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PAPER

Microbial secondary metabolites in school buildings inspected for moisture damage in Finland, The Netherlands and Spain†

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Secondary metabolites produced by fungi and bacteria are among the potential agents that contribute to adverse health effects observed in occupants of buildings affected by moisture damage, dampness and associated microbial growth. However, few attempts have been made to assess the occurrence of these compounds in relation to moisture damage and dampness in buildings. This study conducted in the context of the HITEA project (Health Effects of Indoor Pollutants: Integrating microbial, toxicological and epidemiological approaches) aimed at providing systematic information on the prevalence of microbial secondary metabolites in a large number of school buildings in three European countries, considering both buildings with and without moisture damage and/or dampness observations. In order to address the multitude and diversity of secondary metabolites a large number of more than 180 analytes was targeted in settled dust and surface swab samples using liquid chromatography/mass spectrometry (LC/MS) based methodology. While 42%, 58% and 44% of all samples collected in Spanish, Dutch and Finnish schools, respectively, were positive for at least one of the metabolites analyzed, frequency of detection for the individual microbial secondary metabolites – with the exceptions of emodin, certain enniatins and physcion – was low, typically in the range of and below 10% of positive samples. In total, 30 different fungal and bacterial secondary metabolites were found in the samples. Some differences in the metabolite profiles were observed between countries and between index and reference school buildings. A major finding in this study was that settled dust derived from moisture damaged, damp schools contained larger numbers of microbial secondary metabolites at higher levels compared to respective dust samples from schools not affected by moisture damage and dampness. This observation was true for schools in each of the three countries, but became statistically significant only when combining schools from all countries and thus increasing the sample number in the statistical analyses.

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Environmental impact

A multitude of different microbial secondary metabolites, often referred to as 'microbial toxins', are part of the complex exposures encountered in indoor environments. This study shows that moisture damage and dampness observed in school buildings link to an increase in the number of different mycotoxins present at elevated levels in settled dust; however, it is evident that microbial toxins are also present in non-damaged buildings as part of the 'normal' microbial flora indoors. This extensive study lays the grounds for future attempts to measure indoor exposure to microbial toxins and to assess the health risks related to such exposure.

Introduction

Indoor air quality (IAQ) has a great impact on our health and well-being since we spend up to 90% of our time indoors. A good amount of documentation links dampness, moisture and mould problems to adverse respiratory health effects, both in children and adults.[1,2] Moisture damage to buildings may occur for example due to construction flaws, plumbing leakages, and flooding, as well as because of moisture accumulation due to energy-effective ways of construction, insufficient airing, and insufficient maintenance of plumbing, heating and air conditioning systems.[3,4] Little is known, however, if and how biological and chemical agents associated with moisture damage and dampness play a role in the observed adverse health effects, such as respiratory symptoms, respiratory infections and exacerbation of asthma.[5] Mycotoxins, which are toxic secondary metabolites produced by many moulds, mostly as a means to mediate competitive interactions, are among the suspected causative agents.[3,6–9] It has been shown that secondary metabolites produced by metabolically active fungi and bacteria can act synergistically with other bioactive microbial components (*i.e.* allergenic proteins, cell wall components with inflammatory potential, microbial volatile organic compounds) in amplifying cellular responses in *in vitro* and *in vivo* models.[10–13] Water activity is a key parameter in defining the microbial flora, especially fungi.[14] Thus, an increasing water activity following building dampness may shift the fungal spectrum from the common airborne genera found indoors, *e.g. Penicillium*, *Aspergillus* and *Cladosporium*,[15,16] to typically more toxigenic fungi such as *Stachybotrys chartarum*, *Chaetomium globosum*, *Memnoniella echinata* and several *Trichoderma* species, each producing its own specific mycotoxin and metabolite pattern.[4] In addition to and combination with fungal compounds, toxic bacterial metabolites produced by *e.g. Streptomyces* species have recently been shown to co-occur in moisture damaged buildings.[17]

A variety of analytical methods including thin-layer chromatography, high performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay have been used for the analysis of mycotoxins in indoor environments, for example from ceiling material,[18–20] paper material,[3,21,22] settled dust,[23,24] and indoor air and airborne dust.[25–28] Many have preferred to use mass spectrometry (MS)-based methods, especially tandem MS (MS/MS), because of the high analytical specificity. Vishwanath and co-authors published a method for the simultaneous determination of 186 fungal and bacterial secondary metabolites in indoor matrices using HPLC-MS/MS.[29]

Dampness and indoor mould certainly also occur in school buildings[30,31] and the health risks associated with dampness-related exposures, such as respiratory symptoms and asthma, are likely to be similar to those found in homes.[30,32,33] The HITEA (Health Effects of Indoor Pollutants: Integrating microbial, toxicological and epidemiological approaches) school study aims at clarifying the health impacts of indoor exposures related to moisture damage and dampness on pupils and teachers. The study is conducted in identical setups in Spain, The Netherlands and Finland, thus covering three distinct geographical areas and climatic regions across Europe. Settled dust samples and surface extracts were collected in the course of building inspections from a large number of school buildings affected by moisture damage and dampness (index) and school buildings without such problems (reference). The aim of the present study was to analyze the sample materials for a wide range of toxic fungal and bacterial metabolites by using LC-MS/MS and GC-MS/MS, in order (i) to provide data on the occurrence and prevalence of microbial metabolites in European school buildings; (ii) to measure levels of these metabolites in surface dust from different rooms/location types within these schools; and (iii) to explore the statistical association of metabolite occurrence with moisture damage and dampness observations in the schools. Being the first study to provide such extensive data on the occurrence of microbial metabolites in European school buildings, this work will lay the grounds for future assessments on the relevance of microbial toxins on the health of pupils and teachers.

Methods

Selection and investigation of school buildings

Data on dampness/excess moisture, mould, and pertinent building characteristics were obtained from 85, 92 and 59 primary school buildings in Spain, The Netherlands, and Finland by using an internet based questionnaire addressed to the principals of the schools.[35] In Finland, the data were complemented with a previously completed nationwide, internet based survey. In each country, at least 10 schools with and 10 schools without questionnaire-reported dampness, moisture damage, and/or visible mould that were within a convenient geographical distance to the conducting study centers were selected. Building investigations were performed in these schools by centrally trained field workers following a common protocol and using standardized checklists and surface moisture recorders. Visual observation data were collected on building characteristics, moisture damage, and other potential contributors to poor IAQ including observations on maintenance, sources of dampness/mould, and particles.[34] Sample materials were collected for the microbial toxin analyses in the course of the school building investigations (see the following section). School buildings were categorized as either 'index' or 'reference' buildings based on the number, extent, severity, and location of observations on moisture damage, dampness and visible mould. In unclear cases buildings remained 'not categorized'.

Sampling

Two types of sample materials were collected following a standardized protocol: settled dust swab (SDS) samples and mouldy spot surface swab (MSS) samples. The SDS samples were collected from different locations in the school buildings (*i.e.* classrooms, hallways, teachers' lounges, libraries, dining halls, bath/shower rooms, storage rooms, and other locations), with one integrated SDS sample being collected in each location/room. Settled dust was collected from areas above floor level that are typically less frequently cleaned, for example door and window frames, skirting boards, top of cupboards and shelves, *etc.*, and sampling details (*e.g.* room type, sampling area and height) were recorded. In the index buildings we aimed at collecting approximately 10 samples per school, preferably from locations where dampness and/or moisture damage had been observed during the building investigations. In reference

buildings, a comparable number of samples was collected. We used 7 ml glass vials equipped with Teflon lined caps (Supelco Cat. Nr 27150-U), which were pre-filled with 3 ml HPLC grade methanol (JT Baker Cat. Nr 8402). Foam swabs (Foamtips Pro, Chemtronics Cat. Nr CFP-50) were wetted with methanol and moved across the sampling area. The adhered dust was thoroughly transferred from the swabs into the methanol filled vials; one to several sampling areas were combined into one integrated sample per location. In a few cases, where the sampled dust amounts were large, 3 ml extra of methanol were added to allow suspension and were recorded. The MSS samples were collected following a similar procedure, exclusively from surfaces with visible or suspected microbial growth (*e.g.* from locations with visible moisture damage). Again, one integrated sample was collected per room/location where such observations were made. All vials were transported at room temperature to the study centers on the day of sampling, sealed with parafilm, and stored at −20 °C until analyses.

Sample preparation and analysis of fungal and bacterial metabolites

The HPLC-MS/MS analysis for the detection of 186 fungal and bacterial secondary metabolites was performed as described in detail by Vishwanath *et al.*[29] The methanolic suspensions were shaken for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany). The particulate matter was allowed to settle for 10 min, and 100 µl of the clear upper methanolic layer were transferred into glass vials equipped with glass microinserts. Upon addition of 100 µl water, the diluted raw extract was directly injected into the HPLC-MS/MS instrument.

Detection and quantification in the scheduled Multiple Reaction Monitoring (sMRM) mode was performed with a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C18 column, 150 × 4.6 mm i.d., 5 µm particle size, equipped with a C_{18} 4 × 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, USA). For quantification, external calibration was performed using multi-analyte standards. Results were not corrected for incomplete extraction and/or signal suppression/enhancement due to co-eluting matrix constituents, as matrix-matched calibration was found to be insufficient for correction of these effects in the extremely heterogeneous dust matrix (Vishwanath *et al.* 2009).[29] Positive identification of an analyte was obtained by the acquisition of two sMRM transitions per analyte, which yielded 4.0 identification points according to commission decision 2002/657/EC. In addition, the LC retention time and the intensity ratio of the two sMRM transitions had to agree with the related values of an authentic standard within 0.1 min and 30% rel., respectively.

In the GC-MS/MS analyses, where a GC-triple quadrupole MS/MS instrument (model 1200, Varian Inc.) was used, verrucarol (VER) and trichodermol (TRID) were the target analytes. VER is formed by hydrolysis from macrocyclic trichothecenes, including satratoxins,[24] and can be used to screen all trichothecenes produced by *Stachybotrys* spp. TRID is formed by hydrolysis of its acetate ester, trichodermin, which is a mycotoxin produced by *Trichoderma* and some strains of *S. chartarum*.[35] In brief, methanolic samples were evaporated under a gentle stream of nitrogen and reconstituted in 0.9 ml of dichloromethane. The liquids were applied to polyethyleneimine (1 ml)-bonded silica gel columns (JT Baker, Phillipsburg, NJ) that had been preconditioned with 3 ml of methanol and dichloromethane. After the elution of the sample with 6 ml of dichloromethane, the eluates were evaporated under nitrogen and redissolved in 0.5 ml of methanol. The methanolic samples were mixed with 500 pg of internal standard (1,12-dodecanediol), evaporated under nitrogen, hydrolyzed in 0.2 M methanolic NaOH overnight at room temperature and extracted with water and dichloromethane. The dichloromethane phases were transferred to new vials, evaporated, and stored in a desiccator overnight. Derivatization of the dried extract was performed by adding 80 µl of acetonitrile–toluene (1 : 8, vol/vol) and 20 µl of HFBI followed by heating at 70 °C for 60 min. Details on sample preparation and analytical conditions have been reported previously.[23,24]

Statistical analysis

SAS software package 9.2 was used to produce descriptive statistics and to perform statistical analyses of the microbial metabolite data.

The concentration of individual toxins found varied over several orders of magnitude and hence the concentration data for toxin findings were dichotomised. Differences in the occurrence of individual toxins between moisture damaged index and reference buildings were tested using the Chi-Square test. Where assumptions in the Chi-Square test were not fulfilled, Fisher's exact test was used to calculate *p*-values. Using the same statistical tests, differences in toxin occurrences between different location types in the schools (categorized as classrooms, teachers' rooms, hallways, other locations) and between samples collected in schools of different countries (Finland, Spain, The Netherlands) were tested.

The number of individual metabolites per sample was enumerated, using different concentration cut-offs (>0, >0.1, >1.0, >10 pg cm^{-2}). Using these variables, analyses based on index *versus* reference buildings and damage observation *versus* no damage observation in the sampling location were performed with the Wilcoxon–Mann–Whitney test. This non-parametric test uses rank sums – in this case rank sums of the number of toxins per sample – to explore whether one of the two samples derived from index and reference school buildings tends to have larger values than the other.

Results

Building classification, sampling

An overview of the collected samples and the categorization of buildings based on the school building inspections are shown in Table 1. Samples were collected from a total of 97 school buildings in 66 schools. Of the inspected school buildings, 50 were index buildings, 37 were reference buildings, and 10 were not categorized buildings. 675 settled dust swab (SDS) and 66 mouldy spot surface swab (MSS) samples were collected from different locations; 396 were from index schools, 283 from reference schools, and 62 from schools not categorized (Table 1).

Table 1 School buildings in Spain, The Netherlands and Finland included in the building investigations and sampling: types and origins of sample materials

	Spain	The Netherlands	Finland	Total
	N (%)	*N* (%)	*N* (%)	*N* (%)
Schools/school buildings/samples				
Schools sampled	24	20	22	66
School buildings sampled	32	29	36	97
School buildings defined as index/ reference/not categorized	21/10/1	11/17/1	18/10/8	50/37/10
Total samples analyzed from index/ reference/not categorized school buildings	147 (65.6)/69 (30.8)/8 (3.6)	132 (48.3)/131 (48.0)/10 (3.7)	117 (47.9)/83 (34.0)/44 (18.0)	396 (53.4)/283 (38.2)/62 (8.4)
Sample types				
Settled dust samples (SDS)	212 (94.6)	238 (87.2)	225 (92.2)	675 (91.1)
Surface swab samples[a] (MSS)	12 (5.4)	35 (12.8)	19 (7.8)	66 (8.9)
Total	224	273	244	741
Samples collected from				
Classrooms – all buildings	141 (62.9)	153 (56.0)	151 (61.9)	445 (60.0)
[index/reference/not categorized]	[95/39/7]	[71/77/5]	[69/55/27]	[234/172/39]
Teachers lounges – all buildings	10 (4.5)	26 (9.5)	22 (9.0)	57 (7.7)
[index/reference/not categorized]	[7/3/0]	[13/12/1]	[11/8/3]	[31/22/4]
Hallways – all buildings	32 (14.3)	39 (14.3)	29 (11.9)	101 (13.6)
[index/reference/not categorized]	[19/12/1]	[18/20/1]	[13/8/8]	[50/41/10]
Other locations in the school[b] – all buildings	41 (18.3)	55 (20.1)	42 (17.2)	138 (18.6)
[index/reference/not categorized]	[26/15/0]	[30/22/3]	[24/12/6]	[80/49/9]

[a] Collected from locations with visible moisture damaged and/or suspected mould growth. [b] Other location types include *e.g.* bathroom, shower room, dining hall, library, kitchen, storage room/attic/crawl space, and gym/sports hall.

Microbial secondary metabolite findings

The results of the analyses of 188 fungal and bacterial secondary metabolites from settled dust samples (SDS) are presented in the following on a country level. The term 'metabolites' is here used synonymously for 'secondary metabolites'. Detailed information on the number of positive samples, frequency of detection of individual metabolites and the levels of the individual analytes – typically in the range of low to mid pg per cm^2 sampling area, in a few cases (for physcion) reaching levels of ng per cm^2 surface – can be retrieved from Tables 2–4. In total, 30 different fungal and bacterial secondary metabolites were detected in samples from Spanish, Dutch and Finnish school buildings.

In Spain, 32 school buildings were studied (Table 2). The most commonly found mycotoxins in SDS samples in the index and reference buildings were emodin, physcion, and the enniatins A1, B and B1. Less commonly detected and only occurring in index buildings were alamethicin, meleagrin, griseofulvin, and apicidin. In reference buildings, beauvericin (also in one index building), trichodermol, and verrucarol were detected. Fig. 1 shows the metabolite spectra found in SDS samples in index *versus* reference school buildings and additionally differentiates samples collected from different location types. Findings for samples of non-categorized buildings are listed in Table 2.

In The Netherlands, 29 school buildings were studied. Similar to the situation in Spain, enniatins, physcion, and emodin were most frequently detected in samples from index and reference school buildings (Table 3). Other mycotoxins detected in both index and reference school buildings included beauvericin, meleagrin, 3-nitropropionic acid, and the bacterial toxin valinomycin. Equisetin, griseofulvin, penicillic acid, alternariolmethylether, stachybotrylactam and sterigmatocystin were detected exclusively in index buildings; dihydrosergol, fumigaclavin, roquefortin C, trichodermol and verrucarol were detected in single samples in reference buildings (Table 3; Fig. 1).

In Finland, 36 school buildings were investigated (Table 4). As in the other countries, the predominantly occurring mycotoxins in the index as well as the reference buildings were emodin and the enniatins B and B1 (Table 4). Microbial metabolites only found in index, but not in reference buildings were meleagrin, 3-nitropropionic acid, verrucarol, physcion, alamethicin, trichodermol, and fumigaclavin. Griseofulvin was found exclusively in reference school buildings (Fig. 1).

Table S1 in the ESI† to this paper summarizes the results of the analyses of the mouldy spot surface swab (MSS) samples for the three countries, details on the metabolite findings from these sample materials are described in there. Generally, mycotoxin concentrations in mouldy spot surface swabs were clearly higher compared to settled dust swabs, typically at least by one order of magnitude, with the highest concentrations reaching the sub μg cm^{-2} area. Some of the compounds detected in MSS samples were detectable also in SDS samples taken in the same room/location; however, more often the metabolite findings in the MSS and SDS samples in the same room were not consistent.

Differences in metabolite occurrence in index *versus* reference buildings and between countries

Only a few differences in the occurrence of individual secondary metabolites in settled dust samples of school buildings were found with respect to building category (Fig. 2) and country (Fig. 3). In the combined dataset of the three countries, alamethicin was found only in the index buildings ($p = 0.003$), whereas the occurrence of enniatin B was more frequent ($p = 0.050$) in the reference buildings. Similar results were found for Spain with

Table 2 Fungal and bacterial metabolites detected in settled dust swabs (SDS) collected from 32 Spanish school buildings

	Index school buildings			Reference school building			Non-categorized buildings		
	$N_{samples} = 136$			$N_{samples} = 68$			$N_{samples} = 8$		
	Positive samples (%)[a]	Conc. [pg cm^{-2}] Mean	Max	Positive samples (%)[a]	Conc. [pg cm^{-2}] Mean	Max	Positive samples (%)[a]	Conc. [pg cm^{-2}] Mean	Max
Alamethicin	8 (5.4)	1.09	61.5						
Apicidin	1 (0.7)	0.09	12.1						
Beauvericin	1 (0.7)	0.00	0.34	3 (4.4)	0.01	0.58	1 (12.5)	0.01	0.08
Emodin	30 (20.4)	4.66	316	12 (17.4)	0.96	16.5	4 (50.0)	0.99	2.72
Enniatin A1	2 (1.4)	0.01	1.49	6 (8.7)	0.08	3.71			
Enniatin B	13 (8.8)	0.02	0.69	12 (17.4)	0.05	1.23	2 (25.0)	0.03	0.18
Enniatin B1	9 (6.1)	0.06	2.37	6 (8.7)	0.14	4.29			
Griseofulvin	2 (1.4)	0.08	8.80						
Meleagrin	1 (0.7)	0.65	88.0						
Penicillic acid							1 (12.5)	17.5	140
Physcion	28 (19.1)	180	8916	11 (15.9)	145	5406	6 (75.0)	63.3	139
Trichodermol				2 (2.9)	0.04	2.21			
Verrucarol				1 (1.5)	0.01	1.04			

[a] Relative abundances of positive samples (SDS) calculated according to the number of samples collected in index, reference and non-categorized Spanish school buildings, respectively.

Table 3 Fungal and bacterial metabolites detected in settled dust swabs (SDS) collected from 29 Dutch school buildings

	Index school buildings			Reference school buildings			Non-categorized buildings		
	$N_{samples} = 105$			$N_{samples} = 124$			$N_{samples} = 9$		
	Positive samples (%)[a]	Conc. [pg cm^{-2}] Mean	Max	Positive samples (%)[a]	Conc. [pg cm^{-2}] Mean	Max	Positive samples (%)[a]	Conc. [pg cm^{-2}] Mean	Max
Alternariol-methylether	1 (1.0)	0.01	1.28						
Beauvericin	9 (8.6)	0.07	4.02	5 (4.0)	0.08	9.60	1 (11.1)	0.00	0.03
Dihydrosergol				1 (0.8)	0.00	0.28			
Emodin	24 (22.9)	0.73	16.4	38 (30.7)	0.56	14.3	6 (66.7)	0.42	1.26
Enniatin A	6 (5.7)	0.06	5.14	3 (2.4)	0.00	0.15	2 (22.2)	0.01	0.05
Enniatin A1	5 (4.8)	0.16	13.4	3 (2.4)	0.00	0.19	1 (11.1)	0.04	0.40
Enniatin B	37 (35.2)	0.18	6.25	39 (31.5)	0.07	2.25	4 (44.4)	0.02	0.10
Enniatin B1	14 (13.3)	0.28	11.3	9 (7.3)	0.01	0.34	3 (33.3)	0.02	0.14
Equisetin	4 (3.8)	1.90	193						
Fumigaclavin				1 (0.8)	0.01	0.82			
Griseofulvin	2 (1.9)	0.25	22.5						
Meleagrin	2 (1.9)	0.08	7.60	5 (4.0)	0.12	5.10	3 (33.3)	3.64	29.8
3-Nitropropionic acid	6 (5.7)	1.17	59.4	3 (2.4)	0.04	3.16	3 (33.3)	0.89	3.70
Penicillic acid	1 (1.0)	5.71	600						
Physcion	26 (24.8)	36.3	1094	24 (19.4)	16.2	618	4 (44.4)	18.6	52.5
Roquefortine C				1 (0.8)	0.00	0.42			
Stachybotrylactam	1 (1.0)	5.2	550						
Sterigmatocystin	2 (1.9)	0.05	5.0						
Trichodermol				1 (0.8)	0.05	6.0			
Valinomycin	2 (1.9)	0.02	0.96	2 (1.6)	0.01	0.82			
Verrucarol				1 (0.8)	0.05	6.6			

[a] Relative abundances of positive samples (SDS) calculated according to the number of samples collected in index, reference and non-categorized Dutch school buildings, respectively.

alamethicin and enniatin A1. In Dutch samples, equisetin was found only in the index buildings (Fig. 2).

A number of individual metabolites were significantly more frequently occurring in Dutch *versus* Spanish or Finnish school buildings, including beauvericin, meleagrin, valinomycin, emodin, enniatins A and B, equisetin, nitropropionic acid and physcion (Fig. 3). Alamethicin was significantly more common in dust samples from Spain compared to the other countries.

Statistically significant differences were found in rank sums for the number of microbial metabolites per settled dust swab sample based on the building status (index *versus* reference; Table 5) and the damage status of the sampling location (damage observation *versus* no damage observation in the sampling location; Table S2†). Both building status and damage observations in the sampling location were established in walk-through building inspections by trained personnel. Using higher

Table 4 Fungal and bacterial metabolites detected in settled dust swabs (SDS) collected from 36 Finnish school buildings

	Index school buildings			Reference school buildings			Non-categorized buildings		
	$N_{samples} = 103$			$N_{samples} = 82$			$N_{samples} = 40$		
	Positive samples (%)[a]	Conc. [pg cm^{-2}] Mean	Max	Positive samples (%)[a]	Conc. [pg cm^{-2}] Mean	Max	Positive samples (%)[a]	Conc. [pg cm^{-2}] Mean	Max
Alamethicin	2 (1.9)	0.35	31.7						
Beauvericin							1 (2.5)	0.01	0.42
Emodin	17 (16.5)	0.32	4.64	16 (19.5)	0.85	30.0	8 (20.0)	0.28	2.39
Enniatin A	1 (1.0)	0.08	8.00	1 (1.2)	0.00	0.15			
Enniatin A1	1 (1.0)	0.08	8.64	1 (1.2)	0.01	1.06			
Enniatin B	25 (24.3)	0.65	16.9	28 (34.2)	0.37	12.7	10 (25.0)	0.01	0.13
Enniatin B1	10 (9.7)	1.14	33.9	6 (7.3)	0.29	12.1			
Fumigaclavin	1 (1.0)	0.06	6.48						
Griseofulvin				2 (2.4)	0.44	24.3			
Meleagrin	3 (2.9)	0.21	10.2						
3-Nitropropionic acid	3 (2.9)	0.88	85.0				3(7.5)	0.63	15.0
Penicillic acid							1 (2.5)	1.28	51.0
Physcion	1 (1.0)	0.17	17.3						
Trichodermol	1 (1.0)	0.03	3.13				1 (2.5)	0.01	0.52
Verrucarol	2 (1.9)	0.07	3.96						

[a] Relative abundances of positive samples (SDS) calculated according to the number of samples collected in index, reference and non-categorized Finnish school buildings, respectively.

cut-off values for metabolite enumeration (*i.e.* only analytes exceeding certain concentration per cm^2 sampling area are considered) in the country-combined data, statistically significant higher rank sums for number of metabolites were observed in index compared to reference school buildings (for cut off at >1 and >10 pg cm^{-2}), as well as in locations with damage observations compared to locations without such observations (cut off at >10 pg cm^{-2}). The same trends were visible in the country specific data; however, the differences did not reach statistical significance and were least pronounced for Finnish samples (Tables 5 and S2†).

No statistically significant differences were found between the occurrences of any of the individual metabolites or metabolite sums for the different location types (classrooms, teachers' lounges, hallways, other locations; *data not shown*).

Discussion

Swab samples from settled dust and surfaces with visible mould or suspected microbial growth were collected from school buildings with and without moisture damage and dampness, as categorized upon school building inspections using identical protocols and checklists. Samples collected from classrooms, hallways, teachers' lounges and other locations from index, reference and non-categorized school buildings were analyzed for 159 fungal and 27 bacterial toxins by HPLC-MS/MS.[29] In addition, samples were analyzed for trichodermol and verrucarol using GC-MS/MS for determining trichodermin and screening for *Stachybotrys* macrocyclic trichothecenes.[4] Some of these metabolites, such as sterigmatocystin,[24,36] chaetoglobosin A and griseofulvin[14] and enniatins,[37] were reported earlier to occur in naturally infested indoor materials, whereas others have been shown to be produced by cultures of microbial species known to be prevalent in damp indoor environments *e.g.* stachybotrylactam produced by *Stachybotrys chartarum*, meleagrin and roquefortine C produced by *Penicillium chrysogenum* and valinomycin produced by *Streptomyces griseus*.[38,39]

The frequency of detection of individual microbial metabolites other than emodin, certain enniatins and physcion was rather low in the samples of our study, typically in the range of and below 10% of positive samples. On the other hand, 42%, 58% and 44% of all samples collected in Spanish, Dutch and Finnish schools, respectively, were positive for at least one of the metabolites analyzed. The relatively low prevalence for the majority of individual compounds in our study may be explained by several factors: firstly, the occurrence of well detectable levels of mycotoxins indoors is likely to be largely driven by severe microbial growth on surfaces. Such affected buildings are usually no longer fit for inhabitance or use and certainly should not be used as school buildings. Due to the aims and longitudinal design of the HITEA study, only schools that were in use and did not plan major renovations within the next 12 months were eligible and visited in the course of building inspections. Hence, schools with very severe moisture damage that would require immediate attention were not participating in this survey. Moisture damage and dampness observed in our index schools were rather moderate, which may have resulted in less extensive microbial contamination and lower frequency of detection of individual microbial metabolites, compared to frequencies one might expect based on earlier studies that have focused the sample collection strictly on severely moisture or mould damaged and damp buildings.[17,23,24,28,40,41] Secondly, we followed a non-invasive sampling approach, which focused on collection of dust settled on elevated surfaces in order to (i) allow for maximum comparability between samples/sampling locations in various schools and in different countries; and (ii) assess the occurrence of the target compounds in a sample that potentially reflects airborne exposure. This is very different

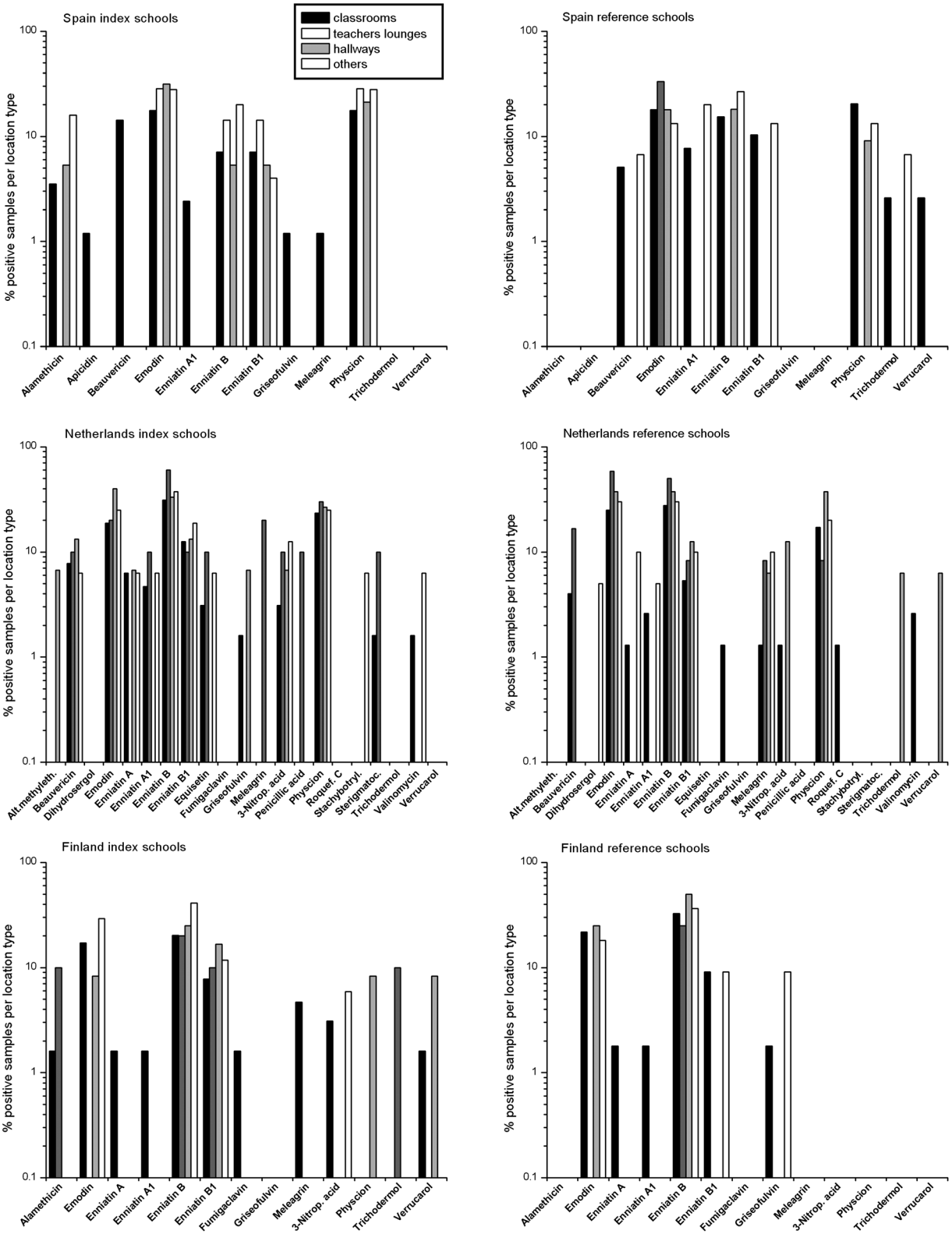

Fig. 1 Frequency of detection of microbial secondary metabolites detected in settled dust swabs (SDS) in different locations in Spanish, Dutch, and Finnish index and reference school buildings.

from a sample collection that targets highly contaminated building materials and samples collected from severely damaged locations only.

Certain mycotoxins were identified in most of the studied buildings, regardless of their categorization as index or reference buildings, or whether the buildings were located in Finland, The

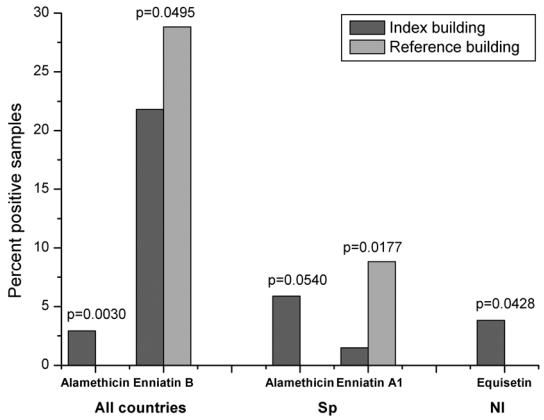

Fig. 2 Differences in frequencies of detection of individual secondary metabolites in settled dust swabs (SDS) collected from index and reference school buildings (all countries combined, as well as country specific data; $p < 0.1$ according to the Fisher's exact test are shown).

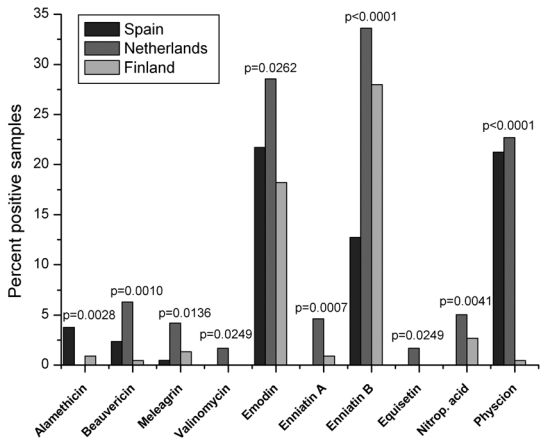

Fig. 3 Differences in frequencies of detection of secondary metabolites in settled dust swabs (SDS) from school buildings in Spain, The Netherlands and Finland ($p < 0.05$ according to the Chi-Square test are shown).

Netherlands, or Spain. Emodin and different enniatins, for example, were found to be among the most prevalent metabolites in all countries and buildings. The higher prevalence of these compounds may, however, be due to the fact that they ionize very well and thus are detectable at very low levels in the analytical method applied, with their detection limits being lower compared to other analytes. Inconsistent detection limits for the multitude of analytes may somewhat bias the frequency of detection of certain compounds, which may also explain the sometimes inconsistent profile of detected substances, for example the finding of tentoxin – a mycotoxin produced by *Alternaria* species along with other secondary metabolites – without the co-occurrence of other *Alternaria* metabolites.

We found few differences in the presence of the individual secondary metabolites with respect to school building status and country. Differences with respect to the detection of individual analytes in index *versus* reference school buildings included alamethicin, which was more frequently detected in index schools in Spain and in the combined dataset, and equisetin, which was more frequent in index schools in The Netherlands. However, these findings are linked to a very low prevalence of the respective analytes (<10%) and thus, these results do not have sound statistical support and may rather be considered as indicative findings. A number of metabolites were detected in different frequencies in the different countries, with The Netherlands ranking the highest in most of these cases. Beauvericin, meleagrin, valinomycin, emodin, enniatin A, enniatin B, equisetin, 3-nitropropionic acid, and physcion were all detected most frequently in samples from Dutch schools. Alamethicin was statistically more frequently found in Spanish schools. The issue of low prevalence of detection of the metabolites also here weakened the statistical basis for the majority of the findings, with exceptions of emodin, enniatin B and physcion. The detected country specific differences in metabolite prevalence may indicate a geographical/climatic dependency of metabolite detection, following the known geographical dependency of the occurrence of fungal and bacterial species.[42,43] However, it is also possible that rather country-specific, school building related factors, such as main building materials, school maintenance and use,[34] may have affected metabolite occurrence. These aspects will be further explored in the longitudinal part of the HITEA study, where microbial metabolites – along with other microbial compounds – will be measured in three subsequent exposure assessments.[44]

In general, we observed a greater variety of different metabolites in the index buildings compared to the reference buildings;

Table 5 Differences in rank sums (Wilcoxon–Mann–Whitney test) for number of metabolites per sample based on building status (index, reference) at increasing cut-off values for the enumeration of metabolites in a sample (all metabolites; metabolites in concentration >0.1, >1.0, and >10 pg cm^{-2})

		All metabolites		Metabolites > 0.1 pg cm^{-2}		Metabolites > 1.0 pg cm^{-2}		Metabolites > 10 pg cm^{-2}	
		Rank sums	*p*-value	Rank sums	*p*-value	Rank sums	*p*-value	Rank sums	*p*-value
All countries combined	Reference	316	0.402	309	0.947	296	**0.045**	296	**0.017**
	Index	305		309		319		319	
Spain	Reference	106	0.451	104	0.752	98	0.327	95	0.093
	Index	101		102		105		106	
The Netherlands	Reference	114	0.830	114	0.865	110	0.218	110	0.094
	Index	116		115		119		120	
Finland	Reference	91	0.619	88	0.211	87	0.093	92	0.690
	Index	95		97		97		94	

this tendency was the clearest in Finnish schools and least pronounced in the Dutch schools. On a country level, certain mycotoxins were detectable only in index, but not in reference schools; however, these findings were rarely consistent among the three countries. For example, the mycotoxins meleagrin and alamethicin, likely to originate from indoor fungal growth, were found exclusively in the index schools in Finland and in Spain (not detected in Dutch schools). Alamethicin is a toxic metabolite that elicits a wide range of biological activities related to its potential to form pores in biological membranes and is known to be produced by *Trichoderma* species that are considered an indicator of moisture damage.[45] On the other hand, enniatins B and A1, potent toxins produced by *Fusarium* species, were found more frequently in reference compared to index buildings. The producing moulds are commonly observed in contaminated grain and thus, grain dust that may be transferred from outdoors to indoors could explain the occurrence of these toxins in high prevalence also in reference schools. Mycotoxin infested insects that end up indoors provide another potential source for low levels of mycotoxins found indoors.[46]

The source allocation of microbial metabolites with respect to indoors *versus* outdoors is not always straightforward. Emodin and physcion are anthraquinone derivatives that are produced by many plants and possess for example anti-cancer, anti-bacterial and immunosuppressive activities. These compounds were among the most frequently detected analytes in index as well as in reference school buildings in all countries – except for physcion in Finnish samples – which rather suggests outdoor sources accounting for their presence. However, also 'on-site' production by common indoor moulds of the genus *Eurotium* cannot be excluded as a source for these mycotoxins. Thus, one question this study raises concerns microbial secondary metabolites that are mainly produced by typical outdoor moulds. In indoor air research focusing on airborne microbes it has for a long time been recognized as vital to include outdoor samples as a reference to indoor findings. Our results demonstrate that a similar approach is also needed for microbial metabolite analysis.

For reasons already stated, differences in metabolite prevalence between the index and the reference school buildings were not evident or statistically not sound at the level of individual secondary metabolites. However, when considering the total number of different metabolites per sample, differences between index *versus* reference buildings and sampling locations with and without moisture damage observations became evident. This was particularly true for the larger dataset of all countries combined and when higher concentration cut-offs were defined for the enumeration of the bacterial and fungal metabolites per sample. These results show that in settled dust derived from moisture damaged or damp indoor environments the number of microbial secondary metabolites present at elevated levels can be expected to be higher compared to indoor environments without moisture damage and dampness.

Conclusions

To our knowledge, this is the most comprehensive study on the presence of mycotoxins and bacterial secondary metabolites in school buildings and in general in indoor environments that were inspected and categorized according to the presence of moisture damage and dampness. Differences in metabolite profiles between Finland, The Netherlands and Spain indicate a certain level of geographical/climatic dependency of metabolite detection. Moisture damage and dampness observed in school buildings and individual rooms seem to link to an increase in the number of different mycotoxins present in higher concentrations in settled dust samples. These aspects will receive further attention in the analyses of subsequent follow-up sampling campaigns in the longitudinal phase of the HITEA study. In addition, the combination of the herein presented data with a rich dataset including other (microbial) exposure data, health data of exposed occupants of the buildings, as well as toxicological data collected in this survey will attempt to elucidate the role of indoor microbial metabolites in adverse health outcomes observed in moisture damaged, damp buildings.

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