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RESEARCH ARTICLE

Toxicity of airborne dust as an indicator of moisture problems in school buildings

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ABSTRACT

Moisture-damaged indoor environments are thought to increase the toxicity of indoor air particulate matter (PM), indicating that a toxicological assay could be used as a method for recognizing buildings with indoor air problems. We aimed to test if our approach of analyzing the toxicity of actively collected indoor air PM *in vitro* differentiates moisture-damaged from non-damaged school buildings. We collected active air samples with NIOSH Bioaerosol Cyclone Samplers from moisture-damaged (index) and non-damaged (reference) school buildings (4 + 4). The teachers and pupils of the schools were administered a symptom questionnaire. Five samples of two size fractions [Stage 1 (>1.9 μm) and Stage 2 (1–1.9 μm)] were collected from each school. Mouse RAW264.7 macrophages were exposed to the collected PM for 24 h and subsequently analyzed for changes in cell metabolic activity, production of nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-6. The teachers working in the moisture-damaged schools reported respiratory symptoms such as cough ($p = 0.01$) and shortness of breath ($p = 0.01$) more often than teachers from reference schools. Toxicity of the PM sample as such did not differentiate index from reference building,s but the toxicity adjusted for the amount of the particles tended to be higher in moisture-damaged schools. Further development of the method will require identification of other confounding factors in addition to the necessity to adjust for differences in particle counts between samples.

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KEYWORDS
Indoor air; moisture damage; PM; school; toxicity; *in vitro*

Introduction

Adverse health effects caused by exposure to the indoor air of moisture-damaged buildings are well known even though the exact causative agents and mechanisms behind the observed effects are still obscure (WHO, 2009). Observations of moisture damage are common both in the Finnish building stock and elsewhere, and are particularly problematic in public buildings such as schools and hospitals (Haverinen-Shaughnessy et al., 2012; Hellgren & Reijula, 2011; Mudarri & Fisk, 2007). Due to the high number of buildings affected by moisture problems at some point in their lifecycle, it is a crucial challenge to identify the buildings that are most likely to cause health problems to the occupants in order to be able to prioritize the renovation efforts.

Despite international guidelines covering both biological and chemical pollutants in indoor air (WHO, 2009, 2010), there is still a basic dilemma of not knowing any reliable health-based method which could be used to estimate the severity of the moisture damage. Identifying the harmful characteristics of the exposure is challenging due to the fact that the indoor environment produces a complex mixture of fluctuating concentrations of a wide variety of components, many of which can potentially cause a harmful response *per se* or be surrogates for other pollutants. The current body of evidence suggests that simultaneous exposure to several exposure agents typically present in moisture-damaged environments exacerbates the effects of a single pollutant, whether it is by affecting the growth and metabolism of microbes (Murtoniemi et al., 2005; Penttinen et al. 2006) or changing the function of the immunocytes of the exposed individuals (Huttunen et al., 2004; Penttinen et al., 2005, Korkalainen et al., 2017).

The concentrations of airborne particles, microbial components or metabolites have all been suggested to be linked with indoor air problems in buildings (Cabral, 2010; Lappalainen et al., 2013; Nevalainen et al., 2015), but none of the approaches quantifying these components can unequivocally differentiate moisture-damaged buildings from non-damaged ones. Assessment of toxicological response to the indoor particulate matter (PM) with a relatively robust cell culture model could serve this purpose, as this approach has been used before when assessing the toxic potential of PM from moisture-damaged offices and schools, animal housing, house dust and emissions from waste handling

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Supplemental data for this article can be accessed here.

(Deschamps et al., 2014; Roponen et al., 2013; Kim et al., 2015; Huttunen et al., 2010). In addition to human and mouse cell lines, household air pollution and effects of moisture-damaged environment have also been studied *in vivo* with mice (Jussila et al., 2003), and *ex vivo* with lung slices and human primary cells (Bernasconi et al., 2010; Punsmann et al., 2013; Rylance et al., 2015).

With the aim of finding a sampling approach suitable for toxicological assays, we have previously assessed different ways of collecting indoor PM, and found that active sampling of relatively small (>1.9 µm) airborne particles was a promising approach to be studied further (Tirkkonen et al., 2016). In this study our aim was to test if analyzing the toxicity of actively collected, size-fractioned airborne PM could differentiate moisture-damaged from non-damaged schools in a cohort of eight buildings, linking measurements also to symptoms reported by occupants of the schools.

Materials and methods

Sampling

Four pairs of moisture-damaged (index) and non-damaged (reference) schools were included in the study. Schools were inspected by a civil engineer for observations of moisture damage prior to sampling. The sampling of the schools was organized pairwise (parallel sampling of one index and one reference school) during five months from January to May 2014. Each of the school pairs had similar construction, were located in the same geographical area and were sampled within a month. The number of pupils varied from 220 to 500 between the schools. All schools had similar ventilation system with mechanically controlled and filtered incoming and outgoing air, and were located either in city centers or population centers.

Airborne PM samples were collected actively with the NIOSH Bioaerosol Cyclone sampler (CDC/NIOSH/HELD, USA) at a flow rate of 3.5 L/min collecting particles in three size categories: Stage 1 (>1.9 µm, 1.5 ml tube), Stage 2 (1–1.9 µm, 1.5 ml tube) and Stage 3 [<1 µm, 0.45 µm polytetrafluoroethylene (PTFE) filter]. Only Stage 1 and 2 samples were analyzed due to insufficient amount of sample material for toxicological assay in Stage 3 samples (Tirkkonen et al., 2016).

The sampling was conducted in classrooms during nine work days, mostly during occupied hours, within a two week period (~66 active hours) in each school. Four classrooms from each school were sampled at a height of 1.5 m, one of them with duplicate samplers, altogether resulting in five sets of samples per school. All samples were stored frozen (−20 °C) until toxicological analysis.

Health questionnaire

Before sampling, the teachers and pupils of the schools were administered questionnaires concerning their health status and indoor air-related symptoms. The teachers and pupils' parents received detailed information on the study and on data protection issues; health questionnaires applied had previously (for the SISU School Intervention Study) been approved by Research Ethics Committee, Hospital District of Northern Savo, Kuopio, Finland, and identical questionnaires were applied in this current study. The parents of the pupils were instructed to fill out the questionnaire together with the child. The questionnaires consisted of questions that were aimed at assessing the indoor air conditions at home and in the school, prevalence of allergic diseases such as asthma, atopy and allergic rhinitis, respiratory symptoms such as wheeze and rhinitis as well as confounding factors such as tobacco smoking and observations of mold in the home.

Altogether 2898 questionnaires were delivered; 165 to the teachers and 2733 to the pupils of the schools. The response rate was clearly lower for the pupils (21%) than for the teachers (63%) but variation between the schools was small (19–23% for pupils and 61–64% for teachers). Due to the low response rate of the pupils, only the results derived from teachers questionnaires are presented here. The majority of the teachers in both index and reference schools were females who were not exposed to tobacco smoke or moisture damages in their home environment (Table 1).

Table 1. Baseline characteristics of the teachers from the index ($N = 56$) and reference ($N = 47$) schools.

Characteristic	Index schools (%)	Reference schools (%)	*p*-Values
Gender			
Female	82%	72%	0.234
Allergic diseases (ever)			
Asthma	18%	21%	0.662
Allergic rhinitis	55%	60%	0.667
Allergic/atopic rash	54%	49%	0.639
Allergic ophthalmitis	36%	37%	0.951
Current moisture and mold damage in the home (past 12 months)			
Water damage	2%	0%	0.453
Visible mold at home	2%	2%	1.000
Mold odor at home	2%	4%	0.591
Damages renovated during the last 2 years	5%	2%	0.624
Other exposures			
Pet keeping	27%	11%	**0.039**
Current smoker	6%	9%	0.703

Bold value represents statistically significant difference between index and reference school ($p < 0.05$.

Number and estimated mass of particles

The number of particles was measured with PAMAS SVSS (PAMAS GmbH, Rutesheim, Germany) with sensor SLS-25/25 (0.5 µm detection limit, max 13 000 particles/ml) and PMA analyzing software. A 50 µl sample of the particulate suspensions from Stages 1 and 2 was diluted in ultraclean water (1:5000) and the particle count was measured in triplicate. The particle concentration in ultraclean water and cell culture media was subtracted from the results. The number of particles is expressed per volume (m^3) of sampled air. Based on the numbers and size distribution of the particles in the sample, we estimated the total particle mass by using standard aerosol density and particle radius derived from measured particle diameter.

$$\text{Total particle mass} = \sum_{i=1}^{n} (V_i \cdot \rho_i)$$

n = particle count, $V_i = 1/6\ \pi d^3$, d_i = diameter of i-th particle,

ρ_i = density of i-th particle = 1000 kg/m^3

Cell culture, sample preparation and exposure of mouse macrophages

RAW 264.7 mouse macrophages (ATCC, Rockville, MD) were cultured as described earlier by Tirkkonen et al. (2016). Briefly, the cells were cultured in a humid atmosphere and in 5% CO_2 in 37 °C using cell culture media (RPMI 1640, Roswell Park Memorial Institute, Gibco, Paisley, UK) supplemented with 10% of heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin-streptomycin. A day before exposure, 0.2×10^6 cells in 0.5 ml aliquots were seeded in 24 well plates (all from Sigma, St. Louis, MO). PM samples were stored in −20° C for a maximum of two weeks and defrosted at room temperature before suspending the particles into 1 ml of cell culture media by sonicating for 15 minutes and shaking for another 15 minutes. The samples were warmed to 37 °C and a dilution series (1:2–1:16) was prepared using complete cell culture media. Before the exposure experiment, the cell culture media was replaced with fresh media and the cells were exposed for 24 hours to four dilutions of dust suspension in duplicate. Similarly treated cell culture media was used as a negative control and lipopolysaccharide (LPS) as a positive control. After the exposure, the cells were gently scraped from the bottom of the culture wells and resuspended into cell culture media. A sample for testing cell metabolic activity was taken and the rest of the cell suspension was centrifuged (5 min at 6082 × *g*, 4 °C) to separate the cells from the media. The concentration of nitric oxide (NO) was measured from the cell culture media and the rest of the media was stored in −80 °C for the analysis of cytokines TNFα and IL-6.

Toxicological analyses

Cell metabolic activity

Cell metabolic activity (CMA) was analyzed by measuring the activity of the cell mitochondria with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay. The MTT assay is based on the ability of the living mitochondria to change color of the tetrazolium salt from yellow to purple. The procedures described earlier (Hansen et al., 1989) were modified by shortening of incubation time to four hours (in +37 °C) after adding sodium dodecyl sulfate (SDS)-buffer. After incubation, the absorbance at 570 nm was measured with a multilabel plate reader (Victor^3, PerkinElmer, Finland) and the cell metabolic activity was calculated by comparing the absorbance of the exposed samples to control cells.

NO production

NO production was measured with a protocol based on the Griess-reagent, as described earlier by Green et al. (1982). NO produced in cell culture media is oxidized to nitrate (NO_3^-) and nitrite (NO_2^-). Nitrate produced by cells can be reduced to nitrite by the Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid). The reagent reacts with arylamine and creates an aniline-colored azo cromophore. Absorbance was measured at 540 nm with a multilabel plate reader (Victor^3, PerkinElmer, Finland) and compared with a standard curve of sodium nitrite.

Cytokine production

Concentration of pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 were both determined with enzyme-linked immunosorbent assays (ELISA) according to manufacturer's instructions (R&D Systems, Minneapolis, MN). Absorbance at 450 nm was measured with a multilabel plate reader (Victor^3, PerkinElmer, Finland) and compared with standard curves.

Statistical analysis

For each endpoint, the average of the five samples collected from each school were compared within the pairs of schools ($N = 5$ per school). For the comparison of the index and reference schools as a group, the average of the five samples was used as a representation of the toxicity level of the school ($N = 4$ per group of schools). The normality of the data was tested with the Shapiro–Wilk test. The statistical significance of the difference between blank and exposed samples was tested with a nonparametric Mann–Whitney *U*-test and Wilcoxon Matched Pairs Signed-Rank Test (SigmaPlot^{TM} version 12.3., Systat Software Inc., San Jose, CA). Basic analyses of differences in individual symptoms reported by teachers in index versus reference schools done using simple cross-tabulation and χ^2-test (IBM SPSS Statistics, Armonk NY, version 20.0). Symptomatic was defined as reporting a given symptom at least once per week over a recall period of three months.

Results

Health questionnaire

The health of the teachers appeared to be affected by the moisture-damaged indoor environment; teachers working in the reference buildings ($N = 47$) reported their overall health status to be better than teachers working in index schools ($N = 56$, $p = 0.09$). Teachers in the index schools reported more absence days due to respiratory infections ($p = 0.02$) as well as respiratory symptoms such as rhinitis ($p = 0.09$), sore or dry throat ($p = 0.01$), productive cough ($p = 0.01$) and shortness of breath ($p = 0.01$). In addition, irritation of eye ($p = 0.04$) and skin ($p = 0.02$) and general symptoms such as fever ($p = 0.05$), muscle pain ($p = 0.06$), fatigue ($p = 0.01$) and concentration difficulties ($p = 0.01$) were reported more often by the teachers from the index schools than teachers from the reference schools.

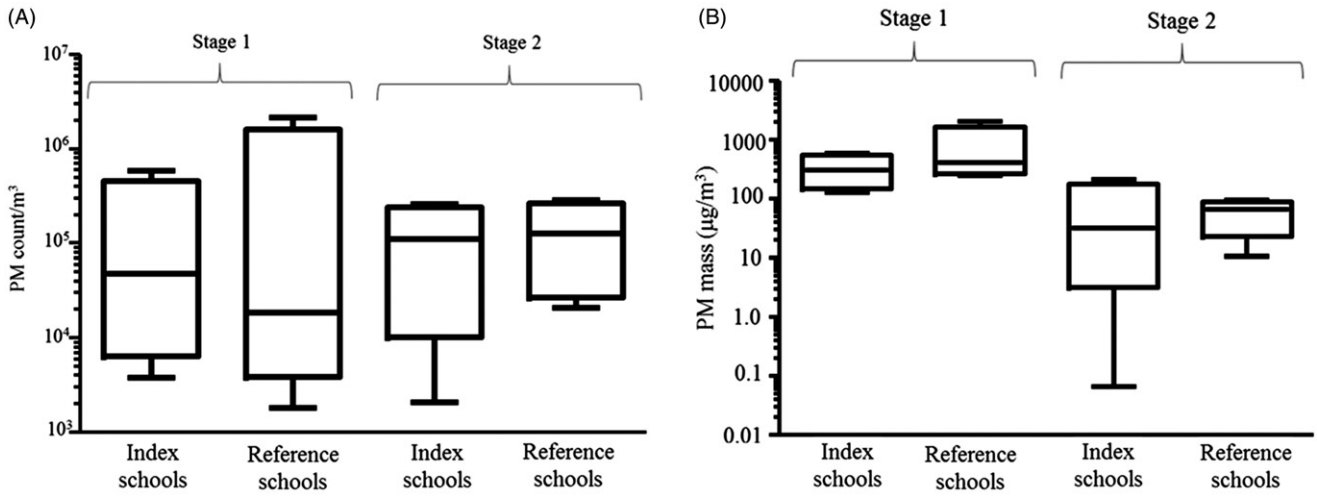

Figure 1. (A) Total particle count of particles found in the aqueous suspensions from Stage 1 and Stage 2 of the NIOSH bioaerosol sampler over the size range 0.5–20 µm (count/m^3) and (B) calculated estimated mass (µg/m^3) of PM collected from the index ($N = 4$) and the reference schools ($N = 4$) with the NIOSH Bioaerosol Sampler (Stage 1 and Stage 2).

Size distribution, number and estimated mass of particles

All studied schools had a similar particle mass distribution based on the calculation of the particle mass in each aerosol sampler stage using the PAMAS particle analyzer (Figure 1(B)). However, the total count of particles in the aqueous suspensions varied between the schools, one reference school having clearly more particles compared to the rest of the schools (Figure 1(B)). The particle size distributions in the aqueous suspensions are shown in the Supplemental data, Figure S1. The samples collected during winter months typically had fewer particles compared to the late spring samples. Statistically significant differences in the median number concentration or the estimated mass concentration of the PM of the schools were not found. The median total particle count of Stage 2 samples was slightly higher than Stage 1 samples, whereas the estimated mass of the Stage 1 samples was clearly higher than Stage 2 samples (Figure 1).

Immunotoxicological effects of the PM samples

Stage 1 (>1.9 µm) samples from both index and reference schools induced dose-dependent and statistically significant increase in the production of NO, TNFα and IL-6 as well as decrease in cell metabolic activity compared to blank samples. The response to the smaller size fraction PM of Stage 2 (1–1.9 µm) particles was less pronounced, reaching statistical significance more often in index schools and only at the highest dose levels. Toxic responses to total amount of neither Stage 1 nor Stage 2 PM differentiated the index buildings from the reference buildings (Figure 2). Pairwise comparison of the schools showed some statistically significant differences, but in both directions; the toxicity of the sample from index school compared to the paired reference school was higher in two cases and lower in two cases (Supplemental data, Tables S1 and S2).

Relative toxicity

In an effort to compare the relative immunotoxicological activity of the samples by adjusting for differing amounts of particles, we also related the measured toxicity to the estimated mass of PM. The samples were first divided into quartiles based on the estimated mass in the sample (Table 2) and then the immunotoxicological activity of the index and reference samples was compared within the quartiles. The comparison showed a trend for higher production of inflammatory mediators and lower cell metabolic activity for the index schools in first three quartiles although the differences between the quartiles were not statistically significant. In the highest quartile the situation was evened out or even reversed (Figure 3). The same trend was seen especially in Stage 1 samples, but was visible also for TNFα and IL-6 in Stage 2 samples.

Discussion

Municipalities are in a dire need of a tool that would allow prioritizing renovations in moisture-damaged public buildings such as schools in their area. Using a toxicity assay to support the findings of technical investigation and microbial measurements could be of special interest in the phase of defining the urgency of actions needed. The possible exposure agents in moisture-damaged buildings are various, including materials such as allergens, pollen, arthropods, chemicals, VOCs, fungal and bacterial components and spores. These components, along with substances such as dirt, skin epithelia and soot can all be found in household dust, which comprises a highly complex mixture of constituents with different toxic potentials. Instead of measuring the concentration of the single components, an integrated effect such as the toxicological response in a cell model could be useful in assessing the possible health risk of indoor environments. The synergy between the components present in moisture-damaged indoor environments has been shown to

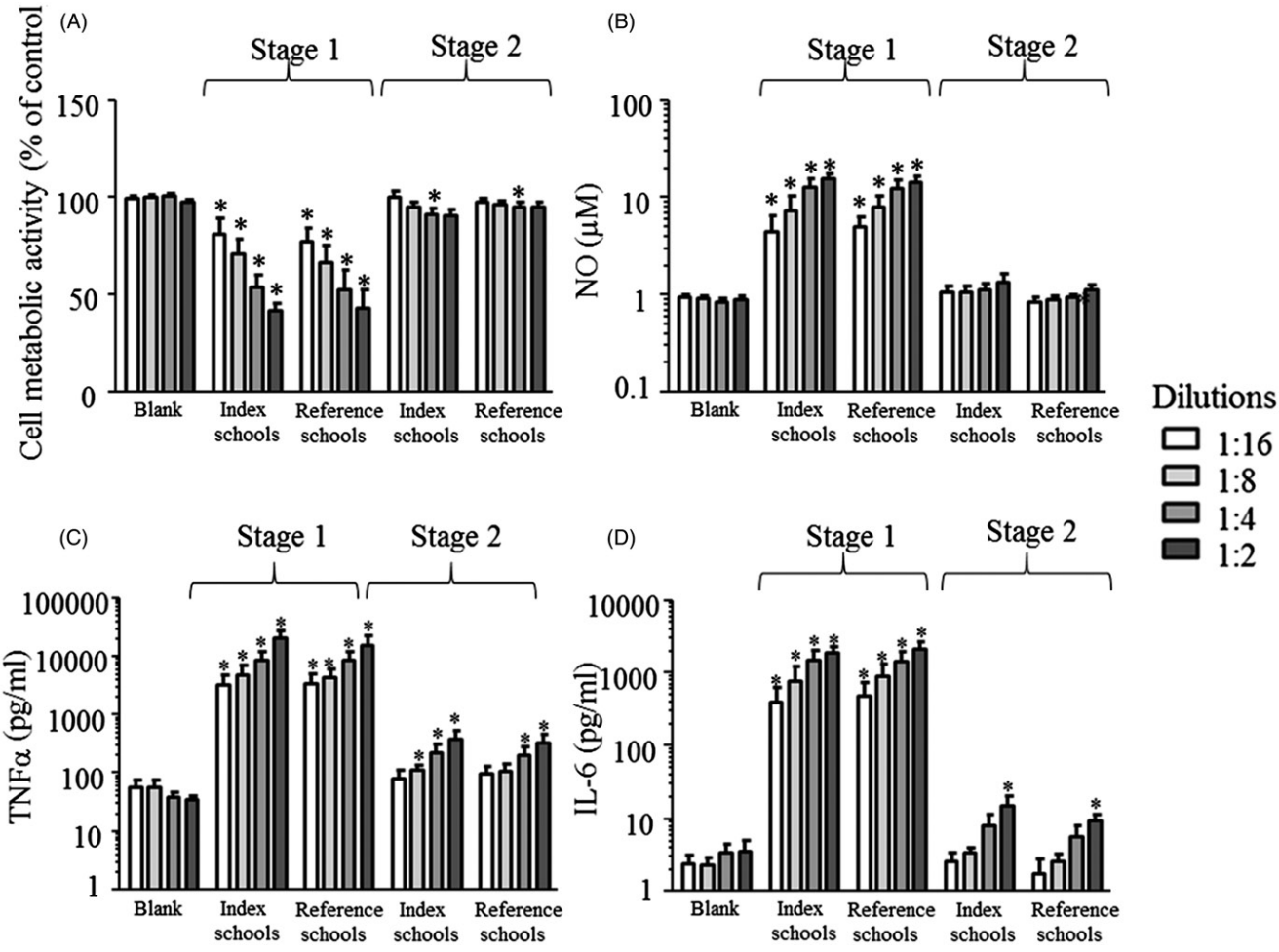

Figure 2. Average (± SEM) (A) Cell metabolic activity and production of (B) NO, (C) TNF-α, and (D) Interleukin (IL)-6 in mouse RAW264.7 macrophages after 24 h exposure to increasing doses (dilutions from 1:16 to 1:2) of PM collected with NIOSH Bioaerosol Sampler (Stage 1 and 2) from index schools ($N = 4$) and of reference schools ($N = 4$). Star (*) indicates a statistically significant difference compared to respective blank samples ($N = 16$) (Mann–Whitney test, $p < 0.05$).

Table 2. Estimated mass of sample material divided in quartiles.

Quartile	Stage 1 (μg/ml)	Stage 2 (μg/ml)
1st	0–440	0–26.8
2nd	440–900	26.8–90.4
3rd	900–2100	90.4–324
4th	2100–14 500	324–1443

increase the effects of exposure, further stressing the importance of a holistic approach (Korkalainen et al., 2017). We evaluated the effectiveness of *in vitro*-based immunotoxicological assay in differentiating moisture-damaged from non-damaged environments in a cohort of eight schools. Our results show that comparing the responses to total collected PM, the specificity of the assay is not high enough to identify the schools with moisture problems, where the teachers reported more symptoms. However, relating the responses to the amount of PM in the sample improved the results, indicating that further standardization of the sampling conditions and taking other confounding factors into account might improve the specificity of the method. Overall, recognition of the dominant factors influencing the responses of the cells – such as other indoor sources of biologically reactive PM – is highly important for further development of this method.

In this study, we chose to focus on sampling schools instead of apartments firstly because schools are an indoor environment where children and school staff spend a significant amount of their time on a daily basis (Salo et al., 2009), making it a very relevant environment for public health. Secondly, we wanted to avoid the variance in microbial quality and quantity typically seen between homes due to differences in aerosol sources (e.g. pets, indoor smoking and fireplaces) (Rintala et al., 2012). Interestingly we saw a trend for lower amounts of airborne PM in the moisture-damaged schools. A similar difference was seen also in a previous study of two schools, where the amount of dust in a moisture-damaged school was found to be lower regardless of the sampling method (Tirkkonen et al., 2016). Higher counts of airborne PM have previously been linked with deteriorated indoor air quality (Lappalainen et al., 2013), which is conceivable given the possible additional sources of particles such as microbial growth in moisture-damaged environments. However, this association might be reversed, for example, by increased cleaning and ventilation in schools with moisture damage. In theory, both the amount and the potency of the sample material should affect the outcome of the exposure. In our study, the differentiation between the schools was improved by taking into account the amount of

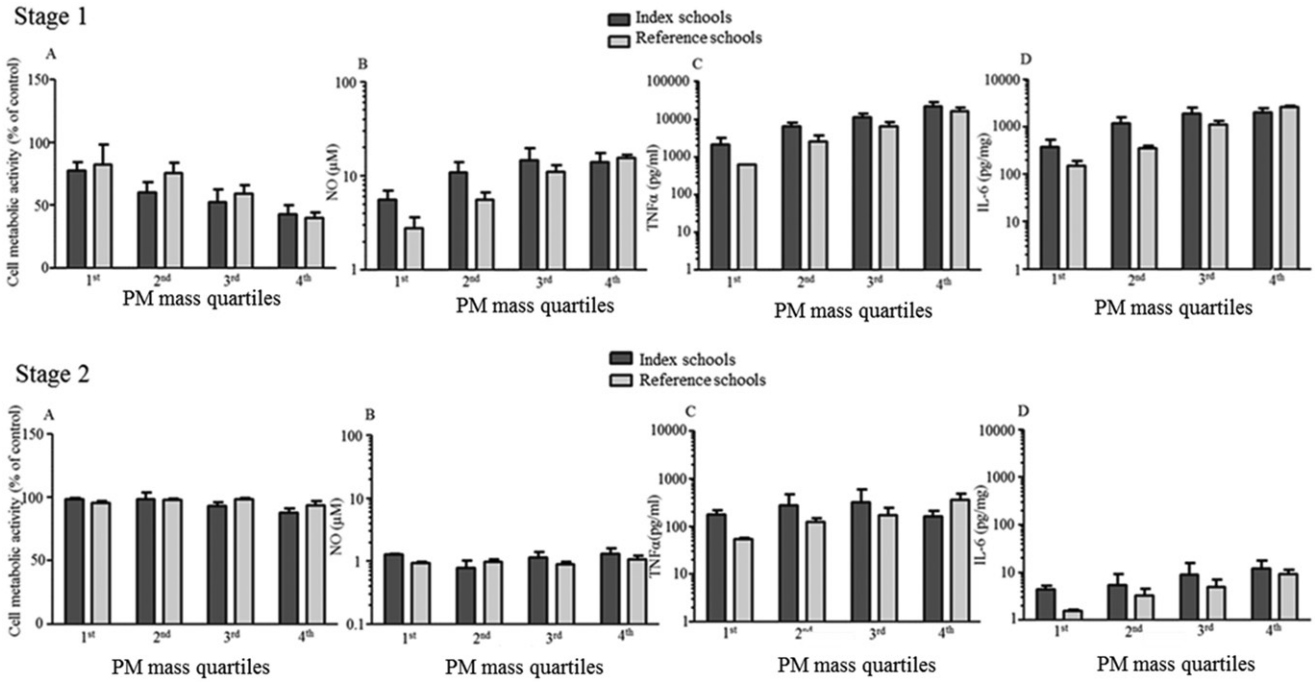

Figure 3. Average (± SEM) (A) Cell metabolic activity and production of (B) NO, (C) TNF-α, and (D) Interleukin (IL)-6 in mouse RAW264.7 macrophages after 24 h exposure to increasing doses [PM mass divided in quartiles] of dust collected with NIOSH Bioaerosol Sampler (Stage 1 and 2) from index schools ($N = 4$) and reference schools ($N = 4$) samples. The differences between the treatments were not statistically significantly different (Mann–Whitney –test).

the sample, i.e. comparing the responses to equal masses of the PM. This suggests that PM collected both from moisture-damaged and non-damaged environment induces immunotoxicological responses, but at lower dose levels the signal from moisture damage tends to be stronger.

Collecting size segregated airborne PM is justified considering that the particles smaller than 10 μm in their diameter can reach a particular region of the respiratory system and potentially deposit there (Heyder, 2004). The NIOSH Bioaerosol Sampler used in this study was able to collect sufficient amount of sample material for the toxicological analyses in both size categories (Stage 1, >1.9 μm and Stage 2, 1–1.9 μm) although responses to Stage 2 samples were clearly lower than Stage 1 samples. Problems with yielding sufficient sample amounts for reliable toxicity testing were seen also earlier with this and other tested methods (Tirkkonen et al., 2016), which drives the need to sample with higher flow or extend the sampling time in future experiments.

The effect of season was seen to some extent within the cohort of eight schools. Seasonal differences in immunotoxicological potency of particular matter has been reported earlier for outdoor air as well as indoor air (Happo et al., 2013; Jalava et al., 2015). However, the seasonal effect does not explain the lack of specificity seen in this study, as each pair of moisture-damaged and non-damaged schools were sampled within the same month and the schools were also located in the same area.

A clear strength of our study design was the pairwise selection of schools which was based on the technical investigation. The data concerning teachers showed that symptoms were indeed reported more often in the moisture-damaged schools than non-damaged schools, even though the possibility of reporting bias cannot be excluded. In Finland, schools that are affected by severe moisture damage will typically be either closed or the activity will be moved away from the affected areas. This means that recruiting occupied school buildings in a study will leave out the worst cases, which may have contributed to the difficulties in separating moisture damaged from non-damaged school environments in this study. We acknowledge that calculating the mass of the sample based on the particle count and size distribution produces an estimate rather than measure of the mass of sample material. However, in this case using the estimate is appropriate considering that pairwise comparisons between the index and the reference schools were made using quartiles of the data rather than exact values and applying the same calculations for both index and reference buildings.

Conclusions

In conclusion, we found that adjustment for differences in the amount of particles was necessary to improve the ability of the tested toxicological assay to differentiate the moisture damage buildings from non-damaged buildings. Along the number of airborne particles, also sampling season and identification of other possible confounding factors are critical points to be addressed in further development of the method.

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