, ω-hydroxyemodine, 2-pyrovoylaminobenzamide*
Penicillium	*brevicompactum*	Botryodiploidin, **Mycophenolic acid***, asperphenamate, brevianamides A*, B* and F*, brevigellide, drimene X, mycochromenic acid, pebrolides, silvatins, Raistrick phenoles
Penicillium	*polonicum*	3-methoxyviridicatin*, nephrotoxic glycopeptides, **verrucosidin***, **normeyhylverrucosidin***, **penicillic acid***, verrucofortine*, anacine*, puberuline*, cyclopenin, cyclopetin, viridicatol, dehydrocyclopeptin
Stachybotrys	*chartarum*[5]	**Macrocyclic trichothecenes** (**satratoxins H*** and **G**, **roridin E**, **verrucarins J** and **B**), esters of **verrucarol** and **trichodermol**, **phenylspirodrimanes**, atranones.
Ulocladium	*chartarum*	Not known

[1] [32, 34, 43, 59]. *Reference standard at IBT.
[2] Toxic metabolites in **bold**.
[3] [60].
[4] Probably only produced by extremely few isolates.
[5] Produces mycotoxins in building, see text for more information on these species.

samples used respiratory filters (A2-P3) capable of retaining both volatile organic compounds and fungal spores.

Fungal identification

Naturally infested materials
Fungi from the infested materials were identified to genus level directly from the sample by phase contrast microscopy (200×, 400× and 1000×) of tape mounts, i.e. transparent adhesive tape gently pressed to the building material and stained with lacto-fuchsin [34]. Contaminants were also isolated on Czapek yeast autolysate agar (CYA), malt extract agar (MEA), oat meal agar (OAT), and V8 agar and identified to the genus level as described above. *Penicillium* and *Aspergillus* isolates were identified to the species level [34] and characterised by secondary metabolite profiling using HPLC-DAD [32, 41]. Representative strains have been deposited in the IBT Culture Collection, Department of Biotechnology, Technical University of Denmark.

Artificially infested materials

39 strains isolated from infested building materials were used in this study: *Penicillium chrysogenum* (6 strains), *Pen. expansum* (1 strain), *Pen. brevicompactum* (6 strains), *Pen. polonicum* (2 strains), *Chaetomium globosum* (6 strains), *Aspergillus ustus* (5 strains), *Asp. niger* (2 strains), *Ulocladium* spp. (4 strains), *Alternaria* spp. (6 strains), and *Paecilomyces* spp. (2 strains). All were identified and cultivated as described by Samson et al. [34] and Simmons [42], and held at the IBT Culture Collection. *Aspergillus* and *Penicillium* cultures were identified to species level using secondary metabolite profiling and traditional identification methods [32, 43].

Cultivation on building materials

New gypsum boards and chipboard sheets 9 mm in thickness were cut into discs 120 mm in diameter. Part of the discs was coated with a thin layer of wallpaper paste (Flügger, Denmark) made of 100% corn starch containing approximately 1% nitrogen [33]. The wallpaper was applied to the pasted surface of the discs which were placed in 140 mm Petri dishes and sterilized using 25 KGy γ-radiation. Autoclaved double-distilled water was added the day before the discs were streak-inoculated by pure conidial suspension containing 10^5–10^7 conidia/ml. The Petri dishes were incubated in a ventilated room at 25 °C, and inspected once a week. Autoclaved double-distilled water was added as the materials dried out (7–14 days) to maintain a high a_w.

Extraction

Mycotoxins were extracted from the infested building materials using the modified method of Nielsen et al. [12]. The fungal material was scraped off using a scalpel and then extracted for ten hours in 10.0 mL methanol (Merck, Gradient Grade) before filtration through 0.45 μm filter (Minisart RC4, Sartorius, Germany). Some extractions were performed with 10.0 mL methanol containing 1.0% v/v formic acid (Merck, Analytical Grade) to make sure that acidic compounds, e.g. tenuazonic acid were extracted. The filter from the sampling device of the vacuum cleaner was extracted with 200 mL methanol. Samples were then evaporated to dryness in a Christ Rotational vacuum concentrator at 30 °C, 1300 rpm, and 1 mbar. Residue was dissolved in 500 μL methanol, except for the *Chaetomium* extracts from the artificially infested materials, which had to be dissolved in 10.0 mL methanol.

Mycotoxin analyses

High performance liquid chromatography
Extracts were analysed on a Hewlett-Packard HP 1100 HPLC equipped with a diode array detector (6-mm flowcell). Scan: 190–600 nm with a bandwidth of 4 nm approx. 2 times per sec. Samples were injected on a HP Hypersil BDS-C_{18} (3 μm particles) 125 × 2 mm column with a 10 × 2 mm HP Supersphere 100 RP-18 guard column. A linear gradient (at 40 °C) of water (double distilled) and acetonitrile (Merck, Gradient Grade) starting with 15% acetonitrile increasing to 100% over 35 min, then isocratic for 5 min. and returning to 15% acetonitrile in 6 min. was used at a flow rate of 0.3 mL/min. Both eluents contained 0.005% (v/v) trifluoroacetic acid. All chemicals were analytical grade.

Reference standards (see Table 1) of appropriate mycotoxins were analysed after the samples. Only chaetoglobosins and sterigmatocystins were quantified, due to the purity and insufficient quantities of many of the standards. Detection limits were ca. 1 ng at 248 nm for the sterigmatocystins and 5 ng for the chaetoglobosins at 220 nm (injected on column with s/n 5).

Thin layer chromatography
Penicillium, *Chaetomium*, *Aspergillus*, *Paecilomyces* extracts were analysed according to Frisvad and Filtenborg [43], and Frisvad and Thrane [32], on Silicagel 60 plates (20 × 20 cm) without fluorescent indicator Merck (No. 5721). CAP (chloroform/acetone/propane-2-ol, 17/3/4 v/v) and TEF (toluene/ethyl acetate/90% formic acid, 5/4/1 v/v) were used as mobile phases. Griseofulvin and external standards of appropriate secondary metabolites were used for confirmation of identity.

TLC plates were evaluated directly after chromatography, then after spraying with $AlCl_3$ and finally after spraying with anis aldehyde, according to Frisvad & Filtenborg [43].

Analysis of *Ulocladium* and *Alternaria* extracts was performed on TLC plates with concentration zone (Whatman, silica gel 60 A, Linear-K pre-absorbent strip, LK6D, No. 4865 821) using TEF and CAM (chloroform/ammonia(28%)/methanol, 135/2/15) as mobile phases. Griseofulvin and standards of secondary metabolites expected to occur in the samples were used as external standards. All chemicals used were Merck analytical grade.

Results

Fungal growth

Penicillium
On the naturally infested materials investigated, *Pen. chrysogenum* was present in almost all samples. Usually a substantial amount of biomass could be seen with the naked eye. Isolation of *Penicillium* conidia onto agar plates often revealed an additional but limited number of *Asp. versicolor* colonies (Table 2).

Table 2. Analysis of materials in a heavily mould infested domestic residence

Room, material[1]	Visual appearance[2]	Isolated fungi	Metabolites[3]
Kitchen, G-F WP	Black discoloring, RV	*Ulocladium* sp., *P. chrysogenum*	
Kitchen, G-F WP	Green spots and red discoloration, RV	*P. chrysogenum*	5-ST (35 ng/cm^2), ST (52 ng/cm^2)
Kitchen, G-F WP	Dark grey discoloring, RV	*P. chrysogenum*	
Hall, G-F WP on WP	Dark green spots	*P. chrysogenum*	
Hall, G-F WP on WP	Dark green spots	*P. chrysogenum, Absidia* sp.	5-ST (0.2 μg/cm^2), ST (1.7 μg/cm^2)
Hall, G-F WP on WP	Black pinpoint colonies	*Chaetomium* sp., *P. chrysogenum*	ST (28 ng/cm^2), CA (0.8 μg/cm^2), CC (0.4 μg/cm^2)
Hall, G-F WP on WP	Black spots	*P. chrysogenum, Ulocladium* sp.	5-ST(45 ng/cm^2), ST (2 μg/cm^2), CA (0.7 μg/cm^2)
Hall, G-F WP on WP	Grey-green spots on wallpaper	*Penicillium chrysogenum*	ST (7 ng/cm^2)
Hall, G-F WP on WP	Dark olive spots	*P. chrysogenum, A. versicolor*	5-ST (0.8 μg/cm^2), ST (1.2 μg/cm^2)
Child's room, WP	Green spots and red discoloration on reversed side	*P. chrysogenum*	5-ST (10 ng/cm^2), ST (2 ng/cm^2)
Office, WP	Dark olive spots	*P. chrysogenum*	ST (0.1 μg/cm^2)
Office, WP	Green spots and red discoloration on reversed side	*P. chrysogenum*	5-ST (75 ng/cm^2), ST (0.2 ng/cm^2)
Office, WP	Dark green spots	*P. chrysogenum*	
Office, WP	Black big pin point colonies, extensive biomass	*Chaetomium* sp.	
Office, WP	Olive green spots	*P. chrysogenum, Chaetomium* sp.	
Office, WP	Black big pin point colonies, extensive biomass	*Sterilia mycelia**	
Office, WP	Olive green spots	*P. chrysogenum*	
Office, WP	Olive green pin point colonies, extensive biomass	*P. chrysogenum, Chaetomium* sp.	CA (27 ng/cm^2)
Office, WP	Black big pin point colonies, extensive biomass	*Chaetomium* sp., *P. chrysogenum*	CA (2.5 μg/cm^2), CC (0.2 μg/cm^{2}g)
Office, WP	Black discoloring with white cotton like colonies, RV	*P. chrysogenum, Chaetomium* sp.	CA (0.2 μg/cm^2), CC (0.1 μg/cm^2)
Office, WP	Dark green	*P. chrysogenum, A. versicolor*	5-st(0.1 μg/cm^2), ST (0.1 μg/cm^2)
Office, WP	Dark green areas, vacuum cleaned	*P. chrysogenum, A. versicolor*	5-st (0.4 ng/cm^2), ST(2 ng/cm^2), meleagrin

[1] WP: wallpaper; G-F WP: glass-fiber wallpaper.
[2] RV: on reverse side of material.
[3] Using HPLC-DAD and TLC; ST: sterigmatocystin; 5-ST is 5-methoxysterigmatocystin; CA: chaetoglobosin A; CC: chaetoglobosin C.
* Unknown non-sporulating fungus overgrew the petri dishes.

Table 3. Evaluation of growth and production of secondary metabolites on gypsum board

IBT No.	Species	Without wallpaper		With wallpaper	
		Growth[a]	Metabolites[b]	Growth[a]	Metabolites[b]
7028	*Alt. arborescens*-group	++	AOH, AME	+++	AOH, AME
7787	*Alt. tenuissima*-group	++	NA[c]	+++	AOH, AME
8478	*Alt. arborescens*-group	+++	NA	NG	AOH, AME
8482	*Alt. tenuissima*-group	+++	AOH, AME	++++	AOH, AME
8542	*Alt. tenuissima*-group	++	NA	++	AOH, AME
8485	*Alt. infectoria*-group	no growth		++	NKMD[d]
15952	*Asp. niger*	no growth		++	NKMD
16066	*Asp. niger*	no growth		++	NKMD
14925	*Asp. ustus*	+	NKMD	++	NKMD
14926	*Asp. ustus*	+	NKMD	++	NKMD
16810	*Asp. ustus*	+	NKMD	++	NKMD
18350	*Asp. ustus*	++	NKMD	++	NKMD
19563	*Asp. ustus*	+	NKMD	++	NKMD
SK 25	*Chae. globosum*	Not performed		++++	Chea A, Chae C
8824	*Chae. globosum*	Not performed		++++	Chea A, Chae C
8825	*Chae. globosum*	Not performed		++++	Chea A, Chae C
8826	*Chae. globosum*	Not performed		++++	Chea A, Chae C
8827	*Chae. globosum*	Not performed		++++	Chea A, Chae C
8828	*Chae. globosum*	Not performed		++++	Chea A, Chae C
17490	*Pae. lilacinus*	++	NKMD	++	NKMD
20167	*Pae. variotii*	+	NKMD	+	NKMD
13972	*Pen. brevicompactum*	no growth		++	NKMD
13981	*Pen. brevicompactum*	no growth		++	NKMD
13982	*Pen. brevicompactum*	no growth		++	NKMD
15953	*Pen. brevicompactum*	no growth		++	NKMD
16085	*Pen. brevicompactum*	no growth		no growth	
17655	*Pen. brevicompactum*	no growth		++	NKMD
15904	*Pen. chrysogenum*	NA		++++	Chrys
17491	*Pen. chrysogenum*	NA		++++	Chrys
17546	*Pen. chrysogenum*	NA		+++	Chrys
19497	*Pen. chrysogenum*	NA		++++	Chrys
19569	*Pen. chrysogenum*	NA		++++	NKMD
20236	*Pen. expansum*	+	NKMD	+	NKMD
14910	*Pen. polonicum*	+	NKMD	+	3MV
14921	*Pen. polonicum*	+	NKMD	+++	3MV, Ves, Vef
7712	*Ulocladium* sp.	++	NKMD	+	NKMD
8822	*Ulocladium* sp.	++	NKMD	+	NKMD
9052	*Ulo. atrum*	++	NKMD	+	NKMD
8051	*Ulo. chartarum*	++	NKMD	+	NKMD

[a] Growth evaluation: ++++: material totally covered by mycelium; +++: 80% to 50% covered; ++: 50% to 20% covered; +: 20% to 5% covered.
[b] Known secondary metabolites: AOH: alternariol; AME: alternariol monomethyl ether; Chea A: chaetoglobosin A; Chea C: chaetoglobosin C; 3MV: 3-methoxy-viridicatin; Ves: verrucosidin; Vef: verrucofortine; chrys: chrysogine.
[c] NA: Not analysed.
[d] NKMD: No known metabolites detected.

Table 4. Evaluation of growth and production of secondary metabolites on chipboard

IBT No.	Species	Without wallpaper		With wallpaper	
		Growth[a]	Metabolites[b]	Growth[a]	Metabolites[b]
7028	*Alt. arborescens*-group	+++	NKMD[c]	+++	NKMD
7787	*Alt. tenuissima*-group	+	NKMD	+++	AOH, AME
8478	*Alt. arborescens*-group	+	NKMD	+++	AOH
8482	*Alt. tenuissima*-group	++	NKMD	++	AOH, AME
8542	*Alt. tenuissima*-group	no growth		++++	AOH, AME
8485	*Alt. infectoria*-group	no growth		no growth	
15952	*Asp. niger*	++++	NγP, TC, Nig	++++	NγP, TC
16066	*Asp. niger*	++++	Orl, NγP	++++	NγP, Nig, Orl, TC
14925	*Asp. ustus*	++++	Kotanines	++	NKMD
14926	*Asp. ustus*	++	NKMD	++++	NKMD
16810	*Asp. ustus*	+++	NKMD	++++	NKMD
18350	*Asp. ustus*	++	NKMD	+++	NKMD
19563	*Asp. ustus*	+++	NKMD	++	NKMD
17490	*Pae. lilacinus*	no growth		no growth	
20167	*Pae. variotii*	no growth		no growth	
13972	*Pen. brevicompactum*	++	MyA	+++	MyA
13981	*Pen. brevicompactum*	+++	NKMD	+++	NKMD
13982	*Pen. brevicompactum*	no growth		+	NA
15953	*Pen. brevicompactum*	+	NA	++	NKMD
16085	*Pen. brevicompactum*	no growth		++	NA
17655	*Pen. brevicompactum*	+	NA	+++	MyA
15904	*Pen. chrysogenum*	++	NKMD	Not performed	
17491	*Pen. chrysogenum*	++	NKMD	Not performed	
17546	*Pen. chrysogenum*	++	NKMD	Not performed	
19497	*Pen. chrysogenum*	++	NKMD	Not performed	
19569	*Pen. chrysogenum*	++	NKMD	Not performed	
20236	*Pen. expansum*	+	NKMD	+	NA
14910	*Pen. polonicum*	++	3MV, Ves, Vef	++	3MV, Ves, Vef
14921	*Pen. polonicum*	++	NKMD	+	NA
7712	*Ulocladium* sp.	++	NKMD	+	NKMD
8822	*Ulocladium* sp.	++	NKMD	++	NKMD
9052	*Ulo. atrum*	no growth		++++	NA
8051	*Ulo. chartarum*	++	NKMD	++++	NA

[a] Growth evaluation: ++++: material totally covered by mycelium; +++: 80% to 50% covered; ++: 50% to 20% covered; +: 20% to 5% covered.
[b] Known secondary metabolites: AOH: alternariol; AME: alternariol monomethyl ether; NγP: naphtho-γ-pyrones; Nig: nigragillin; Orl: orlandin; TC: tetra-cyclic compounds; MyA: mycophenolic acid; 3MV: 3-methoxy-viridicatin; Ves: verrucosidin; Vef: verrucofortine.
[c] NKMD: No known metabolites detected.[d] NA: Not analysed.

On the artificially infested materials *Pen. chrysogenum* grew rapidly and was seen as grey to light green discoloring patches. The materials were overgrown in two to three weeks (Tables 3 and 4), but with a sparse amount of biomass produced on the materials.

On the artificially infested materials it was impossible to differentiate colonies of *Pen. chrysogenum* from those of *Pen. polonicum*, whereas *Pen. brevicompactum* appeared more dark green with a lower growth rate than *Pen. chrysogenum*. The *Pen. expansum* isolate grew poorly on all materials.

Aspergillus

On the materials naturally infested *Asp. versicolor* was isolated very frequently, and appeared either green due to heavy sporulation or as a reddish discoloration with very few conidia.

Growth of especially *Asp. niger* was extremely fast on all artificially infested materials except on gypsum board and the wallpapered gypsum where growth was poor. The other materials were covered by black Aspergilli heads in one to two weeks, and substantial quantities of biomass could be scraped off the material.

Growth of *Asp. ustus* on artificially infested materials was usually seen as large olive green to brown green colonies with large amounts of white-yellow slimy mass with dark green areas with conidia covering approximately 80% of the colony. The fungus grew especially well on the wallpaper and wallpapered chipboard; covering most of the surface after two to four weeks. On the gypsum boards *Asp. ustus* grew poorly producing white solid structures, 1–2 mm in diameter, containing up to 100 Hülle cells.

Chaetomium

Being an ascomycete this species has a very characteristic appearance due to its distinct black or dark green, hairy perithecia visible to the naked eye. On the naturally infested materials it appeared frequently on very wet wallpaper.

On the artificially infested material growth was very fast covering the surface with substantial amounts of biomass after two weeks.

Ulocladium and Alternaria

On the artificially infested materials, *Ulocladium* and *Alternaria* covered the area with a white silky mycelium with black conidia usually covering 20–40% of the total area after two to six weeks.

Paecilomyces

Pae. lilacinus was found to grow poorly on all the artificially infested materials except on gypsum boards, where the characteristic pink cotton-like mycelium was produced, but only in modest amounts.

Pae. variotii grew well on the artificially infested chipboards and wallpapered chipboard, but the quantities of biomass were very limited.

Mycotoxin analyses

Penicillium

Naturally infested materials: The sample from the vacuum cleaned wall infested with *Pen. chrysogenum* revealed meleagrin and ergosterol. The visual observation showed that large quantities of biomass were produced, this was confirmed by a large ergosterol peak in the HPLC chromatogram. Small amounts of sterigmatocystin were also present originating from growth of *Asp. versicolor* [12]. In two samples of glass-fibre wallpaper infested with *Pen. chrysogenum* no known metabolites were detected.

Artifically infested materials: Production of secondary metabolites was low (Tables 3 and 4) in all the *Penicillium* species on building materials compared with the agar media. Nevertheless it was possible to detect production of 3-methoxy-viridicatin, verrucosidin, and verrucofortine from *Pen. polonicum*. *Pen. brevicompactum* produced the mycotoxin mycophenolic acid and two unknown metabolites, metabolite F and O (Figure 1).

From *Pen. chrysogenum* chrysogine was the only known secondary metabolite detected. The TLC analyses showed that all isolates produced an unknown metabolite appearing as dark blue spots after spraying with anis aldehyde. This metabolite was only detected from *Pen. chrysogenum* and had approximately 10% higher R_f-value compared with griseofulvin.

Aspergillus

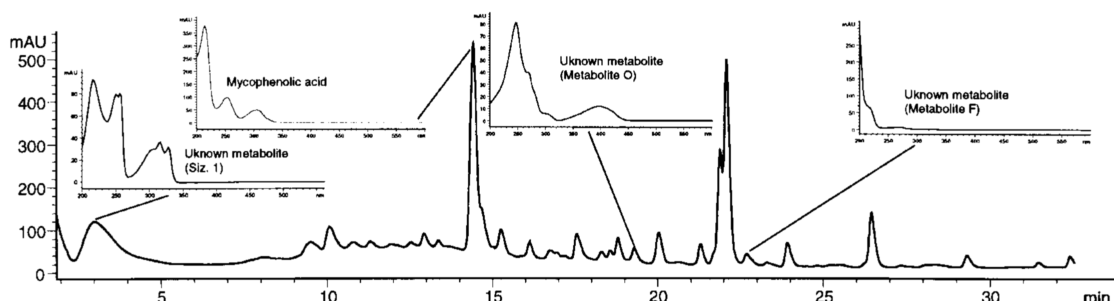
Naturally infested materials: As seen in Table 2, several samples infested with *Asp. versicolor* contained the carcinogenic sterigmatocystins. Red colored areas infested with non or poorly sporulating *Asp. versicolor* biomass contained the largest quantities of sterigmatocystins, whereas areas with many *Asp. versicolor* conidia contained very small quantities.

Artificially infested materials: Asp. niger produced secondary metabolites such as orlandin, nigragillin and more than twenty unknown compounds including several naphtho-γ-pyrones and tetra-cyclic compounds (Figure 1). No ochratoxin was detected.

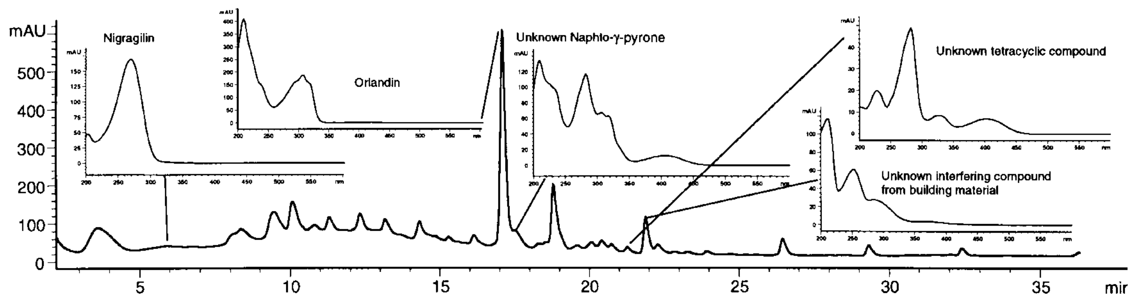
Asp. ustus produced up to forty metabolites on the materials. Two to five structurally unknown kotanins were present. No austamide, austdiol, or austocystins were detected in any of the samples.

Chaetomium

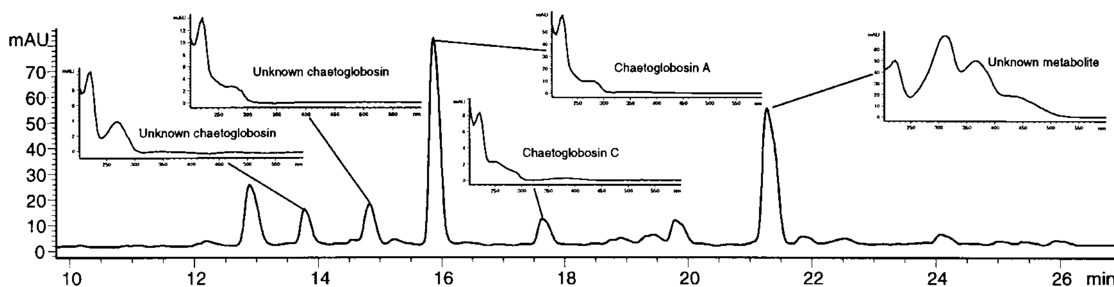
Naturally infested materials: As seen in Table 2, the cytochalasine mycotoxins chaetoglobosins A and C were detected in the naturally infested samples up to 3 μg/cm^2. Besides these, three other chaetoglobosins and approximately 10 unknown metabolites were also detected. Analyses of two old and heavily black-stained building materials (unpublished results) did not reveal any chaetoglobosins A or C, but several



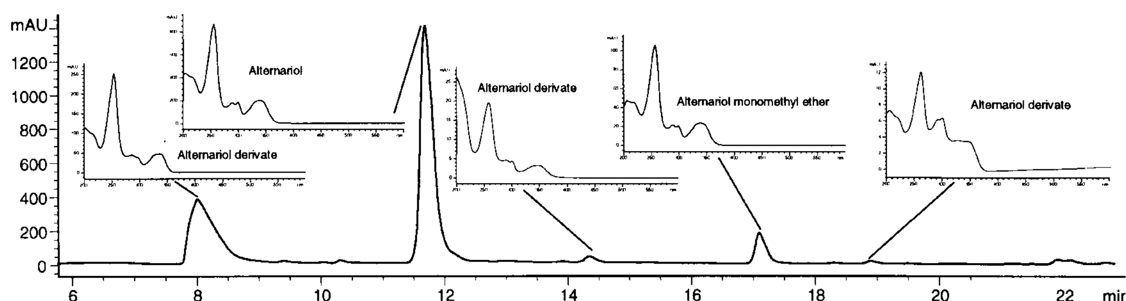
Part of HPLC chromatogram (210 nm) with UV-spectra (200-600 nm) of metabolites inserted, extract of *Penicillium brevicompactum* IBT 13972 on wallpapered chipboard



Part of HPLC chromatogram (210 nm) with UV-spectra (200-600 nm) of metabolites inserted, extract of *Aspergillus niger* IBT 16066 on wallpapered chipboard.



Part of HPLC chromatogram (220 nm) with UV-spectra (200-600 nm) of metabolites inserted, extract of *Chaetomium globosum* IBT 8827 on wallpapered gypsum board.



Part of HPLC chromatogram (256 nm) with UV-spectra (200-600 nm) of metabolites inserted, extract of *Alternaria* IBT 8482 on wallpapered gypsum board.

Figure 1. Selected chromatograms of samples with UV-spectra of major peaks inserted.

other eluted 3–8 minutes before chaetoglobosin A. Generally fewer metabolites were detected in these samples.

Artificially infested materials: The production of secondary metabolites by *Chaetomium globosum* was large. Chaetoglobosin A and C (Figure 1, Table 3) were produced in very similar quantities by all six isolates, 28–50 and 5–7 μg/cm^2 respectively. At least two other compounds with the same chromophores were detected. The unknown metabolite eluting at 21.4 minutes (Figure 1) was detected in all *Chaetomium* extracts except in IBT 7029. Neither chaetocin, sterigmatocystines, or chaetomin were detected in any of the *Chaetomium* extracts.

Paecilomyces

Artificially infested materials: No known mycotoxins were detected from *Pae. variotii*, although the extracts contained several unknown compounds. Extracts of *Pae. lilacinus* did not yield any detectable amount of compounds, in accordance with the small amounts of biomass produced.

Ulocladium and Alternaria

Artificially infested materials: Alternariol and alternariol monomethyl ether and three to five unknown compounds with similar UV-spectra were detected (Figure 1) from all isolates except from the one from the *Alt. infectoria* – group.

No altertoxins or tenuazonic acid were found in any of the extracts, not even in extracts with 1% formic acid in methanol used for extraction.

No mycotoxins or secondary metabolites were detected from *Ulocladium*.

Discussion

It should be noted that the number of true fungal metabolites is uncertain as they are difficult to distinguish from artifacts of the building materials although blank extractions from old and new building materials has been performed.

Also it should be noted that the analytical methods applied does not allow detection of compounds not available as reference standards (Table 1) or proteins and peptides as these are not extracted using the methods described.

Penicillium

The low production of secondary metabolites on building materials compared with the agar media in all the *Penicillium* species is either due to the small amount of biomass present or to the totally different nutrients available [14]. This is in accordance with Larsen and Frisvad [33] who found a weak production of secondary metabolites on wallpaper paste agar compared with a rich medium such as Czapek yeast autolysate agar.

Chrysogine and meleagrin are regarded as non-toxic although it should be noted, that the neurotoxic metabolite, roquefortine C is a precursor for meleagrin.

This raises the question whether the mycotoxins are the pathogenic agents from *Pen. chrysogenum*, as this is the fungus most frequently isolated fungus from infested building materials. Verrucosidin from *Pen. polonicum* is a known mycotoxin, whereas the biological activity of 3-methoxy-viridicatin, mycophenolic acid, verrucofortine, metabolites F and O has not been established [33].

The more pronounced metabolite production by *Pen. brevicompactum* and *Pen. polonicum* compared to *Pen. chrysogenum* is in accordance with experiments on laboratory substrates [10, 33].

Aspergillus

Asp. versicolor is the most abundant species of this genus and the second most frequently isolated fungus from infested building materials, due to its ability to grow on low-nutrient substrates and at low a_w, the high viability of the conidia, and high growth rate on the isolation media [7, 10, 33]. The findings of (Table 2) several natural samples infested with *Asp. versicolor* containing the carcinogenic sterigmatocystins confirm the results of Nielsen et al. [12]. It is interesting that the non-sporulating mycelium contains most sterigmatocystin as the conidia are expected to be the source of exposure.

The isolation frequency of *Asp. niger* is probably over-represented in many reports due to it's ability to overgrow other fungi on the isolation substrates [10, 33, 34], and the compounds detected have not been considered very toxic. But actually few isolates of *Asp. niger* are capable of producing ochratoxin A (Pitt, 1998). Since *Pen. verrucosum* or *Asp. ochraceus* have not been isolated from infested building materials [10, 33], *Asp. niger* would be the probable source, if this toxin is found on building materials. None of the

isolates used in these experiments produced ochratoxins, but a strain that could produce ochratoxin A has been isolated from a Canadian building (M. Gareis, personal communication).

The fact that *Asp. ustus* produced kotanins instead of austamide, austdiol, and austocystins suggests that the *Asp. ustus*-group isolates might be different from the isolates found on cereals.

Chaetomium

The consistent production of the chaetoglobosins and lack of sterigmatocystins in the *Chaetomium* isolates on both naturally and artificially infested materials indicates that it is *C. globosum* [44, 45]. A more thorough identification of the indoor isolates is needed for this genus.

Ulocladium and Alternaria

The production of alternariol from the *Alternaria* isolates are in accordance with Ren et al. [39], who detected only these two toxins on artificially infested cellulosic ceiling tiles. Ren et al. [39] found that their isolates also produced altertoxin I and alternuene on rice. These metabolites were also detected when the isolates used in this study were grown on DRYES (dichloran rose bengal yeast extract sucrose agar) and other laboratory media (results not shown).

Although *Ulocladium* and *Alternaria* have a morphologically similar appearance and share the same major-allergen, no mycotoxins have been detected from *Ulocladium*. A thorough characterisation of the indoor air isolates of this genus is also needed.

Paecilomyces

Only a few isolates of this genus from infested building materials have been preserved at the IBT culture collection. This is insufficient for any conclusions to be drawn concerning mycotoxin production by the two fungal species.

Mycotoxins in buildings

This study, and several others [6, 12, 23, 37, 39, 46], show that many of the fungal contaminants encountered in water-damaged buildings actually produce mycotoxins during growth on building materials. Evidence of inhalative mycotoxicosis due to fungal growth in buildings only exist for *Stachybotrys* [23, 27, 47].

Another paper [48] reports that the dust from a ventilation system contained the trichothecene mycotoxins: roridin A, T-2 tetraol, T-2 toxin, and diacetoxyscripenol. Moreover this paper also reports trichothecenes detected from non-trichothecene producing genera as *Penicillium*, *Aspergillus*, *Alternaria*, and *Ulocladium*. The combination of the reported toxins shows that the findings are not a result of incorrect identification of the fungal cultures, but are due to false positive results using only TLC and HPLC with single-UV wave length detection, which are insufficient specific methods for the detection of trichothecenes [49]. This clearly demonstrates why controversial findings should always be confirmed using a chromatographic method combined with mass spectrometric detection or for compounds having very "specific" UV/VIS spectra a chromatographic method combined with diode array detection.

Data concerning the inhalative toxicity of sterigmatocystin are almost non-existent, although this toxin is a precursor for the aflatoxins and is shown to induce cancer in Danish harbour workers after exposure to dust from peanuts infested with *Asp. flavus* [50]. Pestka and Bondy [51] showed that sterigmatocystin was a stronger inhibitor of cilia from the respiratory tract *in vitro* in one-day-old chickens than aflatoxin B_1.

For the chaetoglobosins no inhalative data have been published. They have low or no ingestive toxicity, but a high toxicity using intravenous injection in animal experiments [44, 45]. The *Chaetomium* ascospores are large (10 μm) and as the deposition of particles in the lungs and upper airways is highest for particles in the range 1–5 μm [4, 52, 53], the *Chaetomium* spores are not deposited in the airways as easily as the *Penicillium* and *Aspergillus* spores (2–5 μm). The *Chaetomium* spores are furthermore produced in asci which again are encapsulated in a perithecium. For this reasons it is doubtful whether the mycotoxins or the volatile compounds contribute to the health problems observed in *Chaetomium* infested buildings.

Data concerning the toxicity of the *Alternaria* mycotoxins are extremely limited, but the quite large spores approximately 10×30 μm, are known to cause Type I allergy, meaning that they are deposited and extracted on the mucosa of the upper airways or in the lung tissue after inhalation [2].

The most frequently encountered species in this study, *Pen. chrysogenum*, did not produce any of the mycotoxins earlier reported. It is suggested that meleagrin, volatiles and the allergenic components of the spores might contribute to an explanation of the ex-

perienced health effects in houses infested with this fungus. Many known and unknown secondary metabolites were detected from the other *Penicillium* and *Aspergillus* species, but as for the rest of the genera studied here, no data on the effects of inhalation of the mycotoxins have so far been published.

It has been shown that crude extracts of *Stachybotrys chartarum*, *Aspergillus versicolor*, *Penicillium chrysogenum*, and *Trichoderma viride* can induce a non IgE mediated histamine release in human mucosal mast cells from the lungs *in vitro* [54].

In cases where the fungal biomass is hidden behind walls or wallpaper, and no obvious route of exposure to the spores exists, MVOCs could be a plausible explanation of reported symptoms, although the observed symptoms cannot be explained by the measured MVOC concentrations, as these are usually 100 to 1000 times below the threshold concentration for irritation [18, 55, 56]. It has been shown that MVOCs from *Trichoderma viride* can induce a non IgE mediated histamine release in human mucosal mast cells from the airways *in vitro* [19].

Conclusion

It should be emphasized that the mycotoxins detected in this study have been obtained on a limited number of materials. As temperature, a_w and material composition vary greatly in buildings, the mycotoxin production might be different when using other materials and extrinsic factors [14].

The presence of the mycotoxins detected in this study, especially the large quantities of chaetoglobosins and sterigmatocystins are of concern as they exhibit a potential risk for people living or working in water-damaged buildings, but risk cannot be assessed until the following data become available:

- The volatility of trichothecenes, sterigmatocystins and other mycotoxins found in water damaged buildings.
- How large are the quantities of spores, hyphal fragments, and mycotoxins that people are inhaling.
- Dose/response relationships and toxic health effects of the mycotoxins by exposure through inhalation.

Additional components such as endotoxins, bacteria, β(1,3)-D-glucanes or allergens could also be causal agents [19, 54, 57, 58].

We recommend that great precautions should be taken when working with microfungi. At the IBT all handling of cultures is completed in laminar flow benches, and cultures waiting for inspection are kept in ventilated benches to protect people from exposure to fungal volatiles as well as particulate matter.

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