

Total viable molds and fungal DNA in classrooms and association with respiratory health and pulmonary function of European schoolchildren

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Abstract

Indoor molds are associated with adverse respiratory effects in children. Although schools are important exposure sources of molds, objective measurements were more often taken in homes. Our aim was to assess indoor molds in schools and related effects on schoolchildren health. The Health Effects of the School Environment study (HESE) included 21 schools (46 classrooms) in Italy, Denmark, Sweden, Norway, and France and 654 schoolchildren (mean age 10 yr). Information on schoolchildren was collected by standardized questionnaires. Measurements of total viable molds (VM, colony-forming units, cfu/m³) and total/specific fungal DNA (cell equivalents, CE/g dust) were taken inside all classrooms in the cold season during normal activities, using the same standardized methodology. Pulmonary function tests were performed on 244 pupils. VM (mean, 320 cfu/m³) and total fungal DNA (geometric mean, $2.2 \times 10^5 \pm 2.1$ CE/g dust) were detectable in all classrooms. The levels were significantly higher in buildings with mold/dampness problems. VM, but not fungal DNA, were inversely related to ventilation rate. VM exceeded the maximum standard of 300 cfu/m³ in 33% of the classrooms. In the past 12 months, dry cough at night (34%) and rhinitis (32%) were the mostly reported. Children exposed to VM levels ≥ 300 cfu/m³, compared with those exposed to lower levels, showed higher risk for past year dry cough at night (odds ratio, OR: 3.10, 95% confidence interval, CI: 1.61–5.98) and rhinitis (OR: 2.86, 95% CI: 1.65–4.95), as well as for persistent cough (OR: 3.79, 95% CI: 2.40–5.60). *Aspergillus/Penicillium* DNA was significantly positively associated with wheeze, and *Aspergillus versicolor* DNA with wheeze, rhinitis, and cough. There were significant inverse associations of *Aspergillus versicolor* DNA with forced vitality capacity (FVC) and *Streptomyces* DNA with both FEV₁ and FVC. In conclusion, indoor VM and fungal DNA were commonly found in monitored European schools and adversely related to respiratory health. Schools should be routinely tested through both culturable and non-culturable methods for global indoor molds' evaluation.

Molds exposure and related health problems are common indoor environmental health issues. Molds exposure was associated with higher risk for respiratory infections, symptoms, and diseases in children and for severe airway

hyperresponsiveness and peak expiratory flow variability in children with asthma. Sensitivity to fungi is a known significant cause of allergic diseases since a long period. Most of the studies were based on visual molds/dampness or mold

odors, as a surrogate for exposures, but the accuracy of questionnaire reports has been challenged (1). Fungi are also present in indoor environments with no reported damp and health outcomes may vary by fungal *genera* (2).

Children spend much time in the schools that are densely populated, making the task of maintaining an acceptable indoor air quality (IAQ) more difficult than in many other indoor environments. Children are more likely to suffer from the consequences of indoor pollutants because they are still physically developing and breathe more than adults during quiet breathing. Although there is evidence that schools are prime candidates for fungal colonization (3), few studies have systematically examined the mycoflora of school buildings and related effects on schoolchildren health. Objective measurements were taken more often in residential settings to assess health effects mainly in asthmatic or atopic children or infants at higher risk of asthma. Most of the studies used culturable methods that detect only live fungi. Viability is important for allergenic expression (many components are released in larger amounts during germination/growth) but non-viable molds (VM), as well as their components, may also have toxic/allergenic properties (4). Recently, non-culture-based methods were developed to measure mold markers in dust/air (i.e., ergosterol), endotoxins, microbial volatile organic compounds, or also fungal DNA (5–10). The benefits of using DNA are time-saving and accuracy, as well as the possibility of identifying also dead or dormant organisms. However, both culturable and non-culturable methods may be useful, because they complement each other, allowing a more comprehensive picture of fungal presence.

The aim of this study was to assess IAQ in European schools as regards total VM and fungal DNA and associated effects on schoolchildren respiratory/allergic conditions.

Methods

Details of the cross-sectional Health Effects of the School Environment (HESE) pilot study have been previously reported (11). Briefly, the study involved six units in five European countries (Italy, France, Norway, Sweden, and Denmark). Twenty-one schools (46 classrooms) with heterogeneous characteristics were selected. The study, approved by the ethical committees of each institution, was carried out in the heating season of 2004–2005 using the same standardized procedure, during a full week in each location.

Information on school/classrooms characteristics was collected through a standardized questionnaire filled in by the teachers. Moreover, detailed inspections were made by technicians, and data on school buildings/classrooms (including construction materials, type of ventilation system, and the presence of visible molds/dampness) were recorded.

Environmental measurements

During normal activities (full classroom) and under representative conditions, standard measurements (including temperature, relative humidity (RH), particles, nitrogen dioxide,

carbon dioxide, ozone, formaldehyde, VM, and dust) were taken, both inside each classroom and outside each school, by personnel from a single center, where the instruments had been calibrated (Uppsala). Data were analyzed at the IVL Swedish Environmental research Institute in Gothenburg (Sweden) and at the Department of Occupational and Environmental Medicine, Orebro (Sweden).

Temperature, RH (%), and carbon dioxide (CO₂, p.p.m.) were measured by Q-Track™ IAQ Monitor (TSI Incorporated, ST Paul, Minnesota, USA). Personal outdoor air supply rate (ventilation rate, l/s per person) was computed from the graphically estimated equilibrium of CO₂ concentration as follows:

$$A = P / (C_{\text{mean}} - C_0) \times 10^6 / 3600$$

where P is the personal emission rate of CO₂ in l/s for sedentary office work at sea level (18 l/h) and C_{mean} and C_0 are the mean CO₂ levels in the classroom and outdoor, respectively.

Total VM were sampled on 25-mm nucleopore filters (pore size of 0.4 μm) with a sampling rate of 1.5 l/min for 4 h and cultivated on two different media (12). The detection limit was of 30 colony-forming units (cfu) per m³ of air.

Dust for analyzing fungal DNA was collected by a vacuum cleaner (Siemens Super XS) provided with a special dust collector (ALK Abello, Copenhagen, Denmark) equipped with a Millipore filter (pore size of 6 μm). Vacuum cleaning was performed for 2 min on the floor and 2 min on desks or chairs (6). From each classroom, two samples of settled dust (from the corridor side and the window side) were collected and sieved through a 0.3-mm-mesh screen to obtain the fine dust that was stored at –20°C until DNA extraction. DNA was extracted from 100 mg dust using the YeaStar™ Genomic DNA Kit (Zymo Research, Orange, CA, USA). Five multiplex reactions were performed in five separate tubes targeting the following species: total fungal DNA, *Aspergillus* spp and *Penicillium* spp (*Asp/Pen* DNA), *Aspergillus versicolor* (*A. versicolor* DNA), *Stachybotrys chartarum* (*S. chartarum* DNA), and *Streptomyces* spp (*Streptomyces* DNA). Primers and probes for total fungal DNA, *A. versicolor* and *S. chartarum* DNA are in the region of internal transcribed spacer 1, 5.8 S rRNA, and internal transcribed spacer 2. Primers and probes for *Asp/Pen* DNA are in the gene for 28S rRNA and for *Streptomyces* DNA in the gene for 16S rRNA. Amplification and detection were performed on a 7300 Real-time PCR Instrument (Applied Biosystems, Foster City, CA, USA) in the Taqman® Universal Master Mix (Applied Biosystems). Standard curves were created for respective analysis using total genomic DNA extracts from pure cultures and were quantified using limiting dilution analysis (13). The reaction targeting *A. versicolor* simultaneously amplified an internal positive control that was used to detect PCR inhibition. The oligonucleotides used for amplification and detection were designed using the software Primer Express 2.0 (Applied Biosystems). The molds level was calculated by fungal cell concentrations for each target mold or molds group. The final result is presented as cell equivalents per gram dust (CE/g dust).

Symptoms/diseases

Data on children were collected through two *standardized questionnaires* filled in by the pupils and their parents, respectively. For the present study, we considered the following outcomes: (i) past year wheeze, (ii) past year dry cough at night (apart from common cold or chest infection), (iii) past year rhinitis (sneezing or runny/blocked nose apart from common cold or flu), and (iv) any current persistent cough (for 4 or more days per week, apart from common colds). Among children with both self-report and parental report (68% of total), the outcomes were present when reported by either children or parents or absent when unreported by both children and parents. Information on children with only self-report (16.4%) or only parental report (15.6%) was also included in the data set to retain the highest number of children and countries in the analyses. This decision produced unbiased results (11). Overall, health status was derived for 654 children.

Clinical tests

Non-invasive clinical tests were performed on five randomized selected pupils in each class. For the present analyses, we focused on forced expiratory volume in 1 s (FEV₁) and forced vitality capacity (FVC). Pulmonary function was measured in 224 children by experienced medical staff using a portable pocket spirometer (Spirobank™; IntraMedic Inc, Sweden). Six children of Reims were excluded from analyses concerning the pulmonary function, because of extremely low values of FEV₁ (likely due to lack of cooperation) that were identified as possible outliers (values with a distance to the next value in the distribution exceeding one-third of the total range of values).

Except for the children of Oslo (where data on height were missing), percent predicted FEV₁ and FVC were computed by using reference equations for European children and adolescents, which take into account gender, age, and height (14).

Statistical analyses

Statistical analyses were performed using SPSS-13.0 release and STATA-9.0 release.

Data on VM and fungal DNA were log-transformed to obtain approximately normally distributed residuals. Pulmonary function tests were normally distributed, and a transformation was not necessary. Based on the standard value suggested for good indoor air by the American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE) (15), VM exposure was defined as *elevated*, for concentrations exceeding 300 cfu/m³; otherwise, it was defined as *low*.

The associations between categorical variables were tested by the chi-square test in contingency tables. Comparisons of continuous variables were made by the analysis of variance (ANOVA), for normally distributed pulmonary function tests, and by non-parametric tests of Kruskal–Wallis (or Mann–

Whitney, for pairwise comparisons). The correlations between environmental continuous variables were analyzed by Kendall Tau-b rank correlation test.

The associations of health outcomes with molds were assessed by logistic regression models with each symptom/disease as the dependent variable (0 = absent, 1 = present) and log-transformed molds as continuous independent variables. VM were entered in the models either as binary exposure variable (*elevated* vs. *low*) or as continuous variable. Besides conventional logistic regression models, we fitted random intercept two-level models for binary dependent variables using the STATA gllamm (generalized linear latent and mixed models) command with logit link function, which estimates the maximum likelihood (numerical integration to evaluate the marginal log-likelihood and numerical derivatives to maximize it). Two hierarchical levels were considered: first level, the child, and second level, the classroom. Both crude and adjusted (for gender, age, passive smoking at home, and asthma in life) odds ratios (OR) and 95% confidence intervals (CI) were reported.

The associations between pulmonary function tests and molds were assessed by linear regression analyses with FEV₁ and FVC as continuous dependent variables and log-transformed molds as independent continuous variables. Both crude and adjusted (for gender, age, height, passive smoking at home, and any of lifetime asthma, dry cough at night, or cough) partial regression coefficients (*B*) and 95% CI were reported. Besides linear regression models, we fitted the generalized least-squares random-effects model (GLS-RE) with fixed effect of the classroom using the STATA xtreg command.

Two-tailed tests with a 5% level of significance were applied in all analyses.

Results

Mechanical ventilation (30% of classrooms) was used in all Swedish and in half Norwegian classrooms (all others were naturally ventilated). Based on ASHRAE standards for indoor comfort level, the temperature slightly exceeded the upper limit of 23°C (74.5°F) in 37% of the classrooms, RH was always below the upper limit of 65%, and the ventilation rate was lower than the minimum school level (8 L/s/p) in 70% of the classrooms. Poor ventilation rate was more prevalent in classrooms with natural than with mechanical ventilation (97% vs. 13%, *p* < 0.001).

Indoor molds

Both VM and total fungal DNA were detectable in all monitored classrooms. Total VM were significantly higher in southern than in northern centers. The lowest concentrations were in Sweden and Norway, and the highest in Reims. Total fungal DNA was significantly higher in Reims than in Siena and Uppsala, i.e., the two centers with the lowest mean values, and in Oslo than in Uppsala (Table 1). In general, also all specific fungal DNA were higher in Reims than in other centers (Table 2). *Asp/Pen* and *Streptomyces* DNA were

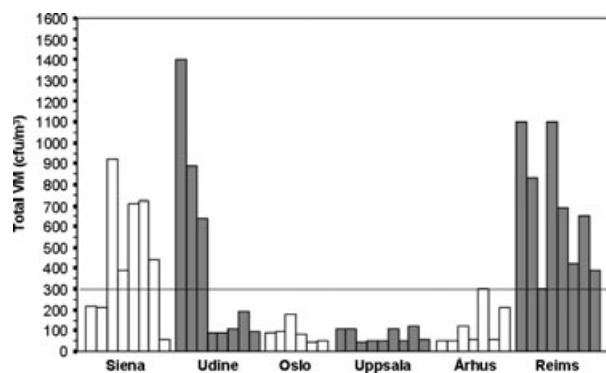
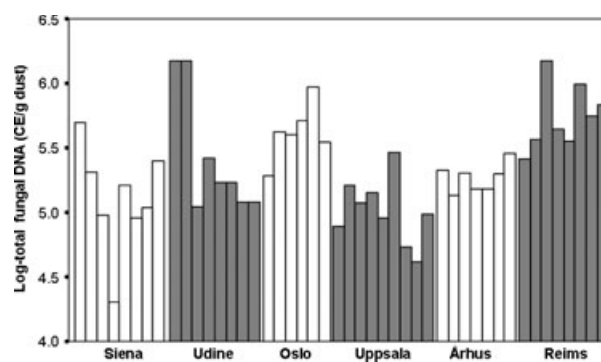
Table 1 Concentrations of total viable molds (VM, cfu/m³) and total fungal DNA (CE/g dust) in the classrooms

	VM	VM	Total fungal DNA
	Mean ± s.d.	GM ± GSD	GM ± GSD
Total (n = 46)	320 ± 351	180 ± 3.0	2.2 × 10 ⁵ ± 2.1
Siena (n = 8)	459 ± 299	350 ± 2.4	1.3 × 10 ⁵ ± 2.6
Udine (n = 8)	438 ± 493	245 ± 3.1	2.7 × 10 ⁵ ± 3.0
Oslo (n = 6)	91 ± 49	81 ± 1.6	4.2 × 10 ⁵ ± 1.7
Uppsala (n = 9)	78 ± 33	72 ± 15.4	1.0 × 10 ⁵ ± 1.8
Århus (n = 7)	120 ± 99	92 ± 2.1	1.9 × 10 ⁵ ± 1.3
Reims (n = 8)	685 ± 310	621 ± 1.6	5.5 × 10 ⁵ ± 1.8

GM, geometric mean; GSD, geometric SD.

detected in all classrooms, and *A. versicolor* and *S. chartarum* DNA in 89% and 24% of classrooms, respectively. The concentrations of VM (Fig. 1) and total fungal DNA (Fig. 2) varied also among the classrooms within the same center. VM exceeded the standard suggested by ASHRAE (15) for good indoor air (300 cfu/m³) in 33% of the classrooms.

Molds growing indoors included *Cladosporium* spp (detectable in 44.3% of classrooms), *Penicillium* spp (34.8%), *Aspergillus* spp (13%), *Alternaria* genera (11%), *Aspergillus versicolor* and *Streptomyces* (6.5%). *Stachybotrys chartarum* was never detected.

**Figure 1** Concentrations of total viable molds in monitored classrooms. The horizontal line indicates the maximum standard value proposed by the American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE) (15) for good indoor air (300 cfu/m³).**Figure 2** Concentrations of total fungal DNA in monitored classrooms.

Visible molds and molds odor were reported for 30% and 33% of the schools, respectively. VM levels were higher in the presence of visible molds than in their absence (mean 425 vs. 254 cfu/m³, $p = 0.04$), as well as total fungal DNA (geometric mean, GM 2.50 vs. 1.59×10^5 CE/g, $p = 0.03$). Total fungal DNA was significantly more concentrated also in the presence of molds odor than in its absence (GM 3.00 vs. 1.51×10^5 CE/g, $p = 0.05$).

Total VM were inversely correlated with ventilation rate (Kendall's tau-b = -0.31 , $p = 0.003$) and positively correlated with indoor RH (0.37, $p < 0.001$). Neither total nor specific fungal DNA were correlated with ventilation rate, and only *A. versicolor* DNA were correlated with RH (0.25, $p = 0.02$). We found significant correlations, although poorly linear, of VM with *A. versicolor* DNA (0.35, $p = 0.001$) and *S. chartarum* DNA (0.26, $p = 0.03$); total fungal DNA with *A. versicolor* and *Streptomyces* DNA (0.21, $p = 0.03$ for both); *A. versicolor* DNA with *Asp/Pen* (0.27, $p = 0.01$) and *S. chartarum* DNA (0.34, $p = 0.004$).

Characteristic of the schoolchildren

Mean age of the children was 9.9 ± 0.8 yr. Compared with the children of the other centers, those of Oslo were significantly older (11.8 ± 0.3), while those of Reims were significantly younger (9.6 ± 0.7). Gender was almost equally distributed in the total sample (Table 3). The highest and lowest prevalence of respiratory symptoms was 34% for dry cough at night and 13% for wheeze. Pairwise comparisons

Table 2 Geometric means ± geometric s.d. of specific fungal DNA (CE/g dust) in the classrooms

	<i>Asp/Pen</i>	<i>A. versicolor</i>	<i>Streptomyces</i>	<i>S. chartarum</i>
Total	$0.3 \times 10^4 \pm 2.6$	$0.3 \times 10^2 \pm 5.6$	$0.2 \times 10^2 \pm 5.2$	2.2 ± 5.6
Siena	$0.2 \times 10^4 \pm 2.3$	$0.5 \times 10^2 \pm 2.6$	$0.1 \times 10^2 \pm 3.3$	2.8
Udine	$0.1 \times 10^4 \pm 2.1$	$0.2 \times 10^2 \pm 3.8$	$0.4 \times 10^2 \pm 1.6$	—
Oslo	$0.4 \times 10^4 \pm 2.7$	$0.4 \times 10^2 \pm 9.5$	$0.1 \times 10^2 \pm 9.7$	1.2 ± 9.6
Uppsala	$0.2 \times 10^4 \pm 2.6$	$0.1 \times 10^2 \pm 5.5$	$0.04 \times 10^2 \pm 4.0$	1.9 ± 4.2
Århus	$0.2 \times 10^4 \pm 1.9$	$0.1 \times 10^2 \pm 1.6$	$1.1 \times 10^2 \pm 1.7$	0.3 ± 6.9
Reims	$0.6 \times 10^4 \pm 2.3$	$1.9 \times 10^2 \pm 3.0$	$0.7 \times 10^2 \pm 3.1$	8.5 ± 1.8

Asp/Pen, *Aspergillus/Penicillium*; *A. versicolor*, *Aspergillus versicolor*; *S. chartarum*, *Stachybotrys chartarum*.

Table 3 Characteristics of the schoolchildren

	Total (n = 654)	Siena (n = 114)	Udine (n = 128)	Oslo (n = 29)	Uppsala (n = 134)	Århus (n = 90)	Reims (n = 159)
Male gender (%)	47.2	32.5	53.1	48.3	52.2	35.6	55.3
ETS (%)	34.3	46.4	28.7	33.3	17.4	39.3	48.4
Lifetime asthma, %	16.4	25.7	7.8	10.3	20.1	17.8	13.6
Outcomes (%)							
Wheeze	13.3	19.3	7.8	10.3	11.3	10.1	17.6
Dry cough at night	34.4	63.2	32.8	31.0	17.2	25.8	35.1
Rhinitis	31.7	47.8	24.4	41.4	25.6	11.1	41.6
Cough	25.9	38.1	22.0	3.4	18.3	10.1	40.0
Pulmonary function* (means $\pm$ s.d.)							
FEV ₁ (l)	2.16 $\pm$ 0.40	2.09 $\pm$ 0.35	2.37 $\pm$ 0.45	2.42 $\pm$ 0.28	2.21 $\pm$ 0.41	1.99 $\pm$ 0.27	1.80 $\pm$ 0.38
FEV ₁ (% predicted)	100.6 $\pm$ 15.4	103.4 $\pm$ 11.3	114.7 $\pm$ 16.6	–	99.5 $\pm$ 11.5	92.9 $\pm$ 9.3	86.5 $\pm$ 15.0
FVC ₁ (l)	2.44 $\pm$ 0.50	2.28 $\pm$ 0.40	2.62 $\pm$ 0.49	2.87 $\pm$ 0.62	2.54 $\pm$ 0.42	2.27 $\pm$ 0.33	1.94 $\pm$ 0.37
FVC (% predicted)	98.6 $\pm$ 13.8	98.5 $\pm$ 10.5	110.4 $\pm$ 15.8	–	99.8 $\pm$ 9.8	92.4 $\pm$ 9.3	87.0 $\pm$ 14.0

ETS, environmental tobacco smoking at home; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

*Total number = 218 (Siena = 40; Udine = 41; Oslo = 29; Uppsala = 39; Århus = 50; Reims = 19).

indicated several significant differences between centers that varied depending on the considered outcome. In general, the significant differences among centers were mainly because of higher prevalence of symptoms/diseases in Reims and Siena than in other centers. About 34% of the children were exposed to environmental tobacco smoke (ETS) at home, with very high prevalence in Reims (48%) and Siena (46%) (Table 3). The prevalence of lifetime asthma was 16%, ranging from about 8% in Udine to 26% in Siena. Both ETS and lifetime asthma significantly affected all considered outcomes, except for ETS and rhinitis.

FEV₁ and FVC significantly differed among centers, mainly because of the lowest and the highest values measured in the youngest and the oldest children of Reims and Oslo, respectively (Table 3). FEV₁ and FVC were positively correlated with age (Kendall tau-b = 0.31 and 0.33, respectively, $p < 0.001$) and height (0.39 and 0.40, $p < 0.001$). After accounting for age and height, FEV₁ and FVC were significantly inversely associated with ETS exposure at home. Female gender and presence of reported lifetime asthma, dry cough at night, and cough were also related to lower values of FEV₁ and FVC, although not significantly. All above-reported results were similar when percent predicted values were considered.

Molds exposure and respiratory symptoms/diseases

After accounting for gender, age, ETS at home, and lifetime asthma, both adjusted conventional and hierarchical regression models indicated that schoolchildren exposed to *elevated* levels of VM (> 300 cfu/m³, $n = 272$, 41.6%), compared with those exposed to *low* levels (≤ 300 cfu/m³), had almost threefold higher risk for dry cough at night and rhinitis and almost fourfold higher risk for cough. Similar results were also found when continuous values of VM were included in the statistical models (Table 4).

Total fungal DNA was positively associated with rhinitis (significantly) and cough (borderline significantly), but only in conventional regression models. The effects of specific fungal DNA substantially differed, depending on the type of outcome and fungal DNA (Table 4). Increased exposure to *Asp/Pen* DNA was significantly and positively related to dry cough at night, cough, and wheeze. The risk for wheeze was confirmed by the hierarchical analysis. Increased exposure to *A. versicolor* DNA was associated with higher risk for all considered outcomes, independent from the statistical model, except for dry cough at night, for which the association was no longer significant in the hierarchical analysis. The exposure to *Streptomyces* DNA did not appear to affect any of the considered symptoms/diseases. Based on the tertile distribution of fungal DNA, dose-response effects were found for all considered outcomes, particularly evident for *A. versicolor* DNA (Fig. 3).

When VM and all specific fungal DNA were included in the same model of logistic regression, accounted for gender, age, ETS at home, and lifetime asthma, VM were significantly related to higher risk for dry cough at night (OR: 2.80, 95% CI: 1.74–4.50), rhinitis (OR: 2.18, 95% CI: 1.36–3.50), and cough (OR: 3.08, 95% CI: 1.84–5.17), whereas *A. versicolor* DNA to higher risk for rhinitis (OR: 1.65, 95% CI: 1.15–2.37). Surprisingly, we found an inverse association between *Streptomyces* DNA and rhinitis (OR 0.66, 95% CI: 0.49–0.88). The results by hierarchical analyses were substantially similar (data not shown).

Molds exposure and lung function

In general, both FEV₁ and FVC were inversely associated with VM and fungal DNA. After accounting for gender, age, ETS at home, and any of lifetime asthma, recent dry cough at night, or cough, the relations were significant or borderline significant (depending on the model of analysis) for *A. versi-*

Table 4 Associations of exposures to total viable molds (VM, cfu/m³) and fungal DNA (CE/g dust) with respiratory disorders in schoolchildren. Odds ratios (OR) and 95% confidence interval (CI)

	Conventional logistic regression		2-levels‡ hierarchical regression
	Crude	Adjusted§	Adjusted§
VM elevated level¶			
Wheeze	1.36 (0.86–2.14)	1.41 (0.80–2.47)	1.41 (0.80–2.48)
Dry cough at night	2.43 (1.73–3.40)***	2.92 (1.95–4.36)***	3.10 (1.61–5.98)***
Rhinitis	1.83 (1.31–2.55)***	2.84 (1.91–4.23)***	2.86 (1.65–4.95)***
Cough	3.15 (2.19–4.54)***	3.70 (2.41–5.69)***	3.79 (2.40–5.60)***
Lg-VM			
Wheeze	1.08 (0.67–1.74)	1.11 (0.62–1.99)	1.12 (0.62–1.99)
Dry cough at night	2.27 (1.60–3.22)***	2.68 (1.77–4.05)***	2.88 (1.41–5.88)**
Rhinitis	1.94 (1.36–2.76)**	2.67 (1.77–4.03)***	2.59 (1.45–4.62)***
Cough	3.32 (2.23–4.92)***	3.48 (2.28–5.46)***	3.60 (2.17–6.00)***
¹ Lg-total fungal DNA			
Wheeze	1.26 (0.74–2.14)	1.72 (0.88–3.38)	1.71 (0.87–3.35)
Dry cough at night	1.06 (0.71–1.59)	1.03 (0.64–1.63)	1.10 (0.51–2.39)
Rhinitis	1.54 (1.04–2.29)*	1.73 (1.09–2.77)*	1.66 (0.80–3.45)
Cough	1.51 (1.00–2.30)*	1.57 (0.96–2.58)†	1.43 (0.69–2.97)
² Lg-Asp/Pen DNA			
Wheeze	1.43 (0.83–2.45)	2.17 (1.06–4.42)*	2.14 (1.05–4.36)*
Dry cough at night	1.40 (0.92–2.12)	1.63 (1.01–2.64)*	1.67 (0.66–4.23)
Rhinitis	1.44 (0.97–2.14)†	1.46 (0.91–2.33)	1.28 (0.60–2.71)
Cough	1.74 (1.14–2.67)**	1.91 (1.14–3.22)*	1.80 (0.87–3.74)
³ Lg- <i>A. versicolor</i> DNA			
Wheeze	1.46 (1.06–2.01)*	1.48 (0.99–2.22)†	1.48 (0.99–2.21)†
Dry cough at night	1.29 (1.02–1.62)*	1.32 (1.00–1.73)*	1.24 (0.75–2.04)
Rhinitis	1.43 (1.13–1.81)**	1.83 (1.38–2.43)***	1.75 (1.18–2.60)**
Cough	1.64 (1.27–2.12)***	1.76 (1.29–2.39)***	1.75 (1.17–2.62)**
³ Lg- <i>Streptomyces</i> DNA			
Wheeze	0.95 (0.68–1.32)	1.01 (0.68–1.49)	1.01 (0.68–1.49)
Dry cough at night	1.06 (0.83–1.35)	1.03 (0.78–1.35)	0.95 (0.56–1.59)
Rhinitis	0.91 (0.71–1.15)	0.80 (0.61–1.04)†	0.71 (0.45–1.12)
Cough	1.15 (0.88–1.50)	1.02 (0.76–1.36)	1.00 (0.64–1.55)

Asp/Pen, *Aspergillus/Penicillium*; *A. versicolor*, *Aspergillus versicolor*.

‡1st level: child, 2nd level: classroom; §analysis accounted for passive exposure to tobacco smoking at home, gender, age, lifetime asthma; ¶>300 cfu/m³.

¹OR calculated for 10⁵ CE/g dust increase; ²OR calculated for 10⁴ CE/g dust increase; ³OR calculated for 100 CE/g dust increase.

Statistical significance – *p < 0.05, **p < 0.01, ***p < 0.001, †0.05 < p < 0.1.

color DNA with FVC and *Streptomyces* DNA with both FEV₁ and FVC (Table 5). Multiple linear regressions with VM and all specific fungal DNA included in the same model as independent variables, and accounted for the above-reported confounders, confirmed the significant inverse associations of *Streptomyces* DNA with FEV₁ (partial regression coefficient, $B = -0.14$; 95% CI -0.22 ; -0.05 , $p = 0.003$) and FVC ($B = -0.13$, 95% CI -0.23 ; -0.04 , $p = 0.005$) and of *A. versicolor* DNA with FVC ($B = -0.12$, 95% CI -0.24 ; 0.02 , $p = 0.05$). VM did not significantly affect pulmonary function of children. Similar results were found when

percent predicted FEV₁ and FVC were considered for statistical analyses (data not shown).

Discussion

Both total VM and fungal DNA were always detectable in monitored classrooms, independent from reported presence of mold problems. VM exposure was related to higher risk of respiratory symptoms/diseases, whereas it did not seem to affect respiratory function of schoolchildren. In general, total fungal DNA significantly affected neither considered symp-

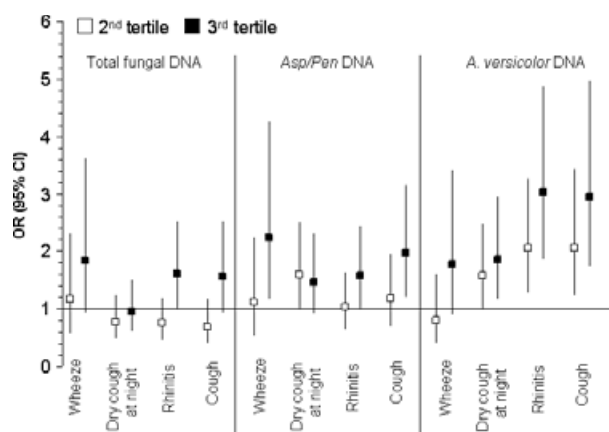


Figure 3 Logistic regression conventional models. Risk (odds ratio, OR) and 95% confidence interval (CI) for reporting respiratory disorders (dependent binary variables, yes/no) by tertile exposure to log-fungal DNA (CE/g dust). Each model is adjusted for gender, age, passive smoking at home, and lifetime asthma. Reference category for molds exposure: 1st tertile of the distribution. For computation of OR, see Table 4.

toms/diseases nor pulmonary function, but significant associations were found when the analyses were conducted on the various fungal genera.

A comparison between previous studies is rarely possible, because of different methodologies and lack of standardization in the terminology used to define health outcomes. Although the HESE study was performed on a non-representative sample of European schools, it provides, for the first time, data on both VM and fungal DNA, and related associations with children health, in a wide range of locations from different countries, using the same standardized procedure.

According to other studies, whether they regarded homes, schools, or working places, molds were always detectable (16–19), although their concentrations were higher in buildings with reported mold problems (9, 20–22). VM levels exceeding the ASHRAE standard for good IAQ in 33% of the classrooms confirmed that the school is an important source of exposure to molds. ‘Non-problem’ buildings should have levels of VM lower indoors than outdoors. In our study, indoor/outdoor ratio (I/O) was higher than 1 in about 70% of the classrooms (32/46, mean I/O = 2.5, median = 1.2, range 0.2–15.7). This is not surprising. Lee et al., for US homes in winter, computed indoor and outdoor median viabilities of 51% and 18%, respectively, for aerosolized fungi (23). Outdoor concentrations, for most genera, are the main source of influence on indoor spores in the warm season (24); we minimized the outdoor influence by performing the study in the cold season (1). A value of I/O higher than 1 was more prevalent in the classrooms with natural than with mechanical ventilation, as well as the median VM concentra-

Table 5 Associations of pulmonary function with total viable molds (VM, cfu/m³) and fungal DNA (CE/g dust). Partial regression coefficients (B) and 95% confidence interval (CI)

	Linear regression models		GLS-RE models
	Crude	Adjusted‡	Adjusted§
Log-total VM			
FEV ₁	−0.11 (−0.24; 0.02)†	−0.05 (−0.07; 0.18)	−0.04 (−0.29; 0.20)
FVC	−0.21 (−0.36; −0.06)	0.01 (−0.12; 0.14)	−0.10 (−0.36; 0.16)
Log-total fungal DNA			
FEV ₁	0.12 (−0.04; 0.28)	0.08 (−0.10; 0.25)	−0.02 (−0.32; 0.29)
FVC	0.18 (−0.01; 0.37)	0.11 (−0.07; 0.29)	−0.01 (−0.32; 0.31)
Log-Asp/Pen DNA			
FEV ₁	0.06 (−0.11; 0.23)	−0.12 (−0.31; 0.08)	−0.21 (−0.55; 0.13)
FVC	0.12 (−0.09; 0.32)	−0.10 (−0.30; 0.10)	−0.23 (−0.58; 0.13)
Log-A. versicolor DNA			
FEV ₁	−0.08 (−0.16; −0.01)*	−0.04 (−0.14; 0.06)	−0.10 (−0.27; 0.07)
FVC	−0.16 (−0.25; −0.06)**	−0.10 (−0.21; 0.01)†	−0.17 (−0.35; 0.003)*
Log-Streptomyces DNA			
FEV ₁	−0.13 (−0.21; −0.04)**	−0.14 (−0.23; −0.05)*	−0.16 (−0.34; 0.02)†
FVC	−0.15 (−0.26; −0.05)**	−0.14 (−0.23; −0.04)**	−0.18 (−0.36; 0.01)†

GLM-RE, generalized least-squares random-effects model.

‡For gender, age, height, passive smoking at home, and any of lifetime asthma, dry cough at night, cough; § as ‡ plus fixed effect of the classroom; Asp/Pen, *Aspergillus/Penicillium*; A. versicolor, *Aspergillus versicolor*; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

Statistical significance – *p < 0.05, **p < 0.01, †0.05 < p < 0.1.

tion in indoor air in excess of outdoor air (161 vs. 23 cfu/m³, $p < 0.001$). These results and the significant inverse correlation of VM with ventilation rate confirmed that inadequate ventilation plays a fundamental role in indoor pollution by biological contaminants. It is to point out that 70% of the classrooms failed to meet the ventilation standard, especially in the absence of mechanical ventilation. However, adequate ventilation is particularly important to avoid the fungal growth. Differently from VM, total fungal DNA was correlated with neither ventilation rate nor indoor RH (in turn related to ventilation rate).

According to Garret et al. (21), RH was a better predictor of higher indoor VM concentrations than temperature. Most indoor fungi grow at 10–35°C and common indoor temperatures are likely not limiting/favoring their growth.

We observed an increasing north–south gradient of VM that might be partially explained by outdoor concentrations significantly lower in northern than in southern centers (GM 64 vs. 206 cfu/m³, respectively, $p < 0.001$) and, mostly, by the presence of mechanical ventilation only in part of northern classrooms. A similar gradient was not found as regards the fungal DNA. On the other hand, indoor VM and total fungal DNA were not correlated, and the most important factors related to VM (e.g., RH and ventilation) did not influence the levels of fungal DNA.

Health effects

Few studies report associations between objective measurements of total VM and children health. Residential exposures to total VM were only marginally associated with respiratory symptoms in Australian children (21) and significantly related to higher report of rhinoconjunctivitis symptoms in German children (16). No associations were found between total VM exposure at home and nocturnal cough in Canadian children (20). Significant relations of total VM with wheeze have been reported, but they regard infants at high risk for asthma (25). The lack of association between total VM and children's health was explained by possible presence of synergistic relationships with allergens, pollutants, and toxins coexisting inside homes (26). In the present study, total VM were significantly and positively related to all considered respiratory outcomes, except for wheeze. Home and school environments highly differ for specific characteristics, and it might be not surprising that our findings contrast to those of previous studies performed in residential settings.

In terms of adjusted ORs, we found similar results whether we included, in statistical analyses, VM as a continuous variable or as a binary exposure variable based on the ASHRAE standard. This might suggest that the threshold of 300 cfu/m³ is too high. The Health Canada guidelines propose a cutoff of 50 cfu/m³, but for single species. Using such a cutoff for total VM, we did not find any significant results. By raising the threshold to 100 cfu/m³, we found significant associations with dry cough at night (OR: 1.78, 95% CI: 1.12–1.83) and cough (OR: 1.92, 95% CI: 1.13–3.26). Finally, rhinitis became significantly related to VM when the cutoff was further raised to 200 cfu/m³ (OR: 1.91, 95% CI: 1.23–3.00).

A criticism would have been the lack of any information on atopic status of examined children. Indeed, for 382 children (58% of the whole sample), we had available information about allergic sensitization to common allergens (i.e., cat, dog, and pollen), as indicated by the parents. After further adjustment for positivity to at least one of cat, dog, or pollen allergens, the reported associations remained significant, and also the relationship between *Aspergillus/Penicillium* and recent rhinitis became significant. It is to point out that any allergy to cat, dog, or pollen was reported for only 51 of 382 children (8.8%).

We found only a recent Malaysian study that assessed the effects of fungal DNA in school on children's health (10), although different outcomes than ours, except for wheeze, were considered. According to the Malaysian study, we found no significant associations between respiratory health of schoolchildren and total fungal DNA, whereas wheezing was significantly and positively associated with exposure to *A. versicolor* DNA. The lack of associations between respiratory symptoms/diseases and total fungal DNA and the presence of significant positive associations with specific DNA (i.e., *A. versicolor* and *Asp/Pen* DNA) confirm and stress the importance of analyzing specific molds. It was shown that *Streptomyces* are potent inducers of inflammatory effects, and we are not able to explain the surprising inverse significant association between recent rhinitis and *Streptomyces* DNA.

To our knowledge, the effects on pulmonary function of the pupils from exposure to measured molds in schools have been never investigated. As regards residential VM, Inal et al. (27) found no significant effects on peak expiratory flow of more susceptible children. In our study, VM exposure did not affect pulmonary function of schoolchildren, whereas significant inverse associations were found with *A. versicolor* DNA and, particularly, with *Streptomyces* DNA. These results confirm the importance of measuring molds independent from their viability when assessing the health effects. It is to point out that the entire method of DNA extraction, cleanup, and quantification by qPCR can be completed in less than a day (28).

The means of percent predicted FEV₁ and FVC resulted quite low in Reims, compared with other centers, and this deserves some comment. There is no reason to believe that there were any methodological differences for Reims as regards pulmonary function. The same protocol was applied in Reims as in the other centers, and regular calibration of the instruments was performed. Moreover, analyses stratified by gender and age showed that for each child of Reims, there was at least one child from other centers with similar spirometric values. Nevertheless, percent predicted in children of Reims were evidently lower than in other centers. There is some evidence that predicted values can be higher than measured ones, but this should be valid for all centers and not only for Reims. Although the equations of Quanier regard specifically predicted values in European children and adolescents, we cannot exclude that such equations are not appropriated for children of Reims. However, it is difficult to understand why. As in other centers, almost all children

of Reims who underwent spirometry were Caucasian (only one was Asiatic). The younger mean age might partially explain the lower measured pulmonary function in children of Reims, but not the lower percent predicted values, because the equations of Quanier take into account the age. When we tried to calculate predicted values using other equations based on the same parameters (i.e., age, gender, and height), we found only little differences with Quanier. It could be that, among the children undergoing spirometry, those of Reims, besides being younger, had also lower mean weight and the equations of Quanier, not including weight, might excessively overestimate the spirometry of Reims. Body mass index seems to affect lung function in children quite differently from adults and there is evidence that FEV₁ and FVC increase proportionally in relation to an increase in BMI (29). Recently, it was found that the measured FEV₁ and FVC values, in normal weight and underweight children, were lower than the values predicted via equations not including weight; in contrast, the values predicted via the equations including the body weight were closer to measured values (29). Unfortunately, we were not able to verify it, because of lack of data on body weight.

In any case, the measured values of pulmonary function in Reims were, on average, the lowest and, coincidentally, the levels of fungal DNA were higher than in other centers. It could rise to the doubt that this fact confuses the relationship between lung function and fungal DNA. Although excluding Reims would have been engendered a bias in our study, which aimed precisely to investigate the association between pulmonary function and mold exposure, it is to point out that, also after excluding from analyses the children of Reims, the inverse association between *Streptomyces* DNA and pulmonary function remained, with only slightly lower significance, likely due to the slightly lower size of the sample (i.e., by adjusted linear regression: $B = -0.07$, 95% CI $-0.14; 0.005$, $p = 0.06$ for FEV₁ and $B = -0.07$, 95% CI $-0.15; 0.002$, $p = 0.05$ for FVC).

Our previous study on the HESE data (11) indicated that increased exposure to carbon dioxide (CO₂) and particulate matter (PM₁₀) was significantly related to higher risk for dry cough at night and lower nasal patency in pupils, respectively. All results of the present study did not change after further accounting for indoor CO₂ and PM₁₀.

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Widespread public attention is currently devoted to the health effects by *S. chartarum*. This enigmatic organism seems to have unique bioactivities compared with other indoor fungi. However, it was shown that the toxins of *S. chartarum* required for producing health effects in animal models exceed the levels measured to date in even the most highly contaminated indoor environments. Indeed, *Stachybotrys genera* proliferate more slowly than other species and require RH around 93% (30). In United States, low levels of *S. chartarum* were detected only in 6% of 1717 buildings (31). This might explain that intensive studies have failed to establish adverse human health effects related to *S. chartarum* exposures in home, school, or office environments (30). We also found detectable values of *S. chartarum* DNA only in 26% of the classrooms, and the levels were indeed very low. For this reason, we did not consider this specific DNA in statistical analyses when assessing the health effects. However, it is to point out that further inclusion of *S. chartarum* DNA in the statistical models did not change the results. Interestingly, in spite of very low levels, there were significant and independent inverse associations of *S. chartarum* DNA with both FEV₁ and FVC.

Conclusions

Both indoor VM and fungal DNA were commonly found in monitored European schools. Because of the negative impact on respiratory symptoms/disease and pulmonary function of schoolchildren, the schools should be routinely tested through both culturable and non-culturable methods for global indoor mold evaluation. Besides visible molds/dampness should be cleaned/removed, a ventilation system should be appropriately designed and maintained to reduce the fungal growth. Vacuum cleaners equipped with high-efficiency particulate air filter should be used to remove also dead or dormant fungal spores and organisms.

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