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Research Paper

Health symptoms, inflammation, and bioaerosol exposure in workers at biowaste pretreatment plants

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ABSTRACT

Biowaste pretreatment plants have been built within the last years in Denmark in order to recycle pre-sorted biowaste from houses, restaurants, and industry. We investigated the association between exposure and health at six biowaste pretreatment plants (visited twice) across Denmark. We measured the personal bioaerosol exposure, took blood samples, and administered a questionnaire. Thirty-one persons participated, 17 of them twice, resulting in 45 bioaerosol samples, 40 blood samples, and questionnaire answers from 21 persons. We measured exposure to bacteria, fungi, dust, and endotoxin, the total inflammatory potential of the exposures, and serum levels of the inflammatory markers serum amyloid A (SAA), high sensitivity C-reactive protein (hsCRP), and human club cell protein (CC16). Higher exposures to fungi and endotoxin were found for workers with tasks inside the production area compared to workers with main tasks in the office area. A positive association was found between the concentration of anaerobic bacteria and hsCRP and SAA, whereas bacteria and endotoxin were inversely associated with hsCRP and SAA. A positive association between hsCRP and the fungal species *Penicillium digitatum* and *P. camemberti* were found, whereas an inverse association between hsCRP and *Aspergillus niger* and *P. italicum* were found. Staff with tasks inside the production area reported more symptoms of the nose than those working in the office area. To conclude, our results indicate that workers with tasks inside the production area are exposed to elevated levels of bioaerosols, and that this may affect workers' health negatively.

1. Introduction

Previous studies on workers collecting organic waste (e.g. Lavoie et al., 2006; Madsen et al., 2021) and from waste recycling plants (Eriksen et al., 2023; Krajewski et al., 2002; Lehtinen et al., 2013) have shown high occupational microbial exposures. In Denmark, due to new requirements from the European Union for the sorting and recycling of waste, several biowaste pretreatment plants have been built in order to meet demands. At these plants, waste collection trucks deliver pre-sorted biowaste from homes, restaurants, and industry, and the biowaste is processed into a biopulp. The biopulp is later used for energy production at biogas plants and the remaining biomass is used as fertilizer, thereby overall fitting into the circular economy framework. At the pretreatment plants, work consists of cleaning or maintenance work inside the production area, transferring and sorting waste by wheel loaders, and work in control rooms, as well as office work. A pilot study at these plants, mainly using stationary samplers, has indicated high microbial exposures and potential associated health effects for the workers (Rasmussen et al., 2021). However, the implications for the workers need to be assessed at a larger scale across more plants and with direct assessments of early signs of health effects, reported health symptoms, and exposure.

Studies on workers in the waste industry have shown a higher prevalence of reported health symptoms connected with bioaerosol exposure (Poulsen et al., 1995). In a review by Poulsen et al. (1995), the authors found that workers involved in manual sorting of domestic waste and workers at compost plants experience symptoms of organic dust toxic syndrome (ODTS), gastrointestinal problems, and irritation of skin, eye, and mucous membranes of the nose and upper airways. Furthermore, Ivens et al. (1999, 1997), in a nationwide study of 2303 Danish waste collection workers, saw a higher reported prevalence of nausea and diarrhoea symptoms in the group of high estimated endotoxin and fungal exposure compared to medium and low exposed workers.

Elevated levels of acute phase response proteins in blood samples can be early signs of health effects. Thus, as a response to infection,

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inflammation, or tissue injury, serum levels of the acute phase proteins C-reactive protein (CRP) and serum amyloid A (SAA) increase (Du Clos and Mold, 2004; Sack, 2018). Increased levels of high sensitivity CRP (hsCRP) is shown to correlate with airflow obstruction and airway inflammation in asthma patients (Takemura et al., 2006), and increased levels of both CRP and SAA have been shown to correlate with acute exacerbations of chronic obstructive pulmonary disease (COPD; Bozinovski et al., 2008). In occupational settings, Madsen et al. (2016) found a positive association between exposure to endotoxin and serum levels of hsCRP and SAA in greenhouse workers. Another marker, which may be used as a sign of effects on human health, is the anti-inflammatory human club cell-16 protein (CC16). The human club cells protect the lung from inhaled toxicants, and as such, CC16 proteins may indicate early changes in lung epithelial barrier function (Broeckaert and Bernard, 2000). Studies have shown an association between reduced serum levels of CC16 and smoking (Bernard et al., 1994; Gribben et al., 2022; Robin et al., 2002), whereas increased levels have been associated with acute or repeated exposures to bioaerosols in wastewater and grain dust exposed workers (Heldal et al., 2013; Steiner et al., 2005; Straumfors et al., 2018).

The aim of this study was to explore the level of bioaerosol exposure for workers at biowaste pretreatment plants, and the association between workers' exposure and its potential association with early signs of health symptoms and self-reported health symptoms. For this, six plants across Denmark were visited twice and the personal bioaerosol exposure was measured (dust, bacterial, fungal and endotoxin exposure, and the total inflammatory potential (TIP) of the bioaerosols).

2. Materials and methods

2.1. Pretreatment plants

The biowaste pretreatment plants receive pre-sorted organic waste from homes, businesses, and restaurants (collection frequency 1–2 weeks), as well as rejected products from the food production industry (typically stored in original packaging). At these plants, biowaste is processed into a biopulp by adding processing water (excess water, e.g. water-runoff from the biowaste), grinding/pulping the material, and removing reject (e.g. plastic bags). The biopulp is stored in a silo and will later be used for energy production at biogas plants. The plants typically consist of a production area, a control room, and a lunch area. The production area includes both waste receiving and processing areas (in one large hall or separated into two different areas within the hall). Control and lunch areas can also be separate rooms or the same room, and are typically on the same floor in the same building as the production area. The rules differ among waste plants when accessing the control and lunch area, such as whether shoes and jackets have to be removed beforehand.

2.2. Study design

The sampling campaign took place at six biowaste pretreatment plants across Denmark from May 2021 to June 2022. Four of the plants (P1, P4-P6) were visited twice during two different seasons (with 5–9 months between visits), whereas plants P2-P3 were visited twice during the same season (summer) one year apart due to planned reconstruction. We collected a total of forty-five personal exposure measurements and forty blood samples. Exposure samples were collected from 31 different staff members, 14 of which participated twice and 17 only once, and blood samples from 27 different staff members, 13 of which participated twice and 14 once. Most plants had a low number of staff hired, and at each plant visit, the number of participants varied from one to six (at plant P5, only one worker was present on a typical workday). Participants consisted of medium to highly exposed staff working inside the production area to various degrees (one hour to full day inside the production area; n = 36 exposure samples from 26 persons; these henceforth called 'exposed') and staff with low exposure who had mainly office work and no tasks inside the production area (n = 9 exposure samples from 5 persons; these henceforth called 'low exposed'). See Table S1 for an overview of the sampling and waste plants.

2.3. Exposure measurements

Participants were fitted with two personal air samplers (Gesamtstaubprobenahme sampler (GSP), BIG Inc., USA) placed in the inhalation zone of the persons (Aizenberg et al., 2001; Frankel et al., 2012). The GSP sampler is designed to collect the inhalable dust fraction of the air by using an intake velocity of 1.25 m/s at a flow rate of 3.5 L/min. This corresponds approximately to the inhalation speed of humans. Samplers were connected to pumps carried in a small backpack. One sampler was mounted with a 37 mm polycarbonate filter (pore size 0.8 µm, SKC) and the other with a 37 mm Teflon filter (pore size 1.0 µm, Merck). Both samplers had a flow rate of 3.5 L/min and the flow was checked and adjusted throughout the sampling period. The average sampling period was 427 min, corresponding to an actual workday (one person 110 min, the remaining persons ranging from 323 min to 553 min).

2.4. Blood sample collection

At the end of the working day, blood samples were taken from participants after they had signed a consent form. The study was approved by the Regional Ethics Committee of the Capital Region in Denmark (H-20060322). Blood samples were collected into BD vacutainer Serum Tubes and kept in coolers on ice during transportation back to the laboratory (within the same day). There, the blood was centrifuged at 4000 rpm for 15 min to separate serum, before freezing serum aliquots at −80 °C.

2.5. Extraction, quantification, and identification of bacteria and fungi

Due to the long travel times from the biowaste pretreatment plants located across Denmark to our laboratory, following sampling, GSP samplers were stored horizontally in closed plastic boxes during transport, followed by storing overnight in a climate controlled room. The polycarbonate filters were extracted the following morning in 5 mL sterile extraction solution (MilliQ water with 0.05 % Tween 80 and 0.85 % NaCl) by orbital shaking at 500 rpm for 15 min at room temperature.

For quantification and identification of bacteria and fungi, suspensions from the polycarbonate filters were plated in serial dilutions on Nutrient agar (NA; Thermo Fisher Scientific Oxoid, Basingstoke, UK) plates with actidione (cycloheximide; 50 mg/L; Serva, Germany) for enumeration of bacteria, on Fastidious Anaerobe Agar with 5 % blood (FAA; SSI Dianostica, Hillerød, Denmark) for enumeration of anaerobic bacteria, and on Dichloran Glycerol agar (DG18; Thermo Fisher Scientific Oxoid, Basingstoke, UK) for enumeration of fungi. NA plates were incubated at 25 °C for seven days, FAA plates were incubated anaerobically in an AnaeroJar with an AnaeroGen sachet (both from Thermo Fisher Scientific Oxoid, Basingstoke, UK) at 37 °C for two days, and DG18 plates were incubated at 25 °C for seven days and at 37 °C for four days. Following incubation, all visible bacterial and fungal colonies were counted. Concentrations of each dilution were calculated as time weighted averages (CFU/m^3), taking into account the CFU count, the volume of extraction solution and plating, the sampling time, and the flow rate. Sample concentrations were calculated as geometric mean (GM) values of appropriate sample dilutions (CFU count between 1 and 200 for bacteria and 1 – 100 for fungi).

A representative dilution (optimal coverage and separation of individual colonies; preferably plates with a CFU count between 50 and 100 for bacteria and 10 and 50 for fungi) was chosen for MALDI identification. Bacterial isolates were identified using the extended direct transfer

method following the manufacturer's instructions in a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) Biotyper System (Bruker Daltonics, Bremen, Germany). Fungal isolates were identified using a modified ethanol extraction protocol. MALDI-TOF MS was analysed on a Microflex LT mass spectrometer (Bruker Daltonics) using the Bruker Biotyper 3.1 software with the BDAL bacterial library and Filamentous Fungi library. The instrument was calibrated weekly using a bacterial test standard (Bruker Daltonics). Isolates were analysed in duplicates on the MALDI-TOF MS using the following cut-offs: isolates with scores lower than 1.70 were unidentified. Isolates with scores between 1.70 and 1.79 were identified to genus level. Isolates with scores of 1.80 or higher were identified to species level (Møller et al., 2022).

2.6. Quantification of dust and endotoxin

Teflon filters were used to determine the dust exposure. Teflon filters (and three blanks on each measuring day) were pre-weighed in a climate controlled weigh-room. After sampling, filters were weighed again after a 16–24 h acclimatization period. Dust mass is presented as time weighted averages (mg/m^3).

After determining the mass of dust, Teflon filters were extracted in 5 mL 0.05 % Tween 20 and 0.85 % NaCl by orbital shaking at 300 rpm for 60 min, followed by a 15 min centrifugation step. The supernatant was frozen at −80 °C until endotoxin analyses. To determine the concentration of endotoxin in samples, we used a recombinant factor C (rFC) assay (Lonza, Walkersville Inc.) following the manufacturer's instructions. Plates were read using a PyroWave XM fluorescence microplate reader (Lonza, Walkersville Inc.). Endotoxin concentrations are presented as time-weighted averages (EU/m^3). As most studies within occupational exposure and risk assessment have previously used the chromogenic kinetic limulus amoebocyte lysate assay (LAL; Kinetic-QCL endotoxin kit, Lonza Walkersville Inc.), we also tested 10 of the samples using this assay. From this, a conversion factor of 10 was determined. This conversion factor is used in tables when presenting geometric means and ranges and for comparison with other studies in the discussion.

2.7. The total inflammatory potential (TIP)

The total inflammatory potential of each air sample was determined using a granulocyte-like cell assay, in which the human promyelocytic leukaemia cell line HL-60 cells (ATCC, CCL-240; kindly provided by E. W. Hansen (Timm et al., 2006)) were exposed to the polycarbonate samples. After exposure, reactive oxygen species (ROS) production was measured by a luminol-dependent chemiluminometric assay (Timm et al., 2009) using a thermostated (37 °C) ORION II Microplate Luminometer (Berthold Detection Systems). Relative-light units per s (RLU/s) were measured for 1 s every 120 s for 180 min. For each sample, RLU over the 180 min period was summed, thereby expressing the total inflammatory potential of the sample as the area under the curve (AUC). AUC was normalised by dividing with the AUC of a within-run reference sample and multiplied by the average AUC of the between-run reference samples (Lu et al., 2020). Afterwards, sample AUCs were expressed as time weighted averages (AUC/m^3).

2.8. Quantification of hsCRP, SAA, and CC16

Serum levels of inflammatory markers were determined by Enzyme-linked Immunosorbent assay (ELISA) according to the specifications by the manufacturer: high sensitivity C-Reactive Protein (hsCRP) by a kit from IBL International GMBH (Hamburg, Germany), Serum Amyloid A (SAA) with a kit from Invitrogen (CA, USA), and Human club cell protein (CC16; also sometimes called Clara cell protein) serum levels was determined with an ELISA kit from BioVendor (Brno, Czech Republic). Absorbance at 450 nm was measured by use of an EPOCH instrument (Biotek Instruments, Inc., Vermont, US).

2.9. Questionnaire

An online questionnaire was sent to 33 staff members, including two persons who did not contribute exposure measurements or blood samples (28 in the exposed group and five in the low exposed group). The questionnaire has previously been used to investigate respiratory health in biofuel workers (Schlünssen et al., 2011), and is a modified version of the the European community respiratory health survey (Burney et al., 1994) with additional questions on e.g. allergy, coughing, asthma, rhinitis, smoking, toxic pneumonitis, and stomach problems. Twenty-one individuals completed the questionnaire (64 % response rate) with 16 from the exposed group (57 % response rate) and five in the low exposed group (100 % response rate). Twelve persons additionally answered a shortened version of the questionnaire as they participated on both sampling days. Aside from the twenty-one persons, ten participants did not provide answers to the questionnaire and two participants gave partial answers, which were excluded from the results. See Table 1 for summaries of participant background information based on questionnaire answers.

The questionnaire included the overall themes: 1) background and education, 2) work history and habits, 3) smoking history, 4) health symptoms within the last week, 5) history of allergies, 6) history of asthma, and the following within the last 12 months: 7) symptoms of the nose, 8) symptoms of the eyes, 9) symptoms of the throat, 10) coughing, 11) wheezing, 12) chest tightness, 13) influenza-like symptoms, 14) diarrhoea, 15) infections, and 16) skin symptoms.

2.10. Temperature and relative humidity

Temperature and relative humidity were measured (at 5 min intervals) inside and outside the waste plants during the sampling period using Tinytag Plus Data Loggers (Gemini Data Loggers, Chichester, UK; Table S1).

2.11. Data analyses

We first analysed how exposure measures differed among those who worked in the production area and those that worked in the office area (i.e. exposure level) using linear mixed effect model. Exposure level was added as a fixed effect and waste plants, seasons, and their interaction as random effects. To determine how microbial communities differed between the two groups, we used redundancy analysis (RDA) on Hellinger transformed concentrations. We further analysed how inflammatory markers were associated with exposure group using the model structure described before, but where we also accounted for age, atopy (yes/no), and sex. We also explored adding BMI and smoking history, but these did not improve the models. Following these models, we investigated how the exposures were associated with levels of inflammatory markers. We here included the concentration of all exposures as fixed effects, and included the remaining variables mentioned above (see Table S2 for a detailed overview of all the models, including transformations). Similar models were also done, but in which the inhaled doses of microorganisms were used instead of the concentrations of the microorganisms. As the models gave very similar results, these are only reported in the supplementary information (Table S3). The inhaled doses were calculated by the following formula: the measured exposure (CFU/m^3) * the inhalation rate (m^3/day, here used 13.68 m^3/day for women and 16.24 m^3/day for men (U.S. EPA, 2011)) * the duration of the workday (day).

For those participants who did not wish to answer the questionnaire and where no information on age and atopy was available, an estimate of the age was used based on the average ages and what we could remember of the person (n = 3). For atopy, we assumed the persons had no atopy if we did not have the information available (n = 4). Model assumptions of homogeneous residuals and variance inflation factors

Table 1
Background information of participants, who answered the questionnaire (all 64 % response rate). The exposed group (57 % response rate) is defined as those working inside the production area (waste receiving and processing area), whereas the low exposed group is defined as those working mainly in the office area (100 % response rate).

	All (n = 21)		Exposed group (n = 16)		Low exposed group (n = 5)	
	n (%)	mean (range)	n (%)	mean (range)	n (%)	mean (range)
Background						
Men	17 (81 %)		15 (93.8 %)		2 (40.0 %)	
Women	4 (19 %)		1 (6.2 %)		3 (60.0 %)	
Age (years)		49.3 (29–63)		49.9 (29–63)		47.4 (33–58)
Height (cm)		178.6 (154–195)		181.0 (163–195)		170.8 (154–184)
Weight (kg)		91.6 (68–120)		93.0 (72–120)		85.8 (68–105)
BMI (kg/m^2)		28.7 (22.7–37.2)		28.5 (22.7–37.2)		29.2 (24.8–33.2)
Current smoker	7 (33.3 %)		6 (37.5 %)		1 (20.0 %)	
Past smoker	6 (28.6 %)		4 (25.0 %)		2 (40.0 %)	
Non-smoker	8 (38.1 %)		6 (37.5 %)		2 (40.0 %)	
Work history						
Years at current workplace		4.9 (<0.5–16)		5.6 (<0.5–16)		2.7 (1.5–4)
Years at waste plant		6.5 (<0.5–21)		7.4 (<0.5–21)		3.5 (1.5–8)
Years at biowaste plant		3.7 (<0.5–15)		4.1 (<0.5–15)		2.7 (1.5–4)
Current work hours						
Total weekly hours		37.4 (15–47)		38.3 (32–45)		34.6 (15–47)
Weekly hours split on tasks*:						
Work in office area	11 (52.4 %)	21.8 (2–37)	6 (37.5 %)	15.2 (2–33)	5 (100.0 %)	29.8 (5–37)
Work in control room	10 (47.6 %)	12.4 (1–25)	10 (62.5 %)	12.4 (1–25)	0 (0.0 %)	0 (0–0)
Work in production area	13 (61.9 %)	18.0 (3–40)	13 (81.2 %)	18.0 (3–40)	0 (0.0 %)	0 (0–0)
Maintenance (production area)	12 (57.1 %)	10.0 (2–36)	12 (75.0 %)	10.0 (2–36)	0 (0.0 %)	0 (0–0)
Cleaning (production area)	9 (42.9 %)	4.7 (0–8)	9 (56.2 %)	5.2 (2–8)	0 (0.0 %)	0 (0–0)
Other tasks	7 (33.3 %)	6.0 (2 – 14)	9 (56.2 %)	4.0 (2 – 10)	2 (40 %)	12.1 (10 – 14)

* for weekly hours split on task, the n and % indicate the number of participants performing those tasks, whereas means and ranges show the number of hours spent on those tasks.

were checked for all models. For samples where the microbial concentrations were below the limit of detection (LOD), a concentration corresponding to 0.5 × LOD was allocated for that sample.

For an overview of whether levels of inflammatory markers were correlated to each other, we ran non-parametric Kendall's rank correlation tests, and correlation matrices were furthermore made for inflammatory markers and exposure concentrations (Fig. S1-S2).

To analyse whether the exposure to specific microbial species was associated with the levels of inflammatory markers, a selection was made from the 172 bacterial and 32 fungal species identified. As many species (including most risk group 2 species) were only found in a few samples, we made a selection of species in which at least 25 % (i.e. 10 samples) of the 40 exposure samples (in which we also had blood serum samples collected) had a minimum concentration of 50 CFU/m^3. This resulted in the following nine species: the fungi *A. fumigatus* (risk group 2 classified in Denmark), *A. niger* (risk group 2 classified in some databases, such as GESTIS), *Penicillium brevicompactum, P. camemberti, P. commune, P. digitatum, P. italicum*, and *P. roqueforti* (most *Penicillium* species are allergenic), and the bacterium *Micrococcus luteus*. We excluded *Micrococcus luteus*, as this species is a common part of the skin microbiome and we assumed it would partly derive from the workers and have minimal effect on the levels of inflammatory markers. Linear mixed effect models were used with the fixed effects of the concentrations of each of the species, including the same covariate and random effect structure as the above models. The concentrations of each species used in the models consisted of the sums of the species based on incubations at both 25 °C and 37 °C. Therefore, as the concentrations of species may be associated with the total fungal concentration, we included the total fungal exposure (sum of the concentrations at 25 °C and 37 °C) as a covariate in the model. In samples where the species were not detected, a value of 0.1 × LOD of the sample was used for those particular species. This was done to avoid too many zeroes, to allow for the log10 transformations, and because using the more conventional 0.5 × LOD might result in too high estimates as several species per sample might receive this value.

For analysing how answers (often = 3, rarely = 2, never = 1) to health related questions in the questionnaire differed among the exposed and low exposed group, we used logistic regression for ordinal data.

All models were conducted in R v. 4.2.1 (R Core Team, 2022) using the *car* package for linear mixed effect models (Fox and Weisberg, 2011), *vegan* for RDA models (Oksanen et al., 2019), and the *ordinal* and *VGAM* packages for logistic regression models for ordinal data (Christensen, 2022; Yee, 2015, 2022; Yee and Wild, 1996).

3. Results

3.1. Exposure measures

Exposed workers, i.e. those that work inside the production area were exposed to higher levels of anaerobic bacteria, fungi, dust, and endotoxin (Table 2, Table S8, and Fig. S3) than workers who mainly worked in the office areas. Bacterial exposures and TIP concentrations did not differ significantly between exposure groups (Table 2, Table S8).

Twohundrednineteen taxa were identified using MALDI-TOF MS, including 15 only to genus level. To species level, we identified 172 bacterial and 32 fungal species. These included five risk group 2 bacteria (*Clostridium perfringens, Enterobacter cloacae*, *Escherichia coli, Klebsiella oxytoca,* and *Staphylococcus aureus)* which all were found in one to three samples and in low concentrations (Tables S4-S5). Two risk group 2 fungi were also found: *A. flavus* was found in exposed workers (11 samples, GM = 362 CFU/m^3 at 25 °C and 82 CFU/m^3 at 37 °C) and *A. fumigatus* was found in nearly all samples (38 samples, exposed workers GM = 1477 CFU/m^3 at 25 °C and 396 CFU/m^3 at 37 °C; low exposed workers GM = 11 CFU/m^3 at 25 °C and 286 CFU/m^3 at 37 °C; Table S6-S7).

A higher fungal species richness was found for exposed workers compared to low exposed workers (Tables S4-S7; Fungi 25 °C: χ^2 = 19.14, df = 1, P < 0.001; Fungi 37 °C: χ^2 = 3.50, df = 1, P = 0.062), however bacterial species richness did not differ between the two groups. Bacterial and fungal communities differed between the exposed and low exposed groups (bacteria 25 °C: F = 3.38, df = 1, df_residuals = 42, P = 0.001; anaerobic bacteria 25 °C: F = 2.40, df = 1, df_residuals = 42, P = 0.002; fungi 25 °C: F = 1.96, df = 1, df_residuals = 43, P = 0.022;

Table 2
Geometric mean (GM) concentrations and ranges of personal exposure and levels of inflammatory markers from serum samples. GM and ranges presented for all workers, for workers with work tasks inside the production area (exposed group), and for samples from workers mainly working inside the office area (low exposed group).

	All (n = 45)		Exposed group (n = 36)		Low exposed group (n = 9)		Exposure level	Outdoor reference	Unit
	GM	range	GM	range	GM	range	P		
Exposure									
Bacteria (25 °C)	2712	104–9.97 × 10^4	3167	104–9.97 × 10^4	1457	444–3534	0.361	40	CFU/m^3
Anaerobic bacteria (37 °C)	566	5–3.83 × 10^4	784	5–3.83 × 10^4	153	18–956	**0.013**	4	CFU/m^3
Fungi (25 °C)	3737	33–9.45 × 10^4	6802	89–9.45 × 10^4	340	33–1625	**< 0.001**	155	CFU/m^3
Fungi (37 °C)	479	5–3.67 × 10^4	858	5–3.67 × 10^4	46	11–493	**< 0.001**	58	CFU/m^3
Dust	0.13	0.01–0.65	0.16	0.02–0.65	0.05	0.01–0.20	**< 0.001**	0	mg/m^3
Endotoxin (rFC assay)	0.91	0.01–49.49	1.57	0.01–49.49	0.1	0.02–0.89	**< 0.001**		EU/m^3
Endotoxin (LAL assay – estimated concentration)	9.08	0.15–494.89	15.71	0.15–494.89	1.01	0.16–8.91		0.3	EU/m^3
TIP	1.55 × 10^7	1.48 × 10^6 - 9.95 × 10^7	1.73 × 10^7	1.66 × 10^6 - 9.95 × 10^7	1.00 × 10^7	1.48 × 10^6 - 2.82 × 10^7	0.770	7.56 × 10^6	AUC/m^3
Inflammatory markers	All (n = 40)		Exposed group (n = 33)		Low exposed group (n = 7)				
hsCRP	1.65	0.07–19.10	1.82	0.07–19.10	1.20	0.17–5.56	0.661		mg/L
SAA	29.72	1.52–352.82	27.54	1.52–352.82	47.58	8.64–166.65	0.648		mg/L
CC16	6.07	2.57–16.25	6.35	2.88–16.25	5.08	2.57–8.51	0.554		ng/mL

Included is P-values from linear mixed effect models on the association between inflammatory markers and exposure level (exposed vs low exposed). Full model outputs can be found in Table S8-S9.
n = the number of exposure or blood samples, TIP = the total inflammatory potential, hsCRP = high sensitivity C-reactive protein, SAA = serum amyloid A, CC16 = human club cell protein 16.
Outdoor reference values based on Rasmussen et al. (2021).

fungi 37 °C: F = 2.09, df = 1, df_residuals = 41, P = 0.072).

3.2. Inflammatory markers

hsCRP and SAA correlated positively (z = 2.18, P = 0.029, Kendalls' tau = 0.24), whereas hsCRP and CC16 correlated negatively (z = -2.92, P = 0.003, Kendalls' tau = -0.32), and SAA and CC16 did not correlate (P = 0.737).

The levels of hsCRP, SAA, and CC16 did not significantly differ between exposed and low exposed workers (Table 2). The levels of hsCRP were positively associated with the exposure to anaerobic bacteria and fungi (25 °C), whereas it was negatively associated with bacteria (25 °C) and endotoxin exposures (Table 3). Similar to hsCRP, SAA levels were also associated positively with the exposure to anaerobic bacteria and negatively with the exposure to bacteria (25 °C) and endotoxin (Table 3). SAA and CC16 levels were positively associated with dust exposures (Table 3). hsCRP levels increased with age and SAA levels were higher in females (Table 3).

From the eight fungal species selected, exposures to *Penicillium camemberti* and *P. digitatium* were significantly associated to hsCRP levels (Table 4). On the other hand, *Aspergillus niger* and *P. italicum* exposures were negatively associated with hsCRP levels. CC16 levels were negatively associated with *P. digitatum* exposures (Table 4).

3.3. Self-reported health symptoms

The exposed group appeared to display more health symptoms than the low exposed group in the last seven days (Fig. 1) and in the last twelve months (Fig. 2). However, only the difference in symptoms relating to nose problems was significantly different between the two groups. For exposed workers, 6 % answered they often sneezed, 75 % that they rarely sneezed, and 19 % that they never sneezed in the last seven days, whereas for low exposed workers, none answered that they often sneezed, 20 % that they rarely sneezed, and 80 % that they never

Table 3
Exposure measures associated with the concentration of the inflammatory markers high sensitivity C-reactive protein (hsCRP), serum amyloid A (SAA), and human club cell protein 16 (CC16). Degrees of freedom, χ^2-values, significance levels (P), and slope estimates are shown. Significant P-values (P < 0.05) are highlighted in bold.

		hsCRP (mg/L)			SAA (mg/L)			CC16 (ng/mL)		
	df	χ^2	P	Estimate	χ^2	P	Estimate	χ^2	P	Estimate
Bacteria (25 °C)	1	17.40	**< 0.001**	−0.456	24.949	**< 0.001**	−0.688	0.01	0.938	0.003
Anaerobic bacteria (37 °C)	1	11.48	**< 0.001**	0.443	31.049	**< 0.001**	0.906	0.36	0.548	0.024
Fungi (25 °C)	1	9.26	**0.002**	0.416	1.164	0.281	0.184	2.07	0.150	0.074
Fungi (37 °C)	1	0.00	0.966	0.005	3.168	0.075	0.272	2.66	0.103	−0.069
Dust	1	0.04	0.835	0.090	6.071	**0.014**	1.242	7.06	**0.008**	0.460
Endotoxin	1	5.09	**0.024**	−0.273	22.169	**< 0.001**	−0.719	0.72	0.397	−0.030
TIP	1	0.26	0.609	−0.072	1.096	0.295	−0.218	1.32	0.251	−0.077
Sex	1	1.59	0.208	−0.313	7.702	**0.006**	−0.772	0.00	0.950	−0.009
Age	1	11.40	**0.001**	0.020	0.037	0.848	−0.001	0.65	0.418	−0.003
Atopy (yes/no)	1	0.37	0.544	0.217	0.825	0.364	0.354	0.05	0.827	0.049

Table 4
Associations between exposure to eight of the most common fungal species and the inflammatory markers high sensitivity C-reactive protein (hsCRP), serum amyloid A (SAA), and human club cell protein 16 (CC16). Degrees of freedom, χ^2-values, significance levels (P), and slope estimates are shown. Significant P-values ($P < 0.05$) are highlighted in bold.

		hsCRP (mg/L)			SAA (mg/L)			CC16 (ng/mL)		
	df	χ^2	P	Estimate	χ^2	P	Estimate	χ^2	P	Estimate
Aspergillus fumigatus	1	0.12	0.725	−0.019	1.64	0.201	−0.115	1.96	0.162	−0.030
Aspergillus niger	1	6.30	**0.012**	−0.117	0.66	0.417	−0.062	2.03	0.154	0.028
Penicillium brevicompactum	1	0.46	0.498	0.036	1.10	0.294	−0.071	2.06	0.151	−0.034
Penicillium camemberti	1	9.49	**0.002**	0.149	3.19	0.074	0.133	0.12	0.734	0.007
Penicillium commune	1	1.29	0.257	0.070	3.15	0.076	0.137	0.00	0.990	0.000
Penicillium digitatum	1	4.89	**0.027**	0.095	0.49	0.482	0.047	4.09	**0.043**	−0.034
Penicillium italicum	1	7.14	**0.008**	−0.133	0.15	0.694	−0.030	0.83	0.363	0.018
Penicillium roqueforti	1	2.87	0.090	−0.051	2.85	0.091	−0.098	3.27	0.071	−0.022
Fungal concentration	1	4.51	**0.034**	0.254	1.01	0.315	0.207	0.22	0.642	0.023
Sex	1	0.00	0.986	0.005	3.04	0.081	−0.499	0.49	0.483	0.098
Age	1	8.27	**0.004**	0.022	0.98	0.322	0.007	0.69	0.408	−0.003
Atopy (yes/no)	1	0.32	0.571	−0.268	1.29	0.255	−0.554	0.04	0.838	0.047

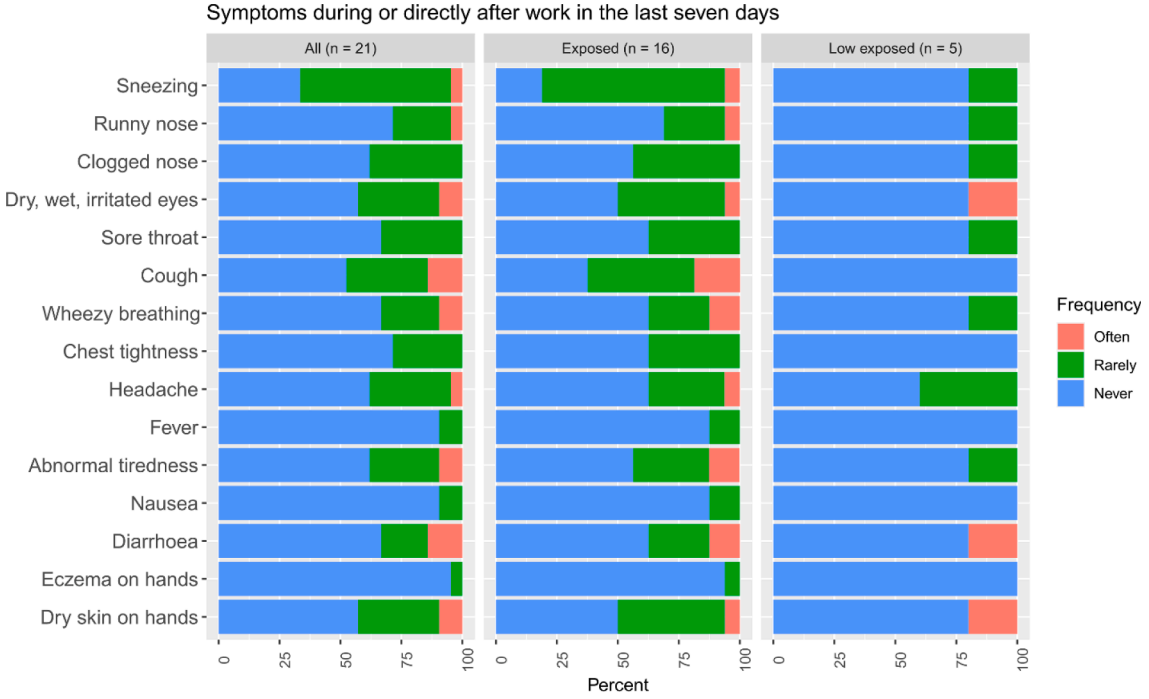

Fig. 1. Self-reported health symptoms displayed in the last seven days.

sneezed; and it was significantly less likely that exposed staff members would answer 'never' to whether they had suffered from sneezing compared to low exposed staff members (estimate −2.21, z = −2.26, P = 0.024, odds ratio = 0.11). Within the last twelve months, 6 % of exposed workers said they often, 88 % said they rarely, and 6 % said they never had symptoms relating to the nose, whereas in low exposed workers none said they often had problems of the nose, 20 % rarely, and 80 % never; and we that it was significantly less likely that the exposed workers would answer 'never' to questions on whether they had nose problems (runny, itchy, clogged nose and sneezing) than low exposed workers (estimate −2.02, z = −2.21, P = 0.027, odds ratio = 0.13). Some of the participants reported that they felt that symptoms displayed within the last twelve months appeared to be related to work inside the production area (nose problems, chest tightness, diarrhoea), whereas other symptoms they felt were related to work inside both production areas, control rooms, and office areas (eye and throat problems).

4. Discussion

We set out to investigate the levels of occupational microbial exposure and how it may influence the health of workers at biowaste pretreatment plants. This was done by determining levels of exposure, the association between exposure and serum levels of inflammatory markers, and self-reported health symptoms.

The group of workers with tasks inside the production area were exposed to higher levels of anaerobic bacteria, fungi, dust, and endotoxin than those working in the office area. The GM exposure to fungi was around 20 times higher in the exposed than the low exposed group and exposures reached levels of 9.5×10^4 CFU/m^3 (geometric mean (GM) 6800 CFU/m^3) and 3.7×10^4 CFU/m^3 (GM 860 CFU/m^3), for fungi incubated at 25 °C and 37 °C, respectively. The fungal levels reported here are comparable to those of workers collecting biowaste and unsorted residual waste (reviewed in Madsen et al., 2021). The exposure ranged among exposed workers, corresponding to the time spent inside the production area vs control room, and workers who mainly work

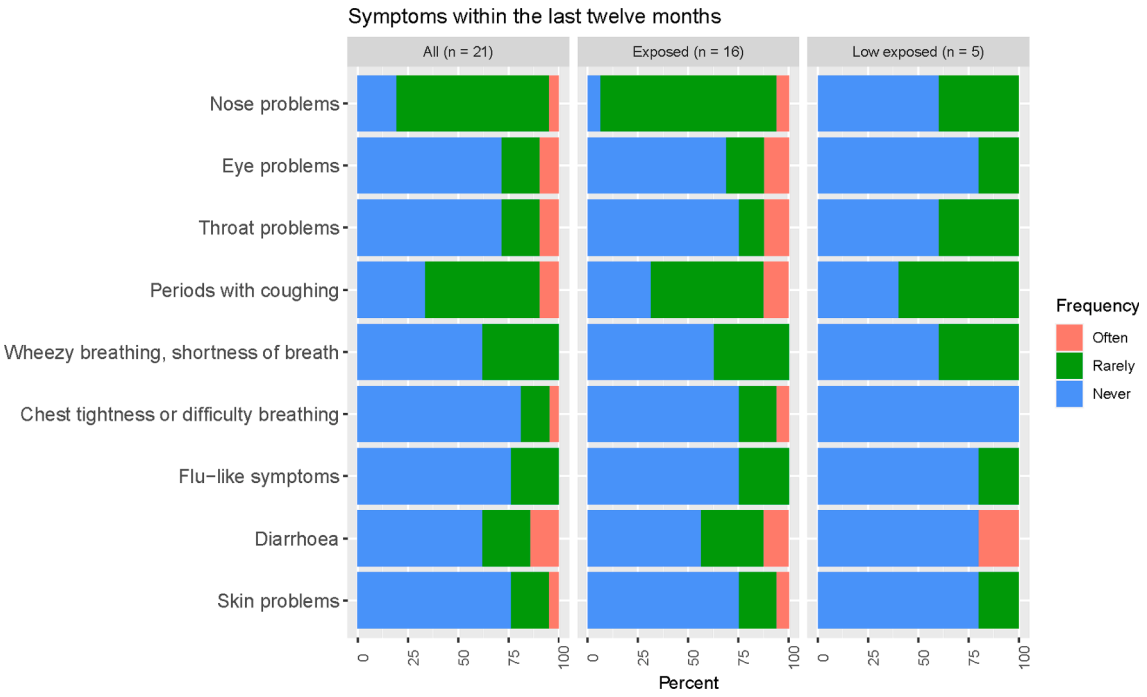

Fig. 2. Self-reported health symptoms displayed in the last twelve months.

inside the production area will therefore be exposed to the highest levels of microorganisms. In a study by Park et al. (2011), in which the authors investigated the exposure to dust, endotoxin, and microorganisms during waste collection and sorting in Seoul, Korea, the authors reported higher exposures during manual waste sorting taking place inside compared to waste collection which takes place outside. Similarly, in an assessment of the bioaerosol exposure in composting facilities, Schlosser et al. (2009) generally found higher microbial exposures in confined areas compared to open air. However, composting materials are typically stored for longer periods with occasional turnings of the composting piles, which may generate large bioaerosol exposures. Biowaste at pretreatment plants is typically processed within a day or two, though it may have been stored for up to two weeks in the waste bin before collection. This may explain the higher fungal exposure levels in composting facilities (e.g. Schlosser et al., 2009) than biowaste pretreatment plants reported here.

Endotoxin and anaerobic bacterial exposures were also higher in samples from the group of workers with tasks inside the production area than those working in the office area, with exposures around five times as high for anaerobic bacteria and 16 times as high for endotoxin. Endotoxin exposures reached levels of almost 50 EU/m^3 using the rFC assay (GM 1.6 EU/m^3). While most studies on occupational exposure have used the LAL assay (see e.g. examples in Duquenne et al., 2012), we here used the rFC assay, as it avoids the use of blood from horseshoe crabs, it reduces interlot variation, and there is no interference from other molecules, such as fungal (1 → 3)-β-d-glucans (Cooper, 1990; Roslansky and Novitsky, 1991; Thorne et al., 2010). Previous results and ours show that the concentrations measured using the two assays correlate well (Madsen et al., 2023; Thorne et al., 2010), though the LAL assay typically measures higher concentrations. This is shown by Madsen et al. (2023), who in a study on wastewater workers, found that LAL assay concentrations of endotoxin were more than four times higher than when measured using the rFC assay. This ratio seems to differ among environments, as we here found that the LAL concentrations measured on a subset of the samples were 10 times as high as concentrations based on the rFC assay. Therefore, when converting the endotoxin concentrations from the rFC assay to an estimate of that measured with the LAL assay, we find concentrations up to almost 500 EU/m^3 and with a GM of 16 EU/m^3, indicating that some workers are exposed to endotoxin levels above the suggested occupational exposure limit of 50 EU/m^3 (Health Council of the Netherlands, 2010). The endotoxin levels found here are higher than in most of the studies reviewed in Madsen et al. (2021) on waste collection workers, where the authors typically find GM or median exposures ranging from 1 to 10 EU/m^3. Compared to composting facilities, which typically find around 200 – 500 EU/m^3 median levels (as reviewed in Schlosser et al., 2009), the biowaste pretreatment plants show lower exposures. The estimated LAL levels found here are, however, comparable to those found by Eriksen et al. (2023), who found GM endotoxin levels of 32 EU/m^3 at manual waste sorting plants and 51 EU/m^3 for fully automatic waste sorting plants. However, we note that the estimated LAL endotoxin concentrations calculated here have to be taken with a measure of caution, as they are calculated estimates.

The GM level of hsCRP was 1.7 mg/L (range 0.1 – 19.1 mg/L), and was for all but one participant below 10 mg/L, which is often used as a clinical reference value (Pagana et al., 2019). The GM level reported here are quite comparable with other studies on occupational exposure, which have found mean hsCRP concentrations of 1.5 mg/L in sewage and compost workers in Norway (Heldal et al., 2016), 1.6 mg/L in Danish greenhouse workers, and 1.9 mg/L (GM) in Danish wastewater workers (Madsen et al., 2023). The GM concentrations of SAA were 29.7 mg/L (range 1.5 – 352.8 mg/L, arithmetic mean 60.6 mg/L) and were higher in women. However, the exposed group mainly consisted of men, whereas the low exposed group was split between men and women, which may bias the results. The levels found is at the high end of what is typically found for healthy adults, which typically range from 1 and 35 mg/L (Ballou et al., 1996; Hijmans and Sipe, 1979; Lannergård et al., 2005; Whicher et al., 1985). Indeed, almost half of the SAA serum samples were above 35 mg/L, showing an inflammatory response. For those that were participating twice, a SAA concentration above 35 mg/L was typically only found in one of the two samples, indicating variable responses over time. In relation to other occupational studies, the levels here are above e.g. wastewater workers (GM 12 mg/L, average 18 mg/L) (Madsen et al., 2023) and greenhouse workers (average 28.3 mg/L, median 15.2 mg/L)(Madsen et al., 2016). The GM concentrations of CC16 were 6.1 ng/mL (range 2.6 – 16.3 ng/mL). These levels are lower

than in e.g. Heldal et al. (2019), who in a study on wastewater workers, found median CC16 concentrations of 27 ng/mL (ranging from 7 to 300 ng/mL) in sewer workers compared to control persons, which had 34 ng/mL (ranging from 5 to 152 ng/mL), but comparable to Widmeier et al. (2007), who found median CC16 levels in waste workers of 9.4 ng/mL (range 4.2–19.1 ng/mL, for workers without asthma), and Heldal et al. (2013) who found mean CC16 levels of 6.4 ng/mL (range 3.0 – 17.1 ng/mL) in sewage workers.

Neither hsCRP, SAA, nor CC16 concentrations differed among exposed and low exposed workers. However, associations between levels of these inflammatory markers and the actual exposure concentrations were found. Thus, a positive association was found between workers' hsCRP and SAA levels and the exposure to anaerobic bacteria (and for hsCRP also for fungi (25 °C)). On the other hand, a negative association was found between hsCRP and SAA and bacteria (25 °C) and endotoxin exposures. The positive association found between hsCRP and SAA levels and anaerobic bacteria and fungi indicates that these microorganisms may induce an inflammatory reaction in workers. To our knowledge, no studies have previously investigated the association between occupational exposure to anaerobic bacteria and hsCRP or SAA. While we found that fungi and hsCRP levels were positively associated, a study in wastewater treatment workers (with low fungal exposure) found the opposite pattern (Madsen et al., 2023). The negative association between hsCRP and endotoxin exposures were surprising, as studies in other environments have found the opposite pattern. The negative association found were based on model estimates after accounting for differences among seasons, waste plants, and physical characteristics of the workers (age, sex, and atopy) and repeated measures on the same person. However, simple correlations without accounting for these factors found the more typical positive correlation between endotoxin exposure and hsCRP and SAA levels (Fig. S1-S2). In studies with several measurements on the same workers, Madsen et al. (2023), found a positive association between hsCRP and SAA and endotoxin after accounting for differences among persons (random effect of person) in a study on wastewater treatment plant workers, and in greenhouse workers, after accounting for both age, sex, asthma, and atopy (Madsen et al., 2016). Differences between this study and studies in other environments with high endotoxin exposure could be due to the use of the rFC endotoxin assay in this study. On the other hand, Madsen et al. (2023) used both assays and while endotoxin measured using the rFC was only nearly significant, the two assays showed the same positive association between endotoxin and inflammatory markers. Nonetheless, similar to the results here, Faruque et al. (2021), in a large cohort study of almost 80 000 persons, found a negative association between CRP levels and estimated occupational exposure to microorganisms, where the exposure was assessed based on current and previous job titles. Due to the relatively low number of participants in the current study, it is difficult to draw too strong conclusions based on the findings of a negative association between exposures and inflammation, and further investigation are needed to examine this further. Nonetheless, the differences found among studies may be due also to differences in microbial species composition, and further studies into the association between bacterial species, the types of endotoxins they produce, and their effect on human health as e.g. measured using inflammatory markers, would be an interesting avenue for future research.

CC16 levels were positively associated with dust exposure, which to some degree matches results from other studies, e.g. Heldal et al. (2013) found that CC16 levels were positively associated with bacterial exposure, though Straumfors et al. (2018) found that CC16 levels did not associate with the exposure measurements, but CC16 levels were higher in the exposed group of workers. We found no associations between CC16 levels and measured exposures. However, Tschopp et al. (2011) found that exposure to splashes and raw sewage was negatively associated with CC16 levels in wastewater workers, though no effect was found due to exposure to garbage dust for waste collectors. Due to the differing findings among studies, it would be interesting to explore the association between the different exposure measures and CC16 levels in a larger group of workers, perhaps with exposure to different microbial communities.

hsCRP, SAA, and CC16 levels were also studied in more detail in relation to the exposure to the eight most common fungal species, where we found a positive association between hsCRP and *Penicillium digitatum*, whereas CC16 and *P. digitatum* showed a negative association. The mycotoxin producing species, *P. digitatum* (Bräse et al., 2009) is a common post harvest plant pathogen, especially on citrus fruit, and it likely explains its high prevalence in the air at the biowaste plants. Infections with *P. digitatum* in humans are very rare with only two cases of pulmonary infections reported (Iturrieta-González et al., 2022; Oshikata et al., 2013). One study found a linear dose response relationship between fungal spore doses of *P. digitatum* and the total inflammatory potential (TIP) of the sample (Lu et al., 2020). For the species *P. commune,* we also found a positive association with SAA concentrations. In a cell based study by Lu et al. (2020), *P. commune* had lower inflammatory potential than some other *Penicillium* species. The species is able to produce mycotoxins (Bräse et al., 2009), but mycotoxins were not measured in this study. Opposite to *P. commune, A. niger* and *P. italicum* were negatively associated with serum levels of hsCRP. Both produce mycotoxins (Bräse et al., 2009), and in the study by Lu et al. (2020), the authors found that *P. italicum* had the highest inflammatory potential (TIP) while *A. niger* was the most cytotoxic. Only few studies have investigated the association between occupational exposure to specific bacterial and fungal species and inflammatory reactions, and further studies in occupational settings as well as controlled exposure studies are needed. Furthermore, to not only explore the live but also dead microorganisms, molecular sequencing approaches could be employed.

Workers were asked to report typical health symptoms displayed within the last seven days and last twelve months of answering the questionnaire. Overall, nose problems and coughing appeared to be the most common symptoms displayed across both groups. Furthermore, workers in the exposed group reported significantly more symptoms of the nose than the low exposed group, both for symptoms in the last seven days and last twelve months. However, only 57 % in the exposed group answered the questionnaire compared to 100 % in the low exposed group. The low response rate in the exposed group means that the health symptoms reported may be seen as a preliminary view of the health symptoms commonly found in this working environment. Nonetheless, the findings of nose symptoms and coughing are in accordance with what has been found in the waste industry (Poole and Basu, 2017; Poulsen et al., 1995; Schantora et al., 2014). This suggests that there might be nose symptoms associated with work in biowaste pretreatment plants, but to corroborate this further, the questionnaire would need to be administered to a larger cohort of workers.

5. Conclusion

We set out to investigate the microbial exposure associated with the recycling of a relatively new waste fraction, pre-sorted biowaste. We found that workers with tasks inside the production area were exposed to higher levels and species of fungi as well as endotoxin compared to the group who mainly have tasks in the office area. The two groups of workers were also exposed to different communities of bacteria and fungi. The endotoxin expoures were higher than what is often found for waste collection workers but lower than what is found for compost workers in other studies. Elevated serum levels of SAA were found in workers, and exposure to anaerobic bacteria, fungi incubated at 25 °C, and dust was associated positively with serum levels of inflammatory markers, indicating that the workers may be negatively affected by the exposures within the plants. Bacteria incubated at 25 °C and endotoxin exposures were surprisingly inversely associated with serum levels of inflammatory markers. Previous studies typically find the opposite pattern, and further studies are needed to explore the negative

association reported here in more detail. Exposed workers reported more health symptoms relating to nose problems than the low exposed group did. All in all, workers working inside the production area of the biowaste pretreatment plants are exposed to high levels of fungi and endotoxin, and this can negatively affect their health.

6. Data availability

Anonymized data is available on request.

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CRediT authorship contribution statement

Pil Uthaug Rasmussen: Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft. **Margit W. Frederiksen:** Data curation, Investigation. **Tanja K. Carøe:** Writing – review & editing. **Anne Mette Madsen:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Anne Mette Madsen reports financial support was provided by The Danish Working Environment Authority.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2023.05.042.

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