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OCCUPATIONAL EXPOSURE TO FUNGI AND PARTICLES IN ANIMAL FEED INDUSTRY

ZAWODOWE NARAŻENIE NA GRZYBY I CZĄSTKI STAŁE W PRZEMYSŁE PASZOWYM

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ABSTRACT

Background: Very few studies regarding fungal and particulate matter (PM) exposure in feed industry have been reported, although such contaminants are likely to be a significant contributing factor to several symptoms reported among workers. The purpose of this study has been to characterize fungal and dust exposure in one Portuguese feed industry. **Material and Methods:** Air and surface samples were collected and subject to further macro- and microscopic observations. In addition we collected other air samples in order to perform real-time quantitative polymerase chain reaction (PCR) amplification of genes from *Aspergillus fumigatus* and *Aspergillus flavus* complexes as well as *Stachybotrys chartarum*. Additionally, two exposure metrics were considered – particle mass concentration (PMC), measured in 5 different sizes (PM_{0.5}, PM₁, PM_{2.5}, PM₅, PM₁₀), and particle number concentration (PNC) based on results given in 6 different sizes in terms of diameter (0.3 μm, 0.5 μm, 1 μm, 2.5 μm, 5 μm and 10 μm). **Results:** Species from the *Aspergillus fumigatus* complex were the most abundant in air (46.6%) and in surfaces, *Penicillium* genus was the most frequently found (32%). The only DNA was detected from *A. fumigatus* complex. The most prevalent in dust samples were smaller particles which may reach deep into the respiratory system and trigger not only local effects but also the systemic ones. **Conclusions:** Future research work must be developed aiming at assessing the real health effects of these co-exposures. Med Pr 2016;67(2)

Key words: occupational exposure, fungi, dust, feed industry, *Aspergillus fumigatus* complex, *Aspergillus flavus* complex

STRESZCZENIE

Wstęp: Dotychczas opublikowano bardzo mało wyników badań dotyczących narażenia na grzyby i cząstki stałe (particulate matter – PM) w przemyśle paszowym, mimo że te zanieczyszczenia mogą być istotną przyczyną zaburzeń zdrowia u pracowników. Celem badania było określenie narażenia na grzyby i pył w jednym z portugalskich zakładów produkujących paszę dla zwierząt hodowlanych. **Materiał i metody:** Próbkę pobrane z powietrza i powierzchni w pomieszczeniach zakładu poddano obserwacji makro- i mikroskopowej. Z powietrza pobrano również dodatkowe próbki w celu amplifikacji genów kompleksów *Aspergillus fumigatus* i *Aspergillus flavus*, a także *Stachybotrys chartarum*, z zastosowaniem ilościowej reakcji łańcuchowej polimerazy (polymerase chain reaction – PCR) w czasie rzeczywistym. Określono też stężenie masowe cząstek (particle mass concentration – PMC) w 5 rozmiarach (PM_{0.5}, PM₁, PM_{2.5}, PM₅, PM₁₀) i stężenie liczbowe cząstek (particle number concentration – PNC) w 6 średnicach (0,3 μm, 0,5 μm, 1 μm, 2,5 μm, 5 μm i 10 μm). **Wyniki:** W powietrzu zakładu najczęściej występowały gatunki z kompleksu *Aspergillus fumigatus* (46,6%), a na powierzchniach w zakładzie – grzyby z rodzaju *Penicillium* (32%). Spośród 3 wybranych do PCR gatunków grzybów w próbkach wykryto jedynie DNA z kompleksu *A. fumigatus*. W próbkach pyłu najliczniejsze były cząstki o mniejszych rozmiarach, które mogą docierać do głębokich rejonów układu oddechowego i wywoływać nie tylko objawy miejscowe, ale również ogólnoustrojowe. **Wnioski:** Konieczne są kolejne badania w celu oceny rzeczywistych skutków zdrowotnych łącznego narażenia na grzyby i pył. Med. Pr. 2016;67(2)

Słowa kluczowe: narażenie zawodowe, grzyby, pył, przemysł paszowy, kompleks *Aspergillus fumigatus*, kompleks *Aspergillus flavus*

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INTRODUCTION

Working in feed industry clearly involves inevitable risks of exposure to grain dust and its microbial contents. Fungal exposure in terms of colony-forming units (CFU)×m⁻³ may range 2.3×10³–4×10⁶ [1–3].

However, very few studies regarding fungal and particles' exposure in this industry have been reported, and none has been performed in Portuguese industries. Grain dust is a complex mixture of fragments of grain, inorganic soil particles and associated organic contaminants. These contaminants may include plant cell debris, insect parts and mites as well as viable and nonviable microorganisms (vegetative cells and spores of fungi, *Actinomyces* and bacteria, and their components such as endotoxins and mycotoxins) [4], all of which represent health hazards upon inhalation [5]. Employees in feed industry may be exposed to large amounts of grain dust during work, frequently leading to pathological symptoms and functional changes of the airways [4]. Moreover, there is substantial evidence that workers handling grains develop allergic respiratory symptoms [4]. Microbiological contaminants, such as fungi, are likely to be a significant contributing factor to these symptoms [4].

Fungal spores are complex agents that may contain multiple hazardous components. Health hazards may differ across species because fungi may produce various allergens and mycotoxins, and some species may infect humans. As it is the case with other occupational settings, there are only some publications reporting fungal burden in feed industry [6], none of which apply conventional together with molecular methods to assess fungal contamination. In addition, this industry generates a large amount of dust [1] and several papers have highlighted the increased health risk due to contamination by particulate matter (PM) in work environments. Indeed, it has been shown that dust might be a vehicle to respiratory airways for other risk factors, such as fungi and their metabolites [7].

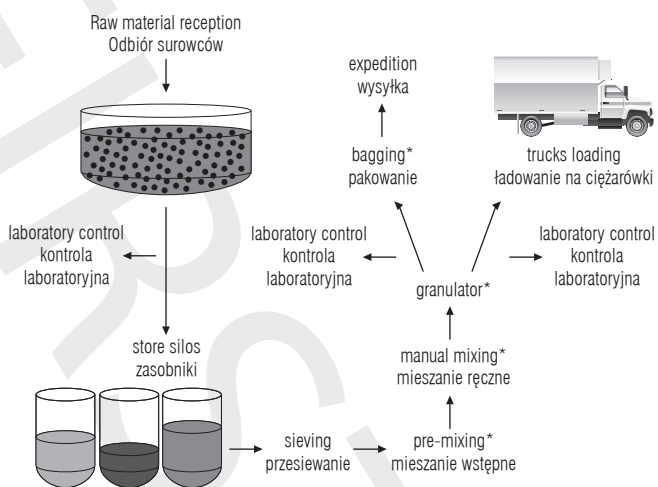
Working in this industry clearly involves inevitable risks of exposure to grain dust and its microbial

contents, being crucial to identify possible sites, tasks, and technical solutions that determine particular high exposure [1,2]. The purpose of this study has been to characterize fungal and dust exposure in different workplaces in one Portuguese feed industry.

MATERIAL AND METHODS

Assessed plant and sampling and measurements strategy

The production process of animal feed in the assessed industry is represented in the Figure 1. The raw materials arrive by train, are cleaned, and stored in silos. In a batch process, the raw materials are grounded and mixed with fats, molasses, and additives, such as vitamins and minerals. The mixture is then usually pressed into pellets and stored in silos again. The animal feed is either packed and shipped in sacks or shipped in bulk trucks. The process is highly mechanized and operates mostly from a central control room. Exposure of workers therefore mainly takes place during unloading, cleaning, maintenance and during manual mixing of some specific components. The unit works 5 days



* Sampling site / Miejsce poboru próbek.

Fig. 1. Production process in the studied animal feed industry plant
Ryc. 1. Proces produkcji w badanej wytwórni pasz

a week in a daily routine of two 8-hour shifts. In all the analyzed workplaces none of the workers used respiratory protection devices.

The workplaces where workers spent most of their time were selected as sampling sites. In addition, we also selected sampling sites where high levels of fungal and particles exposure were expected [8]. As far as fungal assessment is concerned, seven sampling sites were selected as the ones where workers intervene in the production process (Figure 1 and Table 1). In addition to the samples collected within the unit, one outdoor air sample was used as reference.

A task-based approach was used to perform the assessment of exposure to particles and to verify whether measurable differences exist among the different tasks

developed in the workplaces. Before starting the measurements, a flowchart had been done in order to describe the production process, the different workplaces and the respective tasks developed in each one. We defined which tasks should be evaluated in each workplace based on visual observations of work practices on a task-by-task basis, on the time spent by workers in each one, and also on the professional judgment (the task that probably involves higher exposure to particles). After detailed visual observation of each task, we decided to study nine tasks for particle matter assessment. In some cases, the task under consideration corresponded to the only task developed in a specific workplace, such as the manual addition of antibiotics performed in the premixing control room.

Table 1. Sampling sites for fungal and particulate matter exposure assessment in the studied animal feed industry plant

Tabela 1. Miejsca poboru próbek do oceny narażenia na grzyby i pył zawieszony w badanej wytwórni pasz

Workplace Stanowisko pracy	Task Wykonywane zadanie	Fungi Grzyby [n]			Particulate matter Pył zawieszony (N = 11) [n]
		conventional methods metody tradycyjne		molecular biology method (air samples) metoda biologii molekularnej (próbki powietrza) (N = 6)	
		air samples próbki powietrza (N = 7)	surfaces samples próbki z powierzchni (N = 7)		
Bagging (1–3 lines) / Pakowanie (linie produkcyjne 1–3)	machine operating / / obsługa maszyn(y)	1	1	1	n.p.
Bagging (4 and 5 lines) / Pakowanie (linie produkcyjne 4 i 5)	machine operating / / obsługa maszyn(y)	1	1	1	1
Premixing / Mieszanie wstępne	manual adding of antibiotics – control room / ręczne dodawanie antybiotyków – sterownia	1	1	1	2
Filling the silos / Napelnianie zasobników	manual / ręczne	n.p.	n.p.	n.p.	1
	mechanical – machine operating / / mechaniczne – obsługa maszyn(y)	n.p.	n.p.	n.p.	1
Reception of raw materials / / Odbiór surowców	dump truck with soybean – control room / opróżnianie wózka z soją – sterownia	n.p.	n.p.	n.p.	2
Laboratory control / Kontrola laboratoryjna	soybean grinding / mielenie soi	1	1	1	1
Granulator	controlling the process / / nadzorowanie procesu	1	1	1	1
Manual mixing / Mieszanie ręczne	controlling the process / / nadzorowanie czynności	1	1	1	1
Trucks loading / Ładowanie na ciężarówki	loading process / ładowanie	1	1	n.p.	1

N – number of samples/measurements / liczba próbek/pomiarów, n.p. – not performed / nie wykonano.

Sampling collection for fungal assessment

Conventional (cultural) methodologies

Sample collections were performed in February 2015. Air samples of 250 l were collected through an impaction method with a flow rate of 140 l/min onto malt extract agar (MEA) supplemented with chloramphenicol (0.05%) (Oxoid Ltd., United Kingdom), using the Millipore air Tester (Millipore, USA). Surface samples were collected side-by-side from the same indoor sites by swabbing the surfaces with a 10×10 cm square stencil disinfected with a 70% alcohol solution between samples, according to the international standard ISO 18593 (2004) [9]. The obtained swabs were then plated onto the MEA.

Molecular methodologies

Air samples of 300 l were collected using the impinger Coriolis μ air sampler (Bertin Technologies, France), at 300 l/min airflow rate. Samples were collected onto 10 ml sterile phosphate-buffered saline with 0.05% Triton X-100 (Sigma-Aldrich, Portugal), and these collection liquids were subsequently used for DNA extraction using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, United States) according to the manufacturer's instructions.

The samples collected for both conventional and molecular methodologies are listed in the Table 1.

Sample preparation and fungal assessment

Conventional methodologies

All the collected samples were incubated at 27°C for 5–7 days. After laboratory processing and incubation of the collected samples, quantitative (colony-forming units – CFU×m⁻³ and CFU×m⁻²) and qualitative results were obtained, with identification of the isolated fungal species or genera. For the purpose of species identification, microscopic mounts were performed using tease or Scotch tape mounts and lactophenol cotton blue mount procedures. Morphological identification was achieved through macro and microscopic characteristics as noted by de Hoog et al. [10].

Molecular methodologies

Five milliliters from the collection liquid were centrifuged at 2500×g for 10 min, the supernatant was removed and the DNA was then extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit according to the manufacturer's recommendations.

Molecular detection of the DNA from the *Aspergillus fumigatus* complex, and *Stachybotrys chartarum* as well as of toxic strains from *Aspergillus flavus* was achieved by quantitative polymerase chain reaction (qPCR) using the Rotor-Gene 6000 qPCR Detection System (Corbett–Quiagen, Germany) (PCR conditions are referred to in the Table 2). Reactions included 1×iQ Supermix

Table 2. Sequence of primers and TaqMan probes, and conditions of real time quantitative polymerase chain reaction (PCR)

Tabela 2. Sekwencja starterów i sond TaqMan oraz warunki ilościowej reakcji łańcuchowej polimerazy (PCR) w czasie rzeczywistym

Fungi Grzyby	References Piśmiennictwo	Sequence Sekwencja	Reaction conditions Warunki reakcji	
			concentration stężenie [μM]	am T [°C]
<i>A. flavus</i>	Mayer et al., 2003 [11]	F: 5'-GTCCAAGCAACAGGCCAAGT-3'	0.500	53
		R: 5'-TCGTGCATGTTGGTGATGGT-3'	0.500	53
		P: FAM-5'-TGTCTTGATCGGGCCCG-3'-TAMRA	0.375	53
<i>A. fumigatus</i>	Cruz-Perez et al., 2001 [12]	F: 5'-CGCGTCCGGTCCTCG-3'	0.375	52
		R: 5'-TTAGAAAATAAAGTTGGGTGTCGG-3'	0.375	52
		P: FAM-5'-TGTCACCTGCTCTGTAGGCCCG-3'-TAMRA	0.375	52
<i>S. chartarum</i>	Cruz-Perez et al., 2001 [13]	F: 5'-GTTGCTTCGGCGGGAAC-3'	0.150	60
		R: 5'-TTTGCGTTTGCCACTCAGAG-3'	0.150	60
		P: FAM-5'-CTGCGCCCGGATCCAGGC-3'-TAMRA	0.100	60

am T – amplification temperature / temperatura amplifikacji, F – forward primer / starter przedni, R – reverse primer / starter wsteczny, P – probe / sonda.

(Bio-Rad, Portugal), 0.5 μM of each primer, and 0.375 μM of TaqMan probe in a total volume of 20 μl . Amplification followed a 3-step PCR: 40 cycles with denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s. A non-template control was used in every PCR reaction. As far as positive controls for each species are concerned, DNA samples were obtained from reference strains from the Mycology Laboratory from the National Institute of Health Doutor Ricardo Jorge (INSA).

Particulate matter assessment

Regarding particles' exposure assessment, two exposure metrics were considered: particle mass concentration (PMC) – measured in 5 different sizes ($\text{PM}_{0.5}$, PM_1 , $\text{PM}_{2.5}$, PM_5 , PM_{10}) – and particle number concentration (PNC) – based on results given in six different diameter sizes, namely: 0.3 μm , 0.5 μm , 1 μm , 2.5 μm , 5 μm and 10 μm . The measurements were done by means of a direct-reading instrument, handheld particle counter (model 3016/5016, Lighthouse Worldwide Solutions, USA). All the measurements were conducted continuously for the duration of 5 min near the worker's nose and during tasks performance. The same method was used in all collections which were made by the same researcher in order to guarantee reliable and identical sampling methodology.

Data analysis

The data analysis was performed and descriptive statistics was applied, determining the frequency, the median and using graphical representations appropriated to the nature of the data.

In greater detail, the statistical analysis of particles data was performed using the Statistica® software. To compare the PNC and PMC between different workplaces the Kruskal-Wallis test was used, since the normality assumption is not verified ($p < 0.05$). This method was also applied to compare the PNC and the PMC between the different particles' sizes, regardless of the workplace studied as the normality assumption is not verified in this situation as well ($p < 0.05$). Whenever statistically significant differences were detected, the multiple comparison test of Kruskal-Wallis was used.

Results were considered significant for a significance level of 5%.

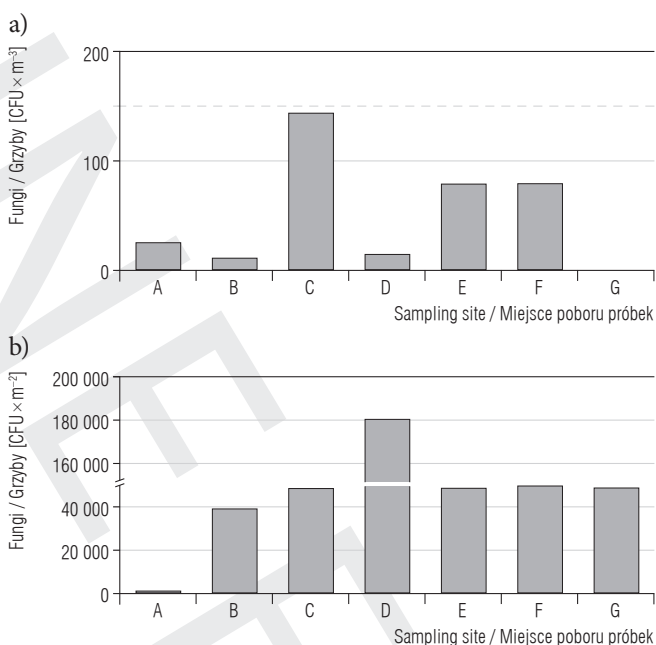
The workplace “premixes (weighing antibiotic medicines)” were not considered in this analysis, since there are only 2 registers which are not enough to produce robust statistical data.

The Mann-Whitney U test was applied to detect statistically significant differences between tasks regarding the different sizes for the PMC and PNC (independent samples).

RESULTS

Fungal load

The fungal load in the air ranged 0–144 $\text{CFU} \times \text{m}^{-3}$ (Figure 2). The surfaces produced results that ranged 0–18 $\times 10^4$ $\text{CFU} \times \text{m}^{-2}$ (Figure 2). None of the sampling sites surpass the guideline proposed by the World Health Organization (WHO) (maximum value of 150 $\text{CFU} \times \text{m}^{-3}$) [14].



A – bagging (4 and 5 lines) / pakowanie (linie produkcyjne 4 i 5), B – premixing / mieszanie wstępne, C – bagging (1–3 lines) / pakowanie (linie produkcyjne 1–3), D – control laboratory / kontrola laboratoryjna, E – granulator, F – manual mixing / mieszanie ręczne, G – trucks loading / ładowanie na ciężarówki.
 CFU – colony-forming unit / jednostka tworząca kolonię.
 The dashed line represents the reference limits by the World Health Organization (WHO) [14] / Przerwana linia pokazuje poziom referencyjny według Światowej Organizacji Zdrowia (WHO) [14].

Fig. 2. Indoor fungal load in the studied animal feed industry plant: a) air, and b) surfaces

Ryc. 2. Zanieczyszczenie grzybami w pomieszczeniach badanej wytwórni pasz: a) w powietrzu i b) na powierzchniach

Six out of the seven samples collected at different sampling sites produced a higher indoor fungal load when compared with the outdoor sample. Moreover, in the same sampling sites, the indoor/outdoor (I/O) ratio was higher than 1.

Fungal identification through conventional methods

Eleven species/genera of filamentous fungi were identified in indoor air samples. Species from the *Aspergillus fumigatus* complex were the most abundant in air (46.6%), followed by those from the *Penicillium* genus (26.7%). But other species such as *Aspergillus ochraceus* complex, *Fusarium graminearum*, *Chryso sporium* sp., *Trichoderma* sp., *Geomyces* sp., *Cladosporium* sp., *Alternaria* sp., *Geotrichum* sp. and *Aureobasidium* sp. (Figure 3 and 4, Table 3) were also detected.

As far as the outdoor sample is concerned, only *Geomyces* sp. was isolated.

Twelve species/genera of filamentous fungi were isolated from surface samples, being the *Penicillium* genus the most frequently found (32%), followed by *Aspergillus flavus* complex (12.5%). Besides these two species, the following species were also detected: *A. ochraceus* complex, *A. niger* complex, *A. fumigatus* complex, *Fusarium verticilloides*, *Rhizopus* sp., *Scopulariopsis candida*, *Chrysosporium* sp., *Chrysonilia* sp., *Ulocladium* sp. and *Aureobasidium* sp. (Figure 3 and 4, Table 3).

The species/complex/genus *S. candida*, *F. verticilloides*, *A. flavus* complex, *A. niger* complex, *Chrysonilia* sp., *Rhizopus* sp., and *Ulocladium* sp. were isolated only in surface samples.

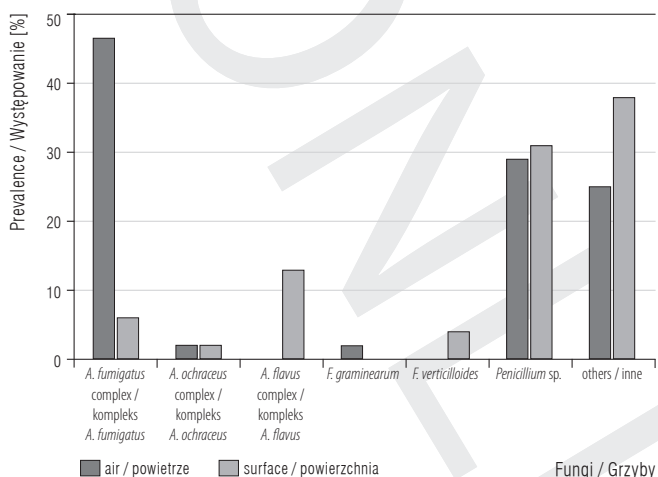
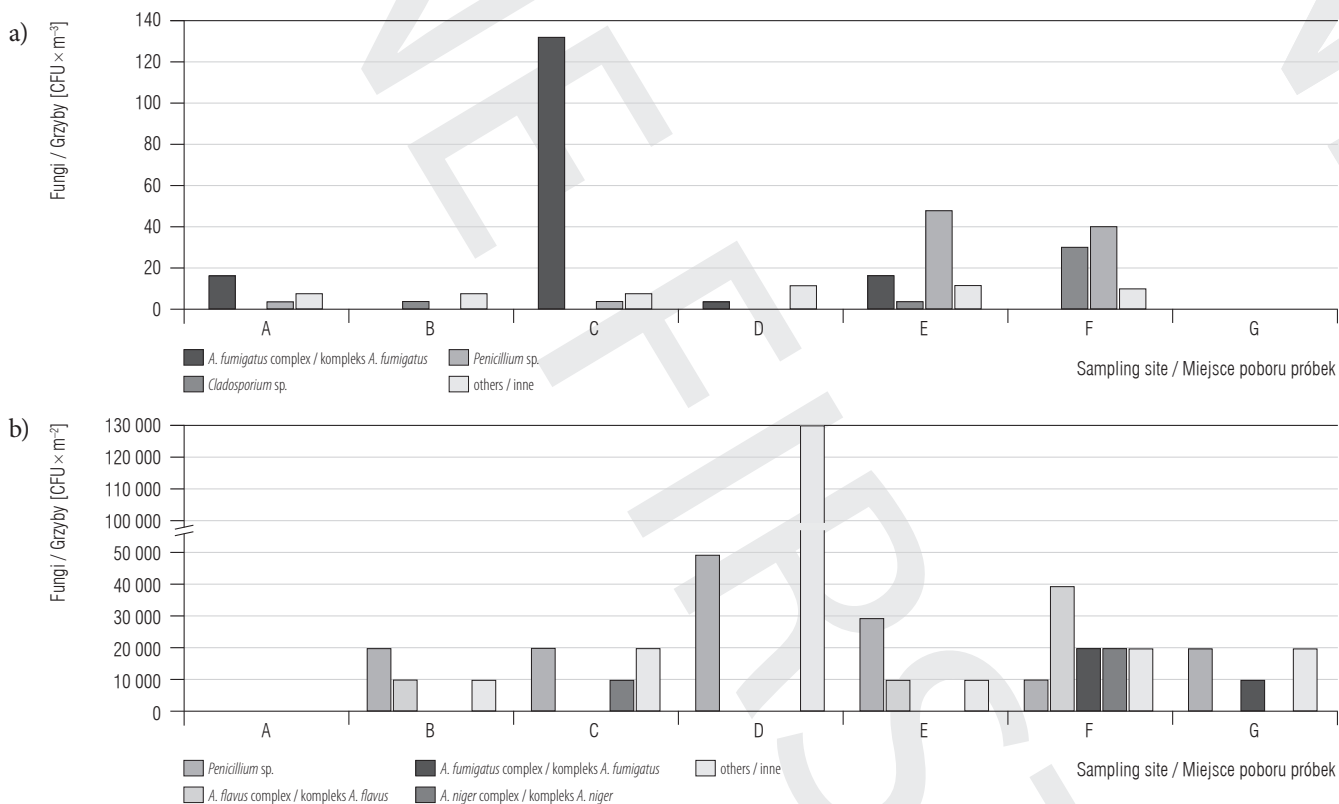


Fig. 3. Prevalent fungi isolated in the indoor air and surfaces in the studied animal feed industry plant

Ryc. 3. Najczęściej występujące grzyby w powietrzu i na powierzchniach w badanej wytwórni pasz



Abbreviations as in Figure 2 / objaśnienia jak na rycinie 2.

Fig. 4. The most prevalent fungi in the studied animal feed industry plant by sampling site: a) air, and b) surfaces
Ryc. 4. Najczęściej występujące grzyby w badanej wytwórni pasz w zależności od miejsca poboru próbek: a) w powietrzu i b) na powierzchniach

Table 3. Most frequently detected fungi indoors in the studied animal feed industry plant
Tabela 3. Najczęściej występujące grzyby w badanej wytwórni pasz

Fungi Grzyby	Prevalence Występowanie
Indoor air samples / Próbki powietrza w pomieszczeniach [CFU×m ⁻³ (%)]	
<i>Aspergillus fumigatus</i> complex / kompleks <i>Aspergillus fumigatus</i>	168 (46.7)
<i>Penicillium</i> sp.	96 (26.7)
<i>Cladosporium</i> sp.	38 (10.6)
others / inne	62 (16.1)
Indoor surfaces samples / Próbki z powierzchni w pomieszczeniach [CFU×m ⁻² (%)]	
<i>Penicillium</i> sp.	150 000 (31.3)
<i>Aspergillus flavus</i> complex / kompleks <i>Aspergillus flavus</i>	60 000 (12.5)
<i>Aspergillus fumigatus</i> complex / kompleks <i>Aspergillus fumigatus</i>	30 000 (6.3)
<i>Aspergillus niger</i> complex / kompleks <i>Aspergillus niger</i>	30 000 (6.3)
others / inne	480 000 (43.8)

CFU – colony-forming unit / jednostka tworząca kolonię.

Fungal identification through molecular methods

We checked for the presence of toxic strains from *A. flavus* complex, *A. fumigatus* complex and *S. chartarum* by real-time PCR. Out of the three selected targeting, we've managed to detect the DNA only from *A. fumigatus* complex and more specifically in the sampling site D, which corresponds to the control laboratory. This was amplified at the cycle threshold (CT) of 36.80, which corresponds to a late amplification, indicating that it is not a highly abundant DNA. We did not manage to detect either *A. flavus* or *S. chartarum*.

Particles results

In general, our results showed that 0.3 µm particles had higher counts and that 10 µm particles obtained the lowest counts (Figure 5). Additionally, significant differences between all the particle sizes were detected ($p < 0.05$), except for the comparisons between 0.5 µm and 1 µm, 1 µm and 2.5 µm, 5 µm and 10 µm ($p > 0.05$).

The PNC results showed, in the particular case of 0.3 µm particle size, that the pre-mixing – control was the task with higher counts showing statistical difference from other tasks ($p = 0.002$), the tasks of trucks loading and granulator were the ones with lower counts (Figure 6).

As for the PMC, and in the case of PM₁₀ results, the pre-mixing (control room) had the highest results together with the mechanical filling (machine operating).

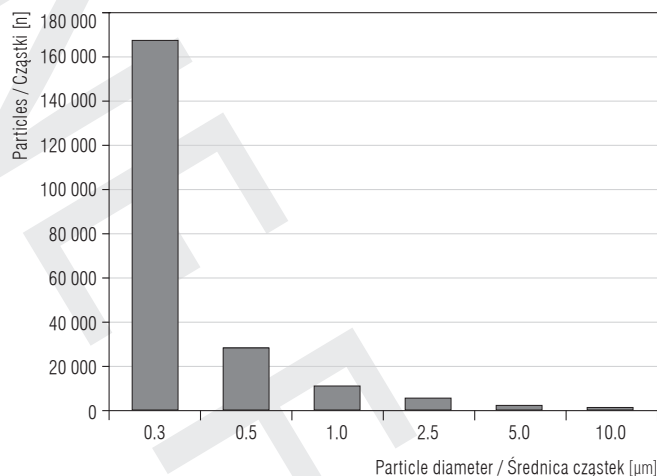
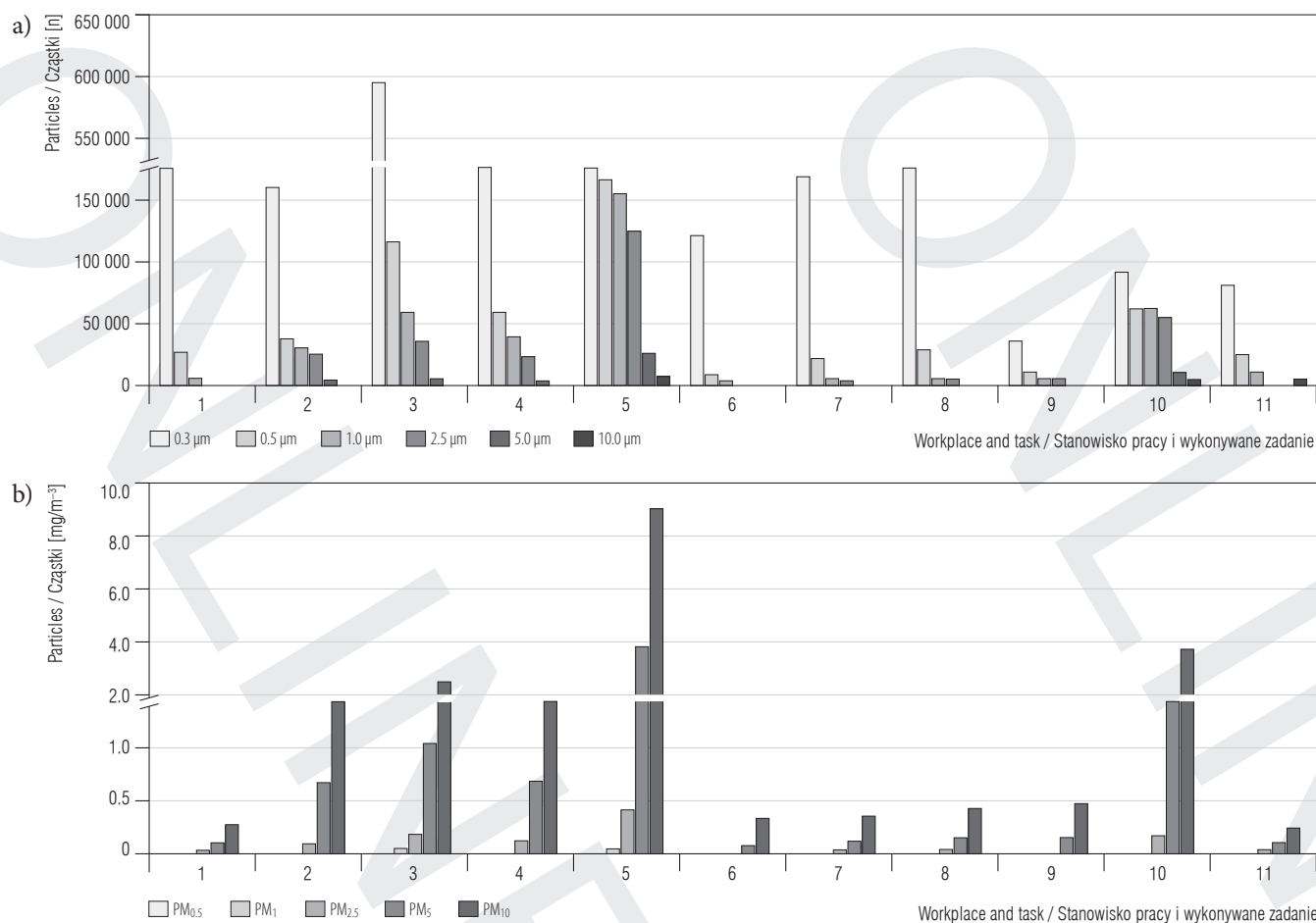


Fig. 5. Median of particle counts in the studied animal feed industry plant depending on the diameter of particles
Ryc. 5. Mediana liczby cząstek w badanej wytwórni pasz w zależności od średnicy cząstek

As far as particles with lower masses are concerned, the tasks of granulation and dumping the truck with soybean were the ones with higher counts (Figure 6).

DISCUSSION

Occupational exposure is rarely associated with one single factor. It is frequently a combination of several risk factors [15]. In this paper we intended not only to characterize fungal occupational exposure but also to understand exposure to other risk factors that may en-



1 – bagging – machine operating / pakowanie – obsługa maszyn(y), 2 – pre-mixing – manual adding of antibiotics / mieszanie wstępne – ręczne dodawanie antybiotyków, 3 – pre-mixing – control room / mieszanie wstępne – sterownia, 4 – manual filling the silos / ręczne napełnianie zasobników, 5 – mechanical filling the silos – machine operating / mechaniczne napełnianie zasobników – obsługa maszyn(y), 6 – reception of raw materials – dump truck with soybean / odbiór surowców – opróżnianie wózka z soją, 7 – reception of raw materials – control room / odbiór surowców – sterownia, 8 – laboratory control – soybean grinding / kontrola laboratoryjna – mielenie soi, 9 – granulator – controlling the process / nadzorowanie procesu, 10 – manual mixing – control room / mieszanie ręczne – sterownia, 11 – trucks loading – loading process / ładowanie na ciężarówki – ładowanie.
PM – particulate matter / pył zawieszony.

Fig. 6. a) Particle number concentration (PNC) and b) particle mass concentration (PMC) in the studied animal feed industry plant by workplace and task

Ryc. 6. a) Stężenie liczbowe cząstek (PNC) i b) stężenie masowe cząstek (PMC) w badanej wytwórni pasz w zależności od stanowiska pracy i wykonywanego zadania

hance fungal exposure, namely aerosolized particles. In the case of fungal assessment, we use the worst case approach that allows the comparison with limit values applying a low cost exposure assessment permitting to prioritize intervention in the assessed setting aiming at the implementation of safety measures [8].

The results of the quantitative analysis should be based on the limits set by respective countries, due to the lack of pan-European standards [16]. However, since in Portugal there are not any guidelines to be used as terms of reference, we decided to apply the guideline proposed by the World Health Organization (WHO) (maximum value of $150 \text{ CFU} \times \text{m}^{-3}$) [14], since it is the most strict one for occupational assessment purposes. Although none

of the sampling sites surpass the proposed guideline, six out of the seven collected air samples presented a higher indoor fungal load when compared with the outdoor sample, which could mean the existence of indoor fungal contamination sources [17]. Results were also compared with others obtained in the same work environment and all the sampling sites presented less fungal load [1,2].

The next stage of risk control in this setting was to verify the existence of indicator species/strains that are representative of harmful fungal contamination in this kind of industry. Verification was carried out applying conventional and molecular methods [18].

As far as the results obtained through conventional methods are concerned, the presence of the most prev-

alent fungi, *A. fumigatus* complex, requires implementation of corrective measures according to the American Industrial Hygiene Association (AIHA 1996) in the Field Guide for the Determination of Biological Contaminants in Environmental Samples [19]. Among *Penicillium* genus, the second one was most frequently found, so we should consider the possibility that several toxic species that are common in feed industry may be present, due to the cereals use as raw materials, such as *P. verrucosum* and *P. griseofulvum* [20]. Other species also affecting the raw materials in this occupational setting, such as the ones belonging to *Aspergillus ochraceus* complex [21] and *Fusarium graminearum* [22] are also known as potentially toxic. Further studies will address the presence of these fungal species in this setting.

Besides air sampling, surfaces samples are crucial to obtain a more complete characterization and assessment of fungal contamination, and should be applied to identify contamination sources [23]. In a previous study, when applied to several occupational settings, the surfaces sampling allowed to realize that there was a high percentage of sampling sites presenting different species in surfaces when compared to the ones identified in the air (64.2%) [24], corroborating the importance of surface analysis as complement to the mycological air characterization. This data confirmed the need to collect both samples to ensure a complete fungal contamination assessment. In our study, seven different species/genera were identified exclusively in surface samples. The second species-complex most frequently found in surface samples was *A. flavus*, known by its aflatoxins production, and recognized as the major aflatoxin B1 (AFB1) producer, a potent carcinogenic agent.

Among the species identified in surfaces, and besides the ones belonging to the *A. flavus* complex, we must point out others with toxic potential namely: *A. ochraceus* complex, *Aspergillus niger* complex and *Fusarium verticilloides* that had been detected in previous studies [25].

In this study we used molecular methods to complement the conventional detection of specific fungal species. Unexpectedly, the most abundant species detected in air by conventional methods, *A. fumigatus* complex, was only detected in one of the samples by molecular methods. Curiously, this species was not present at high levels in this sample when detected by conventional methods. There are several reasons that might serve the basis of these results. In fact the PCR assays for microbial detection may yield false negatives due to several factors, including an inadequate removal of PCR inhibitors in

the sample; ineffective release of microbial DNA content from the cells or poor DNA recovery after extraction and purification steps [26]. In our particular study, one of the inhibition sources could be the presence of particles in the air, as already observed in previous studies [27].

In our study we also accessed the presence of toxic strains by molecular methods. The use of these methods is essential to distinguish strains that produce mycotoxins from those that do not produce them [18]. In our conditions we were not able to amplify the toxic strains from *A. flavus* complex, despite the presence of the non-toxic strains, namely that of *A. flavus* complex as detected by conventional methods. Further studies should be performed to confirm this. To the extent of what refers to *S. chartarum*, its detection might be compromised as there is evidence suggesting that their spores, unlike those from other species, do not remain in the air for a long time [28]. Thus the fact that only air samples were analyzed by molecular methods might account for the lack of detection of this species.

It is noteworthy that only one sample from each plant was taken, and variations in fungal contamination are expected [29]. Nevertheless, the prevalent genera found were common in several other studies applied in settings with grain handling [4].

Exposure assessment to particles was performed based on tasks, allowing a more detailed and comprehensive understanding of exposure. This approach permits the detection of differences between tasks, allowing to identify exposure determinants, engineering controls and other worker protection strategies and focused on those particular tasks and conditions that generate the highest exposures [30]. Knowledge about determinants of exposure is vital for identifying exposure prevention and controlling strategies [31]. This detailed information may also give support in explaining to workers why, when and how they have to change their work practices [32].

Additionally, task-based exposure assessment may provide a better evaluation of exposure variability, instead of assessing personal exposures using continuous 8-hour time weighted average measurements [33] and it also has a lower probability of covering exposure peaks when comparing with the time weighted average exposure over long processes or over work shifts [33]. Moreover, by using direct-reading instruments to perform task-based exposure assessment it is possible to identify more easily the tasks that represent the highest exposure risk and the ones that should have priority for applying control measures [34].

Two exposure metrics were used simultaneously: PNC and PMC. The use of these two metrics simultaneously in occupational exposure assessments has only been done recently and in a small number of studies [35] but it seems of extreme importance because it gives more complete and accurate data to perform risk assessment [36].

Particularly, the PNC data was collected because it might be more closely correlated with the adverse health effects caused by particles. In fact, some studies support that the PNC and size distribution data may have advantages over only the PMC data in the assessment of the health effects caused by airborne particles [37,38].

Although in different contexts, other authors have previously recommended the simultaneous measure of these two exposure metrics to ensure adequate risk assessment [39].

Knowing when and where the exposure to smaller particles occurs with higher intensity gives information regarding where the exposure may have more dramatic health effects. Indeed these particles may reach deeper in the respiratory system indicating that, besides local effects, also systemic effects may be expected [39]. In the studied feed industry it was possible to conclude that particles were present in higher counts at the smaller sizes (Figure 5) and the control room of pre-mixing was identified as the more problematic (Figure 6), since it possessed a higher value of counts for the smaller size of particles. Particularly relevant is the fact that workers from these workplaces do not consider it relevant to use respiratory protection devices which are usually located in a specific room of the process control, away from where workers are. Therefore, this study does not intend to compare results with an occupational exposure limit but to identify the most critical task and the one that should be considered to be a priority to invest in prevention and protection devices.

Besides the PMC and PNC there are other aspects that must be addressed when considering the particles-related health effects: the chemical proprieties [40] that in feed industry vary depending on the raw materials (soya, wheat, corn and others) and the additives (antibiotics and nutrients) that are being handled and applied for each feed product. Additionally, the fact that particles may act as a carrier and a source of nutrients for fungi (such as *Aspergillus*, *Penicillium* and *Mucor* genus) [41] and bacteria [3] also have to be considered. To this end we have to consider that larger particles fractions, such as fungi, are the most relevant ones for allergen exposure [42] and this claims attention to the

fact that even particles from 5 μm and 10 μm sizes are relevant since they are the best carriers for fungi and other allergens present in the workplace.

Particles are also rich in endotoxins from the cell wall of gram-negative bacteria and mycotoxins produced by several fungi [43]. These biologically active compounds adhered to particles, along with coexisting toxicant gases that may be carcinogenic, allergic and irritant are of concern regarding exposure to mixtures and possible additive and synergistic health effects [44].

Unfortunately, all these aspects are not considered for the occupational exposure limits definition and may result in high risk even when exposure to particles and fungi is below the limits defined and recommend for occupational exposures.

CONCLUSIONS

Considering the fungal burden and also the results from particles exposure assessment we think that appropriate safety measures must be taken and may be based on the detail data obtained. These include following preventive measures, such as disinfection and dust reduction through ventilation equipment and also wearing personal protection devices such as filtration half masks and gloves.

Additionally, future research work must be developed to assess the real scenario regarding fungal exposure, maintaining the use of conventional methods and targeting toxic strains from the most prevalent species, and also from others that are common in raw material and feed. This will contribute to the knowledge of co-exposure health effects and, in this way, contribute to the definition of more realistic occupational exposure limits.

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REFERENCES

1. Straumfors A, Heldal KK, Wouters IM, Eduard W. Work tasks as determinants of grain dust and microbial exposure in the Norwegian grain and compound feed industry. *Ann Occup Hyg.* 2015;59(6):724–36, <http://dx.doi.org/10.1093/annhyg/mev012>.

2. Spaan S, Wouters IM, Oosting I, Doekes G, Heederik D. Exposure to inhalable dust and endotoxins in agricultural industries. *J Environ Monit*. 2006;8:63–72, <http://dx.doi.org/10.1039/B509838F>.
3. Halstensen AS, Heldal KK, Wouters IM, Skogstad M, Ellingsen DG, Eduard W. Exposure to grain dust and microbial components in the Norwegian grain and compound feed industry. *Ann Occup Hyg*. 2013;57:1105–14, <http://dx.doi.org/10.1093/annhyg/met036>.
4. Swan JRM, Crook B. Airborne microorganisms associated with grain handling. *Ann Agric Environ Med*. 1998;5:7–15.
5. Young SH, Castranova V. Animal model of (1 \rightarrow 3)- β -glucan-induced pulmonary inflammation in rats. In: Young SH and Castranova V, editors. *Toxicology of 1 \rightarrow 3- β -glucans: Glucans as a marker for fungal exposure*. London: Taylor and Francis; 2005. p. 65–95, <http://dx.doi.org/10.1201/9780203020814.ch4>.
6. Halstensen AS, Nordby KC, Wouters IM, Eduard W. Determinants of microbial exposure in grain farming. *Ann Occup Hyg*. 2007;51:581–92, <http://dx.doi.org/10.1093/annhyg/mem038>.
7. Viegas S, Veiga L, Figueredo P, Almeida A, Carolino E, Sabino R, et al. Occupational exposure to aflatoxin B₁ in swine production and possible contamination sources. *J Toxicol Environ Health A*. 2013;76:944–51, <http://dx.doi.org/10.1080/15287394.2013.826569>.
8. EN 14042:2003. *Workplace atmospheres – Guide for the application and use of procedures for the assessment of exposure to chemical and biological agents*. Brussels: European Committee for Standardization; 2003.
9. ISO 18593:2004. *Microbiology of food and animal feeding stuffs – Horizontal methods for sampling techniques from surfaces using contact plates and swabs*. International Organization for Standardization; 2004.
10. Hoog DC, Guarro J, Gené G, Figueras M. *Atlas of Clinical Fungi*. 2nd edition. Utrecht: Centraalbureau voor Schimmelcultures; 2002.
11. Mayer Z, Bagnara A, Färber P, Geisen R. Quantification of the copy number of *nor-1*, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. *Int J Food Microbiol*. 2003;82:143–51, [http://dx.doi.org/10.1016/S0168-1605\(02\)00250-7](http://dx.doi.org/10.1016/S0168-1605(02)00250-7).
12. Cruz-Perez P, Buttner MP, Stetzenbach LD. Detection and quantitation of *Aspergillus fumigatus* in pure culture using polymerase chain reaction. *Mol Cell Probes*. 2001;15:81–8, <http://dx.doi.org/10.1006/mcpr.2000.0343>.
13. Cruz-Perez P, Buttner MP, Stetzenbach LD. Specific detection of *Stachybotrys chartarum* in pure culture using quantitative polymerase chain reaction. *Mol Cell Probes*. 2001;15:129–38, <http://dx.doi.org/10.1006/mcpr.2001.0347>.
14. World Health Organization. *Guidelines for indoor air quality. Dampness and mould*. Geneva: The Organization; 2009.
15. Libner L, Kuhl K, Kauppinen T, Uuksulainen S; European Agency for Safety and Health at Work. *Exposure to carcinogens and work-related cancer: A review of assessment methods*. European Risk Observatory Report. Luxembourg: Publications Office of the European Union; 2014.
16. Gutarowska B, Skóra J, Stępień Ł, Szponar B, Otlewska A, Pielech-Przybylska K. Assessment of microbial contamination within working environments of different types of composting plants. *J Air Waste Manag Assoc*. 2015;65:466–78, <http://dx.doi.org/10.1080/10962247.2014.960954>.
17. Wouters I, Spaan S, Douwes J, Doekes G, Heederik D. Overview of personal occupational exposure levels to inhalable dust, endotoxin, β (1 \rightarrow 3)-glucan and fungal extracellular polysaccharides in the waste management chain. *Ann Occup Hyg*. 2006;50:39–53, <http://dx.doi.org/10.1093/annhyg/mei047>.
18. Malta-Vacas J, Viegas S, Sabino R, Viegas C. Fungal and microbial volatile organic compounds exposure assessment in a waste sorting plant. *J Toxicol Environ Health A*. 2012;75:1410–7, <http://dx.doi.org/10.1080/15287394.2012.721175>.
19. Hung LL, Miller JD, Dillon KH, editors. *Field guide for the determination of biological contaminants in environmental samples*. 2nd ed. Fairfax (VA): American Industrial Hygiene Association; 2005.
20. Soares C, Abrunhosa L, Venâncio A. [Fungi producers of mycotoxin]. *Microbiol Port Soc Microbiol Mag*. 2013 [cited 2015 Jun 15]. Available from: <http://magazinespm.wordpress.com/2013/07/30/fungos-produtores-de-micotoxinas>. Portuguese.
21. Halstensen AS, Nordby KC, Elen O, Eduard W. Ochratoxin A in grain dust – Estimated exposure and relations to agricultural practices in grain production. *Ann Agric Environ Med*. 2004;11:245–54.
22. Milani JM. Ecological conditions affecting mycotoxin production in cereals: A review. *Vet Med*. 2013;58:405–11.
23. Viegas C, Faria T, Meneses M, Carolino E, Viegas S, Gomes AQ, et al. Analysis of surfaces for the characterization of fungal burden – Does it matter? *Int J Occup Med Environ Health*. Forthcoming 2016.
24. Stetzenbach L, Buttner M, Cruz P. Detection and enumeration of airborne biocontaminants. *Curr Opin Biotechnol*. 2004;15:170–4, <http://dx.doi.org/10.1016/j.copbio.2004.04.009>.

25. Smith JE. Aflatoxins. In: D'Mello JPF, editor. Handbook of plant and fungal toxicants. New York: CRC press, Inc.; 1997. p. 269–85.
26. Yang S, Rothman RE. PCR-based diagnostics for infectious diseases: Uses, limitations, and future applications in acute-care settings. *Lancet Infect Dis*. 2004;4:337–48, [http://dx.doi.org/10.1016/S1473-3099\(04\)01044-8](http://dx.doi.org/10.1016/S1473-3099(04)01044-8).
27. McDevitt JJ, Lees PSJ, Merz WG, Schwab KJ. Inhibition of quantitative PCR analysis of fungal conidia associated with indoor air particulate matter. *Aerobiologia*. 2007;23: 35–45, <http://dx.doi.org/10.1007/s10453-006-9047-6>.
28. Boutin-Forzano S, Charpin-Kadouch C, Chabbi S, Bennedjai N, Dumon H, Charpin D. Wall relative humidity: A simple and reliable index for predicting *Stachybotrys chartarum* infestation in dwellings. *Indoor Air*. 2004;14:196–9, <http://dx.doi.org/10.1111/j.1600-0668.2004.00233.x>.
29. Jantunen E, Ruutu P, Niskanen L, Volin, L, Parkkali T, Koukila-Kähkölä P, et al. Incidence and risk factors for invasive fungal infections in allogeneic BMT recipients. *Bone Marrow Transplant*. 1997;19:801–8, <http://dx.doi.org/10.1038/sj.bmt.1700737>.
30. Goldberg M, Levin SM, Doucette JT, Griffin G. A task-based approach to assessing lead exposure among iron workers engaged in bridge rehabilitation. *Am J Ind Med*. 1997;31:310–8, [http://dx.doi.org/10.1002/\(SICI\)1097-0274\(199703\)31:3<310::AID-AJIM7>3.0.CO;2-0](http://dx.doi.org/10.1002/(SICI)1097-0274(199703)31:3<310::AID-AJIM7>3.0.CO;2-0).
31. Burdorf A. Identification of determinants of exposure: Consequences for measurement and control strategies. *Occup Environ Med*. 2005;62:344–50, <http://dx.doi.org/10.1136/oem.2004.015198>.
32. Meijster T, Tielemans E, Schinkel J, Heederik D. Evaluation of peak exposures in the dutch flour processing industry: Implications for intervention strategies. *Ann Occup Hyg*. 2008;52:587–96, <http://dx.doi.org/10.1093/annhyg/men049>.
33. Bello A, Quinn MM, Perry MJ, Milton DK. Quantitative assessment of airborne exposures generated during common cleaning tasks: A pilot study. *Environ Health*. 2010;9:76, <http://dx.doi.org/10.1186/1476-069X-9-76>.
34. Verma DK, Kurtz LA, Sahai D, Finkelstein MM. Current chemical exposures among Ontario construction workers. *J Occup Environ Hyg*. 2003;18:1031–47, <http://dx.doi.org/10.1080/714044193>.
35. Viegas C, Malta-Vacas J, Sabino R, Viegas S, Veríssimo C. Accessing indoor fungal contamination using conventional and molecular methods in Portuguese poultry. *Environ Monit Assess*. 2014;186:1951–9, <http://dx.doi.org/10.1007/s10661-013-3509-4>.
36. Heitbrink WA, Evans DE, Ku BK, Maynard AD, Slavin TJ, Peters TM. Relationships among particle number, surface area, and respirable mass concentrations in automotive engine manufacturing. *J Occup Environ Hyg*. 2009;6: 19–31, <http://dx.doi.org/10.1080/15459620802530096>.
37. Weijers EP, Khlystov AY, Kos GPA, Erisman JW. Variability of particulate matter concentrations along roads and motorways determined by a moving measurement unit. *Atmos Environ*. 2004;38:2993–3002, <http://dx.doi.org/10.1016/j.atmosenv.2004.02.045>.
38. Wichmann HE, Spix C, Tuch T, Wolke G, Peters A, Heinrich J, et al. Daily mortality and fine and ultrafine particles in Erfurt, Germany. Part I: Role of particle number and particle mass. *Res Rep Health Eff Inst*. 2000;98:5–86.
39. Nygaard UC, Samuelson M, Aase A, Martinus L. The capacity of particles to increase allergic sensitization is predicted by particle number and surface area, not by particle mass. *Toxicol Sci*. 2004;82:515–24, <http://dx.doi.org/10.1093/toxsci/kfh287>.
40. Almeida-Silva M, Almeida SM, Wolterbeek HT. Multi-elemental characterization of indoor aerosols in elderly care centers. *J Radioanal Nucl Chem*. 2014;300:679–84, <http://dx.doi.org/10.1007/s10967-014-2997-1>.
41. Viegas S, Veiga L, Malta-Vacas J, Sabino R, Figueiredo P, Almeida A, et al. Occupational exposure to aflatoxin (AFB₁) in poultry production. *J Toxicol Environ Health A*. 2012;75:1330–40, <http://dx.doi.org/10.1080/15287394.2012.721164>.
42. Raulf M, Buters J, Chapman M, Cecchi L, de Blay F, Doekes G, et al. Monitoring of occupational and environmental aeroallergens – EAACI Position Paper. Concerted action of the EAACI IG Occupational Allergy and Aerobiology & Air Pollution. *Allergy*. 2014;69:1280–99, <http://dx.doi.org/10.1111/all.12456>.
43. Allermann L, Poulsen OM. Inflammatory potential of dust from waste handling facilities measured as IL-8 secretion from lung epithelial cells *in vitro*. *Ann Occup Hyg*. 2000;44:259–69, [http://dx.doi.org/10.1016/S0003-4878\(99\)00100-3](http://dx.doi.org/10.1016/S0003-4878(99)00100-3).
44. Von Essen SG, Donham KJ. Illness and injury in animal confinement workers. *Occup Med*. 1999;14:337–50.