

Oxidative capacity and haemolytic activity of settled dust from moisture-damaged schools

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Moisture damage scoring of schools

The moisture damage score was calculated based on extent, severity and location of damage observations in classrooms or other frequently occupied spaces in school buildings as assessed during walk-through building inspections by trained personnel, utilizing the protocols and surface moisture detection as described by Haverinen-Shaughnessy¹:

- Score = 0 if no moisture damages were observed in the school building
- Score = Score + 1 if moisture damages in locations other than classrooms exceeded 50m²
- Score = Score + 1 if moisture damages in locations other than classrooms exceeded 100m²
- Score = Score + 1 if moisture damages in classrooms exceeded 5m²
- Score = Score + 1 if moisture damages in classrooms exceeded 10m²
- Score = Score + 1 if maximum severity of damage observations in the school building exceeded 2
- Score = Score + 1 if maximum severity of damage observations in classrooms exceeded 1
- Score = Score + 1 if observations of visible mould in classrooms exceeded 1
- Score = Score + 1 if observations of visible mould in other locations than classrooms exceeded 2

The scale of the score was from 0 to 8, but in our dataset the values varied between 0 and 7. In the case of a school with more than one building, the highest value for a building that was included in the pooled sample was chosen. For the statistical analyses, the data was divided in three categories: “no moisture damage” (0), “minor moisture damage” (1-3) and “major moisture damage”⁴⁻⁷.

Questions on health outcomes

- Wheeze: “Has your child had wheezing or whistling in the chest in the last 12 months?”
- Congestion/phlegm: “In the last 12 months, has your child usually seemed congested in the chest or coughed phlegm (mucus) when he/she did not have a cold?”
- Nocturnal dry cough: “In the last 12 months, has your child had a dry cough at night, apart from a cough associated with a cold or chest infection?”
- Rhinitis: “In the last 12 months, has your child had a problem with sneezing or a runny or blocked nose when he/she did not have a cold or the flu?”
- Nasal bleeding: "How many times in the last 12 months, has your child had any of the following symptoms? Nasal bleeding (None, 1 time, 2 times, 3 times or more)*
- Fatigue: "How many times in the last 12 months, has your child had any of the following symptoms? Fatigue (None, 1 time, 2 times, 3 times or more)*

**) Categories 0-2 times were combined and compared with ≥ 3 times in data analysis*

Dust collection and extraction

Each school was sampled during winter/spring 2009. Within each country the sample collection periods were parallelized between the study schools as much as possible. The sampling campaigns were also parallelized between the three countries; however, school-free periods were avoided and local school conditions had to be respected. In each school, approximately 15 locations were sampled, with emphasis on representing well the whole school building / study population. Classrooms were primarily targeted, but also other frequently occupied indoor locations of the school building were considered.

Samples of settled dust were collected in settled dust boxes (SDBs) - polyethylene coated cardboard boxes with dimensions of 45 cm x 20 cm. A similar sampling approach has been described earlier by Wuertz et al.². The boxes were placed in a height of 1.5 to 2.5 m, avoiding locations with major air flow disturbances. After the standardized sample accumulation period of 8 weeks, the SDBs were closed and transported to the local study center by a field worker. In clean laboratory facilities, the settled dust from a single SDB was vacuumed with a pump operated at flow rate of approximately 5 l/min onto one mixed cellulose ester (MCE)-filter cassette (Zefon, 37 mm, 45 μ m). The filter was then suspended in a glass beacher in 5 ml of dilution buffer (1 l deionized water, 0.04 g KH_2PO_4 , 0.25g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.008 g NaOH, 0.2 ml Tween 80 detergent), sonicated for 15 minutes and shaken for another 15 minutes. The

filters were removed; the suspensions were transferred into sterile, pyrogen free 15 ml screw cap plastic vials and stored at -20 °C until further processing.

In order to allow multiple microbial and toxicological analyses from one sample and to limit sample numbers to a feasible quantity, pooling of settled dust suspensions from single locations in the school was performed. For the current analyses, one 'school pool' combining settled dust suspensions from all classrooms/hallways/other locations that were sampled in a given school was used for the toxicological analyses. For the assessment of toxicity, one portion of the pooled dust suspension was filtered through a 70 µm filter to remove the largest particles and resulting in more homogenous sample. The filtered suspension was further aliquotted and stored frozen (-20 °C) until the experiments.

A slightly different sampling approach, but also based on passively collecting settled dust onto a standard surface over an eight week sample accumulation period, was used for the measurement of endotoxin and β-1,3-glucan, as described by Jacobs et al.^{3,4}. Settled dust for these analyses was collected with electrostatic dustfall collectors (EDCs)⁵. While no pooling of the individual samples was performed at the stage of laboratory analyses, we here calculated the mean over the individual locations of each school.

Analysis of microbial components

1.8 ml of settled dust suspensions created from the SDBs were used for DNA extraction. The suspensions were centrifuged (10 minutes at 16.000 * g), the supernatant was reduced to 100 µL and the dust pellet resuspended and transferred into an Eppendorf tube containing glass beads. DNA extraction including a bead-beating step for mechanical cell disruption was performed as described earlier^{6,7}.

To quantitatively measure the concentration of certain microbial groups or species in the dust samples, quantitative PCR assays were performed, targeting different microbial groups. In the current analyses we used following assays: *Penicillium spp./Aspergillus spp./Paecilomyces variotii* group, *Mycobacterium spp.*, and Gram-positive and Gram-negative bacteria. The qPCR primers and probes used in this study have been published earlier⁷⁻⁹. The qPCR laboratory analyses and calculations were performed as described by Kaarakainen et al.¹⁰, using ABI Prism 7000 (Applied Biosystems) and RotorGene 3000 (Corbett Life Science) equipment.

Ergosterol is a compound of the fungal cell membrane and represents fungi in general. Muramic acid is a compound of the bacterial cell wall; however, Gram-positive species have 5-10 times more muramic acid than Gram-negative species and thus, muramic acid is commonly used as a marker of primarily Gram-positive bacterial exposures. 2 and 4 mL of settled dust suspensions were freeze-dried for ergosterol and muramic acid determinations prior to sample preparation. The sample preparation of ergosterol was carried out by modifying the method published by Axelsson et al.¹¹ and of muramic acid by modifying the method presented by Sebastian et al.¹² In our methods, hexane was used instead of heptane. The ergosterol and muramic acid samples were diluted to 100 mL of hexane and to 150 mL of chloroform, respectively, before analysis by gas chromatography tandem mass spectrometry (GC–MS–MS). The analyses were performed with a PolarisQ ion trap mass spectrometer (MS–MS) (Thermo, Austin, TX, USA) equipped with a Trace GC-ultra gas chromatograph (GC) (Thermo Electron Corporation, Milan, Italy) with a DB-5MS fused-silica capillary column (J&WScientific, Loveland, CO, USA)¹³.

The EDC sample processing and analyses of (1-3)- β -D-glucans and endotoxin from those samples have been described previously in detail^{3, 4, 14}. In brief, EDC extracts were tested for endotoxin content using a Limulus Amoebocyte Lysate (LAL) assay (Lonza Group, Basel, Switzerland) according to the manufacturer's protocol. Samples were tested in 1:25 and/or 1:50 dilutions. The resulting endotoxin units (EU) were converted to EU/m²¹⁵. The limit of the assay was assessed at 300 EU/m². Glucans were analyzed with a specific β -(1,3)-glucan sandwich enzyme immunoassay (EIA)¹⁶. The limit for detection of the assay was 200 ng/m².

Analysis of particle counts

Particle counts in settled dust box samples were measured with particle counter PAMAS SVSS (PAMAS GmbH, Rutesheim, Germany) with sensor SLS-25/25 (sixteen size classes between 0.5 and >20 μ m, 0.5 detection limit, max 13 000 particles/ml) and PMA analyzing software. Before measuring particle counts, the samples were diluted in ultra clean water (1:5000). The particle count of clean water was subtracted from results of all samples. The measured sixteen size classes were combined to form one category for all particle sizes.

Scanning Electron Microscopy (SEM)

Nine samples (two index and one reference schools per each country) were selected for detailed imaging with Field Emission-SEM (Philips XL-30, Philips Electron Optics, NL). The dust samples were placed on metal SEM sample stubs and coated twice with 0.5 µm layer of gold in sputter coater machine (EMScope). The images were taken with an accelerating voltage of 10 kV, spot size of 4.0 and a working distance between 10 and 10.2 mm.

Plasmid Scission Assay

The settled dust box samples were diluted in molecular grade water (MB H₂O) and incubated in quadruplicate with 200 ng of plasmid DNA (ϕ 174 RF DNA from Promega, London, UK) in final volume of 20 µl for 6 hours. The same amount of DNA was incubated with MB H₂O and restriction enzyme Pst I (Promega, London, UK) digest to establish negative and positive controls, respectively. The incubated samples were mixed with 3.33 µl of orange/blue loading dye (Promega, London, UK) and electrophoresed on a gel (0.6 % agarose and 0.25 % ethidium bromide) for 16 hours at 30 volts in Tris-borate-EDTA buffer. Finally, the electrophoresed gels were imaged with VisionWorks® software (Ultraviolet Products Ltd, UK) and densitometric analysis was performed with GeneTools® software (Syngene Systems, UK). Gels with less than 10 % damage in the negative controls were accepted for the analysis.

Haemolysis assay

Blood samples were centrifuged at 3750 rpm at 4 °C for 10 minutes. The supernatant along with a thin layer of platelets were discarded, red blood cells (RBC) were resuspended in 0.9 % saline to a final volume of 10 ml, and the sample was centrifuged again. The procedure was repeated two times before preparing a working solution by adding 1 ml of packed RBC to 7 ml of 0.9 % saline. Settled dust samples were diluted 1:2 with 0.9 % saline before the experiments. 125 µl of RBCs were mixed with 125 µl of each dust suspension in triplicate in a 96 well-plate, sealed and shaken for 10 minutes in room temperature. Saline solution and 0.1 % Triton X-100 mixed with RBCs were used as negative and positive controls, respectively. After centrifugation (2000 rpm, 5 minutes, 4 °C), 100 µl of supernatant from each sample was transferred into a corresponding 96-well plate. The optical density (OD) was read at 540 nm by Opsy MR plate reader. The lower limit of detection (percentage at 3 standard deviations over the signal of the negative control) was 1.9 %.

References

1. Haverinen-Shaughnessy U, Borrás-Santos A, Turunen M et al. Occurrence of moisture problems in schools in three countries from different climatic regions of Europe based on questionnaires and building inspections - the HITEA study. *Indoor Air*. 2012;22(6):457-466.
2. Wurtz H, Sigsgaard T, Valbjorn O, Doekes G, Meyer H. The dustfall collector - a simple passive tool for long-term collection of airborne dust: a project under the Danish Mould In Buildings Program (DAMIB). *Indoor Air*. 2005;15:33-40.
3. Jacobs JH, Krop EJ, Borrás-Santos A et al. Endotoxin levels in settled airborne dust in European schools: the HITEA school study. *Indoor Air*. 2014a; 24:148-157.
4. Jacobs J, Borrás-Santos A, Krop E et al. Dampness, bacterial and fungal components in dust in primary schools and respiratory health in schoolchildren across Europe. *Occup Environ Med*. 2014b;71:704-712.
5. Noss I, Wouters IM, Visser M et al. Evaluation of a low-cost electrostatic dust fall collector for indoor air endotoxin exposure assessment. *Appl Environ Microbiol*. 2008;74:5621-5627.
6. Rintala H, Nevalainen A. Quantitative measurement of streptomycetes using real-time PCR. *J Environ Monit*. 2006;8:745-749.
7. Kärkkäinen PM, Valkonen M, Hyvärinen A, Nevalainen A, Rintala H. Determination of bacterial load in house dust using qPCR, chemical markers and culture. *J Environ Monit*. 2010;12:759-768.
8. Haugland R, Varma M, Wymer L, Vesper S. Quantitative PCR analysis of selected *Aspergillus*, *Penicillium* and *Paecilomyces* species. *Syst Appl Microbiol*. 2004;27:198-210.
9. Torvinen E, Torkko P, Nevalainen A, Rintala H. Real-time PCR detection of environmental mycobacteria in house dust. *J Microbiol Methods*. 2010;82:78-84.
10. Kaarakainen P, Rintala H, Vepsäläinen A et al. Microbial content of house dust samples determined with qPCR. *Sci Total Environ*. 2009;407:4673-4680.

11. Axelsson BO, Saraf A, Larsson L. Determination of Ergosterol in Organic Dust by Gas-Chromatography Mass-Spectrometry. *J Chromatogr B*. 1995;666:77-84.
12. Sebastian A, Harley W, Fox A, Larsson L. Evaluation of the methyl ester O-methyl acetate derivative of muramic acid for the determination of peptidoglycan in environmental samples by ion-trap GC-MS-MS. *J Environ Monit*. 2004;6:300-304.
13. Lappalainen MH, Hyvärinen A, Hirvonen MR et al. High indoor microbial levels are associated with reduced Th1 cytokine secretion capacity in infancy. *Int Arch Allergy Immunol*. 2012;159:194-203.
14. Krop EJM, Jacobs JH, Sander I, Raulf-Heimsoth M, Heederik DJJ. Allergens and beta-Glucans in Dutch Homes and Schools: Characterizing Airborne Levels. *Plos One*. 2014;9:e88871.
15. Noss I, Wouters IM, Visser M et al. Evaluation of a low-cost electrostatic dust fall collector for indoor air endotoxin exposure assessment. *Appl Environ Microbiol*. 2008;74(18):5621-5627.
16. Noss I, Wouters IM, Bezemer G et al. Beta-(1,3)-Glucan Exposure Assessment by Passive Airborne Dust Sampling and New Sensitive Immunoassays. *Appl Environ Microbiol*. 2010;76:1158-1167.