BOHS

The Chartered Society for Worker Health Protection

OXFORD

Original Article

Fungal Fragments and Fungal Aerosol Composition in Sawmills

Komlavi Anani Afanou[1,*], Wijnand Eduard[1], Helle Birgit Laier Johnsen[2] and Anne Straumfors[1]

[1]Department of Chemical and Biological Work Environment, STAMI National Institute of Occupational Health, Pb 8149 Dep, 0033 Oslo, Norway; [2]Department of Occupational Medicine and Epidemiology, STAMI National Institute of Occupational Health, Pb 8149 Dep, 0033 Oslo, Norway

*Author to whom correspondence should be addressed. Tel: +4723195247; fax: +4722601170; e-mail: anani.afanou@stami.no

Abstract

Assessment of exposure to fungi has commonly been limited to fungal spore measurements that have shown associations between fungi and development or exacerbation of different airway diseases. Because large numbers of submicronic fragments can be aerosolized from fungal cultures under laboratory conditions, it has been suggested that fungal exposure is more complex and higher than that commonly revealed by spore measurements. However, the assessment of fungal fragments in complex environmental matrix remain limited due to methodological challenges. With a recently developed immunolabeling method for field emission scanning electron microscope (FESEM), we could assess the complex composition of fungal aerosols present in personal thoracic samples collected from two Norwegian sawmills. We found that large fungal fragments (length >1 μm) dominated the fungal aerosols indicating that the traditional monitoring approach of spores severely underestimate fungal exposure. The composition of fungal aerosols comprised in average 9% submicronic fragments, 62% large fragments, and 29% spores. The average concentrations of large and submicronic fragments (0.2–1 μm) were 3×10^5 and 0.6×10^5 particles m^{-3}, respectively, and correlated weakly with spores (1.4×10^5 particles m^{-3}). The levels of fragments were 2.6 times higher than the average spore concentration that was close to the proposed hazardous level of 10^5 spores per m^3. The season influenced significantly the fungal aerosol concentrations but not the composition. Furthermore, the ratio of spores in the heterogeneous fungal aerosol composition was significantly higher in saw departments as compared to sorting of green timber departments where the fungal fragments were most prevalent. Being the dominating particles of fungal aerosols in sawmills, fungal fragments should be included in exposure-response studies to elucidate their importance for health impairments. Likewise, the use of fungal aerosol composition in such studies should be considered.

Keywords: fungal aerosols; fungal fragments; submicronic fragments; field emission scanning electron microscopy; compositional data analysis

Introduction

Bioaerosols are airborne particles originating from various organisms including fungi. Airborne fungal particles are recognized as potential health hazards in environments with heavy fungal contamination (Institute of Medicine, 2004). A number of studies from sawmills have revealed associations between fungal exposure and respiratory impairments such as hypersensitivity pneumonitis, organic dust syndrome (ODTS), and occupational asthma (Belin, 1987; Dahlqvist et al., 1992; Eduard et al., 1992; Halpin et al., 1994; Rask-Andersen et al., 1994; Mandryk et al., 2000; Adhikari et al., 2015). Fungal spores and related biomarkers are commonly measured during fungal exposure assessment whether in indoor or occupational settings. However, the discovery of large numbers of indiscernible submicronic fragments aerosolized from fungal cultures (Kildesø et al., 2000) suggested that fungal particle exposure is more heterogeneous and higher than that commonly revealed by spore measurements (Cho et al., 2005). Measurement of fungal fragments in addition to spores was, therefore, hypothesized as a better approach to investigate fungal particles exposure and the relationship between exposure and diseases (Reponen et al., 2007). Aerosolized fragments are mostly from the vegetative mycelial mass (Afanou et al., 2014). Vegetative fungal biomass dominates filamentous fungal biomass, which represents at least 95% of the fungal biomass (Schnurer, 1993). Release of potentially aerosolizable fraction of fungal particles (spores and fragments) from a given fungal colony is likely dependent on biotic and abiotic stress factors as well as physical forces exerted during various activities performed by workers in occupational settings (Green et al., 2011). Furthermore, the presence of allergens (Mitakakis et al., 2001; Green et al., 2003), antigens (Górny et al., 2002), and mycotoxins (Brasel et al., 2005) in the fragment fraction makes them potentially hazardous for human health.

Diverse methods based on fungal membrane constituents (ergosterol, $(1\rightarrow3)$-B-glucans) (Reponen et al., 2007; Singh et al., 2011), fungal alcohols (arabitol and manitol) (Bauer et al., 2008), enzymes (N-acetyl-D-glucosaminidase) (Madsen et al., 2009), antigens (Green et al., 2006), and DNA (Reponen et al., 2011; Vesper, 2011) have been used as markers for quantification of diverse fungal particles. However, only a FESEM based approach has enabled detailed characterization and measurement of the number of various particle types as well as the compositional fluctuations of fungal aerosols under different experimental settings (Afanou et al 2015a). *In vitro* aerosolized fungal particles is shown to have a complex profile that includes single and aggregated spores, and fragments in various sizes and shapes (Afanou et al., 2015b).

Despite the existing broad knowledge on the adverse health effects associated with fungal spores from non-pathogenic species (Eduard, 2009), the prevalence and the specific role of fungal spores and fragments in the heterogeneous composition of fungal aerosols in different occupational and environmental settings remain unknown. Therefore, characterizing the complex profile of fungal aerosols related to various environmental or occupational exposure determinants represents primary steps for studying the relationship between observed health impairments and fungal aerosol exposure. Moreover, the physical and chemical characteristics of inhalable particles as well as their behavior in the respiratory tract may result in various airway symptoms and health effects. In contrast to the more isometric spores with deposition behaviors well described by their aerodynamic diameter, large spore aggregates, and mycelial fragments have non-isometric morphologies with complex dispersion and deposition behavior. These particles can deposit in the bronchioles and the upper airways triggering specifically bronchitis and asthma development whereas alveolar deposition seems to be typically associated with hypersensitivity pneumonitis and organic dust syndromes (ODTS) (Raulf et al., 2014). Also, the number of particles in the fine fraction (AED ≤ 2.5µm) has been shown as a stronger predictor of adverse immune toxicological effects compared to their mass (Nygaard et al., 2004).

With a recently developed immunodetection method for enumeration of fungal spores and fragments using field emission scanning electron microscopy (FESEM; Afanou et al., 2015a), we aimed to assess the levels and the composition of the fungal aerosols including submicronic fungal fragments (length ≤1 µm), larger fragments (length >1 µm), and spores in personal samples from Norwegian sawmill workers. Furthermore, we investigated how sawmill, season, and department influenced the concentrations and the composition of fungal aerosols. The present study is part of a large project aiming to characterize exposures in sawmills and their association with respiratory and inflammatory effects in sawmill workers over a 5-year period.

Materials and methods

Sampling of thoracic particles in sawmills

We collected 69 personal samples of the thoracic aerosol fraction on 31 workers at two Norwegian sawmills processing spruce during winter (February and March) and summer (June and August) 2013. For the particle

collection, Millipore 37-mm cartridges loaded with polycarbonate filter (0.8 µm pore size) were mounted onto thoracic BGI GK 2.69 cyclones (GK2.69 Cyclone; BGI, Waltham, MA, USA) connected with PVC tubes to a battery-powered pump with an airflow rate of 1.6 l/min. The flow rate was calibrated using a digital flow meter (Defender, SKC Inc., Eighty Four, PA, US) and recorded before and after sampling. Sampling was performed during 8-h day shifts for two consecutive days in summer and winter seasons. The following departments were included: Saw (S), Sawing and sorting of green timber (SSGT), Sorting of green timber (SGT), Kiln drying (KD), Sorting of dried timber (SDT), and Planing (P). The number of samples by departments, season, and sawmill and the process flow are summarized in Table 1.

Particle immobilization and immunolabeling of fungal fragments

Immobilization and immunolabeling of fungal particles was performed on ¼-sector cut out of the sample filter. Sampled particles were first vapor-fixed with 25% glutaraldehyde overnight and immobilized onto the filter

membrane by gently wetting the membrane from beneath with 200 µl 0.1% poly-L-lysine solution (150–300.000 MW, MERCK, Darmstadt, Germany). After drying on LAF bench for 2 h, the membrane was again vapor-fixed overnight with 25% glutaraldehyde. The first fixation aimed to fix soluble antigens whereas the purpose of the second fixation was to link permanently the particles onto the filter membrane through poly-lysine bonds. Subsequently, the filter specimens were mounted onto a 25-mm carbon tab attached to a circular supporting metal grid and placed in a 6-well cell culture plate. Free aldehyde sites were quenched with 2-ml 0.02-M glycine (MERCK). Immunolabeling of fragments was performed as previously described (Afanou et al., 2015a). Briefly, all free binding sites were blocked with 2-ml 5% skimmed milk freshly dissolved in Tris-buffered saline (pH 8, MERCK) containing 0.05% Tween 20 (TBSTSM) for 1 h at room temperature. The samples were then incubated with 1 ml of anti-fungi pIgY antibodies (1:100 equivalent to 120 µg/ml diluted in TBSTSM), washed three times for 5 min with TBSTSM, and subsequently labeled by 1-h incubation with 1 ml of nanogold

Table 1. Number of samples and workers by department, sawmill, and season. The arrow shows the flow of the processes.

Departments		Description	Sawmills	Seasons	Number of workers	Number of samples
	Saw (S)	Sawing of debarked logs into timber	1	Summer	0	0
				Winter	3	6
			2	Summer	2	3
				Winter	4	7
	Sawing & sorting green sorting (SSGT)	Sawing of debarked logs/sorting of green timber	1	Summer	8	15
				Winter	2	2
			2	Summer	0	0
				Winter	0	0
	Sorting of green timber (SGT)	Sorting of green timber based on dimension	1	Summer	0	0
				Winter	1	2
			2	Summer	2	4
				Winter	0	0
	Kiln drying (KD)	Kiln drying of cut timber under controlled temperature and humidity	1	Summer	1	2
				Winter	1	2
			2	Summer	1	2
				Winter	2	4
	Sorting of dry timber (SDT)	Sorting dried timber based on quality and dimension	1	Summer	0	0
				Winter	4	6
			2	Summer	2	4
				Winter	4	8
	Planing (P)	Planing, profiling, and re-sawing of dried timber	1	Summer	0	0
				Winter	0	0
			2	Summer	1	2
				Winter	0	0

(25 nm) conjugated goat anti-IgY secondary antibodies (Aurion, Wageningen, Netherlands) diluted (1:20) in TBSTSM. After the secondary antibody treatment and a second washing step, the filter membranes were rinsed three times for 5 min in water (BPC grade; MERCK) before being subjected to silver enhancement treatment using Aurion kit (Aurion R-gent SE-LM, Wageningen, Netherlands) for 15 min. Thereafter, the membranes were washed three times 5 min with water and air-dried under aseptic conditions. Dried membranes were mounted onto aluminum specimen stubs (25 mm) and coated with 5–6 nm platinum in a Cressington 208HR Sputter Coater (Cressington Scientific Instruments Ltd., Watford, UK). All treatments were performed at room temperature.

FESEM analysis

The composition of fungal aerosols including spores, submicronic fragments (length: 0.2–1 µm), and larger fragments (length: >1 µm) in the samples was assessed using a FESEM (SU 6600 HITACHI, Ibaraki-Ken, Japan) operated in low vacuum (25–30 Pa) and in back scattered electron (BSE) imaging mode. We imaged samples at 6–7 mm working distance and with an acceleration voltage of 15 kV. Fragments were classified as submicronic fragments if the length was ≤1 µm and large fragments if the length was >1 µm. Fragments were identified as of fungal origin when labelled with at least two gold particles for submicronic fragments and at least four gold particles for large fragments (Afanou et al., 2015a). Spores were recognized by their morphology. We counted simultaneously spores and fragments in 100 randomly selected imaged fields at ×3000 magnification and reported the fungal exposure as numbers of spores or fragments per m³ air. The following formulation was used to estimate fungal particle concentration:

$$Fungal\ particles\ per\ m^3 = \frac{n \times exposed\ filter\ area\left(\mu m^2\right)}{k \times FESEM\ area\left(\mu m^2\right) \times Air\ volume\ \left(m^3\right)}$$

n = number of particles counted on the filter
k = number of FESEM fields
Exposed filter area of 37-mm filter = 855×10^6 µm²
FESEM counting area at ×3000 is 1064 µm²

The lowest detectable number of fungal particles on the filter membrane at this magnification was 9.7×10^3 particles per m³ for an average of 8-h air sampling (arithmetic mean of air volume: 0.827 m³). Exposure levels of fungal particles were estimated by the total number of spores and fragments on the filter per m³ of air sampled. One filter without air sampling per season by sawmill used as blank revealed no background levels of fungal particles.

Data analysis

Prior to statistical analysis, all zero counts were arbitrary substituted by 0.5 in order to enable ratio and log calculations. We estimated the levels and the fractions of submicronic fragments (SF), spores (SS), and large fragments (LF) by arithmetic mean (AM) with standard deviation (SD), median with minimum–maximum concentration stratified by sawmill, season, and department. Average mentioned in this paper represents the arithmetic mean. The exposure levels and fractions of each fungal particle type (SS, SF, and LF) were compared between sawmills, seasons (winter and summer), and departments using the non-parametric Kruskal–Wallis (K–W) test since data were not normally distributed (Shapiro test: $p < 0.001$). For multiple categories (departments), we performed a post hoc Wilcoxon rank-sum test (or Mann–Whitney: M–W test) and Bonferroni adjusted the p values (significant p values <0.008). The differences between spores and fragments (SF and LF) were also assessed in similar way but the Bonferroni adjusted significant p values were <0.017. The correlation between different types of fungal particles was estimated by the Spearman rank-sum correlation between different particle types.

The composition of the fungal aerosols was estimated as the percentage of each particle type in the total fungal aerosol. Changes in the fungal aerosol composition (SF, LF, and SS) with respect to sawmill, department, and season were assessed using linear mixed effect analysis of the relationship between centered log ratio (clr) of transformed percentages of particle types and different variables. The clr transformation was used to remove the constant sum constraint of compositional data prior to modeling (Aitchison, 2003; Pawlowsky-Glahn and Egozcue, 2006). Sawmill, department and season were used as fixed effects and the attributed workers identity number as random intercept effect. Clr was calculated as the log of each count ratio divided by the geometric mean of the proportion of the three types of particles. Schematically, the following models were used to assess differences in the fungal particle composition:

$$
\begin{aligned}
\textbf{Sawmills}: \quad Clr =\ & Intercepts + Types \\
& + Types \times Sawmill \\
& + workerID\ number\,(\text{random}) \\
& + residuals
\end{aligned}
$$

$$
\begin{aligned}
\textbf{Departments}: \quad Clr =\ & Intercepts + Types + Types \\
& \times Departments \\
& + workerID\ number\ (random) \\
& + residuals
\end{aligned}
$$

$$\textbf{Seasons}: \; Clr \; = \; Intercepts \; + \; Types \; + \; Types$$
$$\times \; Season \; + \; workerID \; number \; (random)$$
$$+ \; residuals$$

(*Clr*: centered log ratio, *Types:* represent the three types of fungal particles)

Significant changes in the fungal aerosol composition related to the tested fixed effect variables was revealed by significant log-likelihood ratio test between a full model with interaction term versus reduced model without the interaction term. The sign and significance of the regression coefficient of the interaction terms served as indicator for significant change of particle fractions between the levels of the fixed effect variables.

For all comparison tests, a two-sided *p* value of 0.05 was regarded as statistically significant. For a significant log-likelihood ratio test, each department was used as reference in comparison to other departments in order to determine the significant difference. Thereafter, Benjamini and Hochberg false discovery rate (BH-FDR) was used for multiple comparison adjustment (Benjamini and Hochberg, 1995). STATA 13 (StataCorps, College station, Texas USA) was used for statistical analysis.

Results

Fungal particle exposure

Different types of fungal particles including SS, SF, and LF as presented in Figure 1 were detected. We found LF in all samples, whereas SF and SS were detected in 59% and 94% of the samples, respectively. Overall 6% and 41% of the samples had concentrations of SS and SF, respectively, below the detectable level of the FESEM method. As summarized in Table 2, the overall exposure levels of SF and LF were (AM±SD) $0.6 \pm 1.2 \times 10^5$ m^{-3} and $3.0 \pm 3.4 \times 10^5$ m^{-3}, respectively, whereas the spore exposure was $1.4 \pm 2.4 \times 10^5$ m^{-3}. The overall levels of LF were significantly higher than that of spores ($p < 0.001$) and SF, whereas the spore exposure was significantly higher than the exposure for SF ($p < 0.001$). Further, the levels of SS exposure were poorly correlated to the exposure to SF ($r_S = 0.18$, $p = 0.37$) and LF ($r_S = 0.24$, $p = 0.13$). The fragments, however, were strongly correlated ($r_S = 0.78$, $p = 0.01$). The average exposure level of all fragments (SF+LF) was 2.6 times higher than that of spore exposure. As compared to the proposed lowest observed effect level (10^5 spores/m^3) for non-pathogenic and non-toxic fungal exposure (Eduard, 2009), the
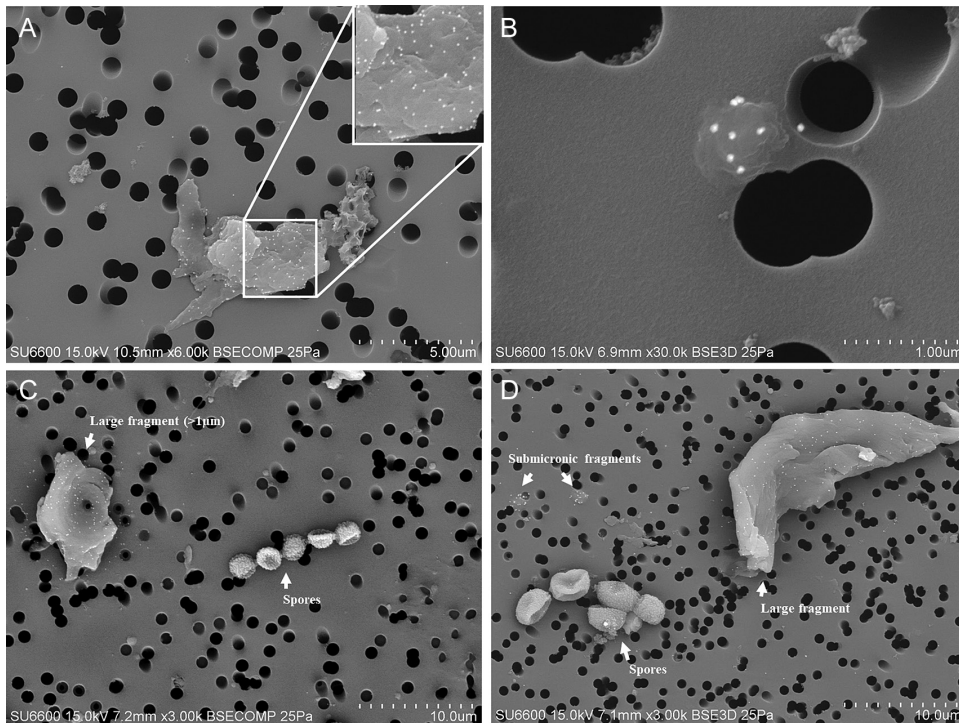

Figure 1. Micrographs of large fragments (A, C, and D), submicronic fragments (B and D), and spores (C and D) in samples collected from sawmills. The white spots represent gold particles.

Table 2. Exposure levels of fungal particles (number of particles × 10⁵ particles per m³) by seasons, sawmills, and departments.

		N	Spores					p value	Submicronic fragments					p value	Large fragments					p value
			AM	SD	Median	min	max		AM	SD	Median	min	max		AM	SD	Median	min	max	
Overall		69	1.4	2.4	0.7	<LDL	12.3		0.6	1.2	0.1	<LDL	6.2		3	3.4	1.9	0.09	21.8	
Seasons	Winter	37	0.6	0.7	0.4	<LDL	4.1	0.0001	0.3	0.5	0.1	<LDL	2.0	0.01	1.9	1.7	1.4	0.09	6.0	0.006
	Summer	32	2.3	3.7	1.1	<LDL	12.3		0.9	1.6	0.2	<LDL	6.2		4.3	4.4	3.3	0.09	21.8	
Sawmills	1	35	1.2	2.1	0.7	<LDL	12.3	0.7	0.6	1.2	0.1	<LDL	6.2	0.2	2.9	2.5	2.6	0.2	11.8	0.4
	2	34	1.5	2.6	0.6	<LDL	11.3		0.5	1.1	0.1	<LDL	5.7		3.1	4.2	1.8	0.09	21.8	
Departments	S	16	0.9	0.9	0.7	<LDL	4.1		0.3	0.5	0.1	<LDL	2.0		1.6	1.8	0.9	0.09	5.7ᵇ	
	SSGT	17	1.7	2.9	0.7	<LDL	12.3		0.98	1.6	0.2	<LDL	6.2		3.6	3.0	2.97	0.5	11.8	
	SGT	6	2.4	4.4	0.7	<LDL	11.3		2.0	2.2	1.4	<0.09	5.7		8.4	7.2	5.8	3.2	21.8ᵃᵇ	
	KD	10	0.5	0.6	0.3	0.1	2.0		0.1	0.1	0.1	<LDL	0.4		1.7	1.3	1.3	0.2	4.2ᵃ	
	SDT	18	1.5	2.6	0.5	0.09	9.9		0.2	0.3	0.1	<LDL	1.0		2.8	2.3	2.15	0.3	7.9	
	P	2	1.3	0.3	1.3	1.1	1.5	0.2	0.2	0.1	0.2	0.09	0.3	0.05	2.7	0.8	2.7	2.1	3.3	0.008

N = number of samples; AM = arithmetic mean; LDL = lowest detectable level; K–W = Kruskal–Wallis test for seasons, sawmills, and departments. S = Saw; SSGT = Sawing and sorting of green timber; SGT = Sorting of green timber; KD = Kiln drying; SDT = Sorting of dry timber; P = Planing. Post hoc test using Mann–Whitney (data with similar letter are significantly different): ᵃ($p = 0.005$); ᵇ($p = 0.004$). Significant level: $p \leq 0.05$.

average spore exposure was slightly higher and 32% of all the exposure measurements exceeded this effect level.

Seasons significantly affected the levels of each type of fungal particles ($p \leq 0.01$) with the highest exposure measured during summer. The average exposure of SS and SF during summer was about 4-fold higher, whereas the exposure to LF was 2-fold higher than that of winter. The exposure to SS, SF, and LF were not significantly different between sawmills. Departments did not influence the concentrations of SS or SF, but the concentration of LF differed significantly between departments ($p = 0.008$, Table 2). The levels of LF exposure were higher in SGT as compared to the levels in KD and S. The gradient of the average exposure levels of LF per departments was as followed from the highest to lowest: SGT≥SSGT≥SDT≥P>KD>S.

Composition of fungal aerosols

LF represented the largest fungal particle fraction in all fungal aerosol samples. The percentage of each of the three fungal particle fractions in the fungal aerosols did not differ significantly between sawmills or departments (Table 3). The percentages of SS and SF, respectively, were also similar during winter and summer. However, the percentage of LF were significantly higher during winter season (Median: 75%, min–max: 6.5–97%) compared to summer (Median: 56%, min–max: 7.4–96%).

A comprehensive analysis of the composition of the aerosols by clr mixed effect modeling, including the fractions of all particle types simultaneously, showed that there were no significant effects of sawmill and season ($p > 0.05$) on the fungal aerosol composition. However, a significant difference in the composition was observed in the SGT department compared to the S department. In the heterogeneous fungal aerosol composition, the average fraction of spores was significantly lower in SGT as compared to S department ($p = 0.003$, Figure 2), whereas the fractions of SF and LF remained unchanged between the six departments (Supplementary Table S01, available at *Annals of Occupational Hygiene* online).

Discussion

In the present study, we documented that workers in sawmills can be exposed to heterogeneous fungal particles including spores and submicronic and large fragments. Workers were exposed to higher levels of fragmented fungal particles than that of spores. The overall composition of fungal aerosols indicated that fragments are the most prevalent fungal particles in sawmills. To the best of our knowledge, no study has assessed exposure to fungal fragments in sawmills. The
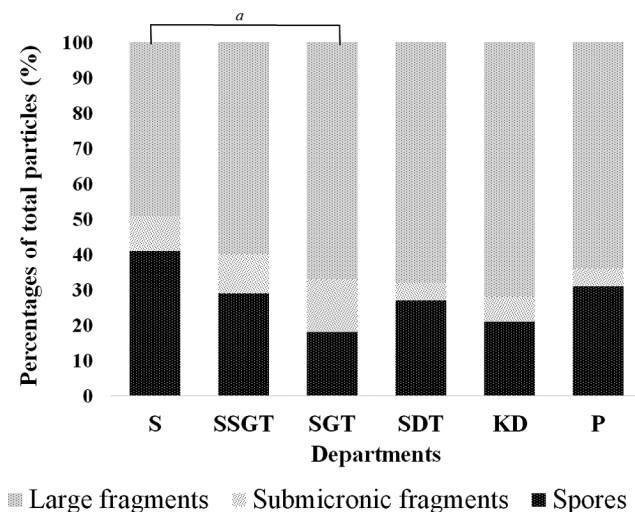
Table 3. Percentages of fungal particles by seasons, sawmills, and departments.

		N	Spores					K–W test p value	Submicronic fragments					K–W test p value	Large fragments					K–W test p value
			AM	SD	Median	min	max		AM	SD	Median	min	max		AM	SD	Median	min	Max	
Overall		69	29	24	22	0.7	92		9	9	5	0.6	42		62	22	67	6.5	97	
Seasons	Winter	37	25	23	17	2	92	0.08	9	8	6	1	36	0.9	66	22	75	6.5	97	0.02
	Summer	32	34	24	33	0.7	89		9	10	5	0.6	42		57	21	56	7.4	96	
Sawmills	1	35	28	24	17	1	83	0.6	9	10	5	0.6	42	0.9	63	22	72	14	96	0.5
	2	34	31	25	25	0.7	92		9	8	6	1	33		60	22	67	6.5	97	
	S	16	41	30	39	2	92	0.3	10	9	7	1	33	0.5	49	25	52	6	80	0.1
Departments	SSGT	17	29	25	16	1	79		11	11	6	0.6	42		60	21	67	21	89	
	SGT	6	18	22	8	0.7	57		15	14	12	2	36		67	17	68	39	89	
	KD	10	21	17	16	2	55		7	5	6	1	17		72	17	78	36	96	
	SDT	18	27	20	23	20	83		5	5	4	1	18		68	20	72	14	97	
	P	2	31	0.1	31	31	32		5	4	5	2	8		64	4	64	61	67	

N = number of samples; AM = arithmetic mean; K–W = Kruskal–Wallis test for seasons, sawmills, and departments; S = Saw; SSGT = Sawing and sorting of raw timber; SGT = Sorting of raw timber; KD = Kiln drying; SDT = sorting of dry timber; P = planing. Significant level: *p* ≤ 0.05.

Figure 2. Variations in the fungal aerosol composition between departments as depicted by diagrams of clr mixed effect modelling. (*a*) Adjusted *p* value < 0.01. S = Saw; SSGT = Sawing and sorting of green timber; SGT = Sorting of green timber; SDT = Sorting of dry timber; KD = Kiln drying; P = Planing.

AM levels of LF detected in sawmills were higher than average levels of morphologically discernible hyphal fragments reported in outdoor air samples by Pady and Kramer (1444 m⁻³; Pady and Kramer, 1960), Delfino and co-workers (203 m⁻³; Delfino et al., 1997), and in strawberry fields (0.41 × 10⁵ m⁻³; Tendal and Madsen, 2011). As compared to other workplaces, LF levels in sawmills were much lower than levels assessed during renovation of moldy walls (6 × 10⁵ m⁻³; Afanou et al., 2015a), grain farming (30 × 10⁵ m⁻³; Halstensen et al., 2007) and seed processing (3.64–86.9 × 10⁵ m⁻³; Madsen et al., 2012). The outdoor levels of fragments reported by Delfino et al. and the workplace levels reported by Madsen and co-authors were associated with asthma symptom exacerbations and acute ODTS, respectively. Based on these results and given that fungal hyphae from common species like *Aspergillus fumigatus* and *Penicillium chrysogenum* are immunologically more potent than their spores (Hohl et al., 2005; Aimanianda et al., 2009; Øya et al., 2018), the exposure levels of fungal fragments observed in sawmills may be linked to adverse health effects in sawmill workers.

Submicronic fragments were also found but at lower concentrations as compared to levels of LF. Although the exposure to submicronic fungal fragments has never been studied in sawmills, to the best of our knowledge, several studies have indirectly investigated their occurrence in various environments. Biomarkers such as beta-glucans or NAHA (N-acetylhexosaminidase) have been measured as surrogate for submicronic fungal particle fraction in size-fractionated dust collected from mold

contaminated indoor environments (Reponen et al., 2007; Madsen et al., 2009; Adhikari et al., 2013; Seo et al., 2014). However, as opposed to the present study, these detection approaches could not confidently confirm the fungal origin of the measured biomarkers because of the imperfect fractionation of SF from spores (Lindsley et al., 2006) and the fact that beta-glucans or NAHA can originate from plants and insects. Further, *in vitro* aerosolization studies using automatic particle counters have reported quite high levels of SF (up to ×400 the levels of spores; Górny et al., 2002; Cho et al., 2005). Our data did not corroborate with these findings as our results showed significantly higher spore concentrations than that of submicronic fragments. The occurrence of low levels of SF in sawmills as compared to laboratory conditions may be due different characteristics of the fungal biomass in the two settings and large variation between factors influencing particles aerosolization. Nevertheless, the documentation of exposure to submicronic and large fragments in sawmills provides a broader dimension of fungal exposure with potential consequences on the exposure–disease relationship at workplaces. SF can stay airborne for longer time than spores, and similar to spores are able to reach the alveolar regions upon inhalation (Cho et al., 2005; Reponen et al., 2007).

Spores are the most studied fungal particles in fungal exposure. Only 32% (22 out of 69) of samples in the present study have spore levels above the suggested LOEL of 10⁵ spores/m³ (Eduard, 2009), but overall, the average spore exposure levels were comparable to previous works that investigated fungal exposure in

sawmills (1.5×10^5 m^{-3}; Oppliger et al., 2005; Rusca et al., 2008). The spore levels in the present study were higher than the concentrations reported from sawmills by Dahlqvist and colleagues (median: 0.3×10^5 m^{-3}; Dahlqvist et al., 1992), and Klarić et al. (max: 0.7×10^5 m^{-3}; Klarić et al., 2012). Several other studies from sawmills have shown quite higher concentrations of spores that were associated with airway impairments among workers (median: 1.3×10^5 m^{-3} (Johard et al., 1992); 2×10^5 m^{-3} (Halpin et al., 1994); 5×10^5 m^{-3} (Dahlqvist and Ulfvarson, 1994); 4×10^5 m^{-3} (Mandryk et al., 1999); AM: 1.4×10^6 m^{-3} (Eduard et al., 1992). However, much higher exposure levels have been reported at other workplaces including farming: 26×10^5 m^{-3} (Eduard et al., 2004), grain farming: 40×10^5 m^{-3} (Halstensen et al., 2007), and cheese factory: $54–1500 \times 10^5$ m^{-3} (Simon and Duquenne, 2014). The differences in spore exposure in sawmills can be attributed to various parameters including sampling conditions (stationary versus personal, inhalable versus thoracic or respirable), modernization levels of facilities that may help reduce fungal colonization of timber and subsequent exposure, or the spatial and temporal variation in fungal growth and sporulation. Of note, we assumed that 1 spore/m^3 is equivalent to 10 colony forming units (CFU) per m^3 for measurements based on cultivation methods (Eduard, 2009).

As most fungal exposure studies have focused on fungal spores, we found it necessary to investigate the correlation between spores and fragments as spore measurements may represent a proxy for fungal exposure. This correlation was weak, indicating that spore concentrations cannot give good prediction of LF or SF levels. Other studies found similar results using beta-glucans as fungal biomarkers in size-discriminated fractions of particles collected during *in vitro* aerosolization (Seo et al., 2009) and in indoor studies (Reponen et al., 2007). This weak correlation may be attributed to different mechanisms governing the release, the dispersion, and the aerodynamic behavior of spores in the air as compared to fragments. However, significant correlation between spores and fragments cannot be totally ruled out due to the limited number of samples in the present study. More importantly, the relationship between spore exposure and health outcomes may differ from fungal fragments or both spores and fragments since the biochemical properties and exposure levels of these particles are different. Therefore, fragments need to be quantified in addition to spores in future epidemiological studies as one approach to elucidate this relationship.

In order to improve our understanding of fungal exposure–disease relation, the characterization of the complex mixture of fungal particles requires broader and holistic analysis approach that includes simultaneously information on various components. Also, because toxic reactions induced by heterogeneous dust particles have been shown to be dependent on various components, origins, and chemical compositions (Kelly and Fussell, 2012), it is likely that any shift in the percentage of each particle type can influence potential adverse health effects induced in the airways of exposed subjects. The exploration of the fungal aerosol composition in the present study showed that the aerosol composition did not change significantly between summer and winter, although the concentrations of SS, SF, and LF varied significantly between seasons. This indicates that the concentrations of SF, LF, and SS are season dependent but their prevalence is not. The composition based approach also revealed a significant change in the fungal aerosol composition in saw departments as compared to the department for sorting of green timber ($p = 0.003$).

In fact, the spore fraction was significantly higher in the saw departments (AM: 41%) as compared to the SGT department (AM: 18%), whereas SF and LF fractions did not show any significant changes. Despite the small number of samples per department, it is likely that workers in sawmills were exposed to fungal aerosols with similar particles type composition, except in the saw departments, where the spore fraction was significantly higher as compared to SGT departments. The observed difference between the saw and the SGT needs, however, to be considered with reservation due to the low number and the unbalanced distribution of samples in the departments. These findings are interesting as the compositional analysis approach revealed new aspects of the exposure dynamic that differs from that observed with concentration data. Further studies with increased sample size and linking exposure data to observed adverse health outcomes will be important to confirm our findings and to investigate how the compositional or the concentration based analyses influence the exposure–disease relationship.

The fraction of LF was significantly higher in winter compared to summer whereas the concentrations of LF were higher in summer. A reason for this difference may be due to the properties of percentage data compared to concentration data. Moreover, we observed large amounts of spores and fragments in the processing stages (SDT and P) following the kiln drying stage. This was most likely due to the colonization of the timber before drying.

Considering the complex composition of fungal aerosols and related toxic or immunogenic constituents, exposure to non-pathogenic fungal species can induce a

wide array of airways symptoms such as mucosal irritation, allergic and non-allergic reactions. Exposure to germinating spores or hyphae have been reported to induce interleukin 4 (IL-4) production and eosinophil mobilization with polarized Th_2 cell allergic inflammation, whereas dormant spores induced interferon gamma (INFγ) production with Th_1 cell responses (Bozza et al., 2002). A recent study exposing differentiated macrophages to spores and mycelial fragments from *A. fumigatus*, *A. versicolor*, *P. chrysogenum*, and *S. chartarum* revealed species and particle type dependent inflammatory potency. Fragments from *A. fumigatus* and *P. chrysogenum* showed higher inflammatory effects compared to spores whereas the inverse was observed with *A. versicolor* and *S. chartarum* (Øya et al., 2018). The presence of hydrophobin rodlet layers on spores has been suggested to explain these differences in immune reactions by shielding immune potent surface constituents on spores from *A. fumigatus*. This layer is absent on the hyphal structures rendering them more exposed to immune cell receptors (Aimanianda and Latgé, 2010). Moreover, spores are mainly phagocytized by airways patrolling phagocytes, but clearance of large hyphae particles often involve polymorphonuclear phagocytes (Brakhage et al., 2010; Knox et al., 2014) with or without the secretion of large amounts of oxidative species intermediates (Park and Mehrad, 2009). Furthermore, elimination of large hyphal particles may induce development of neutrophil extracellular traps (NETs) in a phenomenon known as NETosis. NETosis is characterized by neutrophils that sense the size of microbial particles and release NETs rich in oxidizing enzymes able to degrade large fungal hyphal particles (Branzk et al., 2014). Immunotoxicological responses induced by fungal exposure is likely a multifactorial process with links to particle types, morphology and antigenic constituents as well as the host genetic disposition (Vacher et al., 2015a). The diversity in morphology of spores and hyphal fragments (spherical, oblong, or fiber-like in shape) seems not only to govern the aerodynamic deposition patterns in the human respiratory tract upon inhalation (Cho et al., 2005; Kleinstreuer et al., 2008) but also the type of immune reactions induced through cytokines, chemokines, and from involved immune cells (Vacher et al., 2015b).

The size-dependent immune response supports the need for particle size determination in the characterization of fungal aerosols.

The paradigm of spores as the major agent of exposure to fungal particles bearing allergens and various fungal metabolites changed upon discovery of the liberation of large numbers of submicronic fragments from fungal cultures. These fragments were suggested as the most prevalent fungal particles in indoor exposure (Górny et al., 2002; Reponen et al., 2007). The FESEM based approach used in the present study is relatively tedious but allows specific quantification and classification of fungal fragments (type, size, and shape). In this study, the fraction of submicronic fragments in the fungal aerosol was relatively small, whereas the larger fragments represented the largest fraction. The present study provides useful information in this respect about fungal aerosols.

Conclusions

The personal exposure to fungal particles in sawmills was dominated by large fungal fragments. One-third of workers were exposed to levels of spores that exceeded the suggested effect level of 10^5 spores m^{-3}. The levels of spores and submicronic and large fragments were significantly higher in summer than in winter. The heterogeneous composition of the fungal aerosols was not influenced by seasons or sawmills, but was significantly different between saw departments and the sorting of green timber department. Further studies are needed to elucidate not only the role of fungal fragments, but also of the complex fungal aerosols in health effects observed upon occupational exposure to fungal aerosols.

Supplementary Data

Supplementary data are available at *Annals of Work Exposures and Health* online.

Acknowledgments

References

Adhikari A, Reponen T, Rylander R. (2013) Airborne fungal cell fragments in homes in relation to total fungal biomass. *Indoor Air*; **23**: 142–7.

Adhikari A, Sahu S, Bandyopadhyay A *et al.* (2015). Fungal contamination of the respiratory tract and associated respiratory impairment among sawmill workers in India. *ERJ Open Res*; **1**: 00023–2015.

Afanou KA, Straumfors A, Skogstad A *et al.* (2014) Submicronic fungal bioaerosols: high-resolution microscopic

characterization and quantification. *Appl Environ Microbiol*; **80**: 7122–30.

Afanou KA, Straumfors A, Skogstad A *et al*. (2015a) Indirect immunodetection of fungal fragments by field emission scanning electron microscopy. *Appl Environ Microbiol*; **81**: 5794–803.

Afanou KA, Straumfors A, Skogstad A *et al*. (2015b) Profile and morphology of fungal aerosols characterized by field emission scanning electron microscopy (FESEM). *Aerosol Sci Technol*; **49**: 423–35.

Aimanianda V, Bayry J, Bozza S *et al*. (2009) Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature*; **460**: 1117–21.

Aimanianda V, Latgé JP. (2010) Fungal hydrophobins form a sheath preventing immune recognition of airborne conidia. *Virulence*; **1**: 185–7.

Aitchison J. (2003) *A concise guide to compositional data analysis*. Girona: CDA Work.

Bauer H, Claeys M, Vermeylen R *et al*. (2008) Arabitol and mannitol as tracers for the quantification of airborne fungal spores. *Atmos Environ*; **42**: 588–93.

Belin L. (1987) Sawmill alveolitis in Sweden. *Int Arch Allergy Appl Immunol*; **82**: 440–3.

Benjamini Y, Hochberg Y. (1995) Controlling the false discovery rate: a practical and powerfull approach of multiple testing. *J R Satistical Soc*; **57**: 289–300.

Bozza S, Gaziano R, Spreca A *et al*. (2002) Dendritic cells transport conidia and hyphae of Aspergillus fumigatus from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *J Immunol*; **168**: 1362–71.

Brakhage AA, Bruns S, Thywissen A *et al*. (2010) Interaction of phagocytes with filamentous fungi. *Curr Opin Microbiol*; **13**: 409–15.

Branzk N, Lubojemska A, Hardison SE *et al*. (2014) Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat Immunol*; **15**: 1017–25.

Brasel TL, Douglas DR, Wilson SC *et al*. (2005) Detection of airborne Stachybotrys chartarum macrocyclic trichothecene mycotoxins on particulates smaller than conidia. *Appl Environ Microbiol*; **71**: 114–22.

Cho SH, Seo SC, Schmechel D *et al*. (2005) Aerodynamic characteristics and respiratory deposition of fungal fragments. *Atmos Environ*; **39**: 5454–65.

Dahlqvist M, Johard U, Alexandersson R *et al*. (1992) Lung function and precipitating antibodies in low exposed wood trimmers in Sweden. *Am J Ind Med*; **21**: 549–59.

Dahlqvist M, Ulfvarson U. (1994) Acute effects on forced expiratory volume in one second and longitudinal change in pulmonary function among wood trimmers. *Am J Ind Med*; **25**: 551–8.

Delfino RJ, Zeiger RS, Seltzer JM *et al*. (1997) The effect of outdoor fungal spore concentrations on daily asthma severity. *Environ Health Perspect*; **105**: 622–35.

Eduard W. (2009) Fungal spores: a critical review of the toxicological and epidemiological evidence as a basis for occupational exposure limit setting. *Crit Rev Toxicol*; **39**: 799–864.

Eduard W, Douwes J, Omenaas E *et al*. (2004) Do farming exposures cause or prevent asthma? Results from a study of adult Norwegian farmers. *Thorax*; **59**: 381–6.

Eduard W, Sandven P, Levy F. (1992) Relationships between exposure to spores from Rhizopus microsporus and Paecilomyces variotii and serum IgG antibodies in wood trimmers. *Int Arch Allergy Immunol*; **97**: 274–82.

Górny RL, Reponen T, Willeke K *et al*. (2002) Fungal fragments as indoor air biocontaminants. *Appl Environ Microbiol*; **68**: 3522–31.

Green BJ, Millecchia LL, Blachere FM *et al*. (2006) Dual fluorescent halogen immunoassay for bioaerosols using confocal microscopy. *Anal Biochem*; **354**: 151–3.

Green BJ, Mitakakis TZ, Tovey ER. (2003) Allergen detection from 11 fungal species before and after germination. *J Allergy Clin Immunol*; **111**: 285–9.

Green BJ, Schmechel D, Summerbell R. (2011) Aerosolized fungal fragments. In Adan OCG, Samson RA, editors. *Fundamentals of mold growth in indoor environments and strategies for healthy living*. Wageningen, The Netherlands: Wageningen Academic Publishers. pp. 211–43.

Halpin DM, Graneek BJ, Turner-Warwick M *et al*. (1994) Extrinsic allergic alveolitis and asthma in a sawmill worker: case report and review of the literature. *Occup Environ Med*; **51**: 160–4.

Halstensen AS, Nordby KC, Wouters IM *et al*. (2007) Determinants of microbial exposure in grain farming. *Ann Occup Hyg*; **51**: 581–92.

Hohl TM, Van Epps HL, Rivera A *et al*. (2005) Aspergillus fumigatus triggers inflammatory responses by stage-specific beta-glucan display. *PLoS Pathog*; **1**: e30.

Institute of Medicine. (2004) Human health effects. In *Damp indoor spaces and health*. Washington, DC: National Academies Press. pp. 189–243.

Johard U, Eklund A, Dahlqvist M *et al*. (1992) Signs of alveolar inflammation in non-smoking Swedish wood trimmers. *Occup Environ Med*; **49**: 428–34.

Kelly FJ, Fussell JC. (2012) Size, source and chemical composition as determinants of toxicity attributable to ambient particulate matter. *Atmos Environ*; **60**: 504–26.

Kildesø J, Wurtz H, Nielsen KF *et al*. (2000) The release of fungal spores from water damaged building materials. In Seppanen O, Sateri J, editors. Healthy buildings 2000 *Exposure, human responses and building investigations*. Vol. **1**. Proceedings, Indoor Air Information. OY, Helsinki Finland: pp. 313–8.

Klarić MŠ, Varnai VM, Calušić AL *et al*. (2012) Occupational exposure to airborne fungi in two Croatian sawmills and atopy in exposed workers. *Ann Agric Environ Med*; **19**: 213–9.

Kleinstreuer C, Zhang Z, Li Z. (2008) Modeling airflow and particle transport/deposition in pulmonary airways. *Respir Physiol Neurobiol*; **163**: 128–38.

Knox BP, Deng Q, Rood M *et al*. (2014) Distinct innate immune phagocyte responses to Aspergillus fumigatus conidia and hyphae in zebrafish larvae. *Eukaryot Cell*; **13**: 1266–77.

Lindsley WG, Schmechel D, Chen BT. (2006) A two-stage cyclone using microcentrifuge tubes for personal bioaerosol sampling. *J Environ Monit*; **8**: 1136–42.

Madsen AM, Schlünssen V, Olsen T *et al.* (2009) Airborne fungal and bacterial components in PM1 dust from biofuel plants. *Ann Occup Hyg*; **53**: 749–57.

Madsen AM, Tendal K, Schlünssen V *et al.* (2012) Organic dust toxic syndrome at a grass seed plant caused by exposure to high concentrations of bioaerosols. *Ann Occup Hyg*; **56**: 776–88.

Mandryk J, Alwis KU, Hocking AD. (1999) Work-related symptoms and dose-response relationships for personal exposures and pulmonary function among woodworkers. *Am J Ind Med*; **35**: 481–90.

Mandryk J, Alwis KU, Hocking AD. (2000) Effects of personal exposures on pulmonary function and work-related symptoms among sawmill workers. *Ann Occup Hyg*; **44**: 281–9.

Mitakakis TZ, Barnes C, Tovey ER. (2001) Spore germination increases allergen release from Alternaria. *J Allergy Clin Immunol*; **107**: 388–90.

Nygaard UC, Samuelsen M, Aase A *et al.* (2004) The capacity of particles to increase allergic sensitization is predicted by particle number and surface area, not by particle mass. *Toxicol Sci*; **82**: 515–24.

Oppliger A, Rusca S, Charrière N *et al.* (2005) Assessment of bioaerosols and inhalable dust exposure in Swiss sawmills. *Ann Occup Hyg*; **49**: 385–91.

Pady S, Kramer C. (1960) Kansas aeromycology VI: hyphal fragments. *Mycologia*; **52**: 681–7.

Park SJ, Mehrad B. (2009) Innate immunity to Aspergillus species. *Clin Microbiol Rev*; **22**: 535–51.

Pawlowsky-Glahn V, Egozcue JJ. (2006) Compositional data and their analysis: an introduction. *Geol Soc London Spec Publ*; **264**: 1–10.

Rask-Andersen A, Land CJ, Enlund K *et al.* (1994) Inhalation fever and respiratory symptoms in the trimming department of swedish sawmills. *Am J Ind Med*; **25**: 65–7.

Raulf M, Buters J, Chapman M *et al.*; European Academy of Allergy and Clinical Immunology. (2014) Monitoring of occupational and environmental aeroallergens– EAACI position paper. Concerted action of the EAACI IG occupational allergy and aerobiology & air pollution. *Allergy*; **69**: 1280–99.

Reponen T, Seo SC, Grimsley LF *et al.* (2007) Fungal fragments in moldy houses: a field study in homes in New Orleans and Southern Ohio. *Atmos Environ*; **41**: 8140–9.

Reponen T, Vesper S, Levin L *et al.* (2011) High environmental relative moldiness index during infancy as a predictor of asthma at 7 years of age. *Ann Allergy Asthma Immunol*; **107**: 120–6.

Rusca S, Charrière N, Droz PO *et al.* (2008) Effects of bioaerosol exposure on work-related symptoms among Swiss sawmill workers. *Int Arch Occup Environ Health*; **81**: 415–21.

Schnurer J. (1993) Comparison of methods for estimating the biomass of three food-borne fungi with different growth patterns. *Appl Environ Microbiol*; **59**: 552–5.

Seo S, Choung JT, Chen BT *et al.* (2014) The level of submicron fungal fragments in homes with asthmatic children. *Environ Res*; **131**: 71–6.

Seo SC, Reponen T, Levin L *et al.* (2009) Size-fractionated (1–>3)-beta-D-glucan concentrations aerosolized from different moldy building materials. *Sci Total Environ*; **407**: 806–14.

Simon X, Duquenne P. (2014) Assessment of workers' exposure to bioaerosols in a French cheese factory. *Ann Occup Hyg*; **58**: 677–92.

Singh U, Reponen T, Cho KJ *et al.* (2011) Airborne endotoxin and β-D-glucan in PM1 in agricultural and home environments. *Aerosol Air Qual Res*; **11**: 376–86.

Tendal K, Madsen AM. (2011) Exposure to airborne microorganisms, hyphal fragments, and pollen in a field of organically grown strawberries. *Aerobiologia (Bologna)*; **27**: 13–23.

Vacher G, Ciarlo E, Savova-Bianchi D *et al.* (2015a) Innate immune sensing of Fusarium culmorum by mouse dendritic cells. *J Toxicol Environ Health A*; **78**: 871–85.

Vacher G, Niculita-Hirzel H, Roger T. (2015b) Immune responses to airborne fungi and non-invasive airway diseases. *Semin Immunopathol*; **37**: 83–96.

Vesper S. (2011) Traditional mould analysis compared to a DNA-based method of mould analysis. *Crit Rev Microbiol*; **37**: 15–24.

Øya E, Afanou AKJ, Malla N *et al.* (2018) Characterization and pro-inflammatory responses of spore and hyphae samples from various mold species. *Indoor Air*; **28**: 28–39.