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Revealing *Stachybotrys*-like fungal growth in buildings - Possible exposure highlighted through three case studies

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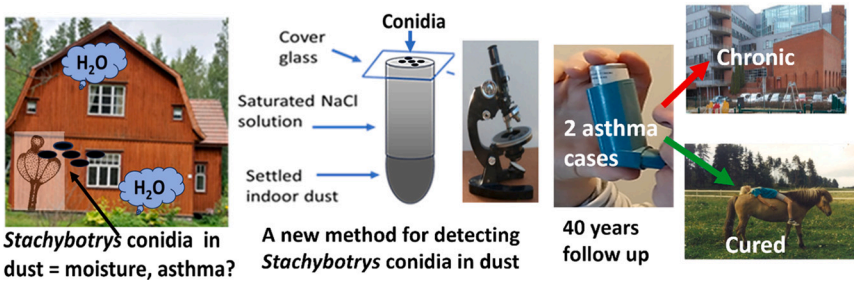
GRAPHICAL ABSTRACT



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ABSTRACT

Genus *Stachybotrys* (*Stachybotryaceae, Hypocreales*) requires high humidity to grow and represents one of the most notorious fungi associated with suspected illness in moist buildings. If *Stachybotrys* conidia are found in settled indoor dusts, their presence may indicate water intrusion and mold infestation revealed after dismantling the building structures. This study describes detection of *Stachybotrys* growth hidden inside the structures of three buildings in Finland. First, a novel microscopic screening method concentrating *Stachybotrys* conidia from settled dust was developed. The method is based on enrichment of conidia floating in the solution of saturated NaCl, separating them from sinking dust particles. Captured conidia were identified based on morphology and cultivated isolates were identified to species or genus level. The second part of the study describes the records of two persons sickened with asthma after exposure to long lasting growth of *Stachybotrys* in two of the buildings. After 38 years of the diagnosis the one person's asthma was declared cured in a medical report. The asthma of the other person developed into chronic illness, diagnosed by The Insurance Court as occupational asthma caused by a moisture-damaged workplace. Diversity and the metabolic activity of the microbes exposing the two persons in

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rural versus urban environments after their asthma diagnosis is offered as a preliminary and hypothetical explanation of the different outcome of the illnesses.

1. Introduction

A narrow range of fungal species grows in damp building material (Loukou et al., 2024). Genus *Stachybotrys (Stachybotryaceae, Hypocreales*) (Wang et al., 2015) includes the most notorious indoor fungi, the *Stachybotrys chartarum* species complex (Andersen et al., 2022; Pestka et al., 2008). The term 'species complex' refers to a group of species morphologically and phenotypically indistinguishable from each other (Binti Mohamad, 2018). *Stachybotrys chartarum* complex is characterized macroscopically by dark growth in cellulose-containing material and microscopically by round conidiophores surrounded by a mucus capsule, large dark spores, 6–9 μm × 4–5 μm × 1–3 μm, which adhere to surfaces and conidiophores consisting of terminal phialides. Phialides, the conidia-forming cells, produce smooth or variously decorated conidia. These features make the genus *Stachybotrys* including the *Stachybotrys chartarum* species complex possible to recognize based on morphology visible by different microscopic techniques (Binti Mohamad, 2018; Lombard et al., 2016; Samson et al., 2004; Wang et al., 2015).

Xerophilic molds such as *Aspergillus* displace *Stachybotrys* growth if the water activity drops below 97 % at 25 °C. Instead, *Stachybotrys* is very competitive in cellulose-containing material with low nitrogen content, that has been moist for a long time ($a_w > 95$ %) (Miller et al., 2003; Mussalo-Rauhamaa et al., 2010). Nitrogen-fixing bacteria in a *Stachybotrys*-infested liner possibly enabled the long lasting growth of *Stachybotrys* (Andersson et al., 1997). With its antagonistic metabolites, *Stachybotrys* can inactivate competing fungal species in the building material and grow as the most dominant species. *Stachybotrys* spores are large, surrounded by a slime capsule and stick to surfaces thanks to their protrusions. Therefore, the spores spread poorly in the air. If *Stachybotrys* conidia are found in dust settled on the upper surfaces of the interior, it may indicate growth in structures and longer-term water damage (Miller et al., 2003; Lombard et al., 2016). *Stachybotrys* spp. grow slower on agar plates than *Aspergillus* and *Penicillium* species. In culture dishes, the antagonism of *Stachybotrys* is depressed and it may be covered by fast-growing molds. *Stachybotrys* growth is easy to detect and identify by direct microscopy, but difficult to find by cultivation, especially in air or dust samples (Miller et al., 2003; Lombard et al., 2016).

The genus *Stachybotrys* includes many species, but from indoor isolates, mostly *Stachybotrys* spp. included in the *S. chartarum* species complex were identified (Cruse et al., 2002; Wang et al., 2015; Lombard et al., 2016; Mussalo-Rauhamaa et al., 2010). Closely related and synonymous species are often combined with it. According to a Finnish study, the prevalence of strains identified as *Stachybotrys chartarum* in Finnish buildings was low, 2,4 % of 1880 samples from water-damaged buildings screened positive by microscopic and cultivation methods (Mussalo-Rauhamaa et al., 2010).

Stachybotrys strains isolated from indoors can be roughly divided into trichothecene and atranone producers (Wang et al., 2015; Lombard et al., 2016). Trichothecenes are the strongest known mycotoxins, and the indoor air problems and health hazards associated with *Stachybotrys* growths have been linked to the production of trichothecenes (Miller et al., 2003; Wang et al., 2015; Piontek and Łuszczyńska, 2021). However, this connection is controversial because the measured amounts of particles and spores containing mycotoxins in the indoor air are estimated to be too small to cause health harm (Miller et al., 2003). Lately, aerosolized bioactive metabolites secreted in guttation droplets by fungi growing indoors have gained interest (Salo, 2022). Atranones are not cytotoxic, so all *Stachybotrys* isolates from the building do not necessarily indicate toxin or trichothecene exposure, but, do clearly indicate a long-term water damage (Miller et al., 2003; Lombard et al., 2016; Piontek and Łuszczyńska, 2021). Strains that produce trichothecenes or atranones may also produce bioreactive and immunotoxic spirocyclic drimans, which are known to cause disorders of the complement system, prevent the release of TNF-α cytokine, are neurotoxic and cytotoxic and affect the activity of various enzymes (Miller et al., 2003; Piontek and Łuszczyńska, 2021). Active *Stachybotrys* growth was shown to secrete bioreactive substances in liquid droplets (Salo, 2022).

Respiratory illness is associated with exposure to dampness and visible mold growth (Caillaud et al., 2018; Hurraß et al., 2024a; Lee et al., 2024; Pestka et al., 2008). The term "visible mold growth" also includes mold infestation hidden in building structure and revealed after dismantling the structures during renovation (Hurraß et al., 2024a; Lee et al., 2024). Mold odor has been connected to unhealthy indoor air and is an indicator of excess and problem-causing moisture, which enables the active fungal or microbial growth (Miller et al., 2003; Mendell and Kumagai, 2017). Actively growing fungal hyphae containing conidiophores, vesicles and vacuoles may indicate metabolic activity, whereas, desiccated conidia and dry hyphal fragments may be less relevant indicators for active fungal growth (Caillaud et al., 2018; Mendell and Kumagai, 2017; Kenne et al., 2014; Kistler and Broz, 2015). 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 (Bennet and Inamdar, 2015; Hurraß et al. 2016, 2024). The metabolic state of the microbes observed in buildings has gained little attention.

This article consists of two separate sub-studies. In the first part of the study, three cases of long-term water damage and *Stachybotrys* growth in building structures is described. A novel, flotation-based screening method for *Stachybotrys* conidia in settled dust was applied for location of the hidden *Stachybotrys* growth based on observation of the conidia in settled dusts. In the second part of the study, the exposure history of two persons diagnosed with asthma after exposures in two of the moist buildings colonized with *Stachybotrys* is compared. The difference in the outcomes of the illness is suggested to be related to qualitative and quantitative differences in airborne microbial exposures in urban and rural indoor environments.

2. Materials and methods

2.1. Three buildings investigated for water damage and microbial growth in building structures

2.1.1. An office in a public urban building in southern Finland

The building was a block house built in the end of 19th century. The intermediate floors of the four-story building were made of wood and insulated with sawdust. The wooden floor planks were covered with an insulation textile, which was covered with a plastic mat. The water damage consisted mainly of leakage of water-filled thermal radiators and water pipes. In the worst affected office, the leaked water penetrated the cracked plastic mat covering the floor. Under the plastic mat, a textile serving as an insulation material was visibly wet and moldy, as well as the wooden floor planks. The water leakage had continued for years unnoticed, and the area of visible water-damaged floor planks and insulation materials included 1–2 m^2.

In 1993, *Stachybotrys* conidia were detected in settled indoor dust. In 1994, the building constructions were opened and *Stachybotrys*-like growth was detected on the floor planks and the insulation material (Fig. 1A, B, a, b). The building underwent an unsuccessful renovation including hypochlorite disinfection, installation of mechanical ventilation and opening of the building structures. After installation of the mechanical ventilation, the situation worsened, 50 % of the personnel

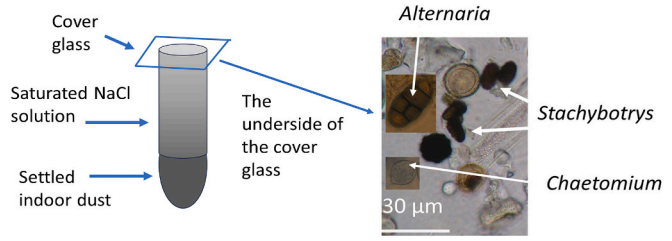

Fig. 1. Experimental design of the floating technique. Settled dust, e.g. 120 mg of dust was mixed with 12 ml of saturated aqueous NaCl solution. A test tube, e.g. 10 ml, was filled with the mixture so that a slightly convex surface was formed, stood for 30–60 min, and a cover glass was placed on top of the surface for 10–20 min. The coverslip was transferred to a glass slide and examined under a phase contrast microscope (Nikon Eclipse E600, Nikon Corporation, Tokyo Japan, 400× magnification). The mold spores enriched on the underside surface of the coverslip were identified to the genus level.

consisting of 35 persons reported workplace-related symptoms. One person was diagnosed with severe asthma. The Insurance Court ruled in 2020 that her asthma, diagnosed in 1994, was an occupational illness caused by exposure in the water-damaged office. From 1994 to 1996 the house was renovated again. Water-damaged material was replaced and the whole personnel was removed to another building, whereafter, reportedly, most of the symptoms of the personnel disappeared except for the chronic asthma of the one person. A complete health follow-up, however, has not been made, so there may be more symptoms than is known at present.

2.1.2. An apartment in an old timber house in southern Finland

An empire-style manor main building from the beginning of the 19th century has been modernized with water pipes and water closets several times since 1950. Paper boards used as insulation materials in ground floor ceilings had moistened due to repeated water leakage from the bathroom in the upper floor (Fig. 1 Panels B, C, b, c). The building had gravity ventilation. Several water leakages were noticed during 1983–1987. The house was inhabited by a family with three children living in the upper floor, suffering from repeated and chronic ear infections and sinusitis. The mother was diagnosed with asthma in 1986. *Stachybotrys*-like conidia were detected in settled dusts and the building was renovated from 1996 to 1998 and several pieces of visible water-damaged paperboards covered with *Stachybotrys*-like growth (1 m^2 × 2.5 m^2) were removed. The family moved to another apartment and the symptoms of chronic infections of the children and the mother disappeared. The asthma of the mother persisted till the end of 1990's, after which it improved and was declared cured in 2020 (in a medical report). A case study of presenting development and improvement of the asthma is presented in a medical journal (Andersson et al., 2018).

2.1.3. Day care center in Helsinki

The day care center was flat roofed, built in 1974 and classified as a moisture problem house by the health authorities. The large areas of visibly moldy building materials and the collected water-damaged building materials are shown in Fig. 1D, E, d, e and the results of microbiological and toxicological analyses were published by Andersson et al. (1997).

2.1.4. Reference buildings

Two nonproblematic urban dwellings and two barns connected to animal sheds in Southern Finland were used as reference buildings. These buildings were not connected to any health complains.

2.2. Collected samples – inspection of settled dusts and building materials

Settled dust samples were swept from surface 1.5–2 m above floor level into sterile Petri dishes. From the three problematic target buildings, dust samples of 200–500 mg were collected. From the four reference buildings, two urban dwellings and two hay barns, collected samples consisted of 200–300 mg dust and 2000–3000 mg dust, respectively. The dusts were inspected by microscopy for presence of conidia of filamentous fungi, focusing on *Stachybotrys*-like conidia. When *Stachybotrys*-like conidia were detected in dust, the emission source was searched for. Samples of visible water-damaged building materials were inspected by microscopy. The morphological life-cycle was photographed in bright field microscope (Olympus model BH2, 1000×, Tokyo, Japan). Dimensions of the conidia and conidiophores were determined in bright field microscope (400× magnification; Olympus CKX41, Tokyo, Japan) and image recording software (cellSens® standard v. 11.0.06, 2012, Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Characterization of *Stachybotrys*-like fungal growth and isolated strains were performed with different microscopic techniques: Morphology of isolated colonies were presented on malt extract agar (15 g malt extract from Sharlab, Spain, and 12 g of agar from Amresco, Solon, OH, USA, in 500 ml of H_2O). Cultivated strains were characterized with a) a fluorescence microscope (Nikon Eclipse E600; Nikon corporation, Tokyo, Japan) at 400× magnification, with filters of BP 330 nm to 380 nm (excitation) and LP 480 nm (emission), b) inspected with bright field and phase contrast optics with 400× magnification (Olympus CKX41, Tokyo, Japan) and image recording software (cellSens® standard v. 11.0.06, 2012, Olympus Soft Imaging Solutions GmbH, Münster, Germany) and c) stereo microscope, Dino-lite portable microscope with Dinocapture imaging software (Dino-light Europe/IDCP B.V. Almere, The Netherlands). Scanning electron microscopy from the gypsum liner from the day care is described in Andersson et al. (1997, 1999).

2.3. Cultivation and identification of fungi

Characterization of the indoor fungal growth on building materials and plate-grown biomass inspected by microscopy allowed identification down to the *Stachybotrys* genus level, to *S. chartarum* species complex level and to *S. chartarum* species level of the strain DSM 12880 isolated from the day care center (Andersson et al., 1997), according to the following criteria: morphology of conidiophores in scanning, electron and light fluorescence microscope, conidiophore morphology and the size of the single-celled conidia. Genus *Stachybotrys* currently includes species representing characteristic morphologies (Samson et al., 2004; Lombard et al., 2016).

From the paper board sampled from the apartment in southern Finland, mold colonies were cultivated on malt extract agar (15 g malt extract from Sharlab, Spain, and 12 g of agar from Amresco, Solon, OH, USA, in 500 ml of H_2O). The plates sealed with gas permeable tape were incubated at 22 °C - 24 °C for 1–3 weeks as described (Andersson et al., 2020). One isolated strain, HJ5, was identified to genus level according to morphology and microscopic inspection.

2.4. Enrichment of Stachybotrys-like conidia from indoor dust using the flotation technique

Since *Stachybotrys* strains hardly grow on standard media used for indoor fungi, a microscopic method for detection of *Stachybotrys* conidia in settled indoor dust was developed. *Stachybotrys* conidia were screened from settled indoor dust by two microscopic techniques: a) from a tape sample taken from the upper surfaces and b) from the settled indoor dust conidia were enriched using the flotation technique. The sensitivities of the tape sampling technique and the flotation technique in the microscopic detection of spores were compared. The flotation technique is based on the differences in the specific gravities (SG) of particles suspended in a solution. Particles with a lower specific gravity than that of the solution are observed floating on the fluid surface, heavier particles sink to the bottom (Pouillevet et al., 2017). A saturated NaCl solution

(SG = 1.2 g ml^{-1}) was used to enrich the *Stachybotrys* conidia (SG = 1 g ml^{-1}) on the underside surface of the cover glass (Pouillevet et al., 2017; China et al., 2018). Experimental procedure of the floating technique is shown in Fig. 1. Dusts investigated with the flotation technique, dust from an urban dwelling and from an animal shed are documented in Alenius et al. (2009) and Andersson et al. (1999).

2.5. Scanning electron microscopy

Procedure for scanning electron microscopy of settled dusts from animal sheds, about 100 mg of dust, was performed as described in detail by Andersson et al. (1999).

2.6. Light microscopy

Actively growing aerial fungal hyphae were stained with the viability stain Hoechst 33342 + propidium iodide (PI). Hoechst 33342 crosses intact and damaged plasma membranes, staining the DNA in nuclei and mitochondria blue in live and dead cells. PI crosses only plasma membranes with disturbed or exceptional permeability, staining DNA and RNA in affected cells red. The staining procedure and inspection of the samples in fluorescence microscope (Nikon Eclipse E600; Nikon Corporation, Tokyo, Japan) at 400× magnification, with filters of BP 330 nm to 380 nm (excitation) and LP 480 nm emission is described by Andersson et al. (2020). An Olympus CKX41 (Tokyo, Japan); magnification 400×, image recording software Cellsense® standard version 11.0.06. was used for bright field and phase contrast microscopy. Stereomicroscopy was performed with Dinocapture imaging software (Dino-Lite Europe/IDCP B.V. Almere, The Netherlands).

2.7. Toxicity analysis

The toxicity tests measuring toxins affecting the cellular energy metabolism, the mitochondria and ion homeostasis were performed as inhibition of the motility of boar spermatozoa (the BSMI assay). The toxins affecting macromolecular synthesis and cytostatic activity based on inhibition of cell proliferation were measured with the somatic cell lines PK-15 and FFL (the ICP assay). The procedure of these tests was previously described in details (Andersson et al., 1997, 2020). Methanol extraction procedure for dusts and building materials were described by Andersson et al. (1997), while ethanol extraction of microbial plate grown biomass and its toxicity testing with the ICP and BSMI assays is described by Andersson et al. (2020) and Castagnoli et al. (2018).

3. Results

3.1. Comparison of the flotation technique and the tape sampling technique

Urban dust, hay barn dust and barn dust mixed with 2 % and 0.05 % (*w*/w) dry *Stachybotrys* spores were studied using the flotation technique. Floating particles in the NaCl solution, enriched on the lower surface of the coverslip, can be seen in Figs. 2 and 3. To compare the flotation technique with the tape sample method, barn dust spread on the bottom of a Petri dish, with *Stachybotrys* spores added at 2 % and 0.05 %, was collected in the tape sample.

In urban dusts, the dominant recognized floating particles were microplastics and pollen (both recognized in 30 % of the inspected microscopic fields). Only 0.15 % of the inspected microscopic fields contained textile fibers (Fig. 2). In barn dusts, the dominant recognized floating particles were fungal spores, recognized in 90 % of the inspected 20 microscopic fields (Fig. 3, Panels A–C). The composition of particles in urban indoor dust and dusts from hay barn differed in particle composition. No microplastics were observed in barn dust.

In barn dusts to which *Stachybotrys* spores had been added, 1–5 of them were visible per field of view, along with other mold spores (Fig. 3, Panels D–F). With the flotation technique it was possible to enrich certain particles under the cover glass, reflecting differences in the particle composition of different dusts. *Stachybotrys* conidia added to the barn dusts apparently also enriched under the surface of the cover glass. The experiment was repeated 3 times, including calculation of *Stachybotrys* conidia from 20 microscopic fields. The average number of detected conidia was 57 (SD ±19) conidia per 20 fields. With the tape-sampling technique, no *Stachybotrys* conidia were detected in a 20 field of view sieve from barn dust to which *Stachybotrys* conidia had been added (data not shown). Based on these preliminary experiments, the flotation technique concentrated the *Stachybotrys* conidia under the surface of the cover slip. The flotation technique seemed to be a more sensitive screen for finding *Stachybotrys* conidia than the tape sample.

3.2. Three cases where the discovery of Stachybotrys conidia led to the discovery of mold growth in structures: an office, a dwelling, and a day care center

Table 1 presents three cases where the finding of *Stachybotrys* spores in settled dust led to the discovery of moisture damage and mold growth in the structures. Three indoor spaces were investigated based on health complaints, Building 1, Mrs. A's study, Building 2, Mrs. B's apartment and Building 3, a day care center. *Stachybotrys* conidia were found in

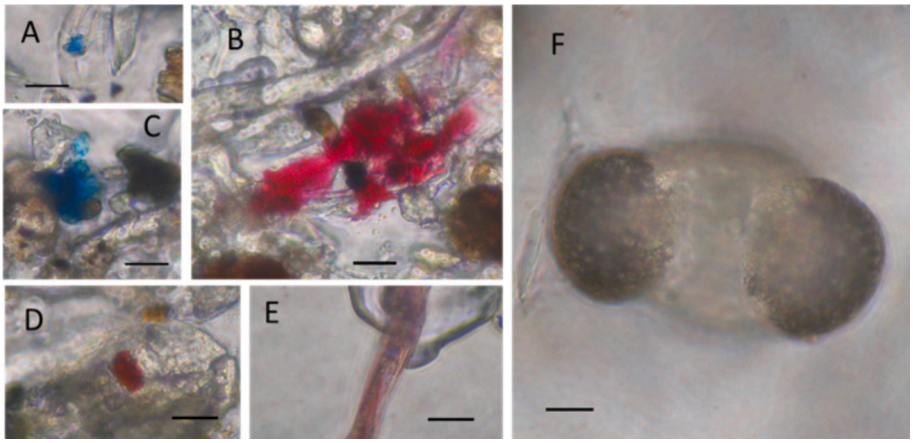

Fig. 2. Light microscope images of dust particles found in dusts, from a nonproblematic urban dwelling, with the flotation technique. Panels A to D show microplastics varying in sizes of 3 μm to >50 μm. Panels E and F show textile fibers and pollen. Out of the 20 inspected microscopic fields, 6 fields contained microplastic particles, 1 field contained textile fibers and 6 fields contained pollen. The bars represent 10 μm.

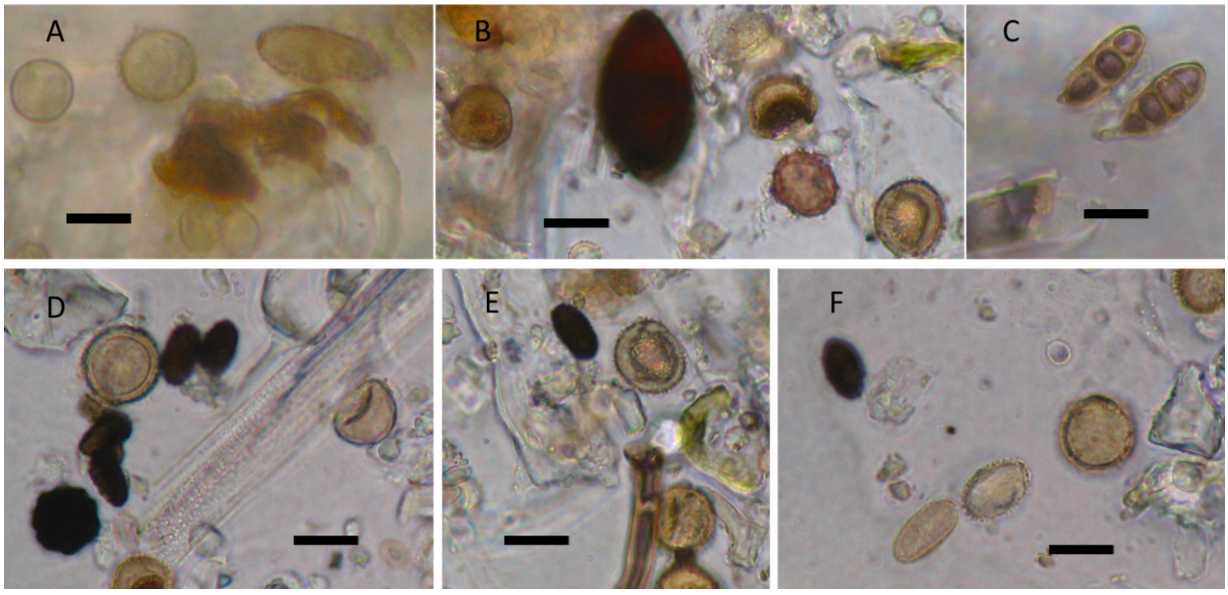

Fig. 3. Light microscope images of dust particles found in barn dusts with the flotation technique. Panels A to C show barn dust containing diverse composition of fungal conidia varying in size and color. Panels D and E show barn dusts with 2 % (w/w), while Panel F with 0.05 % (w/w) *Stachybotrys* spores added, 1–5 dark *Stachybotrys* conidia were visible per field of view. The images represent the average view of 20 microscopic fields of view. The bars represent 10 μm.

Table 1
Comparison of detection methods in the search for *Stachybotrys* growths from settled dust and structures. T = tape sample, F = flotation technique, MEA = malt extract agar[3].

	Conidia in dust		Building material			
	T	F	Cultivation	Toxicity[a]	Identified toxin	T
			MEA	EC_{50} μg ml^{-1}		
Target buildings						
1. Office of Mrs. A	+	+	−	NI (7)		+
2. Dwelling of Mrs. B[1]	−	+	+	>1000 (>100)[1]		+
2. Dwelling of Mrs. B[2]	−	+	−	NI (25)[2]		+
3. Day care center	−	+	−	2 (0.3)[3]	Satratoxin[3]	+
Reference buildings						
1. Urban dwelling	−	−	−			
2. Urban dwelling	−	−	−			
3. Hay barn	−	−	−			
4. Hay barn	−	−	−			

NI=Not investigated. [1] and [2] represent parallel samples from different parts of the building material (Andersson et al., 1997).
[a] Toxicity against somatic cell lines (in parentheses boar spermatozoa).

indoor dust in all sites by microscopy, not by culture on malt extract agar. From dust samples collected from the 4 reference buildings no *Stachybotrys*-like conidia were observed.

When the structures were opened, *Stachybotrys* growths were found in the tape samples of the structures of all sites (Table 1). The collected building materials covered by dark fungal growth are presented in Fig. 4. Finding hidden growth inside building structure is illustrated in Fig. 5.

Stachybotrys colonies were found by cultivation on malt extract agar of building materials in Building 2 (Mrs. B's dwelling). The toxicity of building materials varied between EC_{50} values <1 μg ml^{-1} to >1000 μg ml^{-1}. The most toxic material, the gypsum liner from Building 3, the day care center, contained satratoxin at 17 μg g^{-1} (Andersson et al., 1997). From this liner, a strain DSM 12880, identified as *Stachybotrys chartarum* was isolated on corn meal agar (Andersson et al. (1997).

The characteristics of *Stachybotrys* growth are described in Fig. 6. The figure shows morphology of conidiophores and conidia under light microscope. The dark fungal biomass covering building materials from the three buildings produced dark, oval, usually ornamented single-celled phialoconidia in the size of 2–3 μm × 4–5 μm × 8–10 μm. The conidia and conidiophores of fungal hyphae from the three buildings represented conidial and conidiophore morphology characteristic for fungi belonging to the *Stachybotrys chartarum* species complex (Andersen et al., 2022; Binti Mohamad, 2018; Samson et al., 2004; Lombard et al., 2016), and impossible to distinguish from the strain DSM 12880 identified as *Stachybotrys chartarum* (Andersson et al., 1997). From the samples from the office in southern Finland, no *Stachybotrys*-like colonies grew on malt extract agar. The identification of these strains relayed on the morphology presented in Figs. 4, 5 and 6. Microscopic inspection of the *Stachybotrys*-like growth on building materials of the office revealed structures of hyphae, conidiophores, phialides and conidia similar to those exhibited by the strains DSM 12880 and HJ5.

3.3. The personal history of two persons diagnosed with asthma after exposure in the two water-damaged buildings

The second part of this study describes the personal history of two persons sickened with asthma after exposures in the two water-damaged buildings (Table 1). Fig. 7 shows the personal history of Mrs. A and Mrs. B exposed in Buildings 1 and 2 (Table 1) during a period of 58 years. The figure also shows the progression of the asthma of the two persons 38 years after diagnosis.

Mrs. A and Mrs. B were both exposed in severely moisture-damaged closed spaces, Mrs. A in her office, Mrs. B in her apartment around the clock (she was at home with young children) (Table 1). Fig. 7 shows that both Mrs. A and Mrs. B were exposed to moldy hay in their youth, lived in “hygienic low biodiversity” urban environments as adults, were exposed in damp buildings, and developed asthma. Mrs. A's asthma worsened and after 25 years of chronic illness she had a court ruling by The Insurance Court that her asthma was an occupational illness caused by mold exposure at her workplace. Three years after this, Mrs. A was

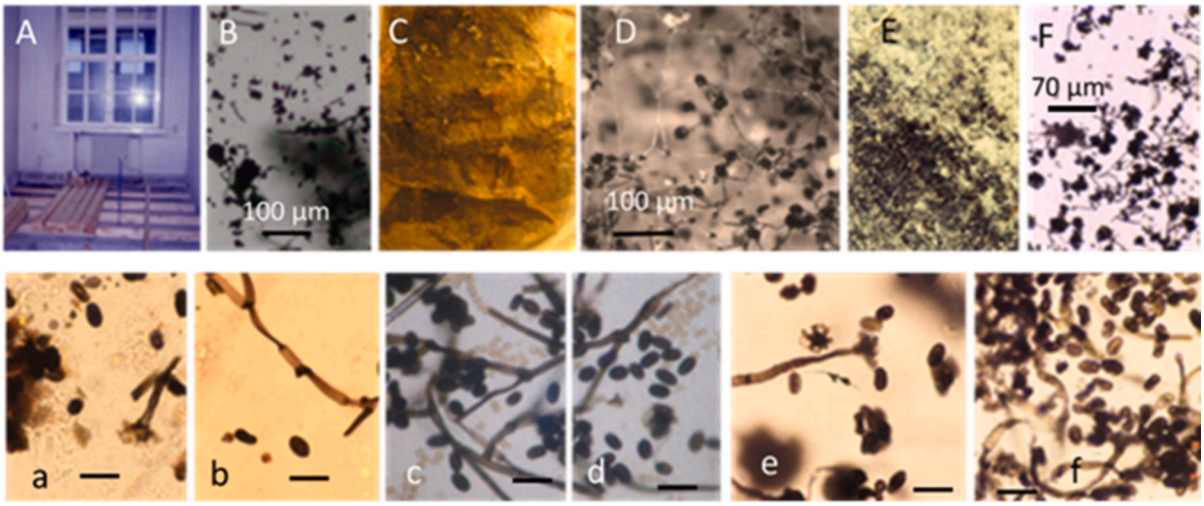

Fig. 4. Building materials from the three water-damaged buildings infested with filamentous fungi. Floor planks and insulation material from an office (A, B, a, b), paper board from an apartment (C, D, c, d) and gypsum liner from a day care center (E, F, e, f). Panels B, D and F and the lower row show bright field micrographs of tape samples from the building materials. The figure shows morphology of conidiophores and conidia under light microscope. The dark fungal biomass covering building materials from the three buildings produced dark, oval, usually ornamented single-celled phialoconidia in the size of 2–3 µm × 4–5 µm × 8–10 µm.

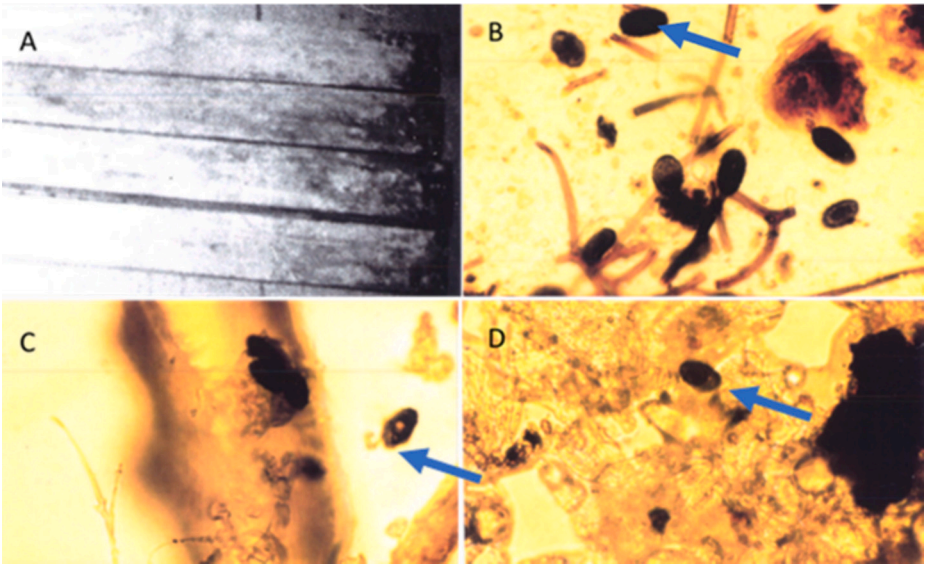

Fig. 5. Finding hidden *Stachybotrys* growth inside the building structures of Building 2. Panel A reveals dark wet wooden floor planks hidden under a covering plastic mat. Panels B and C show bright field micrographs of tape samples from the floor planks and from insulation material covering the planks. Panel C shows a micrograph of a tape sample of dust settled on a surface 1.5 m above floor level (Building 1 in Table 1). The dark spores are 5 µm × 8 µm - 9 µm (arrows).

diagnosed with non-specific interstitial pneumonia (NSIP) according to a medical report. Mrs. B's asthma had been asymptomatic from 2005 and was found to improve and declared cured in a 2020 medical report, even though, Mrs. B had experienced abundant and continuous microbial exposure at work and in animal sheds (Fig. 7).

Results in Table 2 show that the *Stachybotrys*-infested building material exposing Mrs. A was more toxic than that from Mrs. B's dwelling. The other documented differences in life history between Mrs. A and Mrs. B were differences in microbial exposure, blood group and medication. Mrs. A was not exposed to the diverse microbiota in animal sheds, and used a lot of corticosteroids and antibiotics compared to Mrs. B. No allergic responses to indoor dusts, pets, horses, fungi, pollen or food items were recognized in either Mrs. A or Mrs. B.

4. Discussion

The major subject of this study was twofold. Firstly, a novel screening method for enrichment of *Stachybotrys* conidia in settled dust enabled detection of *Stachybotrys* growth hidden inside building structures of three water-damaged buildings. Secondly, the differences in the final consequence of two cases of asthma developed in the *Stachybotrys*-infested buildings and their progress during 38 years after diagnosis were compared. The two persons, Mrs. A and Mrs. B differed in microbial exposures and medications after asthma diagnosis.

The first part of the study dealt with detection of hidden *Stachybotrys* growth, and characterization of the *Stachybotrys*-like fungi in three water damaged buildings. There is a consensus that immediate removal of contaminated materials are crucial for stopping fungal spreading, material degradation and minimizing health risks connected to indoor exposures to actively growing molds (Hurraß et al., 2024a, 2024b; Binti

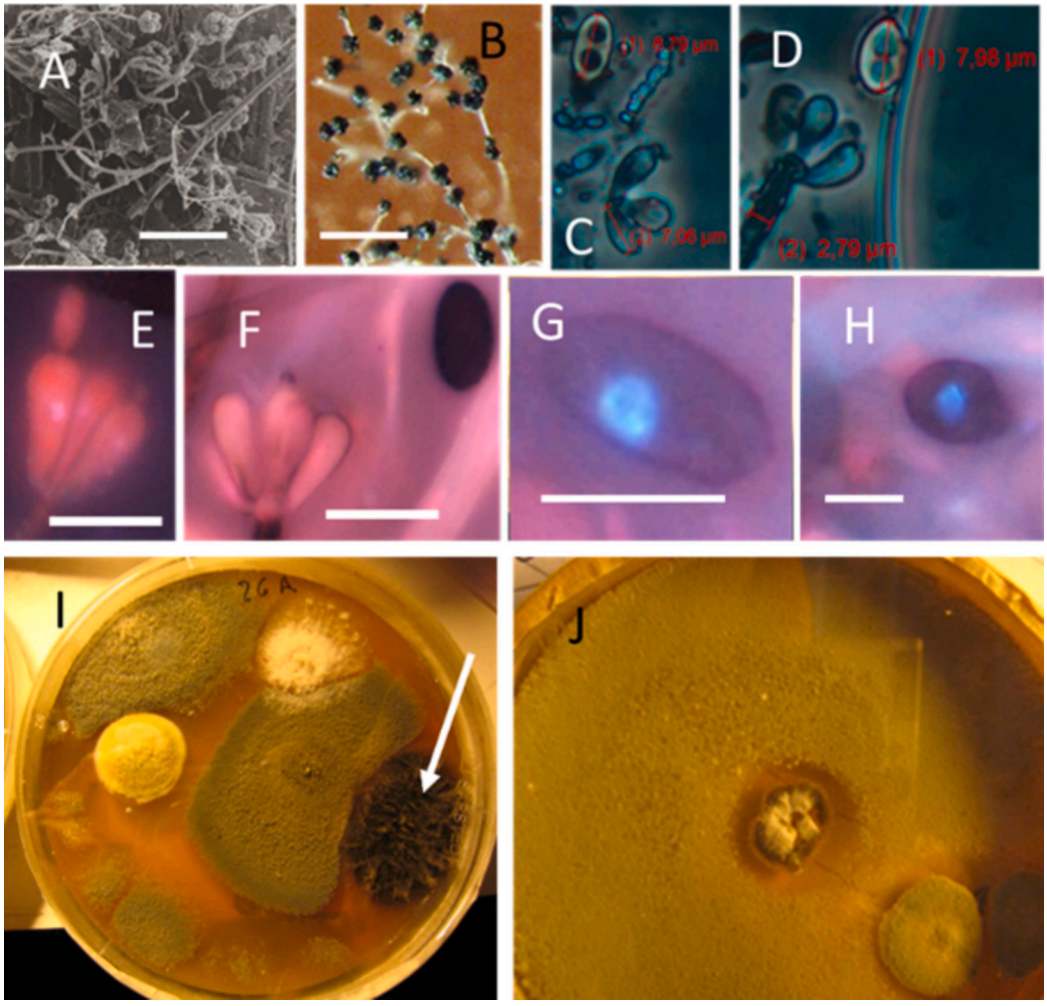

Fig. 6. Morphological criteria for *Stachybotrys*-like growth. Panel A: scanning electron micrograph of the gypsum liner from the day care colonized by *Stachybotrys chartarum* (Andersson et al., 1997). Panel B: stereomicroscopic view of *Stachybotrys* sp. HJ5 isolated from paperboard from the apartment. Panels C and D: phase contrast micrographs of conidiophores, phialides and conidia of strain HJ5. Panels E: and F: fluorescence micrographs of conidiophores of strain HJ5. Panel E: developing conidia on the top of the phialide. Panel F: mature conidia impermeable to the fluorophore. Panels G and H show single-celled phialoconidia with bright fluorescing nuclei. Panels I and J: indoor *Stachybotrys* sp. colonies on malt extract agar. Panel I shows a 3-week-old black *Stachybotrys* colony, while Panel J shows the white-grey *Stachybotrys*-like colony (HJ5) antagonistic to co-growing *Penicillium*.

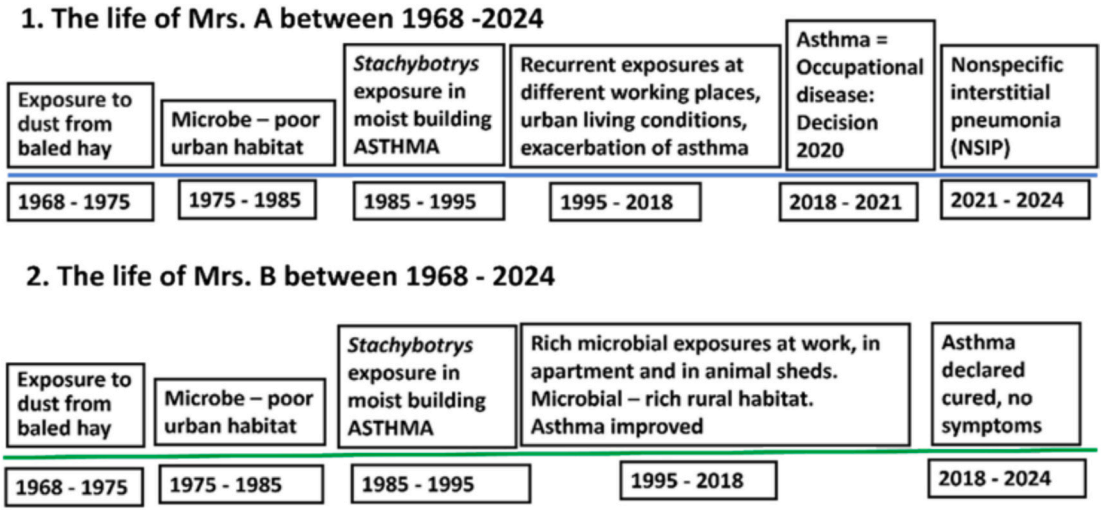

Fig. 7. History of the two exposed persons and progression of their asthma over a period of 58 years. Mrs. A was exposed in Building 1 and Mrs. B was exposed in Building 2 (Table 1).

Table 2
Measured differences between, Mrs. A and Mrs. B., exposed of the indoor environment of closed spaces in water-damaged buildings, and suffering from asthma. The information concerning medications, allergies and blood groups were obtained from medical reports.

	Stachybotrys exposure		Microbial exposure in animal sheds	Medication/year		Other information	
Mrs.	Toxicity of building material[a] EC_{50} = μg ml^{-1}	Time year		Cortisone use[b]	Antibiotic sheds	Allergy	Blood group
A	7[c]	*6*	–	>30	4–5	–	AB
B	25[d], >100[d]	*5*	+[e]	0.5	<1	–	O

[a] Toxicity measured with sperm cells.
[b] Daily use of inhaled corticosteroids per year.
[c] No toxic *Stachybotrys* strains could be isolated.
[d] Toxic *Stachybotrys* strains were isolated, the EC_{50} concentration of the strains was <10 μg ml^{-1}, toxicity was measured both with cell lines and with a sperm test.
[e] Exposre to stable and barn air and dust documented in Andersson et al. (2018) and Alenius et al. (2009).

Mohamad, 2018; Loukou et al., 2024; Zhao et al., 2020). With the novel screening method using the flotation technique, *Stachybotrys* conidia were enriched >100 fold from settled dust (Fig. 3), indicating that the method was more sensitive compared to the tape sampling method. The volume of *Stachybotrys* conidia (9 μm × 5 μm × 3 μm) is 100 $μm^3$–145 $μm^3$ (= 0.1 μl) density of a mold conidia 1 g /cm^3 = 1 g ml^{-1}. One average mold conidium is estimated to weigh 5×10^{-9} g (China et al., 2018) and 0.05 mg conidia were estimated to contain ca. 5×10^4 pieces of conidia. If one conidium was detected in one of the 20 microscopic fields inspected, the detection limit of the method was roughly estimated as ≥100–1000 *Stachybotrys* conidia in 100 mg of settled indoor dust. However, the results represent a pilot study and calibration of the detection limit of the method needs further experiments subjected to statistical analysis.

Drawbacks of the method are also easy to list: The flotation technique needs a lot of settled dust, and conidia of actively growing *Stachybotrys* are slimy and do not easily become airborne (Lombard et al., 2016; Tucker et al., 2007). Also, the method is applicable only for detection of water damages colonized by fungal species with characteristic conidial morphology, unlikely occurrence in outdoor air and high moisture requirements for active growth, features characteristic for the genus *Stachybotrys* (Binti Mohamad, 2018; Dylag et al., 2022). Several indoor species belonging to the genus *Stachybotrys* have been described, but based on microscopic morphology they can be mixed with representants of the related genera *Striatrimotrys, Memnoniella* and *Sirastachys* (Lombard et al., 2016). The number of *Stachybotrys* species present in indoor environments is not clear, *S. chartarum, S. yunnanensis, S. nephrospora, S. microspora, S. elegans,* and *S. chlorohalonata* have been described (Wang et al., 2015).

Another drawback of the flotation method is connected to the sampling of the settled dusts: conidia are not evenly distributed in dusts settled on indoor surfaces. A positive result in the floating test of pooled settled dust certainly indicated *Stachybotrys* growth, whereas negative results may be due to the "hot spots" being overlooked during collection. The floating method described is merely suggested as a low cost method for preliminary screening and as a complement to DNA-, cultivation- and tape sampling-based methods.

In this case report focusing on three heavily contaminated buildings, the test gave positive results in all tested cases, negative results were obtained from the non-problematic reference buildings. Previous studies report that >19 billion spores of *S. chartarum* were formed on an area of 1 m^2 of confluent growth, 0.2 % of these conidia became airborne in 1 h at low-speed airflow producing ca. 40 million potentially allergenic and toxic particles (Lombard et al., 2016; Tucker et al., 2007; Dylag et al., 2022). The airborne toxin load carried by conidial particles was around 4 μg and a person living in a highly contaminated indoor space could accumulate only few nanograms of mycotoxin per day. This level of mycotoxin exposure seems too low for causing the severe symptoms described connected to indoor *Stachybotrys* exposures (Lombard et al., 2016; Tucker et al., 2007; Dylag et al., 2022). Microparticles from toxigenic colonies may boost the concentration of airborne mycotoxins beyond estimates based on airborne conidia as shown earlier (Brasel et al., 2005; Bloom et al., 2007, 2009; Rahman et al., 2024). Also, actively growing *Stachybotrys* may emit bioreactive metabolites in guttation droplets, in addition to those metabolites associated with conidia and small fragments (Salo, 2022; Gareis and Gottschalk, 2014). Bioactive metabolites were detected in vesicles and guttation droplets of indoor isolates of *Penicillium expansum, Trichoderma* strains*, Aspergillus versicolor* and *A. calidoustus* (Salo, 2022; Castagnoli et al., 2018; Mikkola et al., 2023). The presence of *Stachybotrys*-like conidia in settled dusts indicated that the large areas of *Stachybotrys*-contaminated building materials evidently liberated conidia into indoor air. Bioactive substances in guttation droplets secreted by *Stachybotrys* sp. HJ5 were described by Salo et al. (2019). It cannot be excluded that bioactive substances in guttation droplets were secreted from *Stachybotrys*-contaminated building materials into the indoor air of the investigated buildings.

The toxicity profile of the contaminated building materials from the day care (Building 3 in Table 1) and the apartment exhibited greater toxicity in the BSMI assay than in the ICP assay (Table 1). Since the macrocyclic trichothecene mycotoxins produced by *Stachybotrys* were more toxic in the ICP assay than in the BSMI assay, the building materials obviously contained additional bioreactive metabolites possibly produced by *Stachybotrys* or by other microbes or bacteria. Bacteria producing immune-reactive metabolites toxic in cell-based bioassays and antagonistic to fungi were isolated from building materials and characterized. Valinomycin-producing *Streptomyces* strains were found in Building 3, the day care nursery (Table 1) (Andersson et al., 1998; Paananen et al., 2005). *Bacillus amyloliquefaciens* strains producing amylosin, fengycin and surfactin were isolated from Building 1 (Mrs. A's study, Table 1) (Mikkola et al., 2004, 2007; Rasimus-Sahari et al., 2015). No toxic bacteria were searched for in Building 2 (Mrs. B's apartment, Table 1). The presence of *Stachybotrys* in settled dusts and *Stachybotrys* colonizing large areas of building materials indicated long lasting water damage in the three buildings. However, the symptoms of the two asthmatic persons, Mrs. A and Mrs. B were not necessarily caused solely by *Stachybotrys* metabolites, but the symptoms could certainly be connected to the water-damaged building materials and possibly also to the colonizing microbes. The causative agents for health effects and asthma connected to fungal growth in water-damaged constructions inside buildings have been controversial for a long time (Hurraß et al., 2024a, 2024b; Loukou et al., 2024; Nagayoshi et al., 2011; Pestka et al., 2008; Nordin, 2020; Thrasher et al., 2014; Yike and Dearborn, 2011). The connection between asthma and water-damaged and poorly ventilated buildings seems to be generally accepted (Cai et al., 2024; Lu et al., 2022; Norbäck et al., 2018; WHO, 2009; Wong et al., 2016).

In the second part of the study the differences in the final consequence of two cases of asthma developed in the *Stachybotrys*-infested buildings and their progress during 38 years after diagnosis were compared. The worsening of the asthma of the one person, Mrs. A, was connected to repeated microbial exposure in water-damaged urban buildings. She used inhaled corticosteroids for 30 years. The reason for her sickening in chronic progressive lung disease, NSIP is unknown. According to Kankaanranta et al. (2024), prolonged use of inhaled

corticosteroids was associated with several comorbidities in severe asthma patients. The asthma of the other person, Mrs. B, seemed to be cured, despite of high microbial exposures and only short time use of inhaled corticosteroids (Table 2, Fig. 7).

Based on the literature used in the study, a conclusion was drawn concerning qualitative and quantitative differences between emission sources and of bioaerosols in different urban and rural indoor air environments. The sample numbers were small, and the results raised more questions. Briefly, urban indoor spaces contained 100 times less airborne dust per m^3, and >100 times less airborne microbes per m^3 than the air in animal sheds (Andersson et al., 1999, 2018; Peltola et al., 2001; Vornanen-Winqvist et al., 2020). Compared to animal sheds, the dust concentrations and microbial colony numbers in the indoor air of the water-damaged urban office and day care center were presumably too low to cause disease in the exposed occupants. However, the most toxic samples were obtained from urban indoor environments (Andersson et al., 2018). The results presented here (especially the occupational asthma ruling) show that long-term exposure to moisture damage at least in this particular case (Mrs. A in Fig. 7) has led to chronic asthma.

Metabolically active indoor fungal isolates colonizing solid surfaces produced guttation droplets and liquid vesicles containing toxic metabolites and metabolites exhibiting detergent activities (Andersson et al., 2019, 2020; Castagnoli et al., 2018; Gareis and Gottschalk, 2014; Salo et al., 2019; Salo, 2022). Detergents and biocides used in urban indoor environments may potentiate bioreactivity of microbial cell wall components and metabolites (Rook, 2024; Svanes et al., 2018). Therefore, bioreactivity of microbial exposure may be greater in urban indoor environment than outdoors or in animal sheds. More research is needed to confirm these preliminary hypotheses. We aim to determine these preliminary hypothesis in future research. No beneficial effects, but rather the opposite, have been evidenced for exposure to the xenobiotic chemicals or the *Stachybotrys* metabolites detected in airborne indoor urban dusts (Bornehag et al., 2005; Salonen et al., 2024; Rook, 2024; Weschler, 2009; Parks et al., 2020; Wang et al., 2019).

Rural environment connected to rich biodiversity protects against allergy and asthma and increases resilience (Parajuli et al., 2018; Haahtela, 2022; Rook et al., 2017; Schrumpf et al., 2024). Alenius et al. (2009) showed that urban house dust from a dry, non-problematic building caused an allergic Th2 response, stable dust of good quality hay a protective Th1 response. Lichtenstein et al. (2006) speculated that differences in Th1- and Th2-biased immune responses among persons resulting from environmental exposures could contribute to the varied responses observed among individuals exposed to moldy environments (Pestka et al., 2008). Rural lifestyles in biodiverse environments were connected to protection from allergies and asthma in several other reports (Rook, 2024; Pivniouk et al., 2020; Vercelli, 2021; Wallen-Russell et al., 2023). The improvement of Mrs. B's asthma regardless of high microbial exposure in the animal shed supports this explanation. Microbes that actively produce bio-surfactants and harmful substances (Salo, 2022) may in theory affect the reactivity of contaminated indoor air in a different way than dormant airborne microbes emitted from good quality animal feed and bedding (Andersson et al., 2018). Exposure to airborne bacteria may be harmful (Fogelmark et al., 1994), but also beneficial, as especially exposure to lactic acid bacteria has been reported to protect against asthma (Kline, 2007; Adams et al., 2016). The metabolic state of the microbes infesting indoor building materials may affect emission of metabolites, active growth possibly boosts concentrations of harmful substances in indoor air (Andersson et al., 2019). Differences in microbial and chemical exposures, and difference in the medications of the two persons in rural versus urban environments after their asthma diagnosis are presented here as hypothetical contributing causes for the different outcome of the illnesses. Rural environment, contact with animals and exposure to diverse microbiota are known to increase immunological tolerance, to protect from development of asthma and allergy, and to promote mental and physical health (Haahtela et al., 2021; Haahtela, 2022; Rook, 2023, 2024; Wallen-Russell et al., 2023).

5. Conclusions

Microscopy of *Stachybotrys* conidia in settled indoor dust was effective in at least three described cases, and demonstrated to be a quick, low-cost method for detecting severe moisture damage. The occupational asthma diagnosis ruled by the Insurance Court of the first asthma case presented here, shows that long-term exposure to moisture damage may lead to a chronic disease. The other presented case of asthma, on the other hand, shows that asthma developed as a result of exposure to indoor moisture damage may be cured. We suggest that the microbial exposures of urban and rural environments are qualitatively different (concerning diversity and the metabolic state of the exposing microbes). Because of cleaning chemicals and biocides, bioreactivity of microbial exposure may be greater in urban indoor environment than in rural environments or outdoors. Based on these two described extreme cases (including a long term follow up of four decades), health hazards connected to water damages in buildings and the beneficial effects of diverse microbial exposure in the rural environment cannot be ruled out. However, more research is needed to verify the suggested beneficial asthma-curing effects of diverse microbial exposures in rural environments.

CRediT authorship contribution statement

Tuomas Hintikka: Writing – original draft, Conceptualization. **Maria A. Andersson:** Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Tamás Marik:** Writing – review & editing, Validation, Data curation. **Raimo Mikkola:** Writing – review & editing. **Magnus Andersson:** Visualization. **László Kredics:** Writing – review & editing, Validation, Data curation. **Jarek Kurnitski:** Supervision. **Heidi Salonen:** Writing – review & editing, Project administration, Funding acquisition.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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