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Fungi, β-Glucan, and Bacteria in Nasal Lavage of Greenhouse Workers and Their Relation to Occupational Exposure

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The nose and mouth are the first regions of the respiratory tract in contact with airborne microorganisms. Occupational exposures to airborne microorganisms are associated with inflammation and different symptoms of the airways. The purpose of this study is to investigate the relation between occupational exposure to fungi, β-glucan, and bacteria and contents of fungi, β-glucan, and bacteria in nasal lavage (NAL) of greenhouse workers. We also studied whether contents of microorganisms in NAL were related to gender, time of the work week, and runny nose. NAL samples (n = 135) were taken Monday morning and Thursday at noon and personal exposure to inhalable bioaerosols was measured during a working day. The content of fungi and β-glucan in NAL of men was affected by their exposure to fungi and β-glucan. The content of fungi, β-glucan, and bacteria in NAL was higher Thursday at noon than Monday morning. The ratios of fungi in NAL between Thursday at noon and Monday morning were 14 (median value) for men and 3.5 for women. Gender had no effect on the exposure level but had a significant effect on the content of fungi, β-glucan, and bacteria in NAL, with the highest contents in NAL of men. On Thursdays, the median content of fungi in NAL samples of men without runny noses was 9408 cfu per NAL sample, whereas the same content for women was 595 cfu per NAL sample. Workers with runny noses had fewer fungi in NAL than workers without runny noses. A higher content of β-glucan per fungal spore was found in NAL than in the air. This indicates that mainly the larger fungal spores or pollen grains deposit in the nose. The difference between genders and the fact that the content of fungi in NAL was significantly affected by the exposure indicate that the two genders are affected by the same exposure level differently.

Keywords: bacteria; bioaerosols; fungi; nasal deposition; occupational exposure; respiratory symptoms

INTRODUCTION

Evidence from epidemiological or experimental studies supports the hypothesis that exposure to fungi is causally associated with development of hypersensitivity pneumonitis, decline in lung function, severity of asthma, respiratory symptoms, and airway inflammation (Simon-Nobbe *et al.*, 2008). Different forms of fungal diseases affecting the nose and paranasal sinuses are recognized including invasive and non-invasive fungal rhinosinusitis (Ebbens and Fokkens, 2008). Bacteria are implicated as pathogens in chronic rhinosinusitis (Ebbens and Fokkens, 2008), and

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indoor airborne bacteria can be associated with blocked nose (Haverinen-Shaughnessy *et al.*, 2007). Occupational exposure to endotoxin from Gram-negative bacteria is linked to health impairments like fever, nose and throat irritation, chest tightness, cough, shortness of breath, wheezing, acute airway flow restriction, and inflammation (Liebers *et al.*, 2008). In addition, (1→3)-β-D-glucan (β-glucan) from fungi and pollen has been linked to respiratory symptoms, throat irritation, and ear problems (Alwis *et al.*, 1999). Density of nasal hair seems to affect the risk of developing asthma in people with rhinitis (Ozturk *et al.*, 2011). Gender differences have been found in response to both fungal (Jaakkola and Jaakkola, 2004) and endotoxin exposure (Kline *et al.*, 1999). Furthermore, gender differences have been found for nasal hair density (Ozturk *et al.*, 2011) and nose size (Hall, 2005).

The inhalation and sedimentation of fungi and bacteria in the respiratory tract depend on the aerodynamic diameter (d_{ae}) of the fungal or bacterial particle. Particle deposition in the airways is directly connected to damage of the pulmonary system. Therefore, health-related particle sampling should reflect how particles penetrate and deposit in the various regions of the human respiratory system. The fraction able to enter the airways through the nose or mouth is called the inhalable fraction (d_{ae50} = 100 µm). Culturable fungi in aerosols are often present as or associated with particles with an d_{ae} between 2 and 6 µm (Madsen *et al.*, 2009b). Bacterial cells are smaller than fungal spores but culturable bacteria often seem to be aerosolized in clusters or associated with larger particles (d_{ae} 2–8 µm) (Madsen *et al.*, 2009b). The component β-glucan is found in the cell walls of dead as well as living fungi (Fogelmark and Rylander, 1997), in fungal particles smaller than spore size (d_{ae} < 1.0 µm) (Madsen *et al.*, 2009b; Singh *et al.*, 2011), and in pollen (Foto *et al.*, 2004). Most airborne pollen grains are 15–50 µm in diameter (Burge and Rogers, 2000).

In epidemiological studies of exposure, there is no standard method for sampling microorganisms (Lai *et al.*, 2009). The nasal lavage (NAL) procedure allows measurement of presence of fungi and bacteria in the nose. The nose is the primary portal of entry for inspired air, and therefore, the first region of the respiratory tract in contact with airborne fungi and bacteria. Presence of fungi or bacteria has previously been measured in NAL in non-quantitative ways and not as a measure of exposure. Greenhouse workers can be exposed to high concentrations of fungi, β-glucan, bacteria, and endotoxin (Madsen *et al.*, 2009a; Hansen *et al.*, 2011) and have a high prevalence of self-reported respiratory symptoms (Adhikari *et al.*, 2011). In this study, we have measured the exposure to and the content of fungi, β-glucan, and bacteria in NAL of greenhouse workers. Metabolically active microorganisms produce extracellular enzymes, which may be allergenic, and germinating conidia release allergens (Mitakakis *et al.*, 2001; Green *et al.*, 2003). Therefore, exposure has been measured as culturable fungal and bacterial units. To get a measure of the total fungal and pollen exposure, the exposure to β-glucan was also measured. The aim of this study is to investigate the influence of exposure to fungi, β-glucan, and bacteria, and influence of gender and time of the work week on the contents of fungi, β-glucan, and bacteria in NAL of greenhouse workers.

MATERIALS AND METHODS

The greenhouse workers

The greenhouse workers worked for three different companies producing cucumbers, tomatoes, or different potted plants. The workers were from Eastern and Central Europe, Denmark, the Middle East, and Southeast Asia. The workers volunteered to participate in the investigation and we did not exclude any of the participants. On the day the NAL samples were taken, runny nose was reported 31 times (Table 1). Seven men and one woman were smokers. Cigarette smoking within an hour before the NAL samples were taken was reported 21 times. The median ages were 38 and 39 years for men and women, respectively, whereas their median heights were 180 and 170 cm, respectively. The same kinds of work tasks (e.g. packaging of plants, tomatoes, or cucumbers, harvesting, nursing plants, and removing old plants) were done through the week and by both genders.

Nasal lavage

NAL samples were taken twice in the same weeks from 33 greenhouse workers for 18 days; Monday morning between 6:20 and 9:00 (67 samples) and Thursday between 11:10 and 13:20 (68 samples) in 2010 and 2011. From 13 women, a total of 47 samples were taken and from 20

Table 1. Microorganisms and β-glucan in NAL of greenhouse workers with or without runny nose.

Time and gender	Fungi (25°C), cfu		Fungi (37°C), cfu		Bacteria (25°C), cfu		Bacteria (37°C), cfu		β-Glucan, pg	
	Runny nose		Runny nose		Runny nose		Runny nose		Runny nose	
	Yes Median *n*	No Median *n*	Yes Average (positive) *n*	No Average (positive) *n*	Yes Median *n*	No Median *n*	Yes Median *n*	No Median *n*	Yes Median *n*	No Median *n*
Monday										
Women	329 6	15 17	bd (0%) 6	bd (0%) 17	1.91×10^4 6	3.00×10^4 17	2.05×10^4 6	4.64×10^4 17	bd 3	4400 5
Men	108 14	703 29	61 (14%) 14	132 (21%) 29	2.86×10^4 14	2.10×10^5 29	8.07×10^4 14	1.85×10^5 29	1.02×10^4 3	1.38×10^4 10
Thursday										
Women	383 4	595 20	bd (0%) 4	101 (25%) 20	3.82×10^4 4	8.18×10^4 20	2.18×10^4 4	6.68×10^4 20	bd 3	1.20×10^4 15
Men	2324 7	9408 38	119 (25%) 7	127 (31%) 38	2.95×10^5 7	3.23×10^5 38	4.53×10^5 7	3.92×10^5 38	2.34×10^4 7	7.93×10^4 31

n = number of samples, bd = below detection level, cfu = colony forming units present in the 10 ml nasal lavage (NAL), nm = not measured, (positive) = % of samples with fungi able to grow at 37°C.

men, a total of 88 samples were taken. A modification of the method described by Naclerio *et al.* (1983) was used for NAL sampling: workers were asked to incline their heads forward. A nose plug with a rubber tube was inserted into one of the nostrils. The nose was flushed with 5 ml of sterile isotonic saline solution (37°C) using a sterile, disposable syringe. After 30 seconds, the person gently blew the solution into a plastic container, and the solution was immediately put on ice. The procedure was repeated for the other nostril. Solution from the left and right nostrils was mixed. A volume of 0.5 ml of the NAL was used for quantification of fungi and bacteria and 250 µl for β-glucan analysis. The suspensions for measurements of microorganisms were mixed with glycerol (85%, Merck, Germany) to protect the microorganisms when frozen. All samples were frozen at −80°C within 3 h after sampling.

Personal sampling and extraction of inhalable aerosols

Personal exposure was measured during one work day in each week using Gesamtstaubprobenahme (GSP) inhalable samplers (Gesamtstaubprobenahme by BGI, Inc., Waltham, MA, USA). These samplers were chosen as they have a high sampling efficiency (Kenny *et al.*, 1999). Sampling took place from 6:00 or 7:00 to 15:00 or 16:00 during the Wednesdays immediately preceding the Thursday of NAL sampling. Each worker carried a sampler mounted with a polycarbonate filter (pore size 1 µm). In total, 68 samples were taken and the average sampling time was 5 hours and 20 minutes. The dust on polycarbonate filters for quantification of microorganisms and β-glucan was extracted in 10.0 ml sterile 0.05% Tween 80 and 0.85% NaCl aqueous solution by shaking for a 15-min period (500 r.p.m.) at room temperature.

Quantification of β-glucan by the Limulus method

Airborne and NAL-borne β-glucan was extracted from 50 and 78 samples, respectively, in 0.3 M NaOH for 60 minutes. After extraction and dilution, β-glucan was quantified in duplicate using the kinetic Fungitic G Test (Seikagaku Co., Tokyo, Japan). We used a standard curve ranging from 4.0 to 100 pg ml^{-1}. The data are presented as picogram per cubic meter air and as picogram in each 10 ml NAL sample. The detection limit was 180 pg m^{-3} air and 18 pg ml^{-1} NAL.

Quantification of microorganisms in NAL and in airborne dust

The NAL samples were treated 1:1 with 1,4-dithiothreitol (BDH Prolabo, VWR, Belgium) (30 mM) for 15 min to dissolve nasal mucus. The number of fungi in NAL and GSP samples culturable on Dichloran Glycerol Agar (DG18 agar, Oxoid, Basingstoke, UK) at 25°C were counted after 3 and 7 days of incubation. The number of fungi in NAL culturable on Malt Extract Agar (Oxoid,

Basingstoke, UK) with Chloramphenicol (Fluca, USA) at 37°C were counted after 3, 7, and 14 days of incubation. Bacteria were quantified after 3 and 7 days of incubation on 100% Nutrient Agar (Oxoid, Basingstoke, UK) with Actidione [cycloheximide; 50 mg l^{-1} (Serva, Germany)] at 25°C (NAL and GSP samples) and 37°C (NAL samples). A small amount from 50 bacterial colonies able to grow at 25°C (from 20 GSP samples) was Gram stained using a crystal violet solution (Merck, Germany), a Lugol solution (Merck, Germany), and a Safranin solution (Merck, Germany). The Gram-positive and Gram-negative bacteria were counted. Only a subsample of the NAL sample was used for quantification of microorganisms and the detection limit was 3 cfu fungi ml^{-1} at 25°C, 2 cfu fungi ml^{-1} at 37°C, and 300 cfu bacteria ml^{-1} NAL at 25°C and 37°C. For microorganisms in GSP samples, the detection limit was 2 cfu fungi or bacteria ml^{-1}. The data are presented as colony-forming unit in each 10 ml NAL sample and colony-forming unit per cubic meter air.

Treatment of data

Concentrations were approximately log-normally distributed and median values are calculated. For measurements below the detection level, 33% of the detection limit is used. As fungi (37°C) were found in <50% of the samples, these fungi are also presented as averages and as percentage of samples with presence of fungi. The content of β-glucan per fungal spore in NAL versus in inhalable dust was compared using paired *t*-test in SAS version 9.2. Exposure of men and women was compared using PROC GLM. Pearson's correlation coefficients (r^2) were calculated for contents in NAL versus exposure.

The associations of log-transformed exposure to fungi, bacteria, and β-glucan and corresponding log-transformed contents in NAL were estimated in mixed models adjusting for runny nose as a fixed effect and accounting for correlated measures by including individual as a random effect. Associations were also estimated for each gender separately. Similarly, the associations of gender, runny nose, and time of measurement on log-transformed NAL contents were estimated in mixed models accounting for correlated measures by including individual as a random effect. Associations of the three explaining factors with NAL contents were mutually adjusted. Also associations of runny nose and time of NAL sampling were estimated for each gender separately. The PROC MIXED procedure was used for all mixed model analyses.

RESULTS

Contents of fungi, β-glucan, and bacteria in NAL

In the 135 NAL samples of 10 ml, fungi (25°C) (median = 757 cfu; min = below detection (bd); max = 7.5×10^5 cfu per sample) were found in 107 samples and bacteria (25°C) (median = 1.40×10^5 cfu; min = bd; max = 3.46×10^7 cfu per sample) in 121 samples. Fungi (37°C) were only found in 20% of the samples (median = bd; min = bd; max = 3120 cfu). Bacteria (37°C) were also found in 121 NAL samples (median = 1.40×10^5 cfu; min = bd; max = 1.85×10^7 cfu). The component β-glucan was quantified in 78 NAL samples and was above the detection limit in 68 of these samples (median = 3.3×10^4 pg; min = bd; max = 7.6×10^5 pg). Sorted according to time (Monday morning or Thursday at noon), gender, and presence or absence of runny nose, the contents of microorganisms in NAL are presented in Table 1.

Exposure to airborne fungi, β-glucan, and bacteria

All workers were exposed to fungi (25°C) (median = 4.54×10^4 cfu m^{-3}; min = 176 cfu m^{-3}; max = 7.04×10^7 cfu m^{-3}) and bacteria (25°C) (median = 6.79×10^3 cfu m^{-3}; min = 180 cfu m^{-3}; max = 6.41×10^5 cfu m^{-3}) above the detection limit. Fifty samples were analysed for β-glucan, which was present in all samples (median = 1.01×10^5 pg m^{-3}; min = 4172 pg m^{-3}; max = 1.79×10^6 pg m^{-3}). A percentage of 45 of the bacteria (25°C) were Gram-positive. No significant differences in exposure to fungi (25°C) (medians = 3.82×10^4 cfu m^{-3} versus 4.88×10^4 cfu m^{-3}; $P = 0.37$), β-glucan (medians = 1.09×10^5 pg m^{-3} versus 7.11×10^4 pg m^{-3}; $P = 0.44$), or bacteria (25°C) (medians = 8.69×10^3 cfu m^{-3} versus 6.79×10^3 cfu m^{-3}; $P = 0.74$) were found between men and women.

Association between exposure and microorganisms (25°C) in NAL

The association between personal exposure to inhalable microorganisms (25°C) and content of microorganisms in NAL Thursday at noon was studied. The content of fungi (25°C) in NAL of men correlated significantly with their exposure (Fig. 1a). Five women had contents of fungi in NAL below the detection limit; a significant correlation was only found between contents in NAL and exposure when these five values were excluded (Fig. 1b). For bacteria in NAL versus exposure, no

correlation was seen (Fig. 1c,d). The contents of β-glucan in NAL correlated significantly with the personal exposure to β-glucan of men (Fig. 2a) but not of women (r^2 = 0.33, P = 0.21).

In a statistical model, the influence of exposure to fungi, β-glucan, or bacteria on the contents of, respectively, fungi, β-glucan, and bacteria in NAL was studied. The content of fungi in NAL was significantly affected by exposure to fungi (P = 0.0005). The content of β-glucan in NAL was also affected positively and significantly by the exposure to β-glucan (P = 0.0005) (Table 2).

Effects of time, gender, and runny nose on microorganisms (25 and 37°C) in NAL

In a similar model, the effect of gender, runny nose, and time (Monday morning or Thursday at noon) on fungi (25 and 37°C), β-glucan, and bacteria (25 and 37°C) in NAL was studied (Table 3). Gender significantly affected the content of fungi (25°C), β-glucan, and bacteria (25 and 37°C) but not fungi (37°C) in NAL with the higher content in NAL of men.

Runny nose significantly affected the content of fungi in NAL. When each gender was studied separately, the effect of runny nose on fungi (25°C) in NAL was only significant for men, thus men with runny nose had fewer fungi in NAL than men without runny nose.

Time of the week significantly affected the content of fungi (25°C), fungi (37°C) and bacteria (25°C), bacteria (37°C) and amount of β-glucan in NAL with a higher content Thursday at noon than Monday morning. The ratios of fungi (25°C) in NAL between Thursday at noon and Monday

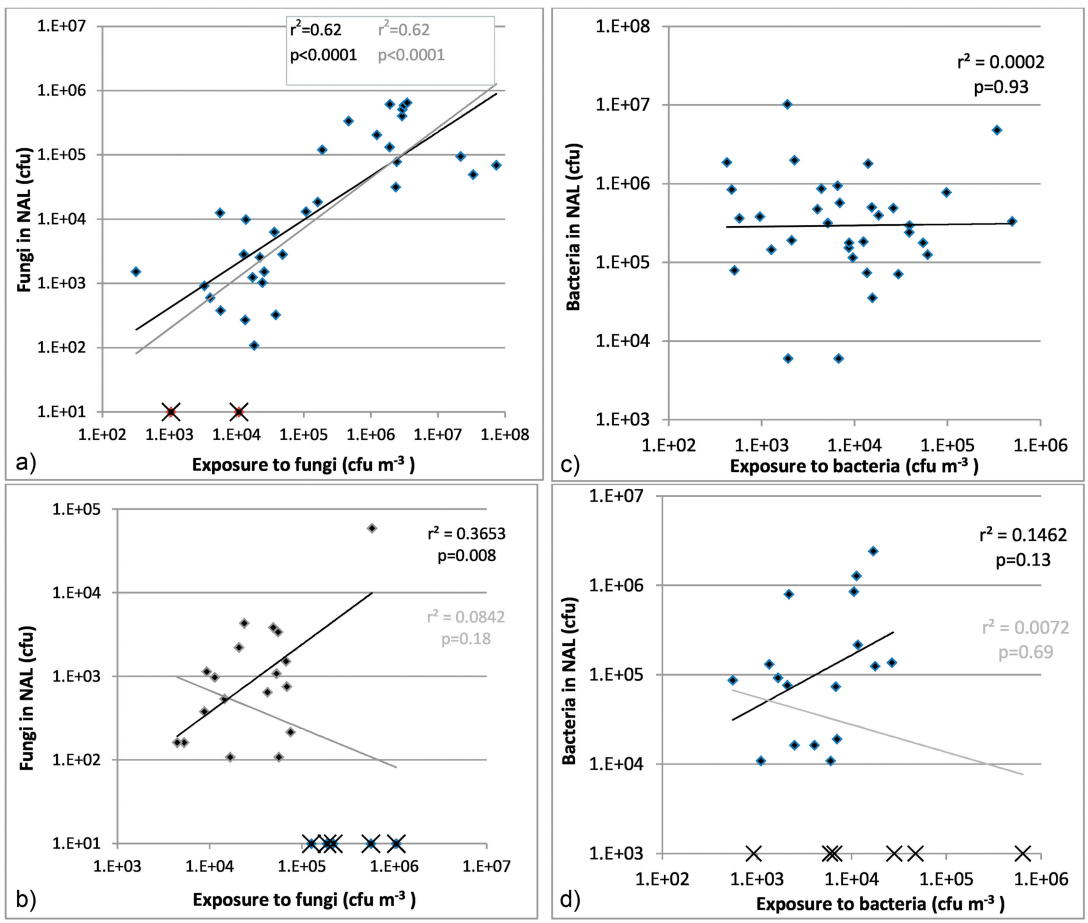

Fig. 1. Relation between exposure to fungi (25°C) (a and b) and bacteria (25°C) (c and d) of men (a and c) and women (b and d) and content of fungi and bacteria in 10 ml NAL. Correlations have been calculated with (grey equation) and without (black equation) values below the detection limit. Points below the detection limits are shown as X.

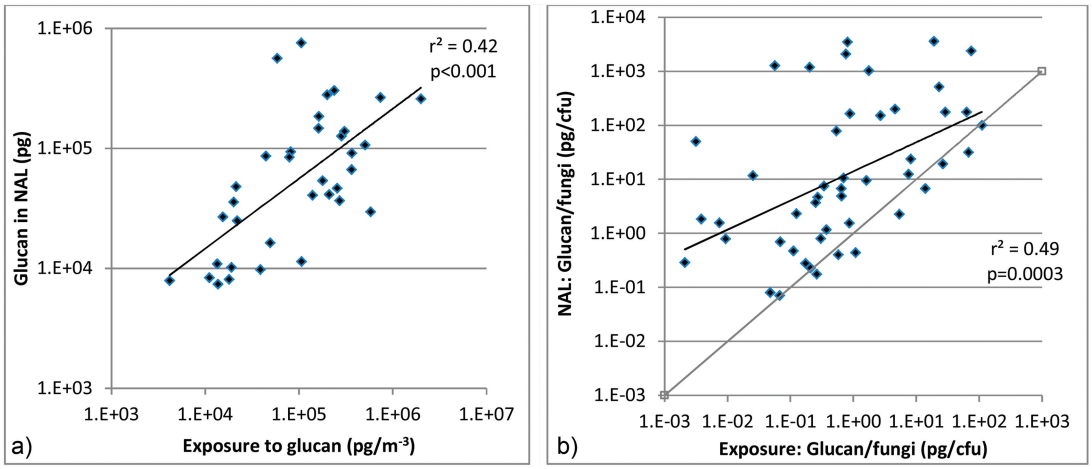

Fig. 2. Correlation between exposure to β-glucan versus content in NAL of men (a) and β-glucan/fungi (25°C) (pg cfu^{-1}) in the exposure versus in the NAL of men and women (b). The grey line is $x = y$.

Table 2. Association between log-transformed contents of fungi, β-glucan, and bacteria in NAL and log-transformed exposure to the same components.

Studied for	Fungi (25°C)	β-Glucan	Bacteria (25°C)
	Estimate* [confidence limits], *P*-value	Estimate [confidence limits], *P*-value	Estimate [confidence limits], *P*-value
Men and women	**0.43** [0.20–0.66], 0.0005	**0.44** [0.26–0.62], 0.0005	−0.099 [−0.52–0.33], 0.65
Men	**0.49** [0.25–0.73], 0.0003	**0.49** [0.21–0.77], 0.0045	0.13 [−0.37–0.62], 0.63
Women	−0.027 [−0.78–0.72], 0.95	0.36 [−0.06–0.78], 0.13	−0.36 [−1.19–0.47], 0.41

*If estimate is larger than 0 and $P < 0.05$, there is a significant positive association between exposure and content in NAL (values in bold). In all analyses, control is made for runny nose and person, and in the non-gender stratified analyses, control is made also for gender.

morning were 14 (median value) for men and 3.5 for women. The ratios of bacteria (25°C) in NAL between Thursday at noon and Monday morning were 4.8 for men and 2.7 for women.

When a similar model was analyzed but with the factor 'smoking versus not smoking within an hour before NAL sampling', no significant effect of smoking on fungi (25°C) (estimate = 1.26, $P = 0.47$) and bacteria (25°C) (estimate = 1.60, $P = 0.70$) in NAL was found.

β-glucan per fungi (25°C)

In order to get an expression of the sizes of the fungal particles and pollen grains accumulating in the nose, we measured β-glucan per cfu of fungi in NAL (median = 6.7 pg cfu^{-1}, average = 56 pg cfu^{-1}) versus in the inhalation zone (median = 1.1 pg cfu^{-1}, average = 12 pg cfu^{-1}). The content was significantly higher in NAL than in the inhalation zone ($P = 0.0004$). However, a significant correlation was found between β-glucan per cfu of fungi in NAL and in the exposure (Fig. 2b). Correlation between β-glucan and fungi in NAL was low though significant ($r^2 = 0.17$, $P = 0.014$).

DISCUSSION

This study shows that the content of fungi and β-glucan in NAL of men is affected significantly by their exposure to fungi and β-glucan. Furthermore, contents of fungi, β-glucan, and bacteria in NAL were higher Thursday at noon than Monday morning. The study also shows that men had more fungi (25°C) (estimate = 12), β-glucan (estimate = 4), bacteria (25°C) (estimate = 4), and bacteria (37°C) (estimate = 4) in NAL than women (Table 3). This difference between genders has not been shown before and may have several explanations. One of the explanations could be nasal anatomy; the larger noses of men (Hall, 2005) and the higher hair density (Ozturk *et al.*, 2011) may constitute a larger area for retention and deposition of microorganisms. Another explanation

Table 3. Association between the levels of fungi, β-glucan, or bacteria in NAL and the factors gender, runny nose, and time (Monday morning and Thursday at noon), respectively. Estimates indicate ratios of content in NAL between the two levels of each factor.

Microorganisms	Studied for	Gender	Runny nose	Time
		Estimate (men versus women) [confidence limits], *P*-value	Estimate (yes versus no) [confidence limits], *P*-value	Estimate (Monday morning versus Thursday noon) [confidence limits], *P*-value
Fungi (25°C) in NAL	Men and women	**11.6** [3.4–39.6], 0.0004	**0.35** [0.13–0.98], 0.049	**0.082** [0.035–0.19], <0.0001
Fungi (25°C) in NAL	Men	—	**0.20** [0.056–0.72], 0.015	**0.058** [0.023–0.15], <0.0001
Fungi (25°C) in NAL	Women	—	1.54 [0.29–8.33], 0.62	**0.15** [0.055–0.60], 0.0029
Fungi (37°C) in NAL	Men and women	1.40 [0.85–2.31], 0.19	0.91 [0.54–1.54], 0.73	**0.56** [0.36–0.87], 0.011
Fungi (37°C) in NAL	Men	—	0.92 [0.46–1.85], 0.82	0.62 [0.34–1.14], 0.13
Fungi (37°C) in NAL	Women	—	0.91 [0.45–1.87], 0.81	**0.49** [0.28–0.84], 0.014
β-Glucan in NAL	Men and women	**4.40** [2.16–9.71], <0.0001	0.52 [0.24–1.22], 0.13	**0.42** [0.22–0.85], 0.014
Bacteria (25°C) in NAL	Men and women	**3.9** [1.18–12.7], 0.034	0.71 [0.28–1.80], 0.48	**0.40** [0.21–0.78], 0.008
Bacteria (25°C) in NAL	Men	—	0.67 [0.22–2.11], 0.50	**0.25** [0.11–0.58], 0.0019
Bacteria (25°C) in NAL	Women	—	0.78 [0.17–3.74], 0.77	1.00 [0.37–2.70], 0.99
Bacteria (37°C) in NAL	Men and women	**3.72** [1.12–12.4], 0.039	0.88 [0.36–2.20], 0.79	**0.45** [0.22–0.91], 0.028
Bacteria (37°C) in NAL	Men	—	0.88 [0.29–2.70], 0.82	**0.31** [0.13–0.78], 0.015
Bacteria (37°C) in NAL	Women	—	0.79 [0.17–3.74], 0.77	0.66 [0.22–1.97], 0.46

Significant values are in bold.

could be the higher respiratory rate of men (Hinds, 1999). Humans mainly breathe through the nose when at rest and increasingly through the mouth with increasing activity level. Under heavy work, the total respiratory rate of men is ~2.1 m^3 h^{-1} (James *et al.*, 1991). However, men seem to have a lesser nasal contribution to breathing during exercise than women (Bennett *et al.*, 2003). All persons in this study performed physical work but the intensity may have differed. When talking, people breathe more through the mouth (Camner and Bakke, 1980), but we did not record talking frequencies. Clearance will also affect the measured content of fungi and bacteria in NAL; however, women and men seem to have similar nasal mucus clearance rates (Ho *et al.*, 2001; Daigle *et al.*, 2003). Age may affect the nasal clearance (Ho *et al.*, 2001); but in this study, women and men had almost the same median ages. Thus, clearance seems not to explain the difference between genders.

During NAL sampling on Thursdays, the greenhouse workers typically had been at work for 5 h. The clearance half-life of fungi (25°C) in the nasal cavity is not known; alginate microspheres with a diameter of 1.3 µm in the nasopharynx have a clearance half-life period of 4 h (Tafaghodi *et al.*, 2004). To calculate the expected content of fungi in NAL, the following equation was used: Expected fungal (25°C) content in NAL = Exposure (cfu m^{-3}) × (inhalation (m^3 h^{-1}) × time at work (h) × deposition (%) × nasal inhalation (%) × clearance (%).

The following assumptions were made for men: exposure = 5×10^4 cfu of fungi (25°C) m^{-3}; inhalation = 1.0 m^3 h^{-1}; 70% of the particles deposit in the nose region; inhalation is 50% by nose; for people without runny noses, 50% is cleared at the time the NAL was taken; all deposited fungi (25°C) can be released into the NAL. This results in the following estimated content in NAL = 5×10^4 cfu m^{-3} × (1.0 m^3 h^{-1} × 5 h × 70% × 50% × 50%) = 4×10^4 cfu of fungi (25°C). In NAL from men

without runny noses, a median content of 9408 fungi (25°C) was measured for Thursday samples. If the same calculations are made for women with the same parameters but with a breathing rate of 0.8 $m^3 h^{-1}$, the expected content of fungi (25°C) in NAL is 3×10^4 cfu. In NAL from women without runny noses, a median content of 595 fungi (25°C) was measured for Thursday samples. There are many uncertainties for each factor in the calculation. However, given the assumptions above, ~22% of the expected fungal content are found in the NAL of men and only 2% in the NAL of women.

The measured exposure levels to fungi, β-glucan (Hansen *et al.*, 2011), and bacteria (Hansen *et al.*, 2010) were within the levels previously found for greenhouse workers. The high and significant correlation between exposure to fungi (25°C) and to β-glucan, and content of fungi (25°C) and β-glucan in NAL suggest that content of fungi (25°C) and β-glucan in NAL from men working in greenhouses may be used as a semi-quantitative estimate of the exposure to fungi (25°C) and β-glucan. The quantification of fungi (25°C) or β-glucan in NAL from men can be a relevant method to evaluate the effect of using personal respiratory airway protection. It is, however, not known if there is a subset of fungi that occur in the nasal cavity with greater adhesion to the mucosa than those that can be collected by lavage and if such fungi may be even more problematic (Sercombe *et al.*, 2006). It will therefore be relevant at species level to compare fungi in NAL with fungi in the air to see which species are not present in the NAL. In a study with NAL samples from healthy subjects, a high diversity of fungal species were found and on average 3.1 different species were found per subject (Braun *et al.*, 2003). In this study, we initially incubated the petri dishes for 2 weeks and counted the colonies every third day. We did, however, not find that the number of colonies increased after 7 days; subsequently we therefore only incubated the cultures for 7 days as we usually do for airborne fungi. Considering that Braun *et al.* (2003) and Lackner *et al.* (2005) found very slow-growing fungi as *Beauveria bassiana* in noses of healthy volunteers (8.7% of 23 persons), it would in hindsight have been relevant to continue the incubation of the cultures for another 2 weeks. However, quantitatively we do not expect *B. bassiana* to constitute a high proportion of the fungi present as *B. bassiana* is not a dominating fungus neither in the air in general (Madsen, 2011) nor in greenhouse air (Hansen *et al.*, 2011).

Greenhouse workers with runny noses had significantly fewer fungi (25°C) in NAL than workers without runny noses. This may be because their noses were runny but it may also be because they more often breathe orally and thus fewer fungi deposit in the nose. Patients with asthma or allergic rhinitis seem to have a tendency to breathe orally more often than healthy people (Chadha *et al.*, 1987; Kairaitis *et al.*, 1999). In this study, we found fungi (25°C) in 67% of 66 samples from Monday morning. In other studies with healthy persons, fungi were found in NAL from 95% of 20 persons (Sercombe *et al.*, 2006), 100% of 10 people (Ragab *et al.*, 2006), 100% of 14 people (Ponikau *et al.*, 1999), and in 91% of 23 people (Buzina *et al.*, 2003). These findings are higher than in this study, which may partly be because we had a much smaller volume of the NAL for cultivation of fungi (25°C) and because 20 samples were from workers with runny noses.

In the present study, the exposure to bacteria (25°C) did not significantly affect the content of bacteria (25°C) in NAL but the content was higher Thursday at noon than Monday morning. We do not know how large a fraction of these NAL bacteria (25°C) comes from the environment. In the inhalation zone, 55% of the bacteria were Gram-negative and according to other studies, Gram-negative bacteria likely come from plant material (Lacey and Dutkiewicz, 1994) in the greenhouse. If the same assumptions are made as for fungi but with a bacterial exposure of 7×10^3 cfu m^{-3}, and if all bacteria in NAL come from the environment, the theoretical content of bacteria in NAL from men is 6×10^3 cfu. For men without runny noses, a median content of 3×10^5 cfu per NAL sample was measured Thursday at noon. The corresponding theoretical value for women is 5×10^3 cfu of bacteria (25°C) per NAL sample, whereas the median measured value was 8×10^4 cfu of bacteria (25°C) per NAL sample. Thus, the measured contents of bacteria (25°C) in NAL are much higher than the calculated. If the measured contents on Monday morning are subtracted from the contents measured Thursday at noon, the measured contents are still higher than the calculated and the bacteria may divide in the nasal cavity. In addition, we may have underestimated the exposure to airborne bacteria as bacteria are sensitive to sampling on filters. On the other hand, bacteria are also sensitive to ultraviolet light (Tong and Lighthart, 1998) and it could be expected that mainly robust bacteria present

in clusters survive the greenhouse air. Sampling directly on agar media could have been done not to stress the bacteria. However, sampling on agar media can only be done for few minutes at time and as the bacterial concentration fluctuates during the working day; direct sampling on agar was therefore not an alternative method.

Most of the bacteria (median = 73%) were able to grow at 37°C, whereas only few fungi (median = 1.9%) were able to grow at 37°C. The content of bacteria (25°C) in NAL was also much higher (median = 100 times higher) than the content of fungi (25°C). It is not known whether fungi (37°C) come from the working environment, but some may because the content in women's NAL was higher Thursday at noon than Monday morning. We do not expect the fungi (37°C) to have infected the nasal cavities as they only constituted a small number fraction of the total number of fungi present in each NAL sample.

The large differences between genders and the fact that the content of fungi in NAL was significantly affected by the exposure indicate that the two genders may be affected differently by the same level of exposure to fungi. If women inhale more through the mouth than men, more fungi enter and deposit deeper in the airways of women according to particle deposition models (James *et al.*, 1991). Several studies have shown that women can be more sensitive to endotoxin exposure (Kline *et al.*, 1999), are particularly susceptible to the adverse effects of workplace indoor molds (Jaakkola and Jaakkola, 2004), more frequently report symptoms of the mucous membranes and general symptoms associated with mold exposure (Ebbehøj *et al.*, 2005), and have an increased susceptibility to rhinosinusitis (Chen *et al.*, 2003) compared with men. In light of the present study, it is relevant to study whether the more frequent symptoms among women are because aerosols deposit in different regions of the airways of men and women.

This is to our knowledge the first study measuring β-glucan in NAL. The content of β-glucan per cfu of fungi was significantly higher in NAL than in the air. This may be because mainly the large spores and pollen grains are retained in the nasal cavity. In NAL, the average content of β-glucan per cfu of fungi was 56 pg and in the exposure, it was 12 pg per cfu of fungi. In airborne inhalable dust in strawberry fields, an average of 21 pg β-glucan per cfu of fungi (25°C) has been found (Tendal and Madsen, 2011). The correlation between contents of β-glucan and fungi in NAL was significant but low. The fact that it was low may partly be because: only culturable fungi were measured, β-glucan also comes from pollen, spores from different fungal species have different amounts of β-glucan per spore (Iossifova *et al.*, 2008), and β-glucan is also present in fungal particles which are smaller than spores and which cannot be cultivated (Madsen *et al.*, 2009b).

In this study, we have shown that the exposure to fungi (25°C) and β-glucan in the studied occupational settings significantly affect the content of fungi (25°C) and β-glucan in NAL. Furthermore, the content of fungi (25°C) or β-glucan in the inhalation zones of men significantly correlates with their fungal (25°C) or β-glucan content in NAL. The content of fungi (25°C) in NAL of women was lower than what could be expected from their exposure. A higher content of β-glucan per fungal spore in NAL than in the air indicates that mainly the larger fungal particles and pollen grains deposit in the nasal cavity. To obtain knowledge about which fungal species pass through the nasal cavity and which deposit in the nasal cavity (and can be released to NAL), fungal species in NAL should be compared with fungal species in the inhalation zone. This study has shown big differences between genders. Thus, the content of fungi (25°C) in NAL of women is less affected by exposure from the environment than that of men. There is inter-individual variability in the route by which people breathe and different factors influence the breathing patterns. Therefore, it will be relevant to confirm this study with more people and in environments with different degrees of exposure and with measurement of physical activity levels. The symptom runny nose was associated with fewer fungi (25°C) in NAL of men. To understand the health effect of this, it is relevant to study whether this is caused by a tendency to breathe more often through the mouth when suffering from runny nose, thus causing a larger deposition in the lower airways and/or by increased clearance due to runny nose.

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