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Original Article

Association of toxic indoor air with multi-organ symptoms in pupils attending a moisture-damaged school in Finland

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Abstract: Background: There is an on-going debate on how best to test toxic indoor air. Toxicological methods based on condensed water samples and cell culture technique are newly introduced research tools which were tested in this study. Methods: Pupils (n=47) from a water-damaged and (n=56) healthy schools were interviewed using a questionnaire. Indoor air was collected with a novel condensed water sampling technique and human THP-1 macrophages were exposed to the condensate. The cytotoxicity of cotton wool swab samples was tested using human BJ fibroblasts. Conventional microbiological culture methods were also performed. Results: Gastrointestinal problems (GI) were reported by 51% from the study cohort but only 4% of the control cohort, relative risk RR=14.30. For any neurological or neuropsychological symptoms, the RR was 63.04, muscular-skeletal pain RR=58.28, headache RR=31.00, respiratory symptoms RR=22.64, fatigue RR=21.45, sub febrility RR=15.49, ear infections RR=7.74, skin rash RR=5.96, all being statistically significant (P<0.001). All indoor air (n=7) and cotton wool samples (n=2) taken from the water-damaged classroom or in proximity of the problematic classrooms were toxic in cell culture assays. Low numbers of moisture-damage indicators were recovered from wall, passive air, and swab samples, namely *Aspergillus ochraceus* species group, *Aspergillus, Eurotium* species group, *Fusarium*, *Tritirachium, Scopulariopsis* genus group and *Aspergillus versicolores* species group. Conclusions: Indoor air toxicity and dampness-related microbiota recovered from the classrooms were associated with multi-organ morbidity of the school occupants. These results corroborated our previous reports from two adult cohorts i.e. evidence of causality. These new toxicological methods based on condensed water and cell culturing techniques seem to be superior to conventional microbiological methods in correlating with clinical symptoms.

Keywords: Dampness and mold, gastrointestinal symptoms, toxicity, moisture damage, indoor air, neurological symptoms, mycotoxins

Introduction

Indoor air has been studied for more than 30 years, but despite ever-increasing number of scientific reports describing clinical presentation and molecular mechanisms related to toxic indoor air, the causality between the toxic dampness microbiota (DM) and multiple health hazards has not yet been clarified. Respiratory adverse health effects have been studied most extensively [1], but neurological, cognitive [2-6] and muscular-skeletal [2, 7, 8] symptoms in occupants exposed to DM have been also reported. Gastro-intestinal (GI) symptoms are more often linked to the alimentary mycotoxin exposure, although there have been reports that indoor DM can also cause this outcome [9]. When the exposure to DM continues, reversible mucosal irritation may be transformed into an irreversible disease that was recently called the Dampness and Mold Hypersensitivity Syndrome (DMHS) [10]. The most recent comprehensive literature review [11] that analyzed all of the published data

between 2011-2018 concluded that of 114 epidemiological studies, the vast majority i.e. 112 (98.2%) supported the claim that the exposure to DM causes single/multi-organ symptoms.

The ecological community of moisture-damaged buildings consists of various fungi, yeast, and gram-positive and gram-negative bacteria: these species might vary over time depending on the competition of microorganisms for the limited availability of nutrition factors [1, 12]. Indoor air may contain a mixture of toxins; each toxin at low concentrations may be non-toxic but when presented as a mixture the components might potentiate each other's effects [13, 14]. Environmental exposure to toxic products in addition to the exposure to nanoparticles from fragments of fungal spores, decay products from construction materials, volatile organic compounds (VOCs) together with biocides used to cleaning have the potential to cause severe morbidity. The effects of secondary metabolic products of DM are known to be variable; some mycotoxins can up- or downregulate genes, inhibit DNA, RNA, and protein synthesis, and cause oxidative stress and inflammation [15, 16]. Other mycotoxins are vasoactive and exert cardiovascular effects, and many affect the immunological and (neuro) endocrine systems [4]. Mycotoxins activate the NLRP3 inflammasome [17], a key activator of innate immunity. The effects of mycotoxins on cellular metabolism are comparable to tobacco, asbestos or even the biological weapon ricin [18]. For example, the trichothecene mycotoxins cause emesis, diarrhea, weight loss, disorders of the nervous and cardiovascular systems, immunodepression, hemostatic dysregulation, decreased reproductive function, and bone marrow damage [19]. Some mycotoxins are even carcinogenic [20, 21].

One important issue that hampers the research into mold-related illness is the current misconceptions about how indoor air should be tested. The prevailing belief has been that there are only gaseous and particulate pollutants, which has led to practicalities to collect and study only these contaminants. It has been hypothesized that mycotoxins migrate with fungal spores. However, the research group of Professor Mirja Salkinoja-Salonen (Finland) in their book "Diagnostic Tools for Building Pathology" [22] shed light on what causes illness in Finnish buildings. They conducted experiments where not only indoor air, but also material samples were collected from a damaged building and cultured followed by an examination of the toxin-producing fungal species. These species produced vesicles, or the so-called "guttation droplets", or exudates on the culture dish. These droplets contained substances that were toxic to all eukaryotic cell types tested at dilutions of 100-20,000 [23-25]. Most of the toxic metabolites produced by microbes have a molecular weight of 300-2000 g/mol, i.e. they are non-volatile. In addition, they are fat-soluble, but move primarily in water vapour, and thus an increased relative humidity of the air promotes their aerosolization (https://aaltodoc.aalto.fi/handle/123456789/13497). It has been demonstrated that *Penicillium expansum* recovered from gypsum boards produced toxic droplets, i.e. exudates, which migrated into the air and were approximately 100-times more toxic in the cell culture assays than indoor air isolates of *Aspergillus, Chaetomium, Stachybotrys* and *Paecilomyces* [26]. It has also been reported that the fungal genus *Trichoderma* isolated from indoor air could produce toxic droplets [25]. This toxic *Trichoderma* thrived in the freshly manufactured dry gypsum board intended to be installed into new buildings [25]. Subsequently, a novel method of collection of indoor air water vapour was developed in 2014 to allow an estimation of indoor air toxicity (https://aaltodoc.aalto.fi/handle/123456789/13497). In this approach, air condensate is collected on a cooled surface of a steel plate which is then tested for toxicity on living human primary cells (US Patent 10,502,722 B2). This innovation has changed the prevailing perception of indoor air pollutants, they are now known to consist of three different types: particles (solids), gases, and liquids.

This study was initiated by the parents of the pupils from one Finnish school who contacted us with a request to investigate the elevated morbidity of their children who presented with multiple non-specific symptoms. The aims of this study were to: 1. record the clinical symptoms due to the exposure to DM in this problematic school and compare the morbidity risks to the children from a healthy school; 2. utilize the novel collection methods for indoor air and the human cell based functional toxicity tests; 3. compare the results from these toxicity tests with the results from traditional microbiological

Table 1. Demographic data and preceding hypersensitivity to allergens among pupils of the study and the control cohorts

		Study cohort		Control cohort	
Age	<12	39	83%	36	64%
	12-15	8	17%	19	34%
	16-19	0	0%	1	2%
Gender	No answer	0	0%	1	2%
	F	28	60%	31	55%
	M	19	40%	24	43%
Allergy to food, plant or animal allergens	no	36	77%	48	86%
	yes	11	23%	8	14%
Pets	no	16	34%	14	25%
	yes	31	66%	42	75%

tests; 4. compare the risks of morbidity of the children calculated from this study with those calculated for adults in our previous investigations. Most importantly, we wanted to tackle the issue of causality because it seemed improbable that such high morbidity among school children occurred simply by chance.

Materials and methods

The cohorts and the symptoms

The study cohort comprised 47 pupils aged 6-15 years. Most pupils presented with symptoms compatible with their exposure to bad quality indoor air. The administration of the school was reluctant to co-operate making it impossible to enroll all the pupils from the problematic school.

The control cohort (n=56) was from a school with no history of water damage that was located in the same region. The age and gender of the responders was matched to the study cohort. Thus, the response rates were 47/400 (12%) and 56/313 (18%) from the damaged and the control schools, respectively. All the respondents were non-smokers, and none reported mold infestation at home. The demographic data for both cohorts, the proportion of respondents with pets at home and underlying allergies are presented in **Table 1**.

Data collection was performed with a previously used questionnaire [2]. The inclusion criteria were that the pupils were attending the moisture-damaged school and were willing to participate in the study. There were no exclusion criteria.

Background of the water-damaged school

A hot water pipe from the district heating network had been leaking into the basement floor of the school and to a music classroom located in the school's basement. The problem was identified in spring 2019, and the space was dried out for 11 months. The use of the music classroom was discontinued in the spring 2019, but the piano was left in the stairway hall and was in use. Due to the complaints from parents and the increased morbidity among the pupils the school was shut down in March 2020. The entrance to the school was closed when we started our investigations and thus samples from the dry food stored in the kitchen (basement floor) are not available for toxicological and microbiological studies. The technical woodworking classroom, the canteen with the kitchen, the corridor between these spaces and the staircase hall where the piano stood, were located in proximity to the water-damaged music classroom, all in the basement. This is a small school with its own kitchen, where food was cooked each day for the pupils and staff. The canteen and the kitchen, located next door to the music classroom, usually have lower indoor air pressure. We suspected that the indoor air from the mold-infested music classroom could have being sucked for a long time into the kitchen thus contaminating the dried foodstuffs stored there.

Microbiological work-up from the moisture damaged school

Sample collection for microbiological studies: The samples for microbiological cultures were collected as follows: A) A piece from the lower edge of the wall of the staircase hall (where the piano was located) in the basement next to the water-damaged space. B) Passive air samples were collected from four classrooms through passive air sampling using the sedimentation method, also called an open-dish method. MEA, DG18 and THG plates (see below) were

kept opened for 1 h to allow the microorganisms or their spores to settle. Passive air samples were taken from i) the first floor of the water-damaged music classroom in the basement, ii) classrooms numbered 123 & 128 on the second floor, and iii) from classrooms located on the third floor C) In addition, a cotton wool swab sample was taken from the surface of a sewer located in the water-damaged music room, and cultivated on MEA, DG18 and THG plates.

Sample processing for microbiological studies: The culture of samples was performed in a microbiological laboratory (Co-op Bionautit, Helsinki, Finland) as instructed [27]. Pieces of wall sample (4.5 g) were dissolved in 40.5 ml solution of 42.5 mg KH_2PO_4, 250 mg $MgSO_4 \times 7H_2O$, 8 mg NaOH dissolved in 1 l water and 0.2 ml Tween 80; [28] and shaken for 60 min. Two series of dilutions were made and plated on malt (MEA; ISO 1600-21, 2013), dichloraneglycerol-18 (DG18; ISO 1600-21:2013), and trypsin-glucose yeast medium (THG). MEA media favour the growth of fungi, THG favours the growth of bacteria, whereas DG18 medium favours fungal species that thrive in dry environments. Colonies were counted for total fungal and bacterial contents after 7, 10 and 12 d of cultivation. Fungal species were identified after 10 d of cultivation. Microbial growth was quantified as a colony forming unit per gram of wall sample (cfu/g).

The number of microbial colonies grown by passive air sedimentation and from cotton wool swab samples were recorded, and fungal colonies were identified both from original and rejuvenated plates. The growth of actinobacteria on THG plates was checked two weeks after the sample inoculation. All plates were cultured at room temperature.

Microbial colonies were identified by microscopic examination, i.e. by studying the morphology of stained samples and photographed.

Toxicity from the moisture damaged school

Sample collection for the toxicological studies: It is noteworthy, at the time of sample collection, an air dryer was in use in the school.

Collection of indoor air samples by the water condensing technique: The condensed water samples were collected as follows: From the basement floor (n=5), 2/5 samples were from the water damaged music classroom i.e. the room in which the dryer was operating, one sample was from the woodworking classroom (opposite to the music classroom), one sample was from the corridor between the music and woodworking classrooms, and one sample was taken from the staircase hall (where the piano was located) between the basement (first and second floors), from where the material sample and swabs were taken. Two samples (n=2) were collected from the second-floor corridor.

The novel principle of indoor air sampling is based on the following approach: Water molecules from the indoor air are frozen on the top of two cold surfaces of metal plates. The frozen samples then melt at room temperature and are collected from a tray below the plates and then the melted water is sent to the laboratory for analysis. The collection comprises the following steps: 1. The temperature and the relative humidity of the room are registered; 2. The steel box, called the “E-collector”, is assembled on a stable stand, e.g. table. 3. A block of dry ice (approx. 1 kg) is carefully placed inside the box with tongs. 4. The steel box is covered with a lid to facilitate freezing. 5. After collection of the frozen specimens, the lid and the dry ice are removed. 6. The frozen water with its content melts and drips into the collection plate. 7. The box is removed from the pedestals and shaken over the collection plate to collect most of the condensate. 8. The pedestals are shaken to collect the maximum amount of condensate. 9. The condensed water sample is transferred e.g. into an Eppendorf tube. 10. The tube is closed, and the sample is ready for shipment. The box can be reutilised after proper washing.

Cotton swab samples

Two cotton wool wipe samples were collected from the furniture in the basement floor; one was from the top of the piano (in the stairway hall) and the other was from the windowsill (in the corridor between music and woodworking classrooms). The stairway hall had poor ventilation and there was an abundance of settled dust. In the corridor, the ventilation was better, and this location was less dusty. The swabs were placed into sterile plastic tubes and sent for toxicological analysis.

Toxicological studies

Toxicological studies were performed at FICAM (University of Tampere. Finland) in a Good Laboratory Practice - compliant laboratory.

Cytotoxicity of the condense water samples from indoor air using human THP-1 macrophages and WST-1 assay: The toxicity of indoor air condensed water samples was studied using human THP-1 macrophage/WST-1 assay. The WST-1 assay [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] is based on the mitochondrial activity of living, metabolically active cells that reduce WST-1 to a coloured product. The optical density of the enzymatic product is measured at 450 nm. The greater the absorbance value, the higher the metabolic activity and thus larger the number of living cells that are present [29]. The viability of cells exposed to water samples is compared to that of the unexposed control cells.

Immediately after arrival, the water samples were sterile filtered (pore size 0.2 μm) and stored at +4°C until use. THP-1 cells (ATCC, #TIB-202) were seeded into 96-well plates at a density of 10000 cells/well, and differentiated into THP-1 macrophages for 48 hrs by challenging to 25 nM Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) in a cell-specific medium [RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (both components from Gibco Invitrogen)], followed by 24 hrs recovery without PMA. The indoor air and control samples (sterile distilled water) were pre-warmed to 37°C for 1 h before use. THP-1 macrophages were exposed to two sample concentrations, 10% and 25% at 6 replicates. Nickel II sulphate hexahydrate (2.0 and 20.0 μg/ml) was used as a positive control of toxicity. Cells were incubated at 37°C, 5.0% CO_2 for 24 hours before the WST-1 assay.

Cytotoxicity of the cotton wool swabs using human BJ fibroblasts and the neutral red uptake (NRU) assay: The toxicity of the samples was interpreted using the neutral red uptake assay (NRU), that assesses lysosomal activity and cell membrane integrity. Only living cells can take up neutral red and thus the absorbance (560 nm) is directly proportional to the number of living cells [30].

The cotton wool swab samples were extracted according to the ISO 10993-12 for medical devices, when applicable. The samples were sterilized by autoclaving and then extracted for 72 h in a cell culture incubator (37°C, 5.0% CO_2), 0.05 g sample/ml in Minimum Essential Medium (MEM) supplemented with 10% FBS, 2 mM L-Glut and 1% non-essential amino acids (all from Gibco Invitrogen). Eight sample dilutions were done using the 2.15 dilution factor. Thus, the concentrations of the final extract in the cell culture ranged from 0.47% to 100%. BJ cells (ATCC #CRL-2522) were seeded into 96-well plates at density of 4000 cells/well in culture medium and exposed to each extract dilution with 6 replicates. The extraction solution without cotton wool was used as a negative control. Extracts of polyurethane film containing 0.1% zinc diethyldithiocarbamate, (Hatano Research Institute, Japan) were used as a positive control. In addition, a control cotton wool (Pharmacare, Finland) extract was used as a reference for the toxicity of cotton wool. Cells were incubated at 37°C, 5.0% CO_2 for 48 h before the NRU assay.

Estimated volume of inhaled vapour

The water content in the indoor air depends on the relative humidity (RH%) and the temperature. The humidity ratio and the density of water in the air in each sampling site were calculated using the Psychrometric Chart (http://www.flycarpet.net/en/PsyOnline). The theoretical daily inhaled water amount was calculated assuming that a person inhales daily approximately 10 m^3 air.

Data analysis

The cohorts and the symptoms: The primary variables were gastrointestinal, respiratory, and neurological or neuropsychological symptoms, headache, fatigue, eye irritation, rash, sub febrility, ear infections, muscular-skeletal pain, multiple chemical sensitivity (MCS) and cardiac symptoms. We also obtained information on diagnosed diseases such as asthma. Risk ratios (RR) were calculated to compare the exposed study cohort to the non-exposed control cohort with respect to the primary variables. The risk ratio is the proportion of subjects with symptoms in the exposed group divided by the proportion of subjects with symptoms in the non-exposed group. For symp-

Table 2. The comparison of risks for symptom manifestations and asthma diagnosis among exposed (n=47) vs. unexposed school children (n=56)

Disease or symptom	Study cohort (exposed) n=47		Control cohort (non-exposed) n=56		Exposed vs. unexposed		
					RR	95% CI	*p*-value
GI- symptoms*	24	51%	2	4%	14.30	(3.73-86.25)	<0.001
Respiratory symptoms**	19	40%	1	2%	22.64	(3.60-453.3)	<0.001
Neurologic symptoms***	13	28%	0	0%	63.04	(3.03-850.2)	<0.001
Headache	26	55%	1	2%	31.00	(5.16-612.4)	<0.001
Fatigue	18	38%	1	2%	21.45	(3.38-430.5)	<0.001
Eye irritation	16	34%	3	5%	6.35	(1.93-26.79)	<0.001
Rash	15	32%	0	0%	5.96	(1.78-25.29)	<0.001
Sub febrility	13	28%	1	2%	15.49	(2.32-316.8)	<0.001
Ear infections	13	28%	2	4%	7.74	(1.82-49.11)	<0.001
Muscular-skeletal pain	12	26%	0	0%	58.28	(2.77-787.1)	<0.001
Asthma	5	11%	3	5%	1.99	(0.43-10.25)	0.32
Multiple chemical sensitivity (MCS)	4	9%	1	2%	4.77	(0.53-4.91)	0.35
Cardiac symptoms****	2	4%	0	0%	10.71	(0.31-155.4)	0.15

*Pain in the abdominal area, nausea diarrhea, vomiting, abdominal swelling, obstipation. **Cough, dyspnea, difficulty in breathing. ***Problems with concentration, brain fog, memory difficulties, muscle traction, dizziness, hearing abnormalities, balance problems. ****Tachycardia, palpitations.

toms with zero prevalence in the non-exposed group, a zero-count adjustment was done by adding 0.25 to all four cell counts. All statistical tests were two-tailed, *and p*-values <0.05 were statistically significant. The analyses were performed using NCSS 2019 Statistical Software (2019). NCSS, LLC. Kaysville, Utah, USA.

Toxicological studies

In the cell culture studies of the indoor air condensates the absorbance values were normalized, i.e., the viability of the untreated control was set as 100%, and all other data were calculated in relation to this control value as either % decrease in cell viability (negative values) or % increase in mitochondrial dehydrogenase activity (positive values).

In the studies with the swabs, the absorbance values were normalized, i.e., the viability of untreated control (0% extract concentration) was set as 100%, and viabilities of the cells treated with different swab extract concentrations were calculated relative to this control value.

Samples were interpreted as being toxic at either concentration when statistically significant changes between the samples of indoor air condensate or cotton wool swabs samples were compared to the respective controls were compared in the Student's t-test (Sigma Plot 14.0) and achieved P<0.05.

Ethical consideration

This retrospective study did not require ethical approval. The Ethical Committee from the Northern Ostrobothnia gave a recommendation to allow the search for biomarkers in mold-related disease among school children, EETTMK 10/2020. All guardians of pupils gave a written informed consent to conduct this study.

Results

The symptomology of the cohorts and cases

The comparisons of the risks for the development of multi-organ symptoms and the diagnosis of asthma are presented in **Table 2**. Importantly, a high frequency of GI-symptoms was reported; 51% of the respondents of the problematic school suffered from at least one of the following symptoms: pain in the abdominal area n=19 (40%), nausea n=15 (32%), diarrhea n=11 (23%), and vomiting n=4 (9%). Thus, the RR for the GI-symptoms was 14.30 (95% CI 3.73-86.25, P<0.001). Respiratory symptoms were also prevalent with an RR of 22.64 (3.60-453.3, P<0.001). However, the statistical analysis also detected highly significant differences between the cohorts in the reported

Figure 1. Microbial species recovered from the moisture-damaged school.

neurological symptoms i.e. brain fog, memory problems, balance problems, dizziness, muscle traction, hearing abnormalities and concentration problems such that their combined risk ratio was as high as RR 63.04 (95% CI 3.03-850.2). The risks for other symptoms were also high: headache RR 31.00 (5.16-612.4), fatigue RR 21.45 (3.38-430.5), eye irritation RR 6.35 (1.93-26.79), skin rash RR 5.96 (1.78-25.29), occasional sub febrility RR 15.49 (2.32-316.8), ear infections RR 7.74 (1.82-49.11), muscle or joint pain RR 58.28 (2.77-787.1), all statistically highly significant, P<0.001. The risks for asthma RR 1.99, multiple chemical sensitivity RR 4.77 or cardiac symptoms RR 10.71 were not statistically significant.

Microbiological work-up

Figure 1. Cultivation of microbes in the water damaged school. According to the results of serial dilutions of the sampled wall material originating from the water-damaged space in the basement, i.e. the area in proximity to the music room contained only a low amount of fungi (110 cfu/g) but all colonies belonged to the *Aspergillus, Eurotium* species group (**Table 3**), species which are mentioned as moisture damage indicators in the Guidelines for the Application of the Housing Health Regulation (https://www.valvira.fi/-/asumisterveysasetuksen-soveltamisoh-1). Inoculation and cultivation of wall material pieces onto agar plates resulted in a small number of fungi but the fungal community composition was more diverse and consisted of four species groups or genera that are also listed as moisture damage indicators: *Aspergillus ochraceus* species group, *Aspergillus, Eurotium* species group, *Fusarium* and *Tritirachium* (**Table 3**). These data suggest that the sampled wall had suffered from moisture but later this had dried out.

The cotton wool swab sample taken from the surface of a dry cement alcove with 150 cm diagonal area located in the music room in the basement produced a low number of colonies on MEA and DG18 plates, but they all belonged to moisture indicator fungi, namely *Aspergillus versicolores* species group (**Table 3**). A passive air sedimentation sample taken from classroom 123, located above the music room, also resulted in a small number of colonies. Out of the four fungal species or genera detected, the *Scopulariopsis* genus group, those growing both on MEA and DG18 plates, represented moisture indicators (**Table 3**). Except for the occasional *Penicillium* colony (**Table 3**), passive air samples collected from classrooms on the first and second floors detected no evidence of growth of fungi.

Direct cultivation of wall pieces produced a small number of bacteria on THG agar and none of those after serial dilution. A few bacterial colonies were found from a passive air sample taken from classroom 123 located above the music room and cultured on MEA (**Table 3**). No bacteria were detected on bacteria favouring THG plates when they had been kept open for 1 h. **Figure 1** illustrates the species recovered from the moisture-damaged school.

To sum up, based on microbial analyses of passive air, material and cotton wool swab samples, the number of fungi or their spores was as low as in that present in buildings not affected by fungi-related damage.

Toxicity of the condensed water samples

The combined results are presented in **Table 4**. All condensed indoor air samples (n=7) were shown to be toxic.

Toxicity of the cotton wool swab samples

The results of the toxicity studies from the cotton wool swabs are shown in **Table 5**.

The swab sample taken from the top of the piano was the most toxic, the control swab

Table 3. Fungal or bacterial species recovered from the wall, passive air sampling and cotton swabs collected from the moisture-damaged school

Sample	Medium	Identified species	Number of colonies	Place
Piece of wall material - serial dilution	MEA		0	Basement, near music room
	DG18	**Aspergillus, Eurotium* species group	110 cfu/g	
	THG		0	
- direct cultivation	MEA	**Aspergillus ochraceus* species group	<20	Basement, near music room
		**Aspergillus, Eurotium* species group	occasional	
		**Fusarium*	<20	
		Penicillium	occasional	
		**Tritirachium*	<20	
	DG18	**Aspergillus ochraceus* species group	<20	
		**Aspergillus, Eurotium* species group	<20	
	THG	bacteria	<20	
Passive air	DG18	*Cladosporium*	1	Classroom 123, the 2nd floor above the moisture-damaged music room (the 2nd floor)
		**Scopulariopsis* genus group	1	
Passive air	MEA	**Scopulariopsis* genus group	1	Classroom 123, the 2nd floor above the moisture damaged music room (the 2nd floor)
		Aureobasidium	1	
		Rhodotorula (yeast)	1	
		bacteria	4	
Passive air	DG18	*Penicillium*	1	Classroom 128 at the 2nd floor
Cotton swab	MEA	**Aspergillus versicolores* species group	1	Surface of a hollow area from music class (the 1st floor)
Cotton swab	DG18	**Aspergillus versicolores* species group	2	Surface of a hollow area from music class (the 1st floor)

Passive air samples were collected for 1 h. *Moisture indicator microbes.

Table 4. Toxicity of the water condensed from indoor air tested in the THP-1 macrophage viability assay

Sample	Conditions at sampling site		Water inhaled ml/day	The % change in THP-1 macrophage viability after exposure to indoor air samples		Interpretation
	RH%[1]	T°C		10% condensate	25% condensate	
Music classroom (basement)	45.5	17.5	109.00	1.80±5.00	-15.90±5.90***	Toxic
Music classroom (basement)	45.5	17.5	109.00	1.50±5.50	-11.10±10.20*	Toxic
Woodworking classroom (basement)	32.5	18.4	43.30	-3.00±5.30	-12.70±3.30***	Toxic
Corridor (basement)	31.2	22.0	127.10	-6.10±1.90**	-5.60±3.30**	Toxic
Staircase hallway (with piano) (basement)	31.7	22.1	128.90	-1.90±2.40	-4.40±2.30**	Toxic
1st floor corridor	33.0	21.8	128.40	-2.90±2.10	-9.60±2.60***	Toxic
1st floor corridor	34.6	21.3	126.50	-2.00±3.70	-7.00±2.20***	Toxic

[1]RH% = relative humidity. Two volumes of the condensate were used: 10% and 25% of the total culture volume. The results are normalized against (untreated) control and expressed as % change in cell viability, mean ± stdev, as compared to the control (0% change in cell viability). Negative values refer to decreased viability, positive values refer to increased mitochondrial activity, both are adverse effects. Each sample was tested at six replicates. The statistically significant changes in viability as compared to the respective control are indicated as *P<0.05; **P<0.01 and ***P<0.001.

sample was the least toxic, and the swab sample from the windowsill was moderately toxic. A swab sample from the piano caused approximately 32% cell death (i.e. 68% viable cells) tested at the concentration of 10.6% condensate. At the same test concentration, a window swab and a control cotton wool swab showed no toxicity. The following 100% extracts were toxic: Piano-top swab specimen caused 77% cell death (23% viability), windowsill swab specimen caused 43% cellular death (57% viable cells), and control cotton wool swab alone caused approximately 41% cell death (59% viable cells). Thus, computationally, dust collected from the piano increased cell death by 36% as compared to the pure cotton wool control.

Estimated volume of inhaled vapour

The calculated daily inhaled water ranged between 43.30-128.90 ml depending on the sampling site. More specifically the volumes are presented in **Table 4**.

Table 5. The toxicity of cotton wool swabs tested in the BJ fibroblast viability assay

Extract concentration %	Control sample (pure cotton wool)	The swab from the window bench (basement corridor)	The swab from the piano (staircase hallway in the basement)
0	100.00±5.72	100.00±4.65	100.00±5.34
0.47	101.27±4.17	107.58±3.61	101.57±6.69
1.01	96.11±3.11	102.67±5.37	99.20±7.26
2.18	101.53±1.76	101.54±3.96	103.90±6.61
4.68	101.56±6.60	98.59±3.03	94.19±4.65*
10.06	99.04±4.11	96.96±2.87	68.08±15.44***
21.6	90.69±3.32**	87.47±4.53***	67.44±18.68***
46.5	79.66±3.98***	80.07±2.31***	42.16±13.33***
100.0	59.45±2.02***	56.79±1.65***	22.93±5.81***

Eight different cotton wool swab extract concentrations, i.e., 0.47-100%, were tested in six replicates each. The results are normalized against the control (0% extract concentration and 100% cell viability) and expressed as % cell viability, mean ± stdev. The statistically significant changes in viability as compared to the respective control are indicated as *P<0.05; **P<0.01 and ***P<0.001.

Discussion

Here, we describe the clinical picture of mold-related illness in children attending a moisture-damaged school. Toxicological and microbiological analyses from the school are also presented. At the first glance, the low cfu of fungi and bacteria isolated from the wall material, air sedimentation and cotton swabs, may erroneously lead to the assumption that the building had not been affected by moisture damage. However, the presence of several fungal species and genera, especially the indicators of moisture-damage (Guidelines for the Application of the Housing Health Regulation (https://www.valvira.fi/-/asumisterveysasetuksen-soveltamisoh-1) such as *Aspergillus ochraceus* species group, *Aspergillus, Eurotium* species group, *Fusarium*, *Tritirachium, Scopulariopsis* genus group and *Aspergillus versicolores* species group indicated the presence of harmful moisture. Although the microbial growth taken from samples from several locations remained small, positive toxicity findings from condensate air samples (n=7) and cotton wool swab samples (n=2) were unambiguous and associated with the symptoms reported by the participants.

In Finland, due to its cold climate, people tend to spend most of the time indoors. In this situation, continuous or cumulative exposure to toxic DM in homes, schools, or workplaces may lead to chronic and poorly specified adverse health effects. Here, children attending the moisture-damaged school did not report multiple chemical sensitivity (MCS), which has been attributed to advanced mold-related disease, or DMHS, as is now designated [10]. In contrast, the calculated risks for MCS have been high in adults of both genders of moisture-damaged buildings occupants [2, 3] indicating that under continuous exposure to moisture-damage in buildings the disease may become chronic in its nature. Importantly, toxic DM may decrease the quality of life i.e. immaterial effects and it may increase health care costs. Direct health care costs can be calculated. For example, when calculated per inhabitant, in 2019 the expenses for annual visits to the doctor's office and health care providers in the town with the water-damaged school were 4-fold higher than in the nearby municipalities i.e. affected school town 171000 € (inhabitants n=7000) vs. 17 000 € (municipality with 2700 inhabitants) or 35000 € (municipality with 5700 inhabitants). There were more visits to pediatric and oto-rhino-laryngological services, i.e. these were 3-5 and 4-5 times more frequent, respectively, in the community with the moisture-damaged school when compared to the neighboring communities (The data are from Financial Statement of the community in question).

It is known that exposure to DM can cause mucosal irritation and lead to chronic inflammation, stimulation and/or disturbances in the functions of immune system [31]. Mucosal dysfunction increases the susceptibility to infections [1-3, 10, 18, 32]. The risk for respiratory symptoms was exceptionally high in our study

cohort, RR 22.64; an observation that corroborated earlier results [2, 3]. In addition, there were other striking symptoms experienced by the children of the moisture-damaged school e.g. neurological symptoms and GI-symptoms with the RR values being 63.04 and 14.30, respectively, and highly statistically significant. The involvement of the GI-tract in the clinical pathology of mold-related disease has been reported [9]. More often, however these symptoms have been attributed to the alimentary entry of mycotoxins through food or feed [33, 34]. The very high prevalence of GI-complaints observed in our study cohort could be interpreted in two ways: The GI-tract is a highly innervated organ, and therefore the complaints may represent autonomous dysregulation that occurs in occupants of mold-infested buildings. The second possibility takes into account the ground plan of the school building and the fact that the lunchtime food was prepared in the school's own kitchen, with dry food being stored in the larder adjacent to the mold-infested classroom, making it difficult to exclude the alimentary route of toxin exposure.

Several Finnish studies have identified a link between muscular-skeletal symptoms and moisture damage in the dwellings [2, 7, 8]. In our schoolchildren the risk for muscular-skeletal pain was also high, RR 58.28, and statistically significant. The interviewed pupils from the problematic school also frequently reported fatigue with a calculated risk of RR 21.45. Fatigue is a common complaint of people living in moisture-damaged buildings [2, 3, 35-37]. Fatigue is a neurological symptom and is reversible but may persist over time even after prolonged avoidance of the insulting agent [10]. Fatigue often associates with poor ventilation and high CO_2 concentrations, i.e. conditions that may have nothing to do with moisture damage [37]. However, when the ventilation is inadequate, hazardous compounds such as DM may become concentrated in the indoor air, thus potentiating the effects of an elevated CO_2 concentration. In our view, this may well be an explanation for the cognitive and emotional impairments reported by our study cohort; these disturbances are not enigmatic; i.e. the so-called "functional disorders" but may have an immuno-toxicological background such as encephalopathy.

Until now, the multicentre HITEA study has been the largest study investigating the relationship between moisture damage, indoor toxic dust and morbidity in school children [38-41]. This study examined primary schools from Finland (n=6), the Netherlands (n=10) and Spain (n=7). The most striking outcome of this investigation was that the levels of endotoxin, used as a surrogate for gram-negative bacteria, were highest in the Dutch schools, whereas the Finnish schools showed the lowest levels [39]. However, the prevalence of respiratory symptoms was more pronounced in the Finnish pupils and wheeze tended to be inversely associated with microbial levels [40]. This apparent contradiction has been addressed in studies of Professor Mirja Salkinoja-Salonen's research group. They reported that several toxin-producing microbial strains could be isolated from the dust present in the kindergartens: avrainvillamide and stephacidin B producing fungi *Aspergillus westerdijkiae* [42] mitochondriotoxic *Bacillus simplex, Streptomyces* and *Nocardiopsis*, and cytotoxic *Trichoderma harzianum Rifai* and *Bacillus pumilus* [43]. In other words, the results of this study indicate that if one wishes to prove causality, then it is essential to consider the potency of the mycotoxins, rather than simply calculating the numbers of the recovered fungi or bacterial colony forming units. This view is new and challenges the existing practices.

There are several limitations in this study that was initiated by the schoolchildren's parents. The administration of the problematic school was reluctant to cooperate. We were able to gather information from only 47/400 (12%) of the pupils, and none of the teachers volunteered to participate in our investigation. We were unable to retrieve dry foods from the kitchen to investigate the possibility of alimentary exposure because after we started our investigation, the entrance to the school became prohibited.

However, there are several strengths of our study. We collected the data on all the symptoms reported by our study participants and did not focus only on asthma and respiratory symptoms, which was the approach applied in the HITEA project. With our set-up, we confirmed that mold-related illness is a multi-organ disease probably related to systemic inflammation and the development of a biotoxicosis [5, 16, 21]. Importantly, we demonstrated that children reacted to toxic DM very similarly to

adults [2, 3], however with symptoms affecting both the central and autonomous nervous systems being remarkably prevalent. The impact of DM on the nervous system has often been overlooked, even ignored. Likewise, children may experience muscular-skeletal pain that is associated with the exposure to DM. When the results from the conventional microbiological tests and those from the human cell based functional tests were combined with the clinical symptoms, concordance can be found. This finding promotes the usefulness of novel indoor air testing to prevent harmful health effects.

In this study, the risk for respiratory symptoms was high and statistically significant, but the risk for asthma was lower than expected. This observation by no means undermines the recognised causality between mold infestation and asthma. If anything, it underlines the fact that different mycotoxins may cause different symptoms and that there will be extensive interindividual variability in the clinical presentation. Exposure to DM may cause cough without diagnosis of asthma [12], but on the other hand, asthma can be accompanied by COPD [44]. Another strength of our study is that we substantiated our clinical findings by careful conventional microbiological investigations which were compared against the novel water condensation technique for indoor air sampling. The latter techniques allowed us to estimate the toxicity of inhaled air at any given moment and to evaluate its relation to the relative humidity. To prove this causality further, it would be imperative to analyse the condensate e.g. by mass spectrometry, to identify the toxins and compare them to the identified toxins from the exudates of fungi [25]. We hope that researchers with access to this specialized equipment will take up this challenge. The methods presented here to collect and test condensed water vapor from indoor air represent the current state-of-the-art.

Taken together, we describe the adverse health effects experienced by the children exposed to toxic DM, mainly impacting on the CNS, GI-tract and muscular-skeletal system. The multi-organ involvement may lead to the assumption that they are suffering a biotoxicosis [21]. The results of toxicological and microbiological investigations supported the presumed causality between toxic building moisture (= the cause) and the higher morbidity in the exposed group (= the outcome). In fact, causality is a philosophical concept, not a mathematical formula. We cannot say with certainty how much evidence needs to be gathered to prove causality. However, spending a long time in a moisture-damaged building results in morbidity, that is irrefutable [2, 3, 45, 46]. It is therefore tempting to conclude that the causality exists and that the outcomes do not occur by chance. Therefore, the collection of clinical evidence focusing only on respiratory symptoms (as in the HITEA study) and the techniques to investigate indoor air fungi by the traditional agar plate collections and short cultivation times seem to be inadequate. To this end, major misconception was the assumption that the toxicity is mediated only by fungal spores (the particulate matter). Our results question the statement that "human mycotoxicosis are implausible following inhalation exposure to mycotoxins in mold-contaminated home, school, or office environments" [47]. There is now an awareness that fungi can expel tiny liquid droplets that aerodynamically migrate into the airways in conditions of increased relative humidity. These data have not yet been incorporated into new mathematical models. In conclusion, we advocate a recognition of DMHS as a systemic multi-organ and multi-systemic disease and recommend that the current practices used to study the toxicity of indoor air should be updated.

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Disclosure of conflict of interest

None.

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