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Airborne Fungal and Bacterial Components in PM_1 Dust from Biofuel Plants

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Fungi grown in pure cultures produce DNA- or RNA-containing particles smaller than spore size (<1.5 μm). High exposures to fungi and bacteria are observed at biofuel plants. Airborne cultivable bacteria are often described to be present in clusters or associated with larger particles with an aerodynamic diameter (d_{ae}) of 2–8 μm. In this study, we investigate whether airborne fungal components smaller than spore size are present in bioaerosols in working areas at biofuel plants. Furthermore, we measure the exposure to bacteria and fungal components in airborne particulate matter (PM) with a D_{50} of 1 μm (called PM_1 dust). PM_1 was sampled using Triplex cyclones at a working area at 14 Danish biofuel plants. Millipore cassettes were used to sample 'total dust'. The PM_1 particles (29 samples) were analysed for content of 11 different components and the total dust was analysed for cultivable fungi, *N*-acetyl-β-D-glucosaminidase (NAGase), and (1 → 3)-β-D-glucans. In the 29 PM_1 samples, cultivable fungi were found in six samples and with a median concentration below detection level. Using microscopy, fungal spores were identified in 22 samples. The components NAGase and (1 → 3)-β-D-glucans, which are mainly associated with fungi, were present in all PM_1 samples. Thermophilic actinomycetes were present in 23 of the 29 PM_1 samples [average = 739 colony-forming units (CFU) m^{-3}]. Cultivable and 'total bacteria' were found in average concentrations of, respectively, 249 CFU m^{-3} and 1.8×10^5 m^{-3}. DNA- and RNA-containing particles of different lengths were counted by microscopy and revealed a high concentration of particles with a length of 0.5–1.5 μm and only few particles >1.5 μm. The number of cultivable fungi and β-glucan in the total dust correlated significantly with the number of DNA/RNA-containing particles with lengths of between 1.0 and 1.5 μm, with DNA/RNA-containing particles >1.5 μm, and with other fungal components in PM_1 dust. Airborne β-glucan and NAGase were found in PM_1 samples where no cultivable fungi were present, and β-glucan and NAGase were found in higher concentrations per fungal spore in PM_1 dust than in total dust. This indicates that fungal particles smaller than fungal spore size are present in the air at the plants. Furthermore, many bacteria, including actinomycetes, were present in PM_1 dust. Only 0.2% of the bacteria in PM_1 dust were cultivable.

Keywords: actinomycetes; aerodynamic diameter of fungi; bacteria; bioaerosol; biofuel; exposure; inhalable dust; PM_1 dust

INTRODUCTION

Fungi grown in pure cultures produce DNA- or RNA-containing non-cultivable particles smaller than spore size (Madsen *et al.*, 2005). Green *et al.* (2005) have described the presence of fragmented conidia in dust collected and cultivated on filters. In indoor air, hyphal fragments constituted 6.3% of the numbers of airborne fungal spores (Li and Kendrick, 1995) and in grain farming, farmers were exposed to 5×10^5 hyphal fragments m^{-3} (Halstensen *et al.*, 2007). When hyphal fragment counts have been incorporated into longitudinal epidemiological studies, associations with asthma severity and peak expiratory flow rates have been identified (Delfino *et al.*, 1997). In these studies with hyphal fragments, the sizes of these hyphal fragments were not described.

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During autolysis, fungal hyphae are seen to release particles smaller than fungal spores (Madsen *et al.*, 2005). It is not known whether these separate observations are part of the same phenomenon or biological process. Fungal particles smaller than fungal spores are generally of interest; one reason is they add to the fungal bioburden, often measured as fungal spores or cultivable fungi. The large surface area relative to mass and the possible alveolar deposition may be important for possible biological effects of these small particles. Furthermore, they are of interest because they are expected to have the ability to stay in the air for a longer time than spores and to penetrate small cracks e.g. in building constructions, which may influence the exposure level.

Evidence from both epidemiological and experimental studies supports the hypothesis that exposure to fungal spores is causally associated with development of hypersensitivity pneumonitis, organic dust toxic syndrome, decline in lung function, severity of asthma, respiratory symptoms, and airway inflammation. Furthermore, a recent review document on fungal spores suggests an occupational exposure limit of 10^5 spores for diverse fungal species in non-sensitized populations (Eduard, 2006). In addition, fungal entities, for example (1 → 3)-β-D-glucan and glucose polymers present in the mycelial cell wall and the fungal spore wall of most fungi, have been shown to have a possible negative impact on health (Douwes *et al.*, 2003). Common fungi such as *Aspergillus*, *Alternaria*, *Penicillium*, and *Trichoderma* secrete the chitinase *N*-acetyl-β-D-glucosaminidase (NAGase) (Lahoz *et al.*, 1976; Claeyssens and Aerts, 1992; Draborg *et al.*, 1995; Hearn *et al.*, 1998). NAGase is also produced by some other organisms (Shi *et al.*, 2007; Zhang *et al.*, 2007) which are not likely to be present in aerosols at biofuel plants and by some bacteria antagonistic to fungi (Manjula and Podile, 2005). Cellulases are secreted by many common bacterial and fungal species (Eriksson, 1981; Bayer *et al.*, 1998). Some cellulases and chitinases are described to be inflammogene or to cause respiratory effects (Losada *et al.*, 1986; Robinson *et al.*, 1990; Jeffrey *et al.*, 1999; Baur, 2005; Allermann *et al.*, 2006). Significant correlations have been found between number of fungi in airborne or settled dust and NAGase activity (Madsen, 2003; Madsen and Würtz, 2005). Thus, NAGase activity may act as a marker of fungi in dust and it may directly add to the inflammatory potency of dust.

At biofuel plants, large amounts of straw are handled and there is a high exposure to inhalable fungal spores (Madsen, 2006). Studies with instillation of respirable dust from a biofuel plant in mice have demonstrated a strong inflammatory response (Madsen *et al.*, 2008). In this study, we want to investigate whether fungal material smaller than spore size is present in the air at working areas at biofuel plants and in which concentrations these particles are present. According to Levetin (1995), fungal spores are between <2 μm and 100 μm. Eduard *et al.* (2001) have studied spores from aerosols by microscopy and they have classified fungal spores as spores >1.5 μm. Table 1 presents aerodynamic diameters (d_{ae}) of fungi in aerosols and shows that fungi are often present as particles with d_{ae} between 2 and 6 μm. The small fungal particles in aerosols from pure fungal cultures have d_{ae} of different sizes and are ~0.4 to 1.1 μm (Table 1). Accordingly, we have tried to sample particles <1 μm to avoid fungal spores and to sample the small fungal particles observed to be released from pure fungal cultures. For this sampling, a Triplex cyclone has been used as it has a sharp penetration curve with a cut point of 1 μm (D_{50}) (Gussman and Kenny, 2000). The Triplex cyclone has previously been used both as a stationary and personal sampler (Davis *et al.*, 2006).

Bacteria including actinomycetes are also present in high concentrations in inhalable and 'total dust' from biofuel (Madsen *et al.*, 2004; Madsen, 2006), but the concentrations of bacteria in particulate matter (PM) with a D_{50} of 1 μm (PM_1) dust have not been described. Spores of actinomycetes have diameters of ~1 μm and spores <1.5 μm have been classified as bacteria (Table 1). Therefore, bacteria can be expected to be present in the PM_1 dust. On the other hand, bacteria seem often to be aerosolized in clusters or associated with larger particles (Table 1). The aim of this study is to investigate whether and in which concentrations fungal components and bacteria are present in airborne PM_1 dust.

MATERIALS AND METHODS

The biofuel plants

The investigation was carried out at 14 Danish biofuel plants. Straw was primarily used to generate energy at the biofuel plants but at one plant wood chips were also used. Measurements were performed for each plant in the winter season in 2004–2006 and each time over two or three successive working days. In the winter time some of the plants converted more straw than in the summer time. The sampling and measurement of numbers of particles were performed in the straw storage room 1.5 m above floor level. In straw storage rooms, people mainly worked with unloading of straw from trucks, measurement of water content of straw, and cleaning of the straw storage room and of the truck body. The employed at the biofuel plants (exclusive people only working in the office) worked on average 56% of their working days in straw storages and 12% of their working days in the boiler rooms. These people did not use any kind of dust protection masks. Earlier measurements show

Table 1. Diameters (d) and aerodynamic diameters (d_{ae}) of microbial particles

	Species	Physical d of spores (μm)	References	d_{ae} of spores or clusters (μm)	References	d_{ae} top of other particles (μm)	References
Fungi	*Aspergillus flavus*	3.5–4.5	Domsch *et al.* (1993)	3.3–3.8[a]	Madelin and Johnson (1992)		
	A. fumigatus	2.5–3.0	Domsch *et al.* (1993)	3.1[b]	Lacey and Dutkiewicz (1976)		
				1.9–2.2[a]	Madelin and Johnson (1992)		
	A. ustus	3.2–4.5	Domsch *et al.* (1993)	2.6[a]	Madsen *et al.* (2005)	0.9[a]	Own observation[c]
	A. versicolor	2–3	Domsch *et al.* (1993)	2[d]	Gorny *et al.* (2002)	0.4[d]	Gorny *et al.* (2002)
				3[a]	Kildesø *et al.* (2003)	1[a]	Kildesø *et al.* (2003)
	Chaetomium globosum	6.5–11	Domsch *et al.* (1993)	2.3[a]	Madsen *et al.* (2005)	0.7[a]	Madsen *et al.* (2005)
	Cladosporium cladosporioides	2–11	Domsch *et al.* (1993)	2.3–2.5[a]	Madelin and Johnson (1992)		
	C. sphaerospermum	3–7	Domsch *et al.* (1993)	3.3[a]	Madsen *et al.* (2005)	1.1[a]	Own observation[c]
	Humicola lanuginose	—	Domsch *et al.* (1993)	5.7[b]	Lacey and Dutkiewicz (1976)		
	Penicillium chrysogenum	2.8–4.0	Domsch *et al.* (1993)	2.8[a]	Madsen *et al.* (2006)	1.0[a]	Madsen *et al.* (2006)
				2.6–3.0[a]	Madelin and Johnson (1992)		
	Stachybotrys chartarum[e]	7–12	Domsch *et al.* (1993)	4.5[a]	Sorenson *et al.* (1987)		
	Scopulariopsis brevicaulis	5–9	Domsch *et al.* (1993)	5.1–5.5[a]	Madelin and Johnson (1992)		
	Trichoderma harzianum	2.8–3.2	Domsch *et al.* (1993)	4.7[a]	Madsen *et al.* (2006)	1.1[a]	Madsen *et al.* (2006)
	Verticillium lecanii[f]	1–10	Domsch *et al.* (1993)	1.2–1.9[a]	Own observation[c]	0.8[a]	Own observation[c]
	Fungi	>1.5	Eduard *et al.* (2001)	2.1–3.3[b]	Lin and Li (1996)		
				<2.1[b]	Yeo and Kim (2002)		
	Fungal hyphal fragments	Length 5–100	Green *et al.* (2005)				
Bacteria	Bacteria	<1.5	Palmgren *et al.* (1986)	1.1–2.1[b]	Meklin *et al.* (2002)		
		<1.5	Eduard *et al.* (1990)	2.7[b]	Lacey and Dutkiewicz (1976)		
				2.1–3.3[b]	Laitinen *et al.* (1994)		
				2.4–4.8[b]	Shaffer and Lighthart (1997)		
				2–6[b]	Lundholm (1982)		
				5–8[b]	Lundholm and Rylander (1983)		
				>8[b]	Bovallius *et al.* (1978)		
	Bacillus subtilis			7[b]	Lighthart *et al.* (1993)		
Actinomycetes	Actinomycetes			0.65–1.1[b]	Meklin *et al.* (2002)		
	Micropolyspora faeni[g]	0.7–1.5	Lacey (1986)	1.9[b]	Lacey and Dutkiewicz (1976)		
	Thermoactinomyces vulgaris	0.5–1.5	Lacey (1989)	0.58[b]	Lacey and Dutkiewicz (1976)		

[a] d_{ae} measured by an aerodynamic particle sizer.
[b] Peak number of cultivable organisms measured by an Andersen sampler.
[c] Methods described in Kildesø *et al.* (2003).
[d] Peak number measured by a particle sizer.
[e] Synonym: *Stachybotrys atra*.
[f] Synonym: *Lecanicilium lecanii*.
[g] Synonym: *Saccharopolyspora rectivirgula*.

a high personal exposure to both fungi and bacteria in straw storage rooms (Madsen, 2006).

Sampling and extraction of PM_1

Airborne particles (29 samples) were sampled using a Triplex cyclone (BGI, MA, USA) with a flow rate of 3.5 l min^{-1}. The Triplex cyclone has a well-defined sharp penetration curve at a flow rate of 3.5 l min^{-1} with a cut point of 1 μm (D_{50}) and ~1% of particles with d_{ae} 1.7 μm penetrate the cyclone. On average, the Triplex cyclones were sampling for 6 h and 18 min day^{-1}. The Triplex cyclone was fitted with a polycarbonate filter with pore size of 1.0. After sampling, the filters were transported carefully to the laboratory. The polycarbonate filters were carefully removed from the samplers 16 h after sampling. We did not see dust deposited on the walls of the cyclones and we have found no papers describing deposition of bioaerosol components on the walls in Triplex cyclones. Following, the dust was extracted in 5.0 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. Parts of the suspensions were immediately used for cultivation of microorganisms and other parts stored at −80°C.

Sampling and extraction of total dust

The main purpose of this study was to characterize particles of PM_1 size and not total dust but to be able to compare PM_1 and total dust. Total dust was sampled in 20 of the areas where PM_1 was sampled simultaneously. Total dust has been defined as the dust collected by a sampler with an entry velocity of 1.25 m s^{-1} (Kenny and Ogden, 2000); we sampled total dust using 25-mm closed-face cassettes (Millipore holder; Millipore, Bedford, MA, USA) with a 5.6-mm inlet and a flow of 1.9 l min^{-1} corresponding to an inlet velocity of 1.25 m s^{-1}. On average, the total dust was sampled for 6 h and 36 min day^{-1}. The samplers were fitted with polycarbonate filters (pore size 0.8 μm) for analysis of cultivable fungi, NAGase, and β-glucan. Polycarbonate filters are known to be non-hygroscopic. The polycarbonate filters were removed from the samplers 16 h after sampling. Following, the dust was extracted in 5.0 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. Parts of the suspensions were immediately used for cultivation of microorganisms and other parts stored at −80°C.

Number and size distribution of airborne particles

At each sample station a Grimm (used during 17 of the sampling days) or an aerodynamic particle sizer (used during 12 of the sampling days) measured particles of different sizes. On average, they were measuring for 6 h day^{-1}. Particles with d_{ae} between 0.75 and 1.0 μm or between 0.487 and 0.523 μm were revealed.

Quantification of microorganisms and DNA/RNA-containing particles

Microorganisms in PM_1 dust and fungi in total dust were quantified using a modified CAMNEA method (Palmgren *et al.*, 1986). The numbers of fungi cultivable on dichloran glycerol agar (DG 18 agar; Oxoid, Basingstoke, UK) at 25 and 45°C were measured. Quantifications were made of the number of bacteria cultivable at 25°C on Nutrient agar (Oxoid) with cycloheximide (50 mg l^{-1}) and of the number of mesophilic actinomycetes (25°C) and thermophilic actinomycetes (55°C) cultivable on, respectively, 10 and 100% nutrient agar with cycloheximide (50 mg l^{-1}). Microorganisms were identified by macroscopic morphology studies and expressed in colony-forming units (CFU) per millilitre of liquid. Actinomycetes were distinguished from bacteria by morphology and by their more firm attachment to the growth medium. The detection limit was 5 CFU ml^{-1}. The airborne concentrations of microorganisms were calculated from the airflow through the filters and were expressed as CFU per cubicmetre. Fungi in total dust were also quantified by cultivation on DG 18 agar. These data were only used to study the correlation between fungi in PM_1 dust and total dust.

Acridine orange is a nucleic acid-selective fluorescent, cell-permeable dye which interacts with DNA and RNA. The PM_1 dust samples were stained in 20 p.p.m. acridine orange (Difco, 212536, Maryland, MD, USA) in acetate buffer for 30 s, with subsequent filtration through a polycarbonate filter (25 mm, 0.4 μm; Nuclepore, Cambridge, MA, USA). After this particles of different sizes were counted at a magnification of ×1250 using epifluorescence microscopy (Orthoplan; Leitz Wetzlar, GMBH Wetzlar, Germany). Thus, the number of fungi (in the following called 'total fungal spores') and bacteria (in the following called 'total bacteria') were determined in 40 randomly chosen fields or until at least 400 cells were counted. In addition, particles stained with acridine orange and which were not fungal spores or bacteria were quantified in three different categories of DNA/RNA particle length: (i) between 0.5 and 1.5 μm, (ii) between 1.0 and 1.5 μm, and (iii) >1.5 μm. Bacteria and fungi were identified by shape and structure and by their size. Bacteria have a diameter <1.5 μm and fungal spores a diameter between 1.5 and 10 μm.

Quantification of (1 → 3)-β-D-glucans, NAGase, and β-glucosidase

Dust suspensions from the polycarbonate filters were used to quantify (1 → 3)-β-D-glucan (in the following called β-glucan) in duplicate using the kinetic Fungitic G Test (Seikagaku Co., Tokyo, Japan). The

triple-helix structure of the β-glucan was made water soluble using 0.3 M NaOH (Fogelmark *et al.*, 2001). The results were expressed in picogram per millilitre of liquid and the detection limit was 4 pg ml^{-1}.

To quantify the activity of NAGase (EC3.2.1.30) and the cellulase β-glucosidase (EC 3.2.1.21) (at 37°C), release of *p*-nitrophenol from *p*-nitrophenol-*N*-acetyl-β-D-glucosaminide or *p*-nitrophenol-*N*-acetyl-β-D-glucopyranoside (Sigma–Aldrich Chemical, Steinheim, Germany) was quantified (Madsen, 2003). Controls were without either the enzyme or the substrate. The results were expressed in picomole per second per millilitre of liquid and the detection limit was 0.02 pmol s^{-1} ml^{-1}. One unit of enzyme activity is defined as the amount of enzyme, which releases 1.0 μmol of *p*-nitro-phenol ml^{-1} enzyme min^{-1}.

The airborne concentrations of these three components were calculated from the airflow through the filters and were expressed as units per cubicmetre.

Treatment of data

For correlation analysis, all data were log transformed, as they were log normally distributed. If the measured component was found in >75% of the samples, the value '50% of limit of detection (LOD) value' was used for measurements below the LOD; if the measured component was found in between 20 and 75% of the samples, only measurements above the detection limit were included; and if the measured component was found in <20% of the samples, the component was not included in the correlation studies.

RESULTS

Airborne PM_1

In general, airborne fungal spores have both a diameter as measured by microscopy and a d_{ae} >1.5 μm (Table 1), while the Triplex cyclone has a penetration cut point at 1 μm (D_{50}). Cultivable mesophilic fungi and 'total fungal spores' were found in, respectively, 6 and 22 of the 29 samples. However, the median concentration of cultivable fungi was below detection level, and no thermotolerant fungi were found. β-Glucan and NAGase were found in all dust samples and thus also in samples with no fungal spores (Table 2). The median concentration of DNA particles >1.5 μm was 4403 m^{-3} and only a fraction (11%) of the number of these particles were identified as fungal spores. On average, 4×10^4 DNA- or RNA-containing particles between 0.5 and 1.5 μm were present in the air and almost 50% of these particles were bacteria. Hence, up to 6.2×10^5 'total bacteria' m^{-3} were found in PM_1 dust.

The median amount of β-glucan and NAGase activity per 'total fungal spore' was, respectively, 7 pg and 3.5×10^{-4} pmol s^{-1} (Fig. 1).

Correlations between components in PM_1 dust

Significant correlations were found between factors mentioned in Table 3; factors not included in the table did not correlate significantly. The fungal components β-glucan and NAGase correlated significantly and the bacterial components such as CFU of bacteria and 'total bacteria' also correlated significantly.

Correlations between fungal components in total dust and components in PM_1 dust

Significant correlations were found between factors mentioned in Table 4; factors not included in the table did not correlate significantly. Fungi (CFU) and β-glucan in total dust correlated with fungal components and with DNA- or RNA-containing particles in PM_1 dust.

Table 2. Components (unit) m^{-3} air of PM_1 samples collected during working days at 14 biofuel plants

Component	Unit	Average	Median	Interval	Positive samples/ total samples
Fungi	CFU	17	Bd	Bd to 105	6/29
Total fungal spores	Number	639	497	Bd to 2377	22/29
Other DNA–RNA, l: >1.5 μm	Number	5624	3906	600–18 659	29/29
β-Glucan	ng	5.6	3.5	0.68–27	29/29
NAGase	pmol s^{-1}	0.21	0.18	0.13–0.45	29/29
Bacteria	CFU	249	48	Bd to 2995	21/29
Thermophilic actinomycetes	CFU	739	178	Bd to 4492	23/29
Mesophilic actinomycetes	CFU	274	19	Bd to 3414	17/29
Total bacteria	Number	1.8×10^5	0.31×10^5	780–6.2×10^5	29/29
Other DNA–RNA, l: 0.5–1.5 μm	Number	2.2×10^5	0.23×10^5	760–19×10^5	29/29
β-Glucosidase	pmol s^{-1}	0.16	0.15	0.099–0.36	29/29
Particles d_{ae} 0.75–1.0 μm	Number	164×10^5	50×10^5	13×10^5–1800×10^5	29/29
Particles d_{ae} 0.487–0.523 μm	Number	247×10^5	150×10^5	23×10^5–850×10^5	14/14

Bd, below detection level; l, length.

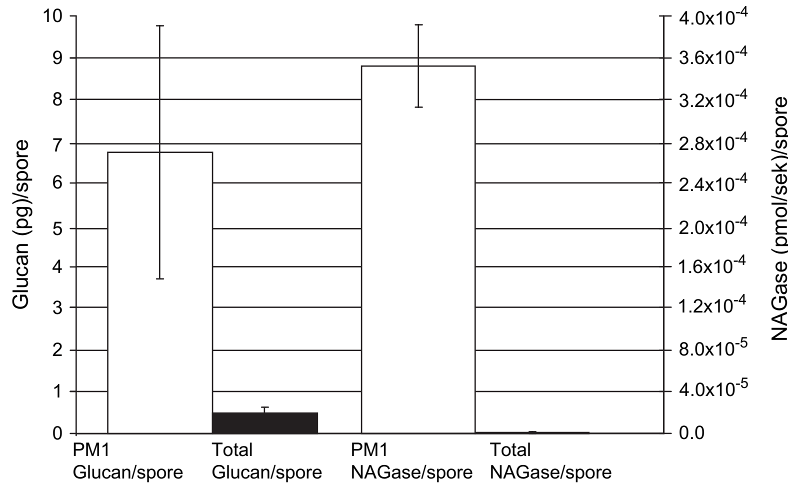

Fig. 1. β-Glucan (pg)/'total fungal spore' in PM_1 and total dust (1. *y*-axis) and NAGase (pmol s^{-1})/total fungal spore in PM_1 and total dust (2. *y*-axis).

Table 3. Factors in the PM_1 fraction correlating significantly

	β-Glucan	β-Glucosidase	Bacteria (CFU)	Thermophilic actinomycetes	Total bacteria
β-Glucan	—	$r = 0.79$, $P < 0.0001$, $n = 29$			
NAGase	$r = 0.83$, $P < 0.0001$, $n = 29$	$r = 0.96$, $P < 0.0001$, $n = 29$			
Bacteria (CFU)			—	$r = 0.72$, $P = 0.0003$, $n = 29$	$r = 0.73$, $P = 0.0012$, $n = 29$
Mesophilic actinomycetes			$r = 0.98$, $P < 0.0001$, $n = 17$	$r = 0.75$, $P < 0.0001$, $n = 17$	

r = Pearson's correlation coefficient, P = level of significance, after the Bonferoni correction only P values $<0.05/12 = 0.0042$ are significant. CFU of fungi was not included as cultivable fungi were only found in six samples.

Table 4. Factors in PM_1 correlating significantly to CFU of fungi and β-glucan in total dust

	PM_1 fraction					
	β-Glucan	Total fungi	NAGase	DNA–RNA L: 1–1.5 μm	DNA–RNA L: >1.5 μm	β-Glucosidase
CFU of fungi in total dust	$r = 0.79$, $P < 0.0001$, $n = 20$	$r = 0.51$, $P < 0.004$, $n = 20$	$r = 0.59$, $P = 0.010$, $n = 20$	$r = 0.50$, $P = 0.016$, $n = 20$	$r = 0.53$, $P < 0.0001$, $n = 20$	$r = 0.54$, $P = 0.020$, $n = 20$
β-Glucan in total dust	$r = 0.71$, $P < 0.0001$, $n = 20$	$r = 0.49$, $P < 0.003$, $n = 20$	$r = 0.62$, $P = 0.007$, $n = 20$	$r = 0.56$, $P = 0.036$, $n = 20$	$r = 0.55$, $P < 0.0001$, $n = 20$	$r = 0.58$, $P = 0.008$, $n = 20$

L = length, r = Pearson's correlation coefficient, P = level of significance, n = numbers of samples. CFU of fungi in PM_1 dust was not included as cultivable fungi were only found in six samples.

The median amount of β-glucan and NAGase per 'total fungal spore' in total dust was, respectively, 0.5 pg and 7.2×10^{-7} pmol s^{-1} (Fig. 1).

DISCUSSION

In this study, we found high concentrations of β-glucan in PM_1 dust (median 3.5 ng m^{-3}) sampled from straw storage rooms where people were handling straw. We have found no other study measuring β-glucan in PM_1 dust. The concentrations were higher than or at the same level as exposure to β-glucan in respirable dust at wood handling plants (0.1–1.76 ng m^{-3}) (Alwis *et al.*, 1999). As expected, the number of 'total fungal spores' was low in PM_1 dust in comparison with what is earlier found in inhalable dust at biofuel plants (Madsen, 2006). Similarly, the number of both cultivable and 'total bacteria' and actinomycetes in PM_1 dust was low in comparison with what is earlier found in inhalable dust at biofuel

plants (Madsen, 2006). The dust sampling in this study was performed in the winter time where the outdoor exposure to bioaerosol is low. Earlier studies have shown higher exposure to microbial components at biofuel plants in spring than in autumn (Madsen, 2006) and storage of biofuels outdoors over summer can cause an increase in the microbiological dustiness of biofuels (Sebastian *et al.*, 2006).

Airborne β-glucan and NAGase were found in PM_1 samples where no cultivable fungi were present, and β-glucan and NAGase were found in higher concentrations per fungal spore in PM_1 dust than in total dust. The concentrations of β-glucan, β-glucosidase, and NAGase correlated highly and significantly (Table 3). This indicates that they all mainly origin from the same fungal particles and that β-glucan is not mainly from bacteria or pollen. Together, this suggests that fungal particles smaller than fungal spore size are present in the air at the plants. Pure cultures of fungi have previously been seen to release particles smaller than spore size (Table 1). DNA or RNA has previously been observed in the small fragments released from fungal cultures (Madsen *et al.*, 2005). We found many DNA- or RNA-containing particles of the size described to be released from fungal cultures. Whether these particles also contain the β-glucan measured in the same samples is not possible to conclude. However, it should be noted that CFU of fungi and β-glucan in total dust correlated positively and significantly with β-glucan and DNA–RNA particles in PM_1 dust (Table 4). On the other hand, the concentrations of these RNA–DNA particles in PM_1 dust did not correlate significantly with the amount of β-glucan in the same samples (Table 3).

Cultivable fungi were found in six of 29 PM_1 samples; the cyclone has a sharp penetration curve and ~1% of the particles with d_{ae} 1.7 μm penetrate the cyclone. Similarly, using the Anderson sampler in indoor environments Pastuszka *et al.* (2000) found between 1 and 100 CFU of fungi m^{-3} and Rautiala *et al.* (1996) found between 10 and 2000 CFU fungi m^{-3} in the size fraction 0.65–1.1 μm. Whether the cultivable fungi found in the six samples in this study and in the two indoor studies are from hyphae, spores, or other fungal components is not known.

β-Glucan has been used as an indicator of fungal exposure, but studies have also shown that it can elicit respiratory inflammation (Rylander, 1999; Thorn *et al.*, 2001; Bonlokke *et al.*, 2006) and systematic inflammation (Sigsgaard *et al.*, 2000). A review concluded that health effects of β-glucans seem plausible, although the underlying inflammatory mechanisms associated with exposure are not clear (Douwes, 2005). Therefore, it is interesting that β-glucan is also present in PM_1 dust.

Due to their small size, fungal fragments can stay in the air longer than fungal spores and can also penetrate and be deposited more deeply in the alveolar region when inhaled. By using simulation models, Cho *et al.* (2005) found that the respiratory deposition of *Stachybotrys chartarum* fragments was 230 times higher than that of spores for adults and even higher for infants. Additionally, air pollution studies have reported that adverse health outcomes, e.g. respiratory and cardiac responses, are associated with both number and mass of particles (Osunsanya *et al.*, 2001), illustrating the importance of including small fungal fragments when assessing mould exposure.

We have measured a high exposure to 'total bacteria' (median = 3×10^4 m^{-3}) with $d_{ae} < 1$ μm, even though cultivable bacteria are often reported as present in clusters or as associated with other particles (Table 1). Exposure to microorganisms present as particles with different d_{ae} has mainly been measured with the Anderson sampler, and using this sampler, only cultivable microorganisms are quantified. Exposure to airborne non-cultivable bacteria present as particles with $d_{ae} < 1$ μm should also be elucidated in other environments.

The median ratio of culturable to 'total bacteria' in the PM_1 fraction in dust from the biofuel plants is 0.2%. This is much lower than seen in inhalable dust from biofuel plants, where 5% of the bacteria were cultivable (Madsen, 2006). This is probably because bacteria present as single or few cells in general are less protected from irradiation and drying than bacteria associated with larger particles or bacteria present in larger clusters. In support of the theory about protection from irradiation, studies of culturable bacteria in the atmosphere show a preponderance of large-sized culturable bacterial particles during clear days; the phenomenon is explained by a higher sunlight sensitivity of airborne bacteria in small particles (Tong and Lighthart, 1998).

The concentration of airborne cultivable actinomycetes was higher than the concentration of other cultivable bacteria. In contrast 'total' and inhalable dust from straw or a straw-converting biofuel plant contains a higher number of cultivable bacteria than cultivable actinomycetes (Madsen *et al.*, 2006). This difference between 'total' and inhalable dust on the one hand and PM_1 dust on the other hand is probably because a larger fraction of the numbers of spores of actinomycetes than of the bacteria are present as single spores. Furthermore, spores of actinomycetes can be smaller than bacteria, so they are present more often in the PM_1 fraction.

CONCLUSIONS

Airborne β-glucan and NAGase were found in PM_1 samples where no cultivable fungi were present, and β-glucan and NAGase were found in higher concentrations per fungal spore in PM_1 dust than in total dust. Furthermore, the number of cultivable fungi

and the amount of β-glucan in the total dust correlated significantly with the number of DNA/RNA-containing particles. A high number of DNA/RNA-containing particles not identified as bacteria or fungal spores were found. Together, this indicates that fungal particles smaller than fungal spore size are present in the air at the biofuel plants. The median exposure to β-glucan (PM_1) was 3.5 ng m^{-3} and may therefore be an important exposure.

A median exposure to 'total bacteria' in PM_1 dust on 3×10^4 m^{-3} was measured. The concentration of airborne cultivable actinomycetes was higher than the concentration of other cultivable bacteria. Hence, the exposure to total bacteria and actinomycetes in PM_1 dust is considerable. The median ratio of culturable to total bacteria in the PM_1 fraction was only 0.2%, showing low viability of bacteria in PM_1 aerosols.

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