

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

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Abstract

Exposure to moisture-damaged indoor environments is associated with adverse respiratory health effects, but responsible factors remain unidentified. In order to explore possible mechanisms behind these effects, the oxidative capacity and haemolytic activity of settled dust samples (n=25) collected from moisture-damaged and non-damaged schools in Spain, The Netherlands and Finland were evaluated and matched against the microbial content of the sample. Oxidative capacity was determined with plasmid scission assay, and haemolytic activity by assessing the damage to isolated human red blood cells. The microbial content of the samples was measured with quantitative PCR assays for selected microbial groups and by analysing the cell wall markers ergosterol, muramic acid, endotoxins and glucans. The moisture observations in the schools were associated with some of the microbial components in the dust, and microbial determinants grouped together increased the oxidative capacity. Oxidative capacity was also affected by particle concentration and country of origin. Two out of 14 studied dust samples from moisture-damaged schools demonstrated some haemolytic activity. The results indicate that the microbial component connected with moisture damage is associated with increased oxidative stress, and that haemolysis should be studied further as one possible mechanism contributing to the adverse health effects of moisture-damaged buildings.

Keywords:

oxidative capacity, reactive oxygen species, settled dust, haemolysis, microbes, moisture

Practical implications: Oxidative capacity of settled dust is associated with moisture score and microbial components, but does not differentiate the moisture damaged from reference buildings. Haemolysis should be considered as one prospective mechanism contributing to the adverse health effects of moisture-damaged buildings.

Introduction

Particulate matter (PM) in the indoor environment may contain several toxic or bioactive components such as metals, polycyclic aromatic hydrocarbons (PAH), pollen, microbes and their metabolites. Increased health risks have been associated particularly to particles originating from combustion processes and microbial growth enabled by excess moisture within the building structures¹. The toxicity of PM is affected by the size, components and emission source of the particles, thus the hazard resulting from exposure is difficult to predict. This has inspired the development of cellular biosensors for assessing the extent and type of toxicity associated with exposure².

Immunotoxicological activity of indoor air PM has been studied most often with immunocytes³⁻⁶ or lung epithelial cells^{7, 8}. A recent study showed an increase in the immunotoxicological activity of dust from school buildings with higher microbial load, although not consistently associated with moisture-damage status of the building or symptoms of the occupants⁹. The failure to reliably identify moisture-damaged indoor environments with bioassays may indicate that the model system simply does not reflect the health responses of occupants accurately enough, but it could also be biased e.g. by other bioactive materials in the sample, the sampling method or measured toxicological endpoints.

The choice of biomarkers of exposure is not straightforward, as the exposure to moisture-damaged indoor environments could cause adverse health effects via several different mechanisms. The strongest evidence is for chronic inflammation and damage to respiratory tissue caused by exposure to fungi and bacteria, although causality remains an issue of debate. Several types of microbial exposure have been connected with indoor air related symptoms¹⁰⁻¹². Importantly, the effect of co-exposure to microbes and their components has

been found to be synergistic^{13, 14}, which supports studying the effects of real life combinations of exposure agents instead of single components.

Production of reactive oxygen species (ROS) is believed to play a pivotal role in the aetiology of PM-induced pulmonary irritation, leading to increased oxidative stress and subsequent inflammatory events locally and systemically¹⁵. PM from ambient air is known to be able to induce production of ROS in different cell types in vitro¹⁶, but information about the oxidative capacity of indoor air particles is limited to studies on specific emission sources such as candles and incenses¹⁷. In addition to combustion particles, microbial components can exert their effect via oxidative stress¹⁸, indicating that PM collected from indoor environment may contain several components with high oxidative capacity.

Several of the microbial secondary metabolites isolated from fungi growing on building materials are potent toxins that can initiate immunomodulatory or cytotoxic effects¹⁹. The prevalence and concentrations of individual toxins tend to be low even in moisture-damaged indoor environments, not differing much from reference buildings^{20, 21}, unless extreme cases such as mould infestation after severe flooding are considered. However, the number of different metabolites detectable at elevated concentrations has been found to increase in moisture-damaged schools and residential buildings^{20, 21}. A likely mode of action of mycotoxins in moisture-damaged indoor environments is to enhance the effect of other exposure agents rather than to cause acute toxicosis, as supported by studies on synergistic effects of co-exposure to mycotoxins and other biological agents¹⁴. Direct evidence of adverse health effects of toxin exposure in moisture-damaged indoor environments at present is scarce, and mostly relating to sporadic cases of particularly serious moisture-damage and the resulting microbial growth. However, studies of these cases have uncovered possible mechanisms of action for toxin-related respiratory distress, e.g. haemorrhage in the lungs caused by metabolites of fungus *Stachybotrys*^{22, 23}.

The current study presents the oxidative capacity and haemolytic activity of a well-characterised set of settled dust samples from moisture-damaged and non-damaged schools from three European countries collected in the context of a pan-European epidemiological study. The results are correlated with the microbial content of samples and respiratory symptom prevalence among children in the sampled schools.

Materials and methods

Study design and respiratory health assessment

The schools participating in cross-sectional “Health Effects of Indoor Pollutants: Integrating microbial, toxicological and epidemiological approaches” (HITEA) -project were selected and categorized as damaged or non-damaged as described earlier^{24, 25}. In addition to the dichotomous categorization of each school, we utilized a moisture-damage score in the analysis (supporting information, p. 1). Settled dust samples were collected from 14 moisture-damaged (index) and 11 non-damaged (reference) schools in three countries: from Spain (5 index + 2 reference schools), The Netherlands (5 + 7) and Finland (4 + 2). For the assessment of respiratory health, all children attending the schools were invited to complete a parent-administered survey based on the validated International Study of Asthma and Allergy in Children questionnaire. Respiratory symptoms (wheeze, congestion/phlegm, nocturnal dry cough and rhinitis) and other symptoms possibly connected to haemolysis (nasal bleeding and fatigue) during the past 12 months were considered as the health outcome (supporting information, p. 2). The association between respiratory symptoms, school dampness and levels of microbial markers in these schools have been reported earlier by Jacobs et al.²⁶ and immunotoxicological potency of the studied settled dust samples by Huttunen et al.⁹.

Dust collection and extraction (supporting information, p.2)

The settled dust samples were collected on cardboard boxes (settle dust boxes, SDBs) and electrostatic dustfall collectors (EDCs)²⁷ placed at a height of 1.5 to 2.5 m for 8 weeks. The processing of SDBs and EDCs has been described earlier²⁶. In brief, the dust accumulated in any given SDB box was vacuumed onto mixed cellulose ester (MCE) filters, suspended in dilution buffer and stored frozen. A target of 15 settled dust box samples from different classrooms and other frequently occupied spaces in each school were pooled and aliquotted for subsequent analyses. The individual EDC cloths were extracted for endotoxin and glucan determinations as described earlier²⁸. The extract was centrifuged, aliquotted and stored frozen in pyrogen-free glass tubes until analysis in central laboratory.

Characterization of the dust sample

The amount of selected microbial groups (fungal group *Penicillium spp./ Aspergillus spp. / Paecilomyces variotii*, the bacterial genus *Mycobacterium spp.*, Gram-positive bacteria, Gram-negative bacteria) in the settled dust box samples were assessed with quantitative PCR assays as described earlier²⁹⁻³² and chemical markers ergosterol and muramic acid by gas chromatography tandem mass spectrometry (GC-MS-MS) with slightly modified methods published earlier^{33, 34}. EDC extracts were analysed for their endotoxin content with Limulus amoebocyte lysate (LAL) assay and glucans with a specific β -(1,3)-glucan sandwich enzyme immunoassay (EIA) as previously described by Noss et al.³⁵ (supporting information, p. 3). The particle count in settled dust box samples was measured with particle counter PAMAS SVSS (supporting information, p. 4). In addition, nine samples (two index and one reference schools per each country) were selected for detailed imaging with scanning electron microscopy (SEM) (supporting information, p. 5).

Plasmid scission assay

Plasmid scission assay (PSA) was performed as described earlier by Koshy et al.³⁶. The settled dust box samples were incubated with plasmid DNA and the relative amount of damaged (relaxed and linearized) DNA was assessed as percentage of all (including supercoiled intact) DNA (supporting information, p. 5). A dose-response curve was fitted on the data and the dilution capable of damaging 50 % of the plasmid DNA (median toxic dilution, TD₅₀) was determined to facilitate the comparison between samples. As the dependency is not linear, it was described with third order polynomial fitted for each school separately. Similarly with the concept of median toxic dose, low median toxic dilution value indicates high bioactivity (Figure 1).

Haemolysis assay

EDTA-anticoagulated blood was collected from two healthy human volunteers after informed consent and with approval of the local ethics committee. Haemolysis of isolated human red blood cells was measured after exposure to dust suspension (supporting information, p. 5). Absorbance of the supernatant was measured and the percentage of haemolysis was calculated as the percentage of absorbance of each sample compared to the positive control. The average of the two tested donors was used to compare the samples with each other.

Statistical analysis

Basic tests and explorative analysis of the data were conducted with IBM SPSS Statistics 23.0 (IBM ®, New York, NY, USA). Since the variables are not normally distributed, differences between damaged and reference schools, countries as well as the comparison to

carrier control were tested using non-parametric Mann-Whitney U-test or Kruskal-Wallis test. Associations between symptoms and haemolytic activity were evaluated using ORs with 95% CI, obtained by multilevel mixed-effects logistic and linear regression. Associations with symptoms were adjusted for gender, age, parental educational level (as an indicator for socioeconomic level) and moisture-damage in the home.

Structural equation modelling is a series of statistical methods that allow complex relationships between one or more independent variables and one or more dependent variables³⁷. A structural model represents hypotheses about effect priority. The model represents the hypothesis that X is a cause of Y. Statistical estimates of direct effects are path coefficients, which are interpreted just as regression coefficients in linear model analysis. A special case of structural equation models, latent variable analysis (LVA) was deployed as the primary analytical technique to assess how the bacterial and fungal markers affect the TD₅₀ values. Latent variables correspond to hypothetical constructs or factors, which are explanatory variables presumed to reflect a continuum that is not directly observable. For example here, we constructed latent variables corresponding to bacterial and fungal effect on the TD₅₀ values, which are not directly observed but can be thought as weighted sums of individual markers. The number of observations did not allow for examining all the variables at the same time, as the model would have had too many estimable parameters compared to the data. However, we were able to construct separate models for fungal and bacterial components, both linked with oxidative capacity. Several criteria were used to evaluate the fit of the final model: e.g. χ^2 with degrees of freedom, the comparative fit index (CFI), and the root mean square error of approximation (RMSEA). These analyses were conducted using lavaan-package³⁸ in the R-software³⁹.

Results

Moisture score, particle count and microbial components

The moisture score was higher in index schools compared to reference schools, reaching statistical significance when the data from all three countries were combined. There were no statistically significant differences in the number of particles or amount of microbial components between the index and reference schools, even though the average amounts of microbial components such as *Penicillium spp./ Aspergillus spp. / Paecilomyces variotii*, muramic acid and *Mycobacteria spp.* as well as Gram positive and negative bacteria were generally higher in moisture-damaged environments (Table 1). The most prominent difference was seen comparing different countries; in most cases, the amount of the microbial components was highest in Spain and lowest in Finland (Figure 2).

Scanning electron microscopy (SEM)

The overall appearance of the samples showed accumulation of fine particulate matter with distinctive geological and biological origins in Spanish samples, heterogeneous material in smaller quantities in Dutch samples and only few dispersed particulates in the Finnish samples (Figure 3). Overall, settled dust samples contained apparent biological structures such as spores, pollen, and hyphae (Figure 4 A-E) as well as inorganic components such as mineral fragments and soot (Figure 4 F). Samples from index and reference schools were not visibly different.

Oxidative capacity

The oxidative capacity of the settled dust from Dutch schools was higher compared to Finnish and Spanish schools, but no statistically significant differences between the schools with and without moisture-damages was found (Table 2, low TD₅₀ values indicate high oxidative capacity).

To study the effects of bacterial and fungal markers on TD₅₀, we constructed two LVA models with latent variables representing “bacterial factor” and “fungal factor”. Amount of Gram-positive and Gram-negative bacteria, *Mycobacteria* spp. and endotoxin in the sample were statistically significantly contributing variables in the construction of latent variable “bacterial factor” whereas the chemical markers ergosterol, glucan and fungal group *Penicillium* spp. / *Aspergillus* spp. / *Paecilomyces variotii* contributed significantly to the “fungal factor”. In the first model, regressing TD₅₀ on the bacterial factor showed a notable negative effect, i.e. increase in bacterial markers lead to lower TD₅₀ and thus higher oxidative capacity of the sample. The number of particles was also inversely associated with TD₅₀. The assessed moisture score of the schools had a direct increasing effect on the bacterial factor (Figure 5A). In the second model both the fungal factor and the number of particles had a clear decreasing effect on the TD₅₀ value, but assessed moisture score of the schools affected the amount of the fungal group *Penicillium* spp. / *Aspergillus* spp. / *Paecilomyces variotii* rather than the fungal factor directly (Figure 5 B). In both models, the country where the samples were collected was a significant factor affecting both TD₅₀ and the bacterial/fungal factor along with moisture score and number of particles. According to fit indices, both LVA models were found to fit well to the data (e.g. RMSEA < 0.1 and CFI > 0.95). Combinations of three or more microbial components significantly improved the fit of the model.

Haemolytic activity

Overall, the haemolytic activity of the dust samples was low but statistically significantly increased levels of haemolysis compared to control samples were seen in some of the moisture-damaged schools, specifically in dust from two out of five studied index schools from Spain (Figure 6). Differences between the index and reference schools or countries were not statistically significant, even though the elevated levels of haemolysis were detected only in moisture-damaged schools from one of the studied countries. Dry cough at night was reported more frequently in the schools with haemolytic samples both when comparing to Spanish reference schools (OR 1.8, 95% CI 1.61-2.01) or all the other Spanish schools (OR 1.49, 95% CI 1.19-1.87). Fatigue was reported less often in schools with haemolytic samples (OR 0.65, 95% CI 0.53-0.79) and no significant associations with wheeze, congestion/phlegm, nasal symptoms or nasal bleeding were seen.

Discussion

There are several possible mechanisms leading to adverse health effects after exposure to indoor air PM in moisture-damaged environments. We aimed to clarify if oxidative capacity or haemolytic activity of indoor air PM are associated with the moisture-damage status of the building or microbial components present in the sample material. While the oxidative capacity of the sample was linked with microbial composition of the sampled school, it did not differentiate the moisture-damaged schools from the non-damaged schools. Structural equation modelling showed that the oxidative capacity was influenced by grouped markers for bacteria (Gram-negative and Gram-positive bacteria, *Mycobacterium spp.* and endotoxin) and fungi (ergosterol, β -glucan and fungal group *Penicillium spp.* / *Aspergillus spp.* / *Paecilomyces variotii*). Haemolytic activity was increased only in two out of 25 schools, but interestingly,

these two Spanish buildings were both moisture-damaged schools and the children attending these schools reported nocturnal cough more often when compared to all other schools in this country; suggesting that on some occasions haemolytic activity could contribute to the adverse effects of indoor exposure.

Comparing the oxidative capacity of the settled dust to the microbial content of the sample revealed that both markers of bacterial and fungal exposure were associated with increased oxidative stress. Importantly, combinations of three or more microbial components significantly improved the fit of the model, indicating that the presence of any single microbial component was not enough to explain the association. The association between the moisture-damage and oxidative capacity is likely confounded by bacterial species or components being more strongly linked with other factors than moisture-damage, such as occupancy, building factors and outdoor levels⁴⁰. More refined classification of the buildings according to moisture observations showed that bacterial indicators as a group increased together with the moisture score, whereas the effect of moisture observations on fungi was reflected in the amount of the fungal group *Penicillium spp. / Aspergillus spp. / Paecilomyces variotii*, that has been shown to be prevalent in moisture-damaged building materials⁴¹. These results support our earlier findings on the immunotoxicological potential of the same samples, showing an association between in vitro responses and particularly bacterial components in the dust and strong effect of the country of origin, but no significant difference between schools classified as moisture-damaged and non-damaged⁹. The low number of observations prevented us from including all variables in the same LVA model, but analysing the data in two parts – separating bacterial from fungal factors - was successful. It would have been interesting to see the interactions also between the fungal and bacterial factors, but this remains to be explored in future studies with larger datasets. Unfortunately, we did not have the possibility to measure all possible factors affecting the oxidative capacity of the PM. For example, combustion exhaust particles have

been previously linked with oxidative stress in several studies⁴², and outdoor emissions are a major contributor to indoor PM¹.

Haemolytic activity of indoor particulate sample is a noteworthy attribute for two reasons: firstly for the unlikely but possible direct adverse health effects of haemolysis such as pulmonary haemorrhage in exposed occupants, and secondly for the indirect effect of increasing the concentration of free haemoglobin in the exposed tissues, leading to potent synergistic activation of innate immunity via Toll-like receptors⁴³. Haemolysins (i.e. proteins and lipids causing lysis of RBC by destroying their cell membrane) were first recognised as the pathogenic product of Gram-positive bacteria even though several fungal species are able to produce haemolysins as well⁴⁴. In the context of indoor air microbial exposure, haemolytic activity has been associated with the fungal genus *Stachybotrys*^{22, 23}. Due to the low number of haemolytic samples in our study it was not possible to link the haemolytic activity with specific microbial components; e.g. *Stachybotrys* was found to be present only in two samples which were not the ones causing haemolysis. However, the results indicate that settled dust from some of the studied moisture-damaged schools was haemolytic, and the activity was not confined to the samples positive for *Stachybotrys* species.

In conclusion, the association of oxidative capacity with the presence of microbial components suggests that in addition to inorganic elements, also organic fraction of the PM contributes to the oxidative stress caused by the exposure. The moisture score of the studied schools was associated with bacterial components as well as with a common group of fungal species in moisture-damaged buildings, but neither oxidative capacity nor haemolytic activity of the samples differentiated the moisture-damaged schools from non-damaged ones. Even though the indication of haemolytic activity of the settled dust was a rare find, haemolysis should be considered as one of the possible mechanisms contributing to the adverse health effects of moisture-damaged buildings.

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Table 1. Average (\pm SE) moisture score, particle count and amount of fungal and bacterial components in settled dust collected from Spain, The Netherlands and Finland. Statistically significant difference between index and reference schools is indicated with a star (*, $p < 0.05$, Mann-Whitney).

| | Spain | | The Netherlands | | Finland | | All countries | |
|---|----------------|----------------|-----------------|----------------|---------------|---------------|----------------|----------------|
| | Reference | Index | Reference | Index | Reference | Index | Reference | Index |
| Number of schools, N | 2 | 5 | 7 | 5 | 2 | 4 | 11 | 14 |
| Moisture score | 1.5 \pm 1.5 | 4.8 \pm 0.6 | 0.3 \pm 0.2 | 2.4 \pm 0.5 | 0.0 \pm 0.0 | 4.0 \pm 0.4 | 0.5 \pm 0.3* | 3.7 \pm 0.4* |
| Particle count (10 ³ /ml) | 1100 \pm 300 | 1300 \pm 400 | 700 \pm 300 | 600 \pm 100 | 200 \pm 20 | 200 \pm 60 | 700 \pm 200 | 700 \pm 200 |
| Fungal components | | | | | | | | |
| Ergosterol (ng/SDB) | 6 \pm 1 | 11 \pm 2 | 15 \pm 3 | 18 \pm 5 | 2 \pm 2 | 3 \pm 1 | 11 \pm 3 | 10 \pm 2 |
| Glucan (ng/m ² EDC) | 3900 \pm 400 | 1700 \pm 100 | 2800 \pm 600 | 3500 \pm 500 | 600 \pm 100 | 800 \pm 200 | 2600 \pm 500 | 2100 \pm 400 |
| <i>Penicillium spp./Aspergillus spp./Paecilomyces variotii</i> (10 ³ CE/SDB) | 40 \pm 5 | 150 \pm 90 | 20 \pm 10 | 70 \pm 20 | 10 \pm 10 | 5 \pm 2 | 20 \pm 10 | 80 \pm 40 |
| Bacterial components | | | | | | | | |
| Muramic acid (ng/SDB) | 110 \pm 10 | 130 \pm 40 | 90 \pm 30 | 180 \pm 30 | 30 \pm 20 | 30 \pm 4 | 90 \pm 20 | 120 \pm 20 |
| Gram positive bacteria (10 ³ CE/SDB) | 400 \pm 7 | 1200 \pm 200 | 200 \pm 80 | 500 \pm 100 | 400 \pm 300 | 300 \pm 200 | 300 \pm 100 | 700 \pm 200 |
| <i>Mycobacterium spp.</i> (10 ³ CE/SDB) | 80 \pm 10 | 150 \pm 60 | 40 \pm 20 | 70 \pm 20 | 30 \pm 10 | 30 \pm 20 | 50 \pm 20 | 80 \pm 20 |
| Gram negative bacteria (10 ³ CE/SDB) | 1200 \pm 80 | 4000 \pm 700 | 400 \pm 200 | 1300 \pm 600 | 400 \pm 200 | 400 \pm 200 | 600 \pm 200 | 2000 \pm 500 |
| Endotoxin (10 ³ EU/m ² EDC) | 4.0 \pm 0.7 | 5.0 \pm 1.8 | 5.9 \pm 0.6 | 11.6 \pm 1.8 | 1.3 \pm 0.3 | 1.3 \pm 0.2 | 4.7 \pm 0.7 | 6.30 \pm 1.5 |

SDB, Settled Dust Box; EDC, electrostatic dustfall collector; EU, Endotoxin Unit; CE, Cell Equivalent; SE, Standard Error

Table 2. Average (\pm SE) oxidative capacity of settled dust from moisture damaged (index) and non-damaged (reference) schools from Spain, The Netherlands and Finland. Oxidative capacity is expressed as toxic dilution $TD_{50} \times 10^{-3} \pm SE$ (n), where lower number indicates higher oxidative capacity of the particulate matter. Statistically significant difference between countries is indicated with a star (*, $p < 0.05$, Kruskal-Wallis).

| Country | Reference schools | Index schools | All schools |
|-----------------|--------------------------|----------------------|--------------------|
| Spain | 64 ± 33 (2) | 55 ± 7 (5) | 58 ± 9 (7) |
| The Netherlands | 33 ± 6 (7) | 27 ± 5 (5)* | 31 ± 4 (12)* |
| Finland | 48 ± 4 (2) | 57 ± 4 (4) | 53 ± 3 (6) |
| All countries | 41 ± 7 (11) | 46 ± 5 (14) | 44 ± 4 (25) |

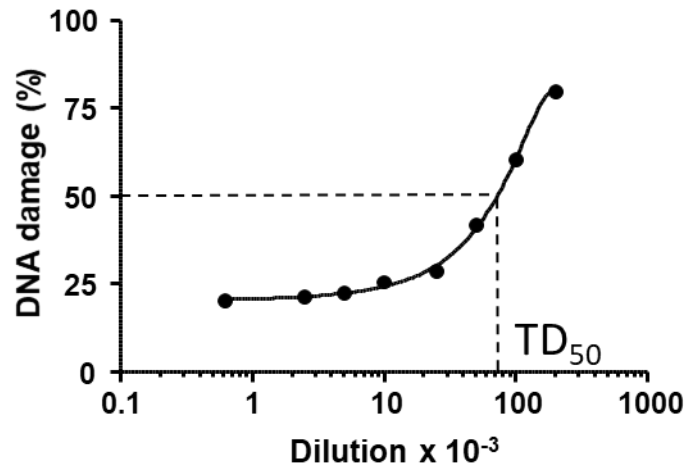


Figure 1. Determination of median toxic dilution (TD₅₀) value from third order polynomial curve fitting to the plasmid scission assay (PSA) data. Example data from one Spanish index school.

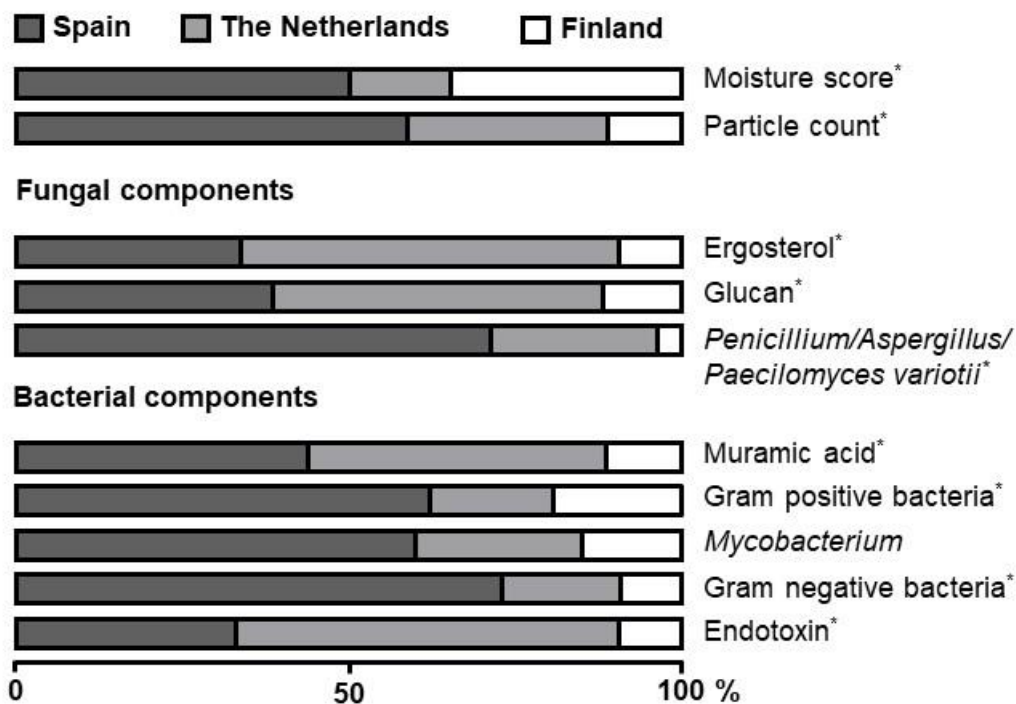


Figure 2. Comparison of average moisture damage score, particle count and microbial components in Spain, The Netherlands and Finland. Figure presents relative contribution of each country to facilitate the comparison between different analytes. Star (*) indicates a statistically significant difference ($p > 0.05$, Kruskal-Wallis) between the countries.

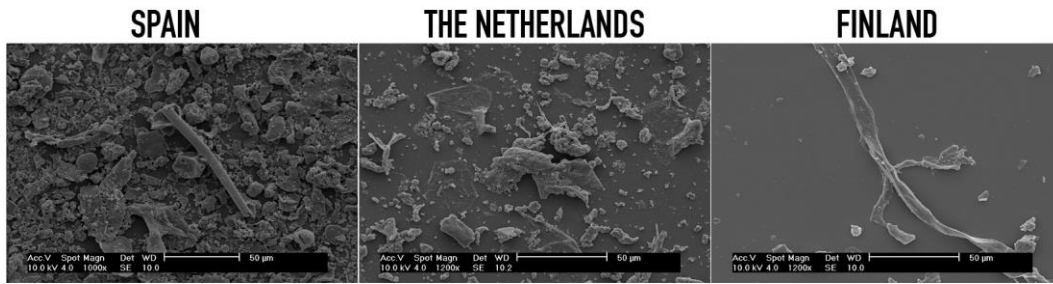


Figure 3. Scanning electron microscopy (SEM) pictures of settled dust samples from moisture damaged schools in Spain (1000x), The Netherlands (1200x) and Finland (1200x).

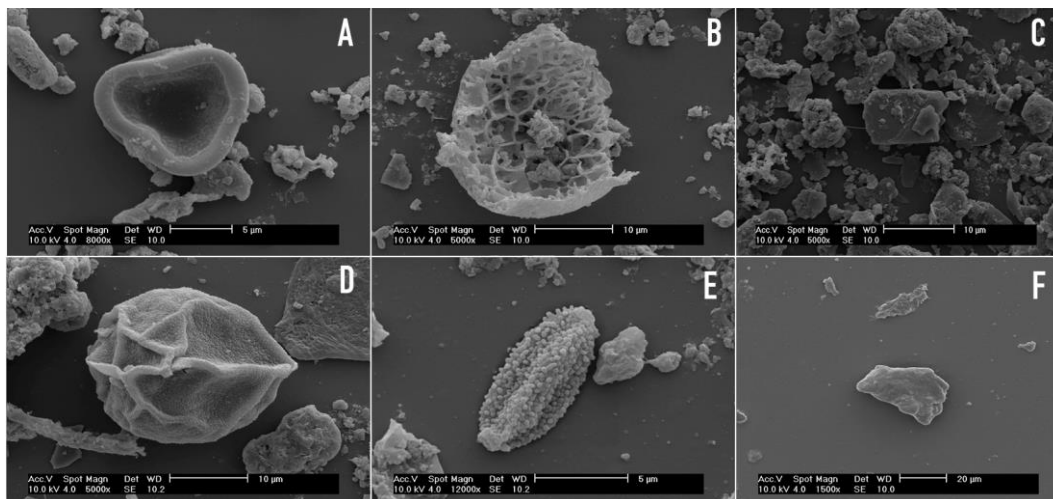


Figure 4. Scanning electron microscopy (SEM) pictures of biological and geological components featured in settled dust samples from schools in Spain (A-C), The Netherlands (D, E) and Finland (F).

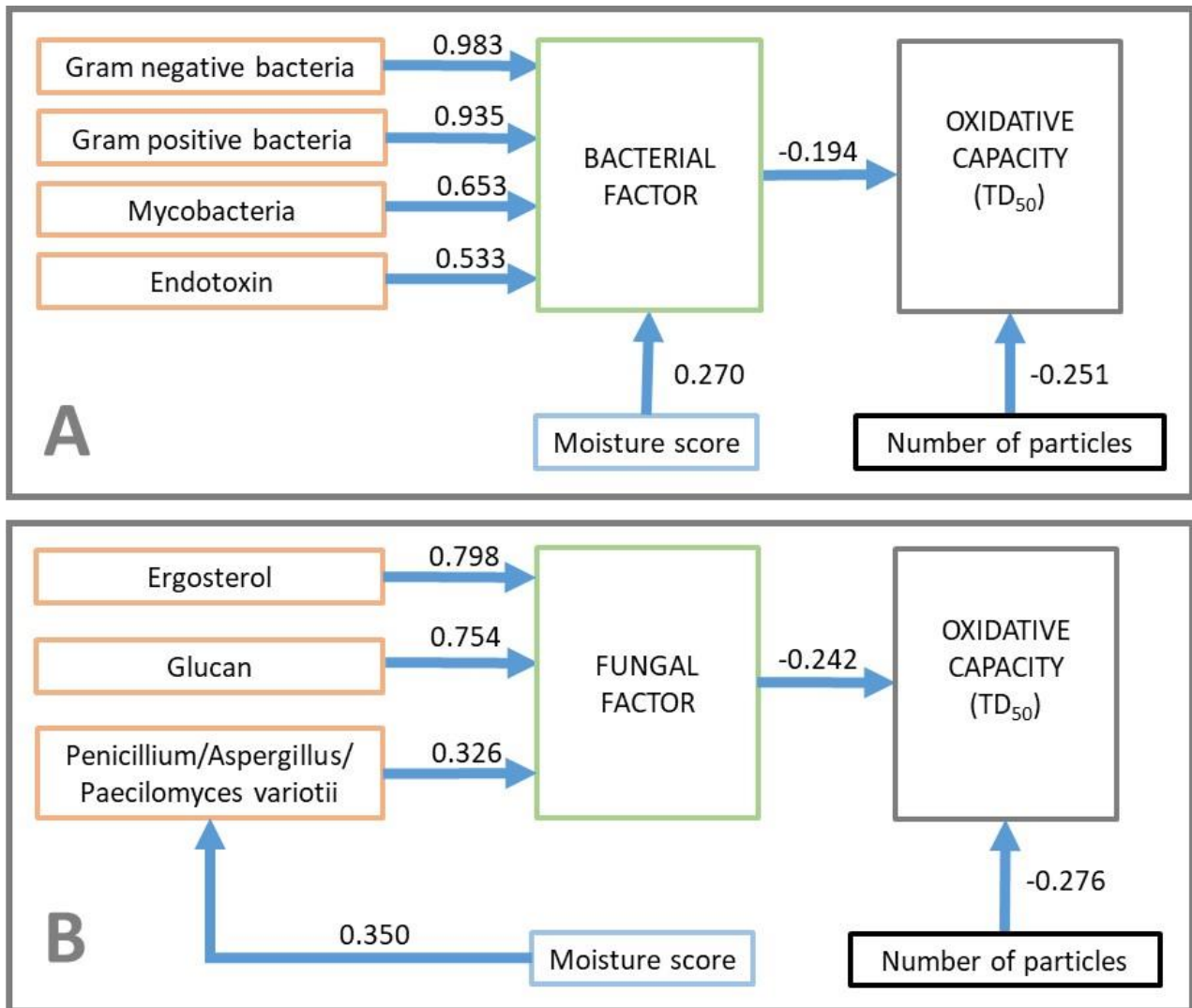


Figure 5. Outcome of structural equation modelling showing standardized estimates of effects between latent variable “Bacterial factor” (A) or “Fungal factor” (B) and oxidative capacity (expressed as toxic dilution TD₅₀, where low value indicates high oxidative capacity), moisture score of the school and number of particles in the sample. Coefficients are standardized linear regression weights, which can be used in examining the possible causal linkage between variables in the latent variable analysis (LVA) modelling approach. Standardized estimates follow normal distribution with mean of zero and variance of one. In addition to the variables in the figure, also country of origin had a significant effect on oxidative capacity, number of particles, moisture score and both microbial factors.

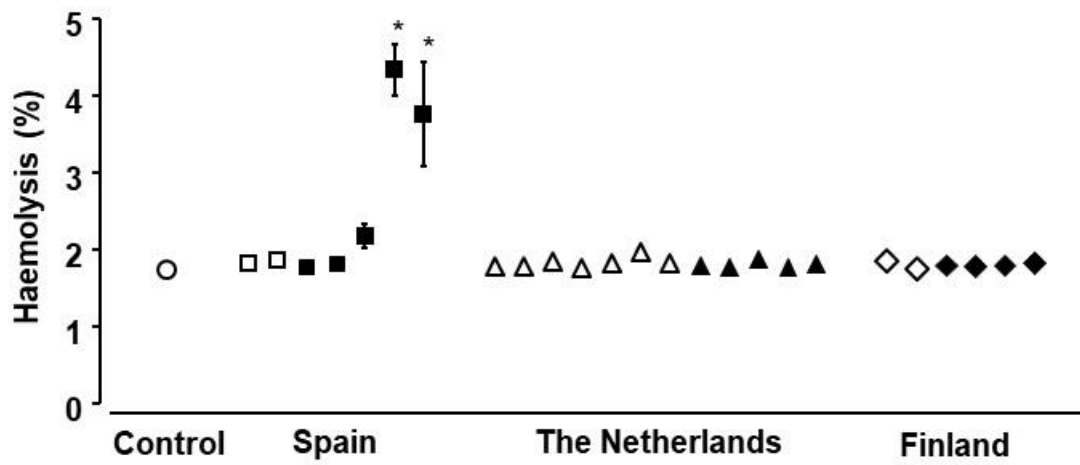


Figure 6. Average (\pm SE) haemolysis after exposure to settled dust collected from 14 moisture damaged (black symbols) and 11 non-damaged (white symbols) schools in Spain (\square), The Netherlands (Δ) and Finland (\diamond). Red blood cells from two donors were analysed in triplicate (N=6). Statistically significant difference ($p < 0.05$, Kruskal-Wallis) compared to control (Dulbecco's Phosphate Buffered Saline, PBS) is indicated with star (*).

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