

# Organic dust toxic syndrome at a grass seed plant caused by exposure to high concentrations of bioaerosols

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We describe an outbreak of sudden health problems in workers at a Danish grass seed plant after exposure to a particularly dusty lot of grass seeds. The seeds are called problematic seeds. The association between development of organic dust toxic syndrome (ODTS) and the handling of grass seeds causing exposure was assessed in a four-step model: (i) identification of exposure source, (ii) characterization of the emission of bioaerosols from the problematic and reference seeds, (iii) personal and stationary exposure measurement at the plant and (iv) repeated health examinations. The grass seeds were identified as the exposure source; the emissions of some bioaerosol components were up to  $10^7$  times higher from the problematic seeds than from reference seeds. Cleaning of the seeds was not enough to sufficiently reduce the high emission from the problematic seeds. Emission in terms of dust was 3.4 times as high from the problematic cleaned seeds as from cleaned reference seeds. The personal exposure reached  $3 \times 10^5$  endotoxin units  $m^{-3}$ ,  $1 \times 10^6$  colony-forming units (cfu) of thermophilic actinomycetes  $m^{-3}$ ,  $8 \times 10^5$  cfu of *Aspergillus fumigatus*  $m^{-3}$  and  $9 \times 10^6$  hyphal fragments  $m^{-3}$ . Several workers working with the problematic seeds had symptoms consistent with ODTS. The most severe symptoms were found for the workers performing the tasks causing highest exposure. Respiratory airway protection proved efficient to avoid development of ODTS. Work with reference seeds did not cause workers to develop ODTS. Exposure was during work with the problematic seeds higher than suggested occupational exposure limits but lower than in studies where researchers for some minutes have repeated a single task expected to cause ODTS. In this study, many different bioaerosol components were measured during a whole working day. We cannot know, whether it is the combination of different bioaerosol components or a single component which is responsible for the development of ODTS. In conclusion, workers developed specific health symptoms due to the high bioaerosol exposure and were diagnosed with ODTS. Exposure to high concentrations of endotoxin, actinomycetes, fungi, hyphal fragments,  $\beta$ -glucan, and *A. fumigatus* occurred when working with a dusty lot of grass seed. Suspicion should be elicited by seeds stored without being properly dried and by seeds producing more dust than usually.

**Keywords:** endotoxin; hyphal fragments; (1  $\rightarrow$  3)- $\beta$ -D-glucan; ODTS; toxic alveolitis

## INTRODUCTION

Grass seed is an important crop in Danish agriculture and Denmark is a leading exporter of grass seeds

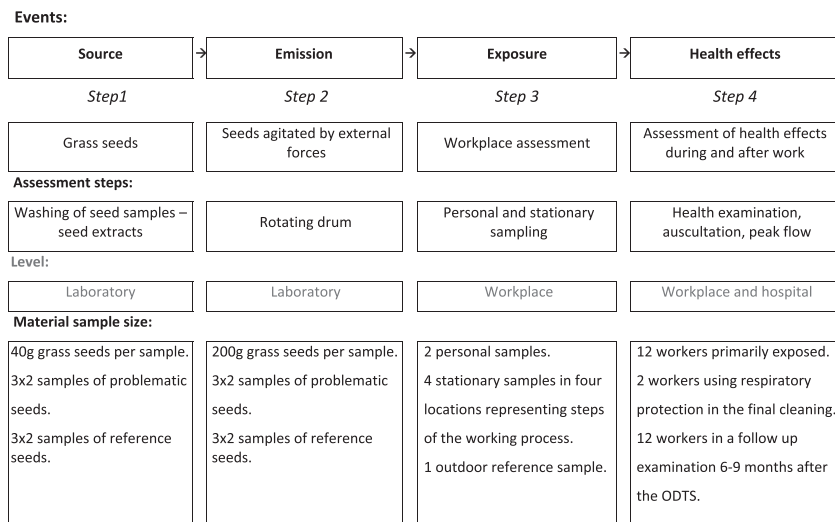
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accounting for 40% of the total grass seed production in the European Union (The Danish Seed Council, 2010). However, little is known about health risks associated with transporting, cleaning, and packaging grass seed. It is expectable that airway symptoms would be frequent in this kind of work as it is in several other occupations in which there is also exposure to bioaerosols (e.g. Sigsgaard *et al.*, 1994; Vogelzang *et al.*, 1999; Von Essen *et al.*, 2007). Endotoxin from bacteria is one of the microbial components which can be present in high concentrations in bioaerosols. Endotoxin has been shown to correlate with acute lung function changes, accelerated lung function decline, work-related and chronic respiratory symptoms, and airway hyper-responsiveness (Rylander *et al.*, 1985; Smid *et al.*, 1992). Exposure to fungi in bioaerosols has been related to symptoms in the eyes and nose and to cough (Melbostad and Eduard, 2001) as well as to asthma symptoms and work-related respiratory symptoms (Eduard *et al.*, 2004; Schlünssen *et al.*, 2011).

Organic dust toxic syndrome (ODTS) is related to exposure to very high concentrations of bioaerosol components. ODTS is also known as inhalation fever or toxic alveolitis, and it is an acute, non-allergic, self-limiting illness characterized by flu-like symptoms: fever, chills, chest tightness, shortness of breath, dry cough, myalgia, and general fatigue. Onset is typically within a few hours after exposure to organic dust and duration of symptoms is often <24 h but may last 5–7 days (Rask-Andersen, 1989; Seifert *et al.*, 2003). Occupational endotoxin exposure seems to increase the risk for ODTS (Basinas *et al.*, 2011) and increased

time of exposure seems to increase the risk for ODTS (Brinton *et al.*, 1987). Exposure to very high concentrations of dust, fungi, and bacteria has been found at a compost plant where a worker developed ODTS or hypersensitivity pneumonitis (HP) (Weber *et al.*, 1993). However, in most studies of ODTS exposure to bioaerosol components have not been measured (Brinton *et al.*, 1987; Rask-Andersen, 1989; May *et al.*, 1990); instead of exposure the content of, for example, fungi in the organic material expected to cause ODTS has been measured in some studies (Boehmer *et al.*, 2009).

ODTS is difficult to measure retrospectively because the symptoms are similar to flu symptoms and because onset is typically a few hours after exposure has occurred and people for these reasons do not necessarily relate the symptoms to an earlier exposure at work. We herein present the first well-documented outbreak of ODTS in a grass seed cleaning facility in Denmark, supplemented with a thorough characterization of the exposure causing ODTS and of seeds causing ODTS versus reference seeds. We used an extended version of a conceptual approach to characterize grass seeds for inhalation exposure risk and associated health effects (Madsen *et al.*, 2006). The concept included in this study a Step 1 used for hazard identification, a Step 2 used to characterize the emission of bioaerosols from the problematic grass seeds and reference seeds, a Step 3 with personal and stationary exposure measurements at the grass seed plant during an entire work day, and a Step 4 with repeated health examinations (Fig. 1).



**Fig. 1.** The conceptual approach used to characterize grass seeds for inhalation exposure risk assessment and associated health effects.

## MATERIALS AND METHODS

### *Description of plant I and plant II*

Plant I receives seeds of grass and clover for cleaning and packaging. Grass seeds were unloaded from lorries inside the plant and mechanically transported into the centre of the plant through a closed system. In an intake hall, where the seeds are either loaded through tubes into open, wooden boxes, which are then covered with a cardboard lid, or transported by a closed conveyor belt to a silo and later transported to the cleaning machines. The boxes with seed are carried by a forklift and emptied 3–4 m above the floor into cleaning machines with a system of large, moving or rotating sieves. The seeds were then moved to the main part of the machines and placed on the floor close to the operator; dust was emitted as these machines were partly open. The cleaned seeds were again loaded into boxes and transported by forklift to another hall, where they are packaged into bags or wooden boxes covered with a lid. Finally, they are loaded through open gates on to lorries to be sold.

Plant II is a similar, recently closed facility but as it was still functional, it was selected for the final cleaning of the specific lot of seeds (in the following called problematic seeds) in an isolated operation. In this plant, the cleaning machines were newer.

### *Tasks in the grass seed facility*

During the day where the problematic seeds were received the 12 employees (called worker 1 to 12) worked with reference seeds or the problematic seeds. During the day of exposure measurement, the two workers, called workers 1b and 2b, worked with the problematic seeds. All 12 workers each carried out one or more of the following tasks:

- Lorry drivers stood beside the lorry when it was emptied by dumping grass seeds into a transport screw in the floor and again when the lorry was loaded.
- Receiving and depositing the seeds in boxes; the worker stood close to big boxes on the floor being filled with seeds from a pipe. Exhaust ventilation was present around the box. Visible dust was released from the falling seeds.
- Driving fork lifts with boxes with seed to be emptied into the cleaning machines and to the packaging facility. The cabins of the forklift trucks were closed, but air intake to the cabin was without a dust filter.
- Packaging: seeds that have been cleaned were emptied into big bags or boxes by an automatized

machine operated by a worker standing some meters away.

All workers had been employed at the grass seed plant for at least 3 years.

### *Health examination of employees*

After only a few hours of unloading and cleaning of the lot of problematic grass seeds 5 out of 12 workers at plant I began to experience airway symptoms and flu-like symptoms along with malaise. All workers were males. Cleaning was stopped the following morning as workers 3 and 4 were brought to the hospital due to respiratory symptoms. The problematic grass seed was reported to produce much more visible dust than normal grass seed. According to the seed company, the problematic seeds had been stored on the farm without being sufficiently dried before storage.

All 10 employees at work during the time the problematic seeds were handled as well as the two truck drivers (workers 11 and 12) transporting the seeds were offered a general health examination at the hospital. The examination included a complete medical history, auscultation, spirometry (Dry 'Vitalograph®', measuring forced expiratory volume in one second (FEV<sub>1</sub>), forced vital capacity (FVC), FEV<sub>1</sub>/FVC, and peak expiratory flow), blood sample (leukocyte count, C-reactive protein [CRP], total IgE, and specific IgE against specific moulds (*Penicillium chrysogenum*, *Cladosporium herbarum*, *Aspergillus fumigatus*, and *Alternaria alternata*; ImmunoCAP®; Phadia, Uppsala, Sweden), grasses, and a standard mix of airborne allergens (Phadiatop®; Phadia). Chest X-ray scans were only performed on workers with symptoms to avoid unnecessary radiation.

After the first health examination, the problematic seeds were cleaned at plant II by two selected workers (workers 1 and 2 and in the final cleaning called workers 1b and 2b). They were supplied with a powered air-purifying respirator with a face shield and helmet (Compact Air mod. Junior A, filter P3, North®; Honeywell, USA) and were examined at the beginning and end of the first work day as well as after 17 days, when cleaning of the problematic seeds was finished. These examinations included health anamnesis, auscultation, inspection of mucosal membranes of the nose, throat and eyes, and spirometry. Furthermore, these two workers were instructed in monitoring expiratory peak flow value (PF) during work and at home, at least six times a day, during the 11 working days the cleaning of the problematic lot was on going. Worker 1b, who

failed to use the respiratory airway protection correctly, had an extra examination after 11 days.

After 6–9 months, all 12 workers went through a follow-up examination.

#### *Seeds used for exposure analysis*

Four sacks of grass seeds (tall fescue, *Festuca arundinaceae*) were obtained from the seed company for analysis: Two sacks of the problematic seeds, one uncleaned and one cleaned, and two sacks of reference seeds, one uncleaned and one cleaned. Three samples of 5 g of each kind of seed were weighed before and after drying in an oven (105°C, 18 h) in order to measure the water content. The moisture content of the grass seeds was between 6.1 and 7.3%.

#### *Inhalation exposure risk assessment*

*Total amount of microbial components in seeds (Step 1).* A subsample of 40 g was taken from each of the four kinds of grass seed samples and shaken (250 r.p.m.) in isotonic water for 15 min on ice (washing) (Fig. 1). The suspensions of seed extracts were used for quantifying colony-forming units (cfu) of fungi and actinomycetes and also for endotoxin analysis. The experiment was performed in triplicate.

*Agitation of seeds in a rotating drum (Step 2).* Emission of dust from each of the four kinds of grass seed samples was determined according to the European standard EN 15051 (CEN—European Committee for Standardization, 2006) using a rotating drum (HSE Rotating Drum Dustiness Tester model EDT38L; JS Holdings, Hertfordshire, UK) (Fig. 1). Bulk samples weighing 200 g ( $\approx 270$  ml) were used, as suggested in MDHS 81 (Health and Safety Laboratory, 1996). The drum was 0.3 m in diameter and equipped with eight internal longitudinal vanes, which lifted the sample and subsequently let it fall at a rate of 4 r.p.m. for 1 min. An air current created by a vacuum pump (flow rate = 38 l min<sup>-1</sup>; mean velocity 0.1 m s<sup>-1</sup> [Pensis *et al.*, 2010]) acted on the suspended material causing dust to be released and collected on a polycarbonate filter at the end of the drum. Each filter was cut into two equal pieces, one piece used for endotoxin analysis, and the other for quantification of microorganisms. Data are presented as concentrations (unit per milligram dust) (Table 2) and as dustiness (amount aerosolised per kilogram grass seeds) (Table 3). The experiment was performed in triplicate.

*Personal and stationary sampling of inhalable aerosols (Step 3).* Personal dust monitoring was conducted using GSP inhalable samplers (CIS by BGI, INC Waltham, MA, USA) from 7 am to 3 pm in

May 2009 (Fig. 1). Each worker carried two GSP samplers (flow rate 3.5 l min<sup>-1</sup>; Apex pumps, Castella, England), one mounted with a Teflon filter (Millipore, Copenhagen, Denmark, pore size 1  $\mu$ m) for gravimetric and endotoxin analysis and one with a polycarbonate filter (pore size 1  $\mu$ m; GE Water & Process Technologies, Trevose, PA, USA) for quantification of microorganisms and pollen. While personal dust monitoring was being performed, we recorded the different tasks being carried out by the two workers. Results are presented as time-weighted averages.

Inhalable bioaerosols were sampled by stationary GSP samplers for  $\sim 7$  h in four working areas representing different steps in the seed treatment process. In addition, an outdoor reference was made upwind from the plant.

*Gravimetric analysis.* The mass of the dust collected on the GSP and rotating drum filters was determined by weighing the filters before and after dust sampling. Before weighing, the filters used for collecting the dust were equilibrated at constant air temperature and humidity for 20–24 h (22°C and 50% relative humidity  $\pm 5\%$ ).

*Extraction of dust.* The dust for endotoxin analysis was extracted in 10.0 ml pyrogen-free water with 0.05% Tween 20 by orbital shaking (300 r.p.m.) at room temperature for 60 min and centrifuging (1000 g) for 15 min. The supernatant was stored at  $-80^\circ\text{C}$ . The dust on polycarbonate filters for quantification of microorganisms 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.

*Determination of endotoxin and  $\beta$ -glucan by the Limulus method.* The supernatant was analysed (in duplicate) for endotoxin using the kinetic *Limulusamoebocyte* lysate test (Kinetic-QCL endotoxin kit; BioWhittaker, Walkersville, Maryland, USA) with  $\beta$ -glucan blocker. A standard curve obtained from an *Escherichia coli* O55:B5 reference endotoxin was used to determine the concentrations in terms of endotoxin units (EU) (10.0 EU  $\approx 1.0$  ng). All samples were diluted at least 10 times before the endotoxin analysis was performed. The limit of detection was 0.05 EU ml<sup>-1</sup>. The data are presented as EU m<sup>-3</sup> air, as EU mg<sup>-1</sup> dust or as EU kg<sup>-1</sup> seeds.

Airborne  $\beta$ -glucan was extracted using 0.3 M NaOH for 60 min. After extraction,  $\beta$ -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<sup>-1</sup>. The data are presented as nanogram per cubic metre air.

*Quantification of microorganisms (CAMNEA).* Microorganisms were quantified using a modified

CAMNEA method (Palmgren *et al.*, 1986). The number of fungi culturable on dichloran glycerol agar (DG18 agar; Oxoid, Basingstoke, England) at 25°C was counted after 3 and 7 days of incubation. In addition, DG18 agar plates were incubated at 45°C to quantify culturable *A. fumigatus*. Mesophilic actinomycetes and thermophilic actinomycetes (55°C) were after 3 and 7 days of incubation quantified on 10 and 100% nutrient agar (Oxoid) with actidione (cycloheximide; 50 mg l<sup>-1</sup>), respectively.

The total numbers of bacteria, fungal spores, hyphal fragments, and grass pollen were determined in samples from Step 3 after staining in 20 ppm acridine orange (Merck, Hellerup, Denmark) in acetate buffer for 30 s with subsequent filtration through a polycarbonate filter (0.4 µm; Nuclepore, Cambridge, MA, USA). Counts were made at a magnification of 1250 times in 40 randomly chosen fields using epifluorescence microscopy (Orthoplan; Leitz, Wetzlar, Germany). The number of fungal spores is called 'total fungi' and the number of bacterial cells is called 'total bacteria'. Total fungi, total bacteria, hyphal fragments, and grass pollen are expressed as number per cubic metre air. The detection limit was 1.6 × 10<sup>4</sup> units m<sup>-3</sup> air.

#### Statistical analysis

Statistical analyses were performed with SAS (Version 8e; SAS Institute, Cary, NC, USA). Analysis of variance (*t*-test, PROC ANOVA) was used on the log-normal distributed data to test the significance of the

differences between the seed samples with regard to concentrations of the different microorganisms, dust, and endotoxin.

## RESULTS

#### Inhalation exposure risk assessment

*Grass seed extracts (Step 1)*. The total amount of endotoxin and microorganisms in seed extracts (Step 1) are presented in Table 1. The concentrations of all components are significantly higher in the extracts from problematic seeds than in reference seeds also after cleaning of the problematic seeds. More endotoxin was aerosolised from uncleaned seeds than from cleaned seeds both when considering problematic and reference seeds. In addition, endotoxin and microorganisms were aerosolised in significantly higher amounts from the cleaned problematic seeds than from uncleaned reference seeds.

*Emissions from grass seeds (Step 2)*. Each grass seed sample was rotated in a drum for 1 min (Step 2) and the concentrations of endotoxin and microorganisms in the released dust are presented in Table 2 and the amounts aerosolised per kilogram seed are termed dustiness and are presented in Table 3.

Significantly higher concentrations of all microbial components were found in the dust from the problematic seeds than in dust from the reference seeds ( $P < 0.0001$  for all components) (Table 2). From the uncleaned problematic seeds, five times

Table 1. Concentrations (amount kg<sup>-1</sup> seed) of endotoxin and microorganisms in grass seed extracts (Step 1).

Source	Endotoxin (EU)	Mesophilic fungi (cfu)	<i>Aspergillus fumigatus</i> (cfu)	<i>Thermophilic actinomycetes</i> (cfu)	<i>Mesophilic actinomycetes</i> (cfu)
Problematic seeds, uncleaned	2.63 × 10 <sup>9a</sup>	2.00 × 10 <sup>10a</sup>	4.50 × 10 <sup>9a</sup>	1.20 × 10 <sup>11a</sup>	2.85 × 10 <sup>1a1</sup>
Problematic seeds, cleaned	1.71 × 10 <sup>9b</sup>	1.18 × 10 <sup>10a</sup>	3.10 × 10 <sup>9a</sup>	1.35 × 10 <sup>11a</sup>	4.36 × 10 <sup>10b</sup>
Reference seeds, uncleaned	8.02 × 10 <sup>7c</sup>	2.70 × 10 <sup>7c</sup>	9.00 × 10 <sup>3b</sup>	3.60 × 10 <sup>5b</sup>	1.80 × 10 <sup>6d</sup>
Reference seeds, cleaned	1.48 × 10 <sup>7d</sup>	4.10 × 10 <sup>8b</sup>	9.20 × 10 <sup>3b</sup>	9.00 × 10 <sup>3c</sup>	4.50 × 10 <sup>7c</sup>

Numbers in the same column followed by the same letter are not significantly different ( $P > 0.05$ ).

Table 2. Concentrations (amount mg<sup>-1</sup> dust) of endotoxin and microorganisms in dust aerosolised from grass seeds ( $n = 3$ ) and amount of aerosolised dust in the rotating drum (Step 2).

Source	Dust (mg)	Endotoxin (EU)	Mesophilic fungi (cfu)	<i>Aspergillus fumigatus</i> (cfu)	<i>Thermophilic actinomycetes</i> (cfu)	<i>Mesophilic actinomycetes</i> (cfu)
Problematic seeds, uncleaned	72.4 <sup>a</sup>	1.05 × 10 <sup>4b</sup>	1.32 × 10 <sup>6a</sup>	4.14 × 10 <sup>5a</sup>	6.22 × 10 <sup>6a</sup>	1.45 × 10 <sup>7a</sup>
Problematic seeds, cleaned	9.9 <sup>c</sup>	1.28 × 10 <sup>4a</sup>	3.03 × 10 <sup>5b</sup>	1.38 × 10 <sup>5b</sup>	7.58 × 10 <sup>5b</sup>	3.79 × 10 <sup>6b</sup>
Reference seeds, uncleaned	14.5 <sup>b</sup>	2.27 × 10 <sup>3c</sup>	8.28 × 10 <sup>3c</sup>	2.61 × 10 <sup>1c</sup>	1.9d	3.82 × 10 <sup>4b</sup>
Reference seeds, cleaned	2.9 <sup>d</sup>	5.49 × 10 <sup>2d</sup>	1.12 × 10 <sup>4c</sup>	9.31 × 10 <sup>1c</sup>	1.45 × 10 <sup>3c</sup>	3.01 × 10 <sup>4b</sup>

Numbers in the same column followed by the same letter are not significantly different ( $P > 0.05$ ).



as much dust was aerosolised as from the uncleaned reference seeds. Even after cleaning, 3.4 times as much dust was aerosolised from the problematic seeds as from the cleaned reference seeds (Table 3). It is notable that especially the thermotolerant fungus *A. fumigatus* and thermophilic actinomycetes were aerosolised from the problematic uncleaned seeds in very high amounts compared to the corresponding reference seeds (Table 3).

*Exposure at plant II (Step 3).* Personal and stationary exposure measured during working hours at plant II and an outdoor reference measurement are presented in Table 4 and Table 5. Personal exposures to all microorganisms and endotoxin exceeded suggested occupational exposure limits (OELs). Stationary measurements showed high concentrations in all areas at the plant, but the seed reception (S1) and the seed silo area (S2) were the most problematic working areas concerning exposure to dust and endotoxin.

#### Health reports and medical examination

Ten people (workers 1–10) worked at the plant the first day the problematic seeds in addition to reference

seeds were handled in the facility. Two drivers (workers 11 and 12) transported the problematic seeds. All 12 underwent a health examination, and seven workers allowed a blood sample to be taken. Workers 1 and 2 are mentioned as workers 1b and 2b in Table 6 when involved in the final cleaning of the seeds.

Seven workers showed respiratory symptoms few hours after working with the problematic seeds and four of them had a fever and six had flu-like symptoms (Table 6). All 12 workers had normal lung function values, leukocyte counts, CRP values and auscultation and in none of the seven workers that were tested were found raised levels of IgE. The found symptoms were consistent with those of ODTS. On five workers, chest X-ray scans were performed. In one worker, clearly exposed and diagnosed with ODTS, suspicion of a minor infiltrate was found; the remaining four had normal chest X-rays.

The workers (workers 1, 1b, 3, 4, 5, and 12) were those receiving the seeds (position 1, Tables 4 and 5), loading them into a silo or boxes (position 2, Tables 4 and 5) and those operating the cleaning machines

Table 3. Dustiness of grass seeds ( $n = 3$ ) in terms of numbers of microorganisms and amount of endotoxin and dust aerosolised during 1 min of rotation in a rotating drum (amount aerosolised  $\text{kg}^{-1}$  grass seed) (Step 2).

Source	Dust (mg)	Endotoxin (EU)	Mesophilic fungi (cfu)	<i>Aspergillus fumigatus</i> (cfu)	<i>Thermophilic actinomycetes</i> (cfu)	<i>Mesophilic actinomycetes</i> (cfu)
Problematic seeds, uncleaned	362 <sup>a</sup>	$3.79 \times 10^{6a}$	$4.77 \times 10^{8a}$	$1.50 \times 10^{8a}$	$2.25 \times 10^{9a}$	$5.25 \times 10^{9a}$
Problematic seeds, cleaned	49.5 <sup>c</sup>	$6.41 \times 10^{5b}$	$1.50 \times 10^{7b}$	$6.82 \times 10^{6b}$	$3.75 \times 10^{7b}$	$1.88 \times 10^{8b}$
Reference seeds, uncleaned	72.5 <sup>b</sup>	$1.62 \times 10^{5c}$	$6.36 \times 10^{5c}$	$2.03 \times 10^{3c}$	$1.57 \times 10^{2d}$	$2.77 \times 10^{6c}$
Reference seeds, cleaned	14.5 <sup>d</sup>	$7.78 \times 10^{3d}$	$1.62 \times 10^{5d}$	$1.35 \times 10^{3c}$	$2.10 \times 10^{4c}$	$4.36 \times 10^{5d}$

Numbers in the same column followed by the same letter are not significantly different ( $P > 0.05$ ).

Table 4. Exposure ( $\text{twa m}^{-3}$  air) to dust, endotoxin and bacteria (Step 3).

Position (s) or worker	Measurement	Dust (mg)	Endotoxin (EU)	Total bacteria (number)	<i>Thermophilic actinomycetes</i> (cfu)	<i>Mesophilic actinomycetes</i> (cfu)
Worker 1b, position S1, S4 and in a truck <sup>a</sup>	Personal	4.15	<b><math>3.22 \times 10^5</math></b>	$1.25 \times 10^8$	<b><math>1.19 \times 10^6</math></b>	$1.26 \times 10^8$
Worker 2b, position S4 and in a truck	Personal	1.52	<b><math>1.40 \times 10^5</math></b>	$1.08 \times 10^8$	<b><math>1.05 \times 10^6</math></b>	$2.45 \times 10^7$
Seed reception (S1)	Stationary	1.49	<b><math>7.44 \times 10^5</math></b>	$8.44 \times 10^7$	<b><math>7.83 \times 10^5</math></b>	$3.43 \times 10^7$
Seed silo (S2)	Stationary	<b>9.10</b>	<b><math>1.34 \times 10^5</math></b>	$1.58 \times 10^8$	<b><math>9.05 \times 10^6</math></b>	$3.05 \times 10^8$
Seed cleaning (S3)	Stationary	1.05	<b><math>4.25 \times 10^4</math></b>	$4.06 \times 10^7$	<b><math>1.53 \times 10^6</math></b>	$1.93 \times 10^7$
Packaging of cleaned seeds (S4)	Stationary	0.26	<b><math>2.39 \times 10^4</math></b>	$2.08 \times 10^7$	<b><math>1.29 \times 10^6</math></b>	$6.80 \times 10^6$
Outdoor reference	Stationary	0.011	2.35	Bd	$9.74 \times 10^2$	$3.82 \times 10^3$

Bd, below detection level; Twa, time-weighted average. Concentrations higher than suggested exposure limits (OELs) are in bold. For dust, it is concentrations larger than  $3 \text{ mg m}^{-3}$  air (Arbejdstilsynet, 2007); for endotoxin, it is concentrations larger than 90 EU  $\text{m}^{-3}$  (Health Council of the Netherlands, 2010) and for cfu of thermophilic actinomycetes, it is concentrations larger than  $2 \times 10^4 \text{ m}^{-3}$  (Dutkiewicz *et al.*, 1994). For cfu of mesophilic actinomycetes, no suggested TLV is available.

<sup>a</sup>Areas the workers reported to be occupied in.

Table 5. Exposure ( $\text{twa m}^{-3}$ ) to fungi, fungal components and grass pollen (Step 3).

Position (S) or worker	Measurement	Total fungi (number)	Hyphal fragments (number)	$\beta$ -glucan (ng)	Mesophilic fungi (cfu)	<i>Aspergillus fumigatus</i> (cfu)	Grass pollen (number)
Worker 1b, position S1, S4 and in a truck	Personal	<b><math>2.46 \times 10^7</math></b>	$8.69 \times 10^6$	$4.84 \times 10^3$	$7.95 \times 10^6$	$8.09 \times 10^5$	$1.45 \times 10^5$
Worker 2b, position S4 and in a truck	Personal	<b><math>1.10 \times 10^7</math></b>	$3.05 \times 10^6$	$2.82 \times 10^3$	$1.62 \times 10^6$	$4.09 \times 10^5$	$2.93 \times 10^5$
Seed reception (S1)	Stationary	<b><math>7.17 \times 10^6</math></b>	$8.52 \times 10^5$	$1.62 \times 10^3$	$1.84 \times 10^6$	$5.02 \times 10^6$	$2.62 \times 10^4$
Seed silo (S2)	Stationary	<b><math>7.01 \times 10^6</math></b>	$1.09 \times 10^6$	$2.22 \times 10^3$	$1.61 \times 10^6$	$6.79 \times 10^6$	$8.11 \times 10^4$
Seed cleaning (S3)	Stationary	<b><math>3.01 \times 10^6</math></b>	$4.33 \times 10^5$	$1.66 \times 10^3$	$1.05 \times 10^6$	$4.12 \times 10^5$	$1.38 \times 10^4$
Packaging of cleaned seeds (S4)	Stationary	<b><math>2.13 \times 10^6</math></b>	$3.64 \times 10^5$	$1.97 \times 10^3$	$6.9 \times 10^5$	$3.20 \times 10^5$	$5.12 \times 10^3$
Outdoor reference	Stationary	Bd	Bd	$1.81 \times 10^1$	$9.74 \times 10^2$	$2.44 \times 10^2$	Bd

Bd, below detection level; twa, time-weighted average. Suggested OEL only available for fungal spores: concentrations larger than  $10^5$  spores  $\text{m}^{-3}$  air are in bold according to a suggested OEL (Eduard, 2009).

(position 3, Table 6). These were also the workers with most pronounced symptoms. Four workers (workers 2, 9, 10, and 11) at work at the time the problematic seeds were received did not work with or near the problematic seeds, but worked with reference seeds and none of them had any health complaints suggestive of airway disease (Table 6).

Workers 1 and 2 (called 1b and 2b), involved in the final cleaning at plant II, had normal auscultation findings and spirometry values before, during, and after the period of work and they had no work-related changes in peak flow values. During the 2 days, worker 1b did not use personal airway protection properly, and he developed symptoms similar to those during the first exposure and compatible with ODTS. However, PF was unchanged and an extra health examination showed normal auscultation and spirometry values.

*Follow-up* At follow-up, 6–9 months after work with the problematic seeds, none of the 12 workers had any respiratory symptoms nor did they report symptoms of ODTS in the period in between. The seven workers with symptoms had a general health examination which was normal in all cases.

## DISCUSSION

In this study, we show that workers at a grass seed plant can be exposed to very high concentrations of different bioaerosol components. Workers were diagnosed with ODTS. The investigation was prompted by health complaints by employees at a grass seed plant. All workers showing symptoms had an obvious exposure to dust from a problematic seed lot. Health examination was almost normal (lung auscultation, spirometry, and X-ray scan) even in workers who experienced clear symptoms and sub-

jective difficulty in breathing. This is in accordance with the definition of ODTS (Seifert *et al.*, 2003). There was a close association between exposure to the problematic seeds and onset of symptoms and after exposure symptoms waned also in accordance with the diagnosis of ODTS. Those at work when the problematic seeds were handled but not working near these seeds showed no symptoms. The severity of complaints from the workers was associated with the degree of exposure, with position 1 (receiving the seeds) and position 2 (loading the seeds into a silo or boxes) being places with high exposure (Tables 4 and 5).

During the final cleaning, worker 1b in Table 6, using airway protection while cleaning the seeds, generally had no complaints but twice showed symptoms of ODTS after a few hours when not using the protection properly. Worker 2b used airway protection correctly at all times during the final cleaning and showed no symptoms. According to Seifert *et al.* (2003), efforts to prevent ODTS should focus on avoiding exposure, which is why the correct use of airway protection is of importance. Investigations of the effects of respiratory devices (disposable particulate respirators or particle-filtering half masks) during exposure to organic dust found that they reduced symptoms in short-term exposures both for healthy participants (Dosman *et al.*, 2000) and for participants already suffering from a dust-related disease (Müller-Wening and Repp, 1989; Müller-Wening and Neuhaus, 1998).

Exposure to a wide variety of microorganisms has been suggested or found to be responsible for more severe diseases, for example, HP, which may lead to chronic lung damage (Selman *et al.*, 2010). Actinomycetes (Bourke *et al.*, 2001) and *A. fumigatus* (Selman *et al.*, 2010) have been mentioned as causing

Table 6. Demographic data and health symptoms of grass seed workers (Step 4)

Worker	Respiratory protection	Age	Years employed	Smoker	Problematic seeds <sup>a</sup>	Dry cough	Mucosal irritation	Shortness of breath	Chest tightness	Fatigue like	Flu like	Fever	Duration <sup>b</sup>	X-ray scan	Spirometry	Auscultation	IgE	ODTS
1	-	39	3	+	+	+	+	+	+	-	+	+	1 week	N	N	N	Negative	Yes
1b	(+)	39	3	+	+	+	+	+	-	-	(+)	-	1 day	n.p.	N	N	n.p.	Possible
2	-	52	>5	-	((+))	-	-	-	-	-	-	-	0	n.p.	N	N	Negative	-
2b	+	52	>5	-	+	-	-	-	-	-	-	-	0	n.p.	N	N	n.p.	-
3	-	45	>5	-	+	+	+	+	+	-	+	+	1 week	Possible infiltrate	N	N	Negative	Yes
4	-	41	>5	+	+	+	+	+	+	+	(+)	+	1 week	N	N	N	Negative	Yes
5	-	59	>5	-	(+)	+	+	+	+	-	+	+	3 days	N	n.p.	N	Negative	Yes
6	-	45	>5	-	(+)	-	-	-	(+)	-	(+)	-	1 day	n.p.	N	N	n.p.	Possible
7	-	47	>5	-	(+)	-	+	+	+	-	-	-	1 day	N	n.p.	N	Negative	Possible
8	-	66	>5	-	(+)	-	-	-	-	-	-	-	0	n.p.	N	N	n.p.	-
9	-	58	>5	-	((+))	-	-	-	-	-	-	-	0	n.p.	N	N	Negative	-
10	-	35	5	+	((+))	-	-	-	-	-	-	-	0	n.p.	N	N	n.p.	-
11	-	58	>5	-	((+))	-	-	-	-	-	-	-	0	n.p.	n.p.	N	n.p.	-
12	-	34	4	-	(+)	+	+	-	(+)	-	-	-	3 days	n.p.	N	N	n.p.	Possible

Yes; partly; very limited; - no; N, normal; n.p., prescribed, but not performed due to worker's wish. Workers number 1 and 2 are also included as workers 1b and 2b as they, after primary exposure, later performed final cleaning of seeds. Fever considered 'suspected', if felt by the worker but not measured.

<sup>a</sup>Work with problematic seeds.

<sup>b</sup>Duration of any symptom of ODTS.



HP, and we found *A. fumigatus* and actinomycetes to be released in very high quantities from the problematic seeds. The health examinations do, however, not indicate further health complications, and findings of the follow-up examination also gave no reasons to suspect chronic changes.

There are no internationally accepted threshold limit values (TLVs) or OELs for endotoxin, actinomycetes, and fungal spores. Suggested TLVs or calculated 'no effect values' for inhalable or 'total endotoxin' exposure are between 30 and 2000 EU m<sup>-3</sup> (Haglund and Rylander, 1984; Rylander *et al.*, 1985; Kennedy *et al.*, 1987; Castellan *et al.*, 1987; Smid *et al.*, 1992; Smid, 1993; Michel, 1997; Rylander, 1997; Donham and Cumro, 1999; Donham *et al.*, 2000; Health Council of the Netherlands, 2010). A review of the literature on human challenge experiments suggests a no-effect level for endotoxin exposure in relation to development of toxic pneumonitis of 200 ng endotoxin m<sup>-3</sup> ( $\approx 2000$  EU m<sup>-3</sup>) (Rylander, 1997). Based on a review study, it has been suggested that an exposure level of 10<sup>5</sup> fungal spores per cubic metre air would be an appropriate baseline for an OEL for spores from various fungi (Eduard, 2009). Exposures larger than  $2 \times 10^4$  cfu of thermophilic actinomycetes m<sup>-3</sup> have been suggested as a TLV (Dutkiewicz *et al.*, 1994). All stationary and personal measurements of exposure were higher than the highest suggested limit of 200 ng m<sup>-3</sup> for endotoxin, higher than the 10<sup>5</sup> fungal spores m<sup>-3</sup> and higher than the  $2 \times 10^4$  cfu of thermophilic actinomycetes m<sup>-3</sup>. As these suggested OELs all are well below the measurements at the grass seed plants and below what has been measured in two other environments where workers developed ODTS—at a compost plant (Weber *et al.*, 1993) and at farms (Malmberg *et al.*, 1993)—keeping below the OELs is expected to protect the workers from developing ODTS. In other environments where plant materials such as seeds are handled, very different exposure levels to endotoxin have been found (reviewed in Madsen *et al.*, 2009). High endotoxin exposure has been found in cucumber and tomato nurseries (median = 320 EU m<sup>-3</sup>, max = 4020 EU m<sup>-3</sup>) (Madsen *et al.*, 2009), grain farming (GM = 5900 EU m<sup>-3</sup>) (Halstensen *et al.*, 2007) and cotton mills (median = 438 ng m<sup>-3</sup>, max = 6936 ng m<sup>-3</sup>) (Simpson *et al.*, 1999), and a grass seed processing plant (78 000 EU m<sup>-3</sup>) (Smit *et al.*, 2006) but the exposure at the Danish grass seed plant was higher. The exposure of grass seed workers to  $\beta$ -glucan was also higher than earlier found for compost workers (median = 0.79 ng m<sup>-3</sup>, max = 14.45 ng m<sup>-3</sup>) (Hryhorczuk *et al.*, 2001).

In a study of the effect of various airborne particles on asthma severity, hyphal fragments caused the most consistently significant effects on the parameters used to define asthma severity (Delfino *et al.*, 1997). Most studies, however, do not include hyphal fragments, but Halstensen *et al.*, (2007) argue that hyphal fragments should be included in microbial analyses as they contain many of the same properties as fungal spores. In this study, the exposure to hyphal fragments was up to  $8.7 \times 10^6$  fragments m<sup>-3</sup>, which appears high when compared with other studies (Pady and Kramer, 1960; Delfino *et al.*, 1997; Tendal and Madsen, 2011). Also the exposure to grass pollen was high and it greatly exceeded what is usually found in outdoor air (Sommer and Rasmussen, 2009; Tendal and Madsen, 2011).

This investigation clearly demonstrates that this particular lot of grass seeds produced much more dust than normal, comparable seeds. Simulated handling of grass seeds (Step 2) caused an aerosolisation of  $\sim 5$  times as much dust, 23 times as much endotoxin, 750 times as many mesophilic fungi, 10<sup>4</sup> times as many *A. fumigatus* and 10<sup>7</sup> times as many thermophilic actinomycetes as reference seeds. In studies on a farm (Malmberg *et al.*, 1993) and a compost plant (Weber *et al.*, 1993), exposure events, which had led to ODTS, were reconstructed. The mean spore (bacterial, fungal, and actinomycetes) concentration during a 5- to 15-min measurement was  $1.3 \times 10^{10}$  spores m<sup>-3</sup>, which was 110 times higher than the concentration measured during exposure on reference farms for 1–2 h (Malmberg *et al.*, 1993). During a 20-min exposure assessment during a worst case scenario at a compost plant, the exposure to dust was 149 mg m<sup>-3</sup>, the exposure to fungal spores was  $3.7 \times 10^9$  spores m<sup>-3</sup> and the exposure to endotoxin  $\sim 2 \times 10^4$  EU m<sup>-3</sup> (Weber *et al.*, 1993). On the grass seed plant where we measured during a whole working day (Step 3), we found that handling of the seeds causing ODTS led to a corresponding exposure of  $1.3 \times 10^8$  bacterial (including actinomycetes) cells plus fungal spores m<sup>-3</sup>. Thus, a lower level of exposure to fungi or fungi plus bacteria than at the farms and a lower concentration of fungal spores and dust than at the compost plant seem to cause ODTS for the grass seed workers. However, the very high exposure to endotoxin of  $3.2 \times 10^5$  EU m<sup>-3</sup> is probably also involved in the development of ODTS. Furthermore, our exposure measurement was performed during a whole working day and not only during working processes expected to cause the highest exposures. The stationary measurements in the different areas of the plant

revealed different exposure levels but all can be considered as high.

Field exposure measurements (Step 3) reflects a combination of the contribution from the handled seeds, how and for how long time the seeds are handled and the admixture of bioaerosols from neighbouring areas while measurements using the rotating drum (Step 2) reflects the concentrations that can during certain conditions be aerosolised from the seeds. Step 1 shows that the seeds are the source of the bioaerosol exposure, and the low outdoor reference measurement shows that there is no notable outdoor contribution to the high indoor exposure—outdoor air will dilute the exposure. The outdoor reference exposure to endotoxin and  $\beta$ -glucan was on the same level as other outdoor reference measurements of endotoxin (Madsen, 2006a) and  $\beta$ -glucan (Madsen *et al.*, 2011). Step 2 shows that the exposure from handling cleaned problematic seeds (Table 3) is significantly reduced in comparison with handling uncleaned seeds. However, the bioaerosol contribution from cleaned problematic seeds is high and cleaning of problematic seeds is not enough to reduce the exposure to the level caused by work with reference seeds.

Comparison of concentrations of microorganisms in Step 1 versus Step 2 (Table 1 versus Tables 2 and 3) shows that only a small fraction of the present microorganisms are aerosolised during mechanical handling for 1 min. This is in accordance with what is seen for straw and wood chips (Madsen *et al.*, 2006) and indicates that repeated exposure will occur if seeds are handled repeatedly. The dustiness (Step 2) of uncleaned problematic seeds in terms of all the studied microorganisms and endotoxin was higher than what has earlier been measured for wood chips and straw (Madsen *et al.*, 2004). The concentration of endotoxin in extracts from the problematic seeds was higher than what has earlier been seen in grass seed extracts; the concentration in reference seed extract (Step 1) is at the level of what has earlier been found for grass seed extracts (Smit *et al.*, 2006).

In most environments where organic material is handled, personal measurements of exposure to bioaerosol and dust show higher levels than stationary measurements (Ogden *et al.*, 1993; Madsen, 2006b; Madsen *et al.*, 2009). However, at the grass seed plant exposure to dust and some bacteria at the seed silo area (S2) was higher than personal exposure. This may be because some of the automatic processes generated dust.

## CONCLUSION

Exposure to very high concentrations of bioaerosol components can occur when working with grass seeds.

The high exposure to bioaerosols and symptoms of grass seed workers was in accordance with the diagnosis of ODTs. Up to  $10^7$  times as many microorganisms were aerosolised from the seeds causing ODTs as from reference seeds. Suspicion should be elicited by seeds producing more dust than usually or not having been sufficiently dried, and precautions should be taken.

The conceptual approach used in this study proved to be good for both hazard identification and for determination of the influence of handling problematic seeds versus reference seeds on the exposure and health effects. The exposure assessment and health examination showed that it was likely the simultaneous exposure to high levels of many different bioaerosol components that caused the development of ODTs. This serves to emphasize the importance of measuring a wide range of bioaerosol components in studies of ODTs. If we had measured only one component as, for example, endotoxin, actinomycetes or fungi, we could have been misled to conclude that it was this one component which caused the ODTs. But we cannot know, whether it is the combination of different bioaerosol components or a single component which is responsible for the development of ODTs.

The much lower dustiness of reference seeds and the absence of health symptoms when only reference seeds were handled show that handling of normal grass seeds may not cause health problems. However, it is not known how often the plant receives problematic grass seeds and a more comprehensive investigation of the exposure and general health of employees in the grass seed industry is still lacking and indeed of relevance.

In this study, personal respiratory airway protection proved efficient to avoid development of ODTs and should always be at hand in facilities handling grass seed. It should be used consistently at all stages when handling seeds which seem to be dustier than usually. All direct handling of the problematic seeds seemed to imply a serious exposure even after cleaning. Thus, precautions should also be taken when handling the cleaned problematic seeds and it should also be considered whether such seeds might pose a risk to the consumer.

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