Safety and Health at Work 13 (2022) 9–16

Contents lists available at ScienceDirect

Safety and Health at Work

journal homepage: www.e-shaw.net

Original article

Bioaerosol Exposure and *in vitro* Activation of Toll-like Receptors in a Norwegian Waste Sorting Plant

Elke Eriksen*, Pål Graff, Ine Pedersen, Anne Straumfors, Anani K. Afanou

STAMI, National Institute of Occupational Health, Gydas Vei 8, 0363 Oslo, Norway

ARTICLE INFO

Article history:
Received 31 May 2021
Received in revised form
22 September 2021
Accepted 23 September 2021
Available online 29 September 2021

Keywords:
Occupational exposure
Waste Management
Dust
Bacteria
Fungi
Toll-Like Receptors

ABSTRACT

Background: The global shift toward greener societies demands new technologies and work operations in the waste-management sector. However, progressive industrial methods do not necessarily consider workers' health. This study characterized workers' exposure to bioaerosols and investigated the bioaerosols' potential to engage the immune system *in vitro*.

Methods: Full shift personal aerosol sampling was conducted over three consecutive days. Dust load was analyzed by gravimetry, fungal and actinobacterial spores were analyzed by scanning electron microscopy, and endotoxin by limulus amebocyte lysate (LAL) assay. *In vitro* exposure of HEK cells to airborne dust samples was used to investigate the potential of inducing an inflammatory reaction.

Results: The total dust exposure level exceeded the recommended occupational exposure limit (OEL) of 5.0 mg/m^3 in 3 out of 15 samples. The inhalable endotoxin level exceeded the recommended exposure level by a 7-fold, whereas the fungal spore level exceeded the recommended exposure level by an 11-fold. Actinobacterial spores were identified in 8 out of 14 samples. *In vitro* experiments revealed significant TLR2 activation in 9 out of 14 samples vs. significant TLR4 activation in all samples.

Conclusion: The present study showed that the dust samples contained potentially health-impairing endotoxin, fungi, and actinobacterial levels. Furthermore, the sampled dust contained microbial components capable of inducing TLR activation and thus have the potential to evoke an inflammatory response in exposed individuals.

1. Introduction

The ongoing global shift toward greener societies, aiming for the sustainable use of natural resources, demands climate and environmentally friendly restructuring in various fields. The waste-management sector is particularly important in this respect, attempting to meet sustainability goals by the sorting and recycling of materials from the waste back into the value chain, implementing new technologies, and adapting work operations. However, progressive industrial methods, although beneficial from an environmental point of view, do not necessarily favor occupational health for workers.

Waste management plays a central part in the global shift toward circularity and sustainability. To increase the efficiency of waste sorting, state-of-the-art waste sorting facilities utilize optical sensor recognition in combination with ballistic- and selective air-blast separation to sort residual waste in unlike fractions, such as plastics, metal, and paper. Despite the fully automated sorting processes, manual labor is paramount in regards to cleaning and maintenance. During the sorting process with air-pressure guns, dust is dispersed in the work atmosphere. This airborne dust may contain various hazardous components, such as heavy metals, bioaerosols, volatile organic compounds (VOC), and other particles [1–3]. Previous exposure studies of domestic waste handling have indicated that the inhalation of bioaerosols containing microorganisms, such as bacteria and fungi, and corresponding toxins, have been associated with adverse health effects [4], particularly symptoms from the respiratory system, such as asthma [5–7].

Exposure to microorganisms engages the human immune system by activating toll-like receptors (TLR) that initiate

Elke Eriksen: https://orcid.org/0000-0002-9868-1347
* Corresponding author. STAMI, National Institute of Occupational Health, Gydas Vei 8, 0363 Oslo, Norway.
E-mail addresses: Elke.Eriksen@stami.no (E. Eriksen), Pal.Graff@stami.no (P. Graff), Ine.Pedersen@stami.no (I. Pedersen), Anne.Straumfors@stami.no (A. Straumfors), Anani.Afanou@stami.no (A.K. Afanou).

2093-7911/$ – see front matter
https://doi.org/10.1016/j.shaw.2021.09.002

inflammatory responses. Such exposure responses can be investigated *in vitro* in reporter cell systems. TLRs, which can be found on immune cells, e.g. peripheral mononuclear leukocytes and T cells, are integral parts of the innate immune system and play a major role in pathogen recognition [8,9]. So far, only a few studies have attempted to investigate the exposure to bioaerosols and biogenic gasses, as well as the immunological properties during the sorting of residual waste from private homes [10–12].

This study characterized the workers' exposure to dust, endotoxins, fungal, and actinobacterial spores, as well as TLR2 and TLR4 activation properties.

2. Methods

2.1. Study site and population

This study was conducted in February/March 2017 at a fully automated waste sorting plant that handles domestic waste from 95,000 Norwegian homes. Maintenance and cleaning of sorting machines are executed on a daily basis on a two-shift schedule, five days a week. The investigated work operations were categorized as follows: (a) Cleaning of sorting machines, conveyor belts, and the ground floor level using air-pressure blowers and brooms, (b) maintenance, (c) driving the excavator in the waste reception hall or the truck in the storage hall, (d) manual removal of large items from the conveyor belt that connects the shredder in the waste reception hall to the automated sorting lines, and (e) miscellaneous, e.g. supervision from the control room, office-related work, and breaks.

Eight males (out of a total of 9 workers in the sorting plant) participated over a period of three consecutive days, three persons three days, one person two days, and four persons one day, resulting in a total of 30 samples, 15 for total dust and 15 for endotoxin.

2.2. Sampling methods

Each participant was equipped with two personal sampling devices that were placed in the breathing zone. Total dust, fungal, and actinobacterial spores were sampled on 25 mm hydrophilic polycarbonate membrane filters with a pore size of 0.8 μm (Merck KGaA, Darmstadt, Germany) that were mounted in antistatic polypropylene filter cassettes (Pall Laboratories, Port Washington, NY, US).

Endotoxins were sampled on 25 mm glass-fiber filters (1 μm, GF/A, Whatman, UK) mounted in PAS-6 filter cassettes [13]. All filter cassettes were attached to air pumps (GS5200, GSA Messgerätebau GmbH, Ratingen, Germany) that were carried in a backpack and operated at an average flow rate of 2.0 (±10%) liters per minute. Airflow was measured prior to and after sampling, using a Defender 510 (TPF Control B.V., The Netherlands). The sampling time varied between individuals from 1.8 to 8.9 hours, with a mean sampling time of 6 hours per shift.

2.3. Gravimetric analyses

The total dust load per air filter was determined by gravimetry using a microbalance (Sartorius AG, MC210, Göttingen, Germany). Filters were weighed in a climate-controlled weigh-room at standard laboratory conditions with a mean temperature of 20 ± 1°C and relative humidity of 40 ± 2% prior to, and after exposure. An acclimation period of at least 48 hours preceded the gravimetric analyses in all cases. Unexposed blank field filters were included for each 10^{th} field sample. The detection limit was estimated as three times the standard deviation of blank filters at 0.02 mg/filter.

2.4. Endotoxin analysis

Endotoxin-loaded filters were washed in 5 ml endotoxin-free water with 0.05% Tween-20 by orbital shaking for 1 hour. After the extraction step, the suspensions were centrifuged at 1,000 g for 15 minutes. The supernatant was aliquoted and stored at -20°C until analyses. Prior to analyses, the suspensions were diluted 30 times. Subsequently, the Limulus amebocyte (LAL) kinetic-QCL assay was applied to all samples according to the manufacturer's recommendations (Lonza Ltd., Basel, CH). Parallel controls of spiked (50 EU/ml) samples and blanks were included. The final endotoxin concentration was estimated by spectrophotometry (BioTek Instruments Inc., VT, USA) in reference to a five-point standard curve with concentrations ranging from 0.005 to 50 EU/ml. The detection limit for endotoxin was 0.75 EU/filter.

2.5. Dust suspension for microscopic analysis and cell assay

The filters were washed prior to analyses as 14 out of 15 PC filters were overloaded and could not directly be analyzed with a field emission scanning electron microscope (FESEM). The filters were placed in 15 mL centrifuge tubes and submerged in a 5 ml dispersion medium (PBS+0.1%BSA). Subsequently, the filters were sonicated for 5 min and agitated by orbital shaking for 60 min. The dust suspensions were transferred to new tubes, and the orbital-agitation process was repeated for 25 min using a 2 ml dispersion medium. After pooling the dust suspensions, 1 ml aliquots were prepared in sterile, endotoxin-free cryo-tubes and kept at -20 ℃ until FESEM analysis, DNA extraction, and *in vitro* cell assays.

2.6. FESEM analysis of spores from fungi and actinobacteria

Two hundred microliters from each aliquot were filtrated onto a 25 mm polycarbonate filter (pore size: 0.45 μm, Merck KGaA, Darmstadt, Germany). Subsequently, the filters were air-dried under sterile conditions and then mounted onto 25 mm diameter aluminum pin stubs (Agar Scientific Ltd., Stansted Essex, United Kingdom) using double-sided carbon adhesive discs. The filter samples were coated with 5-6 nm platinum in a Cressington 200HR Sputter Coater (Cressington Scientific Instruments Ltd., Watford, UK). Spores of bacterial and fungal origin were identified based on morphological characteristics. Spores were counted in 100 randomly selected imaged fields at ×3,000 magnification and reported as the number of spores m^{-3} air. The number of spores was estimated by extrapolating the counts in the selected fields to the whole filter using the following formula:

$$\text{Spores per } m^3 = \frac{n \times \text{filtration filter area } (\mu m^2) \times \text{Dilution factor}}{\text{k} \times \text{FESEM image field area } (\mu m^2) \times \text{sampled air volume } (m^3)} \quad (1)$$

where
n = number of particles counted on the filter; $k = 100$; filtration filter area of 25 mm filter = 227×10^6 μm^2; FESEM Image field area at 3,000 = 1064 μm^2. Dilution factor: 35 [14]. The lowest detectable number of particles (LOD) with FESEM was 3.73×10^4 m^{-3} at a total air sampled volume of 1 m^3.

2.7. DNA extraction and ddPCR

DNA extraction by cell lysis and spin-column separation using a Qiagen isolation kit (DNeasy Plant Mini Kit, Qiagen GmbH, Hilden, Germany) was conducted on 14 field-sample extracts as previously

described by Straumfors et al. [15]. The DNA yield was evaluated with a QBit 4 Fluorometer (Thermo Scientific, DE, USA). Extracted template DNA varied between 0.023 and 0.26 ng/µl.

For fungal amplification, 20 µl of reaction mixtures containing 5.2 µl purified water, 10 µl EvaGreen SuperMix, 0.4 µl of each the forward (FF390) and reverse (FR1) primer (10 µm) [16], and 4 µl genomic template DNA were used, partitioned into droplets (Bio-Rad QX200 droplet generator, Bio-Rad Laboratories Inc., CA, USA), and then thermocycled at 95°C for 10 minutes, 40 cycles at 95°C for 30 seconds, 50°C for 30 seconds and 60°C for 1 minute, ending with a final stabilization step at 4°C for 5 minutes.

For bacterial amplification, 20 µl of reaction mixtures containing 5.6 µl purified water, 10 µl EvaGreen Supermix, 0.2 µl of each, the forward (341F) and reverse primer (R806) [17] and 4 µl template DNA were used, partitioned into droplets, and thermocycled for 5 minutes at 95°C, 40 cycles alternating between 5 minutes at 4°C and 5 minutes at 90°C. Amplification was followed by two 5-minute stabilizing steps, at 4°C and 90°C, respectively. Quality scores of PCR products were measured with a Bio-Rad QX200 droplet reader. For controlling uncertainty, the ddPCR threshold for recognition of positive droplets was set at 9,000 for fungi and 11,500 for bacteria.

2.8. TLR2 and TLR4 activation assays

The experiments with TLR2 and TLR4 HEK reporter cells (Invivogen, France) were conducted in a 96 well plate following the procedure described by Brummelman et al. [18]. Briefly, resuspended cells in 180 µl of fresh cell medium (DMEM + 10% fetal bovine serum + diverse HEK Blue selection antibiotics) at 2.8×10^5 cells ml^{-1} density were exposed to 20 µl of the dust suspensions and incubated at 37°C for 22h. 20 µl of the cell supernatant were then transferred to new Nunc plates, and 180 µl Quanti-Blue (Invivogen, France) was added. After 180 min incubation, the color developed was measured spectrophotometrically at 649 nm using SpectraMax i3 with SoftMax Pro 6.3.1 software (Molecular Devices LLC, San Jose CA, US). Each sample was run in duplicate, and the whole experiment was repeated once. The data were reported as the arithmetic mean values of the four absorbance measurements. Negative controls (PBS + 0.1% BSA) and positive controls for TLR2 (LTA, lipoteichoic acid; InvivoGen, France) and TLR4 (LPS, lipopolysaccharide; InvivoGen, France) activation were included in the experiment.

2.9. Data analyses and statistics

All data analyses were performed in R/RStudio (R version 4.0.2) using the stats [19] and rstatix package [20] for statistical analyses and ggplot2 [21] for data visualization.

An initial investigation (Shapiro-Wilk's normality test) revealed that total dust, endotoxin, fungal spore, and actinobacterial levels, and the number of bacterial and fungal DNA copies were not normally distributed ($p < 0.01$). These data were log transformed prior to analyses. Multiple correlations of the exposure measures and cell-based immunological effects was done by pairwise correlation (Bonferroni corrected Pearson correlation). Actinobacterial levels below the detection levels were arbitrarily replaced by $x = (LOD/2^{0.5})$/air volume. Summary statistics were reported by workday and across workdays.

TLR activation was normally distributed. Significant activation was determined by comparing the TLR2 and TLR4 to the HEK null control cells using a pairwise t-test with a p-value below 0.05.

3. Results

3.1. Dust exposure

Substantial variation in total dust exposure between individuals and workdays (Fig. 1) was identified, and 3 out of 15 samples, with an estimated dust exposure of 5.62, 7.41, and 18.93 mg/m^3, respectively, were particularly high. Dust exposure was significantly higher on Monday (GM: 3.37 mg/m^3) compared to Tuesday and Wednesday with a GM of 2.67 and 0.89 mg/m^3, respectively. The geometric mean (GM) for total dust exposure across all samples and sampling days was 2.00 mg/m^3.

3.2. Endotoxin exposure

Endotoxin levels varied noticeably between samples, ranging from 223 to 5277 EU m^{-3} (Table 1). The average exposure values did not vary significantly between sampling days with a geometric mean of 864, 529, and 637 EU m^{-3} on Monday, Tuesday, and Wednesday, respectively. The estimated geometric mean across all three sampling days was 663 EU m^{-3}.

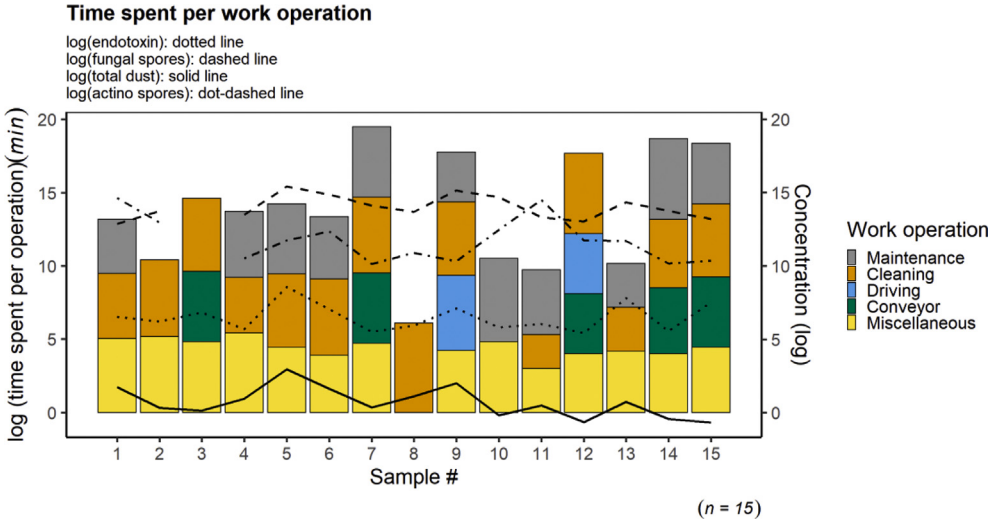


Fig. 1. Time per work operation. Monday: sample 1–5, Tuesday: sample 6–10, Wednesday: sample 11–15.

3.3. Fungal spore exposure

The number of identified fungal spores m^{-3} varied between 3.93×10^5 and 50.1×10^5 between samples (Table 1). Exposure levels were on average higher on Tuesday: 19.9×10^5 compared to the other sampling days (GM Monday 10.7×10^5, GM Wednesday: 7.56×10^5). However, the difference in fungal spore exposure between workdays was statistically not significant. The geometric mean for fungal spores m^{-3} was 11.8×10^5.

3.4. Actinobacterial spore exposure

Actinobacterial spores were identified in 8 out of 15 samples. The average exposure across all sampling days was 1.27×10^5 actinobacteria spores m^{-3} with highest exposure values measured on Monday (GM: 2.59×10^5) (Table 1). The difference between sampling days was statistically not significant.

3.5. Fungal and bacterial DNA copies

The number of bacterial and fungal DNA copies m^{-3} varied between the 14 samples. The GM for fungal DNA copies m^{-3} air was 2.45×10^5 across all samples. For bacterial DNA copies, the GM was 0.61×10^5 copies m^{-3} air (Table 1). The concentration of fungal DNA was higher than bacterial DNA in all but one sample (sample #9) (Fig. 2).

3.6. Activation of TLR2 and TLR4 receptors by dust samples

SEAP enzyme activity in TLR2 and TLR4 HEK reporter cells varied significantly between samples. The TLR2 reporter cells were activated in 9 out of 14 samples, whereas all samples provoked a stimulation of TLR4 reporter cells (Fig. 3). Activation patterns differed significantly between TLR2 and TLR4 reporter cells in 11 out of 14 samples. Three of the samples (#5, #9, and #10) activated both receptors with similar intensity. No significant activation of TLR2 and TLR4 could be seen with the washing buffer (PBS + 0.1% BSA) and the control media.

3.7. Relation between exposure parameters and the cell-based immunological effects

The total dust level was significantly correlated to the endotoxin and fungal spore level (56% and 57%, respectively, Table 2). SEAP activation of TLR2 and TLR4 receptor cells was significantly correlated to the total dust level (72%, and 72%, respectively). A significant correlation between TLR2 activation and fungal spore levels (69%), as well as fungal DNA copies (−61%), was observed. SEAP activation did not correlate significantly with any other exposure components.

4. Discussion

This study measured the occupational exposure to biological agents in a newly established waste sorting facility. The results showed that the Norwegian occupational exposure limit for total dust was exceeded in 20% of the samples, and the recommended exposure limits for endotoxins and fungal spores were exceeded by many folds in all samples. Total dust levels were highly correlated to endotoxin and fungal spore concentrations. Furthermore, high amounts of bacterial and fungal DNA were recovered from the sample material. *In vitro* reporter cell experiments indicated that

Table 1
Summary statistics for exposure parameters by workday and across workdays. Summary statistics based on absolute values. *p < 0.05 **p < 0.01 ***p < 0.001

		Monday (n = 5)	Tuesday (n = 5)	Wednesday (n = 5)	Across days (n = 15)
Dust (mg m^{-3})	Arithmetic mean (±SE)	5.93 * (±3.35)	3.55 (±1.21)	1.07 * (±0.32)	3.52 (±1.22)
	Geometric mean (GSD)	3.37 (3.15)	2.67 (2.45)	0.89 (1.94)	2.00 (2.84)
	Median (min, max)	2.56 (1.15, 18.93)	3.01 (0.84, 7.41)	0.64 (0.51, 2.05)	1.63 (0.51, 18.93)
Endotoxin (EU m^{-3})	Arithmetic mean (±SE)	1532 *** (±942)	660 (±212)	1024 (±456)	1072 (±343)
	Geometric mean (GSD)	864 (2.99)	529 (2.11)	637 (3.05)	663 (2.58)
	Median (min, max)	677 (297, 5277)	376 (242, 1206)	418 (223, 2421)	494 (223, 5277)
		(n = 4)	*(n = 5)*	*(n = 5)*	*(n = 14)*
Fungal spores (m^{-3})	Arithmetic mean (±SE)	7.6×10^5 *** ($\pm 10.8 \times 10^5$)	22.7×10^5 ($\pm 11.9 \times 10^5$)	8.54×10^5 ($\pm 50.9 \times 10^5$)	16.2×10^5 ($\pm 3.78 \times 10^5$)
	Geometric mean (GSD)	10.7×10^5 (2.97)	19.9×10^5 (1.81)	7.56×10^5 (1.7)	11.8×10^5 (2.25)
	Median (min, max)	8.23×10^5 (3.93×10^5, 50.1×10^5)	24.0×10^5 (8.68×10^5, 38.2×10^5)	6.22×10^5 (4.5×10^5, 17.0×10^5)	9.41×10^5 (3.93×10^5, 50.1×10^5)
Actinobacterial spores (m^{-3})	Arithmetic mean (±SE)	7.18×10^5 *** ($\pm 5.28 \times 10^5$)	1.21×10^5 ($\pm 5.20 \times 10^5$)	4.54×10^5 ($\pm 3.79 \times 10^5$)	$4.1 (\pm 1.97 \times 10^5)$
	Geometric mean (GSD)	2.59×10^5 (5.83)	0.76×10^5 (3.04)	1.20×10^5 (5.63)	1.27×10^5 (4.5)
	Median (min, max)	2.76×10^5 (<LOD, 22.8×10^5)	0.54×10^5 (<LOD, 2.6×10^5)	1.20×10^5 (<LOD, 19.7×10^5)	1.23×10^5 (<LOD, 22.8×10^5)
Fungal DNA copies (m^{-3})	Arithmetic mean (±SE)	4.71×10^5 ($\pm 0.49 \times 10^5$)	1.04×10^5 ($\pm 0.35 \times 10^5$)	7.07×10^5 ($\pm 3.25 \times 10^5$)	4.25×10^5 ($\pm 1.30 \times 10^5$)
	Geometric mean (GSD)	4.66×10^5 (0.12)	0.87×10^5 (1.87)	4.15×10^5 (3.51)	2.45×10^5 (3.08)
	Median (min, max)	4.82×10^5 (3.47×10^5, 5.85×10^5)	0.76×10^5 (0.47×10^5, 2.42×10^5)	4.42×10^5 (0.74×10^5, 18.7×10^5)	2.94×10^5 (0.47×10^5 $18. \times 10^5$)
Bacterial DNA copies (m^{-3})	Arithmetic mean (±SE)	1.28×10^5 ($\pm 0.66 \times 10^5$)	0.59×10^5 ($\pm 0.30 \times 10^5$)	1.56×10^5 (±0.87)	1.13×10^5 ($\pm 0.37 \times 10^5$)
	Geometric mean (GSD)	0.85×10^5 (2.79)	0.30×10^5 (3.89)	0.93×10^5 (2.99)	0.61×10^5 (3.37)
	Median (min, max)	0.78×10^5 (0.37×10^5, 3.17×10^5)	0.17×10^5 (0.062×10^5, 1.61×10^5)	0.89×10^5 (0.25×10^5, 5.01×10^5)	0.75×10^5 (0.062×10^5, 5.01×10^5)
TLR2 (absorbance)	Arithmetic mean (±SE)	1.15 (±0.31)	1.34 (±0.19)	0.50 (±0.12)	0.99 (±0.15)
	Geometric mean (GSD)	1.05 (1.63)	1.30 (1.32)	0.46 (1.57)	0.84 (1.83)
	Median (min, max)	0.94 (0.67, 2.06)	1.25 (1.02, 2.07)	0.37 (0.33, 0.97)	1.00 (0.33, 2.07)
TLR4 (absorbance)	Arithmetic mean (±SE)	1.93 (±0.10)	1.89 (±0.14)	1.33 (±0.12)	1.70 (±0.10)
	Geometric mean (GSD)	1.93 (1.11)	1.87 (1.20)	1.31 (1.20)	1.66 (1.27)
	Median (min, max)	1.87 (1.77, 2.24)	1.96 (1.38, 2.16)	1.22 (1.15, 1.81)	1.81 (1.15, 2.24)

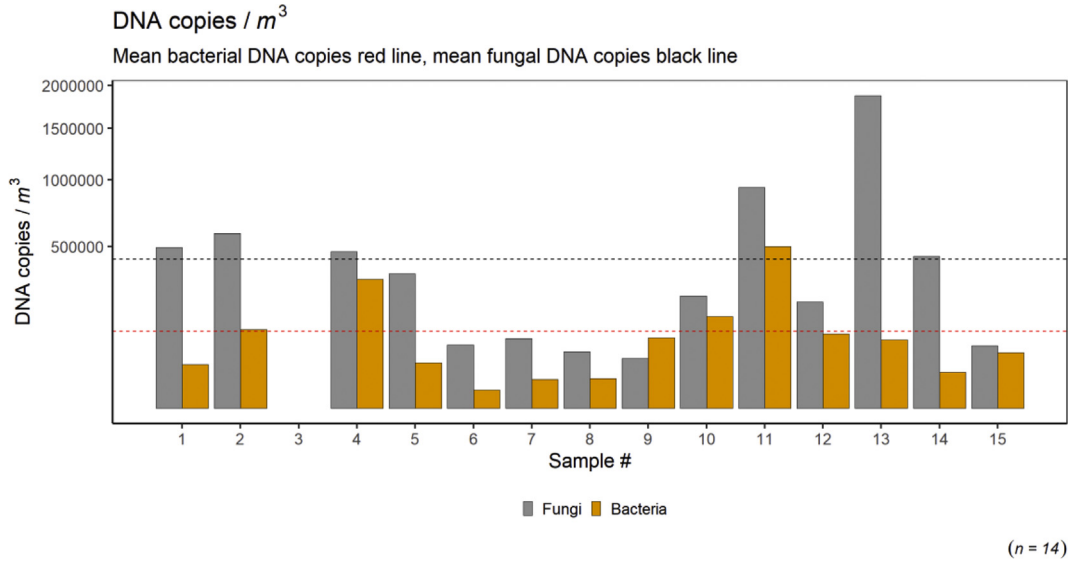


Fig. 2. Comparison of bacterial and fungal DNA copies m^{-3} after ddPCR. Sample #3 was not available for DNA extraction.

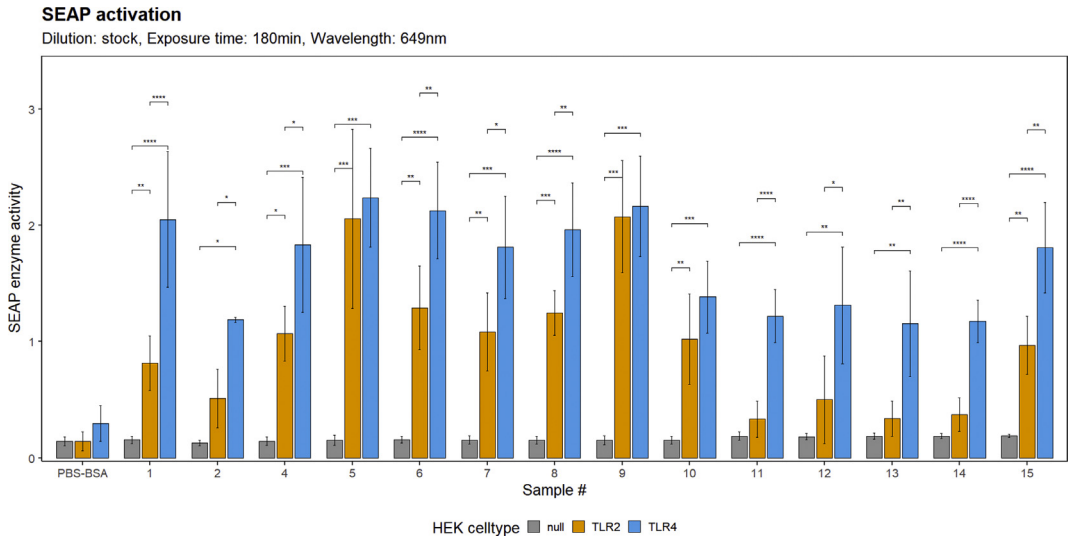


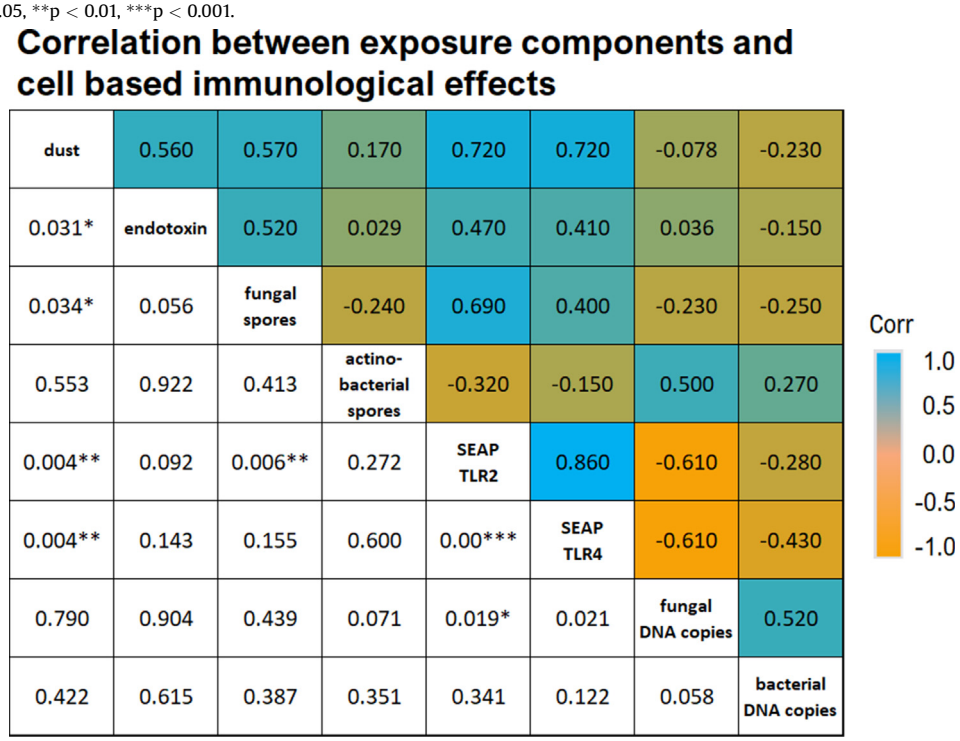
Fig. 3. Comparison of SEAP activity in HEK cells. HEK null: gray, TLR2: orange, TLR4: blue. Error-bars indicate standard deviation. Asterisk indicates significance levels: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

the dust samples contained ligands capable of stimulating TLR2 and TLR4 receptors, and thus, had the potential to evoke an inflammatory response in exposed workers. These results reflect the complexity of work environmental air samples and indicate that a substantial part of the sampled material was of microbial origin.

Inhalable dust may contain microbes, such as bacteria and fungi, as well as their associated fragments. In the past decades, evidence from epidemiological studies revealed that exposure to dust could cause adverse health effects in humans [22–24], such as mucosal irritation, discomfort, or even illness [25]. The majority of the workers who participated in this study reported to frequently (weekly) suffer from adverse health symptoms, such as upset stomach and diarrhea that can be attributed to work-related exposure (unsystematically collected data). The estimated mean exposure levels of total dust, endotoxin, and fungal spores varied between workdays (Fig. 1), with significantly elevated exposure levels on Monday (Table 1). On Monday, all workers engage in cleaning and maintenance tasks that include the removal of settled dust from sorting machines and conveyor belts by air-pressure blowers and brooms, during which a large amount of dust is dispersed in the work atmosphere. On the remaining workdays, the workers rotate between various work operations. It can be assumed that exposure to dust is higher during the manual removal of large items from the conveyor belt that connects the shredder in the waste reception hall to the automated sorting lines, compared to driving a truck with an in-cab air filtration system or control room and office work. Furthermore, high exposure moments occur during cleaning tasks with air-pressure guns, in which settled dust is dispersed in the work environment. As shown in Figs. 1 and 2 there was some variation in exposure levels between workers. However, time spent at different work tasks had no statistical support to elucidate these differences. This may be due to the low sample size or a lack of statistical significance of work task as an explanatory variable, per se.

Organic dust currently has an effective OEL of 5 mg/m^3 (total dust fraction; [26]). The total dust levels measured in the current

Table 2
Pairwise correlation of exposure measures and cell-based immunological effects (Pearson coefficient: upper corner, p-values: lower corner). Exposure measures: total dust, endotoxin, fungal spores, and actinobacterial spores, as well as fungal and bacterial DNA copies, are log transformed. TLR2 and TLR4 activation in absolute values. Significance levels indicated by *p < 0.05, **p < 0.01, ***p < 0.001.



study varied between individuals and sampling days with an average of 2.00 mg/m^3, which is below the OEL. In only 3 out of 15 samples, the OEL was exceeded. However, exposure below the given OEL may cause symptoms in individuals due to interaction effects between different irritants, as well as differences in individual sensitivity [27]. The average exposure level appears relatively low in comparison to an average dust exposure of 7.7 mg/m^3 measured in waste sorting and collecting reported by Krajewski et al. [28]. In another study conducted by Park et al. [29], an average dust exposure level of 0.9 mg/m^3 was reported for waste collectors.

Occupational exposure to endotoxins has been linked to various negative health outcomes, such as fever, headache, wheezing, as well as diseases of the respiratory system [30,31]. The health-based recommended occupational exposure limit (HBROEL) of 90 EU/m^3 per 8-hour TWA, recommended by the Health Council of The Netherlands [32], is generally used as a reference value in Norway. The average time-weighted endotoxin concentration (GM: 458 EU/m^3) measured in the current study exceeded the recommended HBROEL by a 5-fold. Equally, high exposure levels have been reported in studies conducted in waste collecting and sorting facilities [29,33].

Just like endotoxins, fungal spores are omnipresent in facilities that handle the residual waste. Occupational exposure to fungi has been correlated to adverse health effects of the respiratory system, such as reduced lung function, inflammation of air-ways, as well as allergic responses [34,35], and a recommended exposure limit of 1×10^5 fungal spores m^{-3} for nonpathogenic and nonmycotoxin producing species has been proposed [36]. The time-weighted fungal spore concentration observed in this study varied between a minimum of just below 1.5×10^5 and a maximum of over 37×10^5 spores m^{-3}. Both these measures exceed the recommended exposure limit. These high levels are in line with exposure levels reported in other studies that investigated occupational exposure in residual waste handling [37,38]. Based on the strong correlation between the fungal spore level in the given sample material and the *in vitro* TLR2 activation, there is a risk that exposed individuals develop exposure-related symptoms.

Actinobacteria have been reported to be one of the prevailing phyla identified in air samples from waste sorting plants [39,40]. Actinobacteria do not only play a major role as human pathogens [41] but have also been identified to contribute to the development of antibiotic resistance by transferring antibiotic resistance genes (ARGs) to pathogenic bacteria [42]. Heldal et al. [37,43] reported a significant exposure-response relationship between actinobacterial spores and respiratory symptoms such as cough, as well as a significant decrease in work shift lung function. In the present study, actinobacterial spores were found, in rather high numbers, in 8 out of 14 dust samples. The high levels of actinospores found in these eight samples give reason to believe that workers in the given facility may develop exposure-related symptoms [37]. However, to further assess exposure-related health risks, such as infection, identification of actinobacteria at the species level and their viability would be needed. Further, the LOD is relatively high in this study but far below the advised exposure level in Norway of 10^5 spores/m^3. The use of the FESEM quantification method will therefore be limited to exposure levels above the LOD, thus giving no possibility to assess possible risk effects associated with exposure levels below the LOD, particularly in case of mixture exposure with other microorganisms. To fully understand the effects of exposure to bioaerosols, it is necessary to investigate the microbial biodiversity in the work environment. This study estimated the amount of bacterial and fungal DNA copies m^{-3} work-air by applying a multitemplate ddPCR, using primer sets that provide the most acceptable trade-off between amplicon size, primer specificity, and coverage of bacteria and fungi [44]. The mean of 0.61×10^5 DNA copies m^{-3} for bacteria and 2.45×10^5 DNA copies

m^{-3} for fungi suggests that the collected material contains a substantial amount of microbial DNA.

While TLR2 activation is known to promote pro-T_H2 cytokines, TLR4 activation is known to induce the innate immune system and promote the production of proinflammatory cytokines and chemokines that shape the adaptive immunity toward T_H1 effector cells [45,46]. Workers in the waste industry have previously been shown to develop both lung-specific and systemic inflammation and cross-shift reduced lung function associated with microbial exposure [37,43]. Despite the limited sample size, the current study showed that the dust samples contained a wide array of TLR2 and TLR4 agonists that are capable of engaging downstream cascades that have the potential to promote an inflammatory response in exposed workers. The overall effect of TLR receptor-ligand interaction on the workers' health is complex and will strongly depend on exposure levels and bioavailability of the different components, genetic factors, and the workers' individual innate immune defense, as well as the balance in the cellular response-counter-response [47–49]. The immune responses in the present study were limited to the activation of TLRs, primarily induced by microbial components. Other irritants or chemicals that also may cause inflammatory responses were not assessed.

Despite the limited sample size in this study, the high exposure levels of endotoxins and fungal spores indicate a need for preventive measures to reduce individual work exposure. This should include personal protection equipment, such as respirators and protective clothing, as well as hygiene measures, such as hand sanitation and designated clean areas. Furthermore, work operations that disperse dust in the work environment, such as the use of air-pressure guns during cleaning, should be avoided. Alternatively, the use of a centralized vacuum cleaning system can be advised. Also, when planning waste sorting facilities, it is important to incorporate measures to reduce occupational exposure, such as adequate ventilation systems (both general and directed), as well as enclosure of waste sorting lines and other high exposure areas.

To the best of our knowledge, the present study is the first to assess TLR2 and TLR4 activation properties of personal air samples from waste sorting facilities. The *in vitro* results give reason to believe that exposed workers may develop inflammatory symptoms as a response to occupational dust exposure. Despite the limited sample size, the obtained results give adequate reason to further investigate the effects of occupational exposure to microorganisms and associated toxins on the species level.

5. Conclusion

This study investigated exposure to dust, endotoxins, fungal, and actinobacterial spores in a fully automated waste-sorting facility. The concentrations of endotoxin and fungal spores exceeded recommended effect levels in all samples. The Norwegian OEL for organic dust was exceeded in 20% of the samples. Furthermore, 64% of the samples activated TLR2 and significant TLR4 receptor activation in all samples. These results justify further and more detailed investigations of the exposure to biological agents in waste sorting facilities with the aim to identify species diversity and abundance, as well as high-exposure work operations.

Ethical statement

This study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The participants were informed about the study aims prior to the study gave their consent. Participation was voluntary, and participants could quit at any time.

Funding source

This work was financially supported by Romerike Avfallsforedling IKS (ROAF). The founding sponsor had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the publication of the results.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

Ragnhild Beate Strand Østrem, and Wijnand Eduard at STAMI and Kari Kulvik Heidal, and Thea Haugesten Johansen, formerly at STAMI, are greatly acknowledged for participating in the fieldwork. Thanks are due to Oda Astrid Haarr Foss for guidance and help with ddPCR analyses. We also thank employers and employees at the participating facility for their cooperation.

References

[1] Lehtinen J, Tolvanen O, Nivukoski U, Veijanen A, Hänninen K. Occupational hygiene in terms of volatile organic compounds (VOCs) and bioaerosols at two solid waste management plants in Finland. Waste Manag 2013;33(4):964–73.
[2] Viegas S, Almeida-Silva M, Viegas C. Occupational exposure to particulate matter in 2 Portuguese waste-sorting units. Int J Occup Med Environ Health 2014;27(5):854–62.
[3] Rahkonen P. Airborne contaminants at waste treatment plants. Waste Manag Res 1992;10(5):411–21.
[4] Poulsen OM, Breum NO, Ebbehoj N, Hansen AM, Ivens UI, van Lelieveld D, et al. Collection of domestic waste. Review of occupational health problems and their possible causes. Sci Total Environ 1995;170(1–2):1–19.
[5] Abou-ElWafa HS, El-Bestar SF, El-Gilany AH, Awad El-Toraby Eel S. Respiratory disorders among municipal solid waste collectors in Mansoura, Egypt: a comparative study. Arch Environ Occup Health 2014;69(2):100–6.
[6] Hagemeyer O, Bunger J, van Kampen V, Raulf-Heimsoth M, Drath C, Merget R, et al. Occupational allergic respiratory diseases in garbage workers: relevance of molds and actinomycetes. Adv Exp Med Biol 2013;788:313–20.
[7] Jahangiri M, Neghab M, Nasiri G, Aghabeigi M, Khademian V, Rostami R, et al. Respiratory disorders associated with occupational inhalational exposure to bioaerosols among wastewater treatment workers of petrochemical complexes. Int J Occup Environ Med 2015;6(1):41–9.
[8] Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4(7):499–511.
[9] Liu G, Zhao Y. Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4+ CD25+ T cells. Immunology 2007;122(2):149–56.
[10] Rasmussen PU, Phan HUT, Frederiksen MW, Madsen AM. A characterization of bioaerosols in biowaste pretreatment plants in relation to occupational health. Waste Manag 2021;131:237–48.
[11] Madsen AM, Frederiksen MW, Jacobsen MH, Tendal K. Towards a risk evaluation of workers' exposure to handborne and airborne microbial species as exemplified with waste collection workers. Environ Res 2020;183:109177.
[12] Viegas C, Caetano LA, Cox J, Korkalainen M, Haines SR, Dannemiller KC, et al. The effects of waste sorting in environmental microbiome, THP-1 cell viability and inflammatory responses. Environ Res 2020;185:109450.
[13] van der Wal J. Comparative measurements of the total dust concentration at the work place with different samplers – part I. Staum Reinhalt Luft 1983;43:4.
[14] Afanou KA, Straumfors A, Skogstad A, Nilsen T, Synnes O, Skaar I, et al. Submicronic fungal bioaerosols: high-resolution microscopic characterization and quantification. Appl Environ Microbiol 2014;80(22):7122–30.
[15] Straumfors A, Foss OAH, Fuss J, Mollerup SK, Kauserud H, Mundra S. The inhalable mycobiome of sawmill workers: exposure characterization and diversity. Appl Environ Microbiol 2019;85(21):1448.
[16] Vainio EJ, Hantula J. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol Res 2000;104(8):927.
[17] Herlemann DPR, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. ISME J 2011;5(10):1571–9.
[18] Brummelman J, Veerman RE, Hamstra HJ, Deuss AJM, Schuijt TJ, Sloots A, et al. Bordetella pertussis naturally occurring isolates with altered lipooligosaccharide structure fail to fully mature human dendritic cells. Infect Immun 2015;83(1):227–38.
[19] R Core Team. R. A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2020.

[20] Kassambara A. rstatix: pipe-friendly framework for basic statistical tests; 2020.
[21] Wickham A. ggplot2: elegant graphics for data analysis. New York: Springer-Verlag; 2016.
[22] Habybabady RH, Sis HN, Paridokht F, Ramrudinasab F, Behmadi A, Khosravi B, et al. Effects of dust exposure on the respiratory health symptoms and pulmonary functions of street sweepers. Malays J Med Sci 2018;25(6):76–84.
[23] Nordby KC, Fell AKM, Notø H, Eduard W, Skogstad M, Thomassen Y, et al. Exposure to thoracic dust, airway symptoms and lung function in cement production workers. Eur Respir J 2011;38(6):1278.
[24] Melbostad E, Eduard W. Organic dust-related respiratory and eye irritation in Norwegian farmers. Am J Ind Med 2001;39:209–17.
[25] Poulsen OM, Breum NO, Ebbehøj N, Hansen AM, Ivens UI, van Lelieveld D, et al. Sorting and recycling of domestic waste. Review of occupational health problems and their possible causes. Sci Total Environ 1995;168(1):33–56.
[26] The Norwegian Labour Inspection Authority. Regulations concerning action and limit values for physical and chemical agents in the working environment and classified biological agents FOR-2011-12-06 NO. 1358-ed.. Oslo, Norway: Ministry of Labour and Social Affairs; 2013.
[27] Johnston JR. Hazard prevention and control in the work environment: airborne dust. Protection of the human environment occupational health and environmental health Series, Geneva, 1999, World Health Organization WHO/SDE/OEH/99.14: English Only. Ann Occup Hyg 2000;44(5):405.
[28] Krajewski JA, Tarkowski S, Cyprowski M, Szarapinska-Kwaszewska J, Dudkiewicz B. Occupational exposure to organic dust associated with municipal waste collection and management. Int J Occup Med Environ Health 2002;15(3):289–301.
[29] Park DU, Ryu SH, Kim SB, Yoon CS. An assessment of dust, endotoxin, and microorganism exposure during waste collection and sorting. J Air Waste Manag Assoc (1995) 2011;61(4):461–8.
[30] Kumar S, Adhikari A. Dose-dependent immunomodulating effects of endotoxin in allergic airway inflammation. Innate Immun 2017;23(3):249–57.
[31] Eduard W, Pearce N, Douwes J. Chronic bronchitis, COPD, and lung function in farmers: the role of biological agents. Chest 2009;136(3):716–25.
[32] Health Council of The Netherlands. Health risks associated with livestock farms; 2012. The Hague, The Netherlands.
[33] Kozajda A, Jezak K, Cyprowski M, Szadkowska-Stanczyk I. Inhalable dust, endotoxins and (1-3)-beta-d-glucans as indicators of exposure in waste sorting plant environment. Aerobiologia (Bologna) 2017;33(4):481–91.
[34] Rivera A, Ro G, Van Epps HL, Simpson T, Leiner I, Sant'Angelo DB, et al. Innate immune activation and CD4+ T cell priming during respiratory fungal infection. Immunity 2006;25(4):665–75.
[35] Viegas C, Faria T, de Oliveira AC, Caetano LA, Carolino E, Quintal-Gomes A, et al. A new approach to assess occupational exposure to airborne fungal contamination and mycotoxins of forklift drivers in waste sorting facilities. Mycotoxin Res 2017;33(4):285–95.
[36] Eduard W, Heederik D, Duchaine C, Green BJ. Bioaerosol exposure assessment in the workplace: the past, present and recent advances. J Environ Monit: JEM 2012;14(2):334–9.
[37] Heldal KK, Halstensen AS, Thorn J, Eduard W, Halstensen TS. Airway inflammation in waste handlers exposed to bioaerosols assessed by induced sputum. Eur Respir J 2003;21(4):641–5.
[38] Madsen AM, Alwan T, Orberg A, Uhrbrand K, Jorgensen MB. Waste workers' exposure to airborne fungal and bacterial species in the truck cab and during waste collection. Ann Occup Hyg 2016;60(6):651–68.
[39] Degois J, Clerc F, Simon X, Bontemps C, Leblond P, Duquenne P. First metagenomic survey of the microbial diversity in bioaerosols emitted in waste sorting plants. Ann Work Expos Health 2017;61(9):1076–86.
[40] Veillette M, Bonifait L, Mbareche H, Marchand G, Duchaine C. Preferential aerosolization of Actinobacteria during handling of composting organic matter. J Aerosol Sci 2018;116:83–91.
[41] Sowani H, Kulkarni M, Zinjarde S, Javdekar V. Chapter 7 – Gordonia and related genera as opportunistic human pathogens causing infections of skin, soft tissues, and bones. In: Kon K, Rai M, editors. The microbiology of skin, soft tissue, bone and joint infections. 2. Academic Press; 2017. p. 105–21.
[42] Jiang X, Ellabaan MMH, Charusanti P, Munck C, Blin K, Tong Y, et al. Dissemination of antibiotic resistance genes from antibiotic producers to pathogens. Nat Commun 2017;8(1):15784.
[43] Heldal KK, Madsø L, Eduard W. Airway inflammation among compost workers exposed to actinomycetes spores. Ann Agric Environ Med 2015;22(2):253–8.
[44] Chemidlin Prévost-Bouré N, Christen R, Dequiedt S, Mougel C, Lelièvre M, Jolivet C, et al. Validation and application of a PCR primer set to quantify fungal communities in the soil environment by real-time quantitative PCR. PLoS One 2011;6(9):24166.
[45] Zakeri A, Russo M. Dual role of toll-like receptors in human and experimental asthma models. Front Immunol 2018;9(1027).
[46] Netea MG, Van der Meer JWM, Sutmuller RP, Adema GJ, Kullberg B-J. From the Th1/Th2 paradigm towards a Toll-like receptor/T-helper bias. Antimicrob Agents Chemother 2005;49(10):3991–6.
[47] Poole JA, Romberger DJ. Immunological and inflammatory responses to organic dust in agriculture. Curr Opin Allergy Clin Immunol 2012;12(2):126–32.
[48] Boasen J, Chisholm D, Lebet L, Akira S, Horner AA. House dust extracts elicit Toll-like receptor–dependent dendritic cell responses. J Allergy Clin Immunol 2005;116(1):185–91.
[49] Rylander R, Michel O. Organic dust induced inflammation—role of atopy and TLR-4 and CD14 gene polymorphisms. Am J Ind Med 2005;48(4):302–7.