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A cohort study of cucumber greenhouse workers' exposure to microorganisms as measured using NGS and MALDI-TOF MS and biomarkers of systemic inflammation

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ABSTRACT

Work in greenhouses entails exposure to airborne fungi and bacteria. The aims of this study are to obtain knowledge about whether exposure to fungal and bacterial genera and species during work in a cucumber greenhouse is affected by work tasks, and whether a cohort of greenhouse workers' serum levels of serum amyloid A (SAA) and C-reactive protein (CRP), biomarkers of systemic inflammation, are associated with this. Data on personal exposure to airborne fungal and bacterial species measured over 4 years as well as serum levels of SAA and CRP sampled over two years were analyzed. For data analysis, the main work tasks were grouped into three different groups, called 'grouped work task'. Microorganisms were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) and next-generation sequencing (NGS). The 'daily exposure' of greenhouse workers' were as follows: 4.8×10^4 CFU bacteria/m^3, 1.4×10^6 CFU fungi/m^3, and 392 EU/m^3 of endotoxin. Workers were exposed to many different microbial species including several species within the genera *Acinetobacter*, *Bacillus*, *Microbacterium*, *Pseudomonas*, *Staphylococcus*, and *Streptomyces*. The genera *Ralstonia* and *Cladosporium* were found in most samples. The exposure levels as well as the microbial composition were associated significantly with grouped work task and season with high exposures during tasks in close contact with mature and old plants and in the autumn. CRP and SAA levels were also associated with exposure level and grouped work tasks. The Shannon-Wiener indices were not different in the 3 'grouped work tasks'. Several specific species including e.g. *Halomonas elongata*, *Stenotrophomonas maltophilia*, *Podosphaera fusca*, and *Wallemia* spp. were found frequently or in high concentrations in the exposures associated with the highest levels of CRP and SAA. The microorganisms *S. maltophilia*, *P. fusca*, and *Wallemia* spp. were also found on the cucumber plant leaves. In conclusion, both exposure level and the species composition seem to have an effect on the serum levels of CRP and SAA of exposed workers. The greenhouse workers were exposed to only a few species characterized as human pathogens.

1. Introduction

Work in greenhouses is associated with elevated exposure to airborne fungi, bacteria, dust, endotoxin (Hansen et al., 2010; Madsen et al., 2009), and plant allergens (Raulf-Heimsoth et al., 2017), and can be associated with development of acute and chronic respiratory symptoms (Zuskin et al., 1993) including occupational rhinitis (Gerth van Wijk et al., 2011), allergy (Thilsing et al., 2012), and asthma (Farruggia et al., 2001; Mons, 2004; Monsó et al., 2002). Elevated levels of the inflammatory marker C-reactive protein (CRP) have been found in serum samples of bioaerosol exposed wastewater treatment plant workers (Heldal et al., 2010; Mattsby et al., 1978; Thorn et al., 2004)

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and farmers (Larsson et al., 1994). For people working in wastewater treatment plants, elevated levels of CRP are associated with impaired lung function (Heldal et al., 2019). Increased serum levels of CRP and serum amyloid A (SAA) have been associated with airway inflammation (Takemura et al., 2006), asthma (Kilic et al., 2012), and chronic obstructive pulmonary disease (Bozinovski et al., 2008). Elevated SAA and CRP levels have also been associated with increased risk of cardiovascular disease in a prospective epidemiological study of nurses (Ridker et al., 2000). *In vitro* and *in vivo* studies with single microbial species have shown that purified human SAA causes cell death of the yeast *Candida albicans* (Gong et al., 2019), and CRP binds to metabolically active conidia of e.g. *Aspergillus fumigatus* (Richardson et al., 1991) and some bacteria (Sproston et al., 2018) and facilitates phagocytosis.

Although work in greenhouses is associated with elevated and highly variable exposure levels to fungi, bacteria, and endotoxin, a previous study showed no clear association between exposure to bacteria and fungi and greenhouse workers' serum levels of CRP and SAA, while a significant association was found for endotoxin exposure and serum levels of CRP and SAA (Madsen et al., 2016b). For waste collection workers exposed to elevated levels of fungi and bacteria, no significant association has been found between exposure and inflammation of the lower airways (Heldal et al., 2003), and no difference in CRP levels of workers in water-damaged schools versus reference schools has been found (Purokivi et al., 2001). Some health effects, such as ODTS (Organic Dust Toxic Syndrome), are associated with short-term exposure to very high levels e.g. 10^5 EU/m^3 and 10^8 bacteria/m^3 (Madsen et al., 2012). Hypersensitivity pneumonitis and reduced lung function can develop after long-term exposure to high concentrations of fungi (10^7 spores/m^3) (Eduard, 2009), and low-grade chronic inflammation seems to be a risk factor for atherosclerosis (Packard et al., 2008; Shah et al., 2019), and therefore both peak and long-term exposures are relevant to investigate.

Recent studies from farm stables (White et al., 2019), cannabis harvest (Green et al., 2018), the drilling waste industry (Daae et al., 2019), grass seed plants (Madsen et al., 2015), dental clinics (Adhikari et al., 2017), and a underground subway (Dybwad et al., 2012) show that the microbial exposure or potential exposure is composed of many different fungal and bacterial species. Gram-negative bacteremia induces higher blood levels of CRP than gram-positive bacteremia (Abe et al., 2010). Studies based on cell lines and mice as model organisms have compared microbial species, and show that different fungal and bacterial species have different inflammatory and cytotoxic potential (Baseler et al., 1983; Fogelmark et al., 1991; Huttunen et al., 2003; Madsen et al., 2020). A literature study reveals that some microbial species are reported to be associated with occupational health problems (Madsen et al., 2020). As examples, cases of hypersensitivity pneumonitis among greenhouse workers resulting from inhalation of *A. niger* (Hamaguchi et al., 2009) and *A. fumigatus* (Yoshida et al., 1993) have been reported. The knowledge about occupational exposure to bacterial and fungal species during work in greenhouses is very limited, but presence and concentrations of specific species or specific species compositions may affect the greenhouse workers' occupational health. A study with grass seed workers indicates that not only the concentration of microorganisms and concentrations of microbial enzymes, but also the microbial composition is different in organic dust causing airway symptoms from that of reference dust (Madsen et al., 2015).

The exposure levels to bioaerosols in cucumber and tomato greenhouses are high and dependent on e.g. the age of plants and work task. The tasks packing of cucumber and clearing of old cucumber plants are associated with exposure to bacteria respectively ~100 and ~10^3 times higher than outdoor reference measurements while exposure to fungi during the same two tasks are ~100 and ~10^4 times higher than outdoor reference measurements (Madsen et al., 2014). We hypothesize that different exposure levels or tasks are associated with different species compositions and that the species composition may influence the health effects of the exposure. At world level the production of cucumbers has increased from 2.2×10^7 tons in 1994 to 7.5×10^7 tons in 2018 (FAO, 2020). In this study, we investigate the exposure to fungal and bacterial species during work in a cucumber greenhouse from 2007 until 2012. As the cucumbers were grown in a soil-less growth medium using a hydroponic system and as the cucumbers were the only crop produced in this greenhouse, we expect that there is one main source of exposure, the cucumber plants. The aims of this study are to obtain knowledge about whether exposure to fungal and bacterial genera and species during work in a cucumber greenhouse is affected by work tasks and season, and to study whether serum levels of SAA and CRP are associated with microbial composition and work task. We also wanted to obtain knowledge about whether the workers were exposed to human pathogenic species. We have chosen the approach to measure exposure during randomly selected days for 5 years to get a measure of long-term exposure and task- or season-related exposure, and to relate this to serum levels of SAA and CRP in blood samples from the workers taken over 2 years. Bioaerosols are characterized using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and next-generation sequencing (NGS).

2. Methods

2.1. The greenhouse, work tasks, and sampling dates

The greenhouse produced cucumbers (*Cucumis sativus* L.) and had up to 20 employees, and we measured exposure of greenhouse workers from 2007 to 2012. The study includes 11 measurements rounds (Tables s1), and in the first weeks with blood sampling the workers received a schedule to fill out about the work tasks they have done every day. An example of a completed schedules can be found in Fig. s1. The work tasks performed were: 'planting young plants', 'distributing biological and chemical pesticides' (hereafter referred to as 'distributing pesticides'), 'harvesting cucumbers', 'pruning off leaves from growing plants', 'severing the plant', which was done just above the roots to dry the plants out, 'driving the forklift or truck', 'packing cucumbers', 'clearing old plants' (hereafter referred to as 'clearing'), which was done when the harvest season was over, and 'removing strings and driplines' while colleagues were clearing the plants. Some of the workers were present on several of the days of exposure assessment.

2.2. Exposure assessment

Personal exposure was measured repeatedly on 31 different workers; in total 81 personal samples were collected. Each workday typically comprised 1 or 2 work tasks for each person, and if a worker had a new work task after the lunchbreak, a new sampler was mounted on the worker. The average sampling time of each sample was 294 min (GM = 275 min, range = 47–443 min). Sampling was done using GSP samplers (Gesamtstaubprobenahme, CIS by BGI) which samples inhalable particles (airflow 3.5 l/min), and which have been described to have a good sampling efficiency (Kenny et al., 1999). The airflow of the samplers was checked every hour. Two GSP samplers were attached to each worker's clothing within the breathing zone; one sampler with a polycarbonate filter (pore size 1 μm, Osmonics Inc., Minnetonka, MN, USA) for culturable counts, MALDI-TOF identification, and sequencing, and one with a Teflon filter (pore size 1 μm; Millipore, Bedford, MA, USA) for endotoxin analysis. Each sampling day, one Teflon filter and one polycarbonate filter were brought as blind filters and subsequently used for endotoxin analysis and culturing of microorganisms, respectively.

Stationary samples were taken in three areas; once in the cucumber-packing hall for 282 min while 'packing cucumbers', once in the greenhouse while 'clearing of old plants' for 262 min, and twice 'harvesting cucumbers' for 188 and 235 min. Outdoor reference samples were taken during 9 of the sampling days (Table s1). Data on exposure to unidentified fungi and bacteria and to endotoxin have been part of published studies (Madsen et al., 2009, 2014).

2.3. Extraction of dust containing microorganisms and endotoxin assay

The day after each sampling, dust collected on filters was extracted. The dust on the Teflon filters was extracted in 6.0 mL pyrogen-free water with 0.05% Tween 20 by orbital shaking (500 rpm) at room temperature for 60 min and centrifuging (1000×*g*) for 15 min. The supernatant was stored at −80 °C until it was used for the endotoxin assay. The supernatants were analyzed for endotoxin using the kinetic Limulus assay as described previously (Madsen et al., 2014).

The dust collected on polycarbonate filters for personal and stationary samples was extracted in 10 mL and 5 mL sterile solutions (0.05% Tween 80, 0.85% NaCl), respectively, by orbital shaking for 15 min (500 rpm) at room temperature. Extracts were aliquoted in 1 mL volumes in cryotubes (Sigma Aldrich, Germany) with 0.5 mL of 85% glycerol. Aliquots were then stored at −80 °C until culturing and/or sequencing.

2.4. Leaves

Two cucumber leaves, an old and a young leaf, were carefully collected from old plants in September 2012. The leaves were placed in clean plastic boxes and transported to the laboratory. The microorganisms on each leaf were extracted in 100 mL extraction solution (0.05% Tween 80, 0.85% NaCl) by orbital shaking for 15 min (500 rpm) at room temperature, and stored as explained for the microorganisms on the polycarbonate filters.

2.5. Next-generation sequencing

DNA was extracted from personal samples ($n = 9$) from one worker, from two stationary samples, and the two leaf samples using the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer's protocol with 400 μL of resuspended settling dust used for extraction. The samples from this particular worker were selected as he performed different work tasks during different days, was part of the study for the entire duration, and gave blood samples. His exposure levels resembled those of his colleagues, Table s2). Extracted DNA was then amplified in a two-step PCR targeting the V4 variable region of the bacterial 16S rRNA gene and the internal transcribed spacer (ITS) region of fungal ribosomal cistron. The primers targeting the V4 region were: 515F: GTGCCAGCMGCCGCGGTAA and 805R: GGACTACNVGGGTWTCTAAT and for the ITS region, primers used were ITS1F: CTTGGTCATTTAGAGGAAGTAA and ITS1R: GCTGCGTTCTTCATCGATGC (Caporaso et al., 2011; Ghannoum et al., 2010). Amplicon library PCR was performed using 10 ng of extracted DNA as template per 25 μL PCR reaction (400 nM of each dNTP, 1.5 mM $MgSO_4$, 2 mU Platinum Taq DNA polymerase High Fidelity, and 1 × Platinum High Fidelity buffer (Thermo Fisher, USA) and 400 nM of barcoded library adapter pair (Illumina, USA). Thermocycler settings included initial denaturation at 95 °C for 2 min followed by 35 cycles of 95 °C for 20 s, 56 °C for 30 s, 72 °C for 60 s and final elongation at 72 °C for 5 min.

All amplicon PCR reactions were run in duplicate and pooled. Amplicon libraries obtained were purified using AMPure XP bead protocol (Beckman Coulter, USA) with the following modifications: The sample:bead ratio was 5:4, and the purified DNA was eluted in 23 μL nuclease-free water. Library concentrations were measured with Quant-iT HS DNA Assay on a Qubit 3.0 fluorometer (Thermo Fisher, USA) and quality checked using D1000 ScreenTapes and a TapeStation 2200 (Agilent, USA). Library PCR was performed by using 2 μL of purified Amplicon PCR product as template per 25 μL PCR reaction (1x PCRBIO Reaction Buffer (PCRBIO, UK), 1U PCRBIO Hifi Polymerase (PCRBIO), and 400 nM of barcoded nextera adaptor mixes (Integrated DNA Technologies, Belgium). Thermocycler settings included initial denaturation at 95 °C for 2 min followed by 8 cycles of 95 °C for 20 s, 55 °C for 30 s, 72 °C for 60 s and final elongation at 72 °C for 5 min. Amplicon library cleanup was performed as described previously.

Samples were pooled in equimolar concentrations, and the library pool was sequenced on the MiSeq platform (Illumina, USA) using reagent kit v3 (2 × 300 PE) with a 20% Phi-X spike-in.

All sequenced sample libraries were processed using the AmpProc pipeline v5.1 (https://github.com/eyashiro/AmpProc) Taxonomy was assigned using SILVA release S132 (Quast et al., 2012) and UNITE (v8.0, 2019-02-02) as reference databases for bacterial and fungal taxonomy respectively (Kõljalg et al., 2014). Taxonomy of chloroplast and mitochondrial zero-radius operational taxonomic units (ZOTUs) were manually curated using BLAST. The obtained raw sequence data is available at the European Nucleotide Archive (ENA) under project accession number PRJEB38777.

2.6. Plating on agar for counting

The dust suspensions from the filters and leaves were plated on dichloran glycerol agar with 0.1 g chloramphenicol/l (DG-18 agar; Thermo Fisher Scientific Oxoid, Basingstoke, UK), and nutrient agar (NA; Thermo Fisher Scientific Oxoid, Basingstoke, UK) plates with actidione (cycloheximide; 50 mg/l; Serva, Germany) for quantification of fungi and bacteria. The plates were incubated at 25 °C for 7 days and were inspected every day and colonies were counted.

2.7. Identification by MALDI-TOF MS

Samples from 5 workers representing different work tasks and seasons, three stationary samples, nine outdoor reference samples (Table s1), and the two leaf samples (pooled) were thawed and plated on DG-18 agar, bird seed agar with 0.1 g chloramphenicol/l (BSA; based on *Guizotia abyssinica* (L.f.) Cass. seeds; also called niger seeds), NA, and SSI agar (SSI Diagnostica, Copenhagen, Denmark) for identification and quantification of fungi and bacteria. The plates were incubated at 25 °C for 14 days and were inspected every day. Data for the two media for fungi and the two media for bacteria are presented together.

Fungi and bacteria were identified using MALDI-TOF MS Biotyper System (Bruker Daltonics, Bremen, Germany) as described previously (Madsen et al., 2015). The MALDI-TOF MS analysis was performed on a Microflex LT mass spectrometer (Bruker Daltonics) using the Bruker Biotyper 3.1 software with the BDAL standard library and filamentous library 1.0. A bacterial test standard (Bruker Daltonics) was used to calibrate the instrument. The data from air samples are presented at species level as time-weighted average exposures in CFU/m^3 air. A fungus with white colonies found repeatedly on DG-18 could not be identified by MALDI-TOF, and it was identified to genus level using microscopy and a key (Domsch et al., 1993).

2.8. Blood sample collection and analysis

Six to 12 blood samples were taken from each worker (1 woman and 7 men), and in total 60 blood samples were collected from September 2010 to September 2012. Blood samples were drawn Monday morning after work had begun and the following Thursday at noon. Blood sampling was only performed if the worker was at work. Blood samples were drawn into plain vacutainer tubes (BD Vacutainer® gold top serum separator), inverted 5 times, left to clot for a minimum of 60 min and centrifuged at 3000 rpm for 15 min to separate serum. Serum was frozen at −80 °C until analysis.

Serum levels of SAA were determined by enzyme-linked immunosorbent assay (ELISA; Invitrogen, CA, USA) according to the manufacturer's specifications. The range of the standard solutions was 0–600 ng/mL. Sensitivity was given by the manufacturer as 4 μg/l. Two controls consisting of recombinant human SAA1 in a tissue culture matrix (19 mg/l and 332 mg/l) were provided with the kit and included as samples (1:1000 dilution) in all runs. The detection limit (LOD) was 4 μg/l; one concentration was below this value and was given a random number

between 1 and 4. Serum levels of CRP were determined by ELISA from IBL International GMBH (Hamburg, Germany) according to the manufacturer's specifications. The range of the standard solutions was 0–10 μg/mL. Sensitivity was given by the manufacturer as 0.02 μg/mL. Two controls (0.5 μg/mL and 5 μg/mL) were prepared in dilution buffer from human CRP control material (code 85/506, NIBSC) and included in all runs. None of the samples had concentrations below the LOD of 0.02 μg/mL. The CRP and SAA data have been part of another study with workers from three commercial greenhouse growers (Madsen, et al., 2016b).

In patients with community-acquired pneumonia, cancer, and non-malignant disease, the half-life of SAA and CRP has been measured to be 1 to 1½ days and 2 days, respectively (Raynes et al., 1983; Takata et al., 2011). CRP has no diurnal variation (Meier-Ewert et al., 2001). In the statistical analysis, we have used the Thursday concentrations and also the average of the Monday and Thursday concentrations – called CRP-weekly or SAA-weekly. None of the SAA and the CRP levels were as high as during e.g. flu (Whicher et al., 1985); none of the workers reported flu or other infections, and none were diagnosed with asthma. Three of the workers were atopic.

2.9. Calculation of numbers of inhaled microorganisms

Cucumber production in the greenhouse was discontinued in the winter with no plants in the greenhouse. To estimate the 'daily exposure' 1) we calculated the GM exposure for each work task based on the measured exposure during the 3 seasons combined with time spent on the work tasks performed on the 10 days with exposure assessment; 2) as 1) but here the GM of exposure is normalized for numbers of samples taken each season; thus if a task was performed during the 3 seasons and the number of samples from each season were not equal one sample taken in e.g. spring counted as much as two samples taken in the autumn etc. In expression 2) also the work task schedules are included in the calculations. During the harvest time, some of the workers had a 6-day workweek. In order to estimate the inhaled number of microorganisms per working life we used the following assumptions based on their working conditions: a ventilation rate of 12 l/min, a working period of 8 h/day for 21.5 days/month, 9 working months/year for 40 years combined with the measured amount of time spent on the eight different main tasks and the exposures during these tasks. This gives an inhalation rate of 4.6×10^4 m^3 air per working life of 40 years. To calculate the inhaled volume during free time, we used the following assumptions for 1) sleeping: a ventilation volume of 6 l/min, for 8 h/day, for 365 days/year for 40 years which is 4.2×10^4 m^3 air; and for 2) free time spent awake, we used an average inhaled volume of 8 l/min for 8 h for 193.5 work days/year and 16 h per day for 166.5 days/year for 40 years, which sum up to 3.0×10^4 m^3 air. Based on previous measurements of indoor and outdoor exposures (Frankel et al., 2012; Madsen, 2006; Rao et al., 1996), we used the following exposure levels for time spent awake but not at work: 1.2 EU/m^3, 100 CFU bacteria/m^3, 200 CFU fungi/m^3, and the following values for sleeping: 0.5 EU/m^3, 300 CFU bacteria/m^3, and 100 CFU fungi/m^3.

2.10. Data treatment

The work tasks were grouped into three main categories where 'A' is no direct contact with the mature cucumber plants, 'B' is mainly contact with the cucumbers post-harvest, and 'C' is in close contact with the mature and the old plants. Thus 'A' covers 'packing cucumbers', 'planting young plants', 'distributing pesticides', and 'driving the forklift or truck' and combinations of these four tasks, while 'B' covers 'packing cucumbers' for 4 h or more combined with less than 2 h of 'harvesting cucumbers' or 'pruning off leaves from growing plants', and 'C' covers 'harvesting cucumbers', or 'pruning leaves from growing plants' for 4 h or more, or 'removing old plants, driplines, or strings' or 'severing the plant' maybe combined with each other or with 'packing cucumbers' for less than 3 h. Numbers of samples from each grouped task can be found in Table s1. The exposure data from 2010 to 2012 were analyzed separately and together with data from 2007 to 2010. Exposure data were log-transformed. Exposure to endotoxin, bacteria, and fungi in groups A, B, and C were compared using General linear model (GLM). The potential effects of grouped work tasks and season on exposure to endotoxin, bacteria, and fungi were studied in a mixed model with random effect of person.

Pearson correlation coefficients (r) on log-transformed levels of SAA, CRP, endotoxin, bacteria, and fungi were calculated. We compared serum samples collected Monday and Thursday for log-transformed levels of SAA and CRP using a paired *t*-test. The associations between log-transformed exposure to endotoxin, bacteria, and fungi, season, and grouped work task and the corresponding log-transformed level of SAA and CRP were estimated in mixed models with random effect of person. In addition, we have used backward stepwise regression analysis. Data were analyzed using SAS (version 9.4).

The NGS and MALDI-TOF data were analyzed using R (3.6.1) (Team, 2013) in RStudio (February 1, 5001; www.rstudio.com), using the package ampvis2 (Andersen, et al., 2018a). Visualizations were generated using ggplot2 (Wickham, 2016). Ordination of the microbial communities were performed using canonical correspondence analysis (CCA) plots where the data was either constrained against the grouped work task, or the task depending on the analysis. Statistical comparisons between clusters was performed using the envfit function as part of the R package vegan (Oksanen et al., 2013) where the environmental fit factor was either the grouped work task or the task. The Shannon-Wiener index values were calculated using the ampvis2 package using the diversity function in the R package vegan.

3. Results

3.1. Exposure levels and inhaled amount

The personal exposure levels were between 595 and 1.1×10^6 CFU bacteria/m^3 (av = 4.8×10^4), 1620 and 8.7×10^7 CFU fungi/m^3 (av = 1.2×10^6), and 47 and 3727 endotoxin units (EU)/m^3 (av = 392) (Table s2). Based on the measurements throughout all years, 'packing cucumbers' was the most time-consuming work task followed by 'harvesting cucumbers'. Based on the measured exposure levels and the described assumptions the workers will on average inhale a total of 2.2×10^9 CFU bacteria, 6.3×10^{10} CFU fungi, and 1.8×10^7 EU (endotoxin) during their working hours through an entire 40 year working life (Table s2).

The concentrations of airborne bacteria, fungi, and endotoxin in reference samples taken outside the greenhouse were between below detection (bd) and 788 CFU bacteria/m^3 (GM = 104), 93 and 2.3×10^4 CFU fungi/m^3 (GM = 811), and for endotoxin between 0.38 and 5.8 EU/m^3 (GM = 1.63).

3.2. Association between grouped work tasks and exposure levels

Schedules of work tasks done each work day for 4 × 14 days periods and notes about work tasks during each day of exposure assessment showed that each worker often had the same two work tasks during all days within the same week. The work tasks were grouped into three main categories, and the category a worker belonged to during the day of exposure measurement was the same as based on the schedules (Table s1). The exposure levels to endotoxin, bacteria, and fungi were different in grouped work tasks A, B, and C with C having the highest exposure, which was seen for all data from 2007 to 2012 analyzed together ($p < 0.0001$ for all exposures) and for data from 2010 to 2012 analyzed separately ($p < 0.0001$ for all exposures) (Fig. s2ab). Statistical analysis (random effect of person) showed a significant association between task and season vs exposure, where grouped work tasks 'C' was associated with the highest exposures to endotoxin, bacteria, and fungi. Concordance was found between data from 2007 to 2012 and all data

Table 1
β-coefficients for the strength of the effects of grouped work tasks and season on the log transformed values of endotoxin, bacteria, and fungi exposure studied for the two fixed factors separately and in a backward stepwise regression.

	Data from persons analyzed for SAA and CRP ($n = 29$)						Data for all persons ($n = 69$)					
Fixed factor	Endotoxin		Bacteria		Fungi		Endotoxin		Bacteria		Fungi	
	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value
Grouped work task A B C	-	**0.026**	-	**0.0002**	-	**0.035**	-	**<0.0001**	-	**<0.0001**	-	**<0.0001**
A	**−0.41**	**0.018**	**−1.17**	**<0.0001**	**−1.93**	**0.0089**	**−0.55**	**<0.0001**	**−0.91**	**<0.0001**	**−1.96**	**<0.0001**
B	**−0.31**	**0.036**	**−0.72**	**0.0015**	−0.67	0.22	**−0.39**	**<0.0001**	**−0.32**	**0.037**	**−1.41**	**<0.0001**
C	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
Season	-	**0.012**	-	**0.0008**	-	**0.0072**	-	**0.015**	-	**0.043**	-	**<0.0001**
Spring	0.016	0.93	−0.30	0.33	−1.31	0.10	−0.27	0.20	−0.67	0.067	−0.58	0.33
Autumn	**0.35**	**0.041**	0.55	0.055	0.48	0.49	**0.21**	**0.046**	−0.18	0.26	**0.80**	**0.0009**
Summer	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
Significant in stepwise												
Grouped work task A B C	–	–	-	**0.0002**	–	–	-	**<0.0001**	-	**<0.0001**	-	**<0.0001**
A	–	–	**−1.17**	**<0.0001**	–	–	**−0.55**	**<0.0001**	**−0.91**	**<0.0001**	**−1.57**	**<0.0001**
B	–	–	**−0.72**	**0.0015**	–	–	**−0.39**	**<0.0001**	**−0.32**	**0.037**	**−1.25**	**<0.0001**
Cr	–	–	Ref.		–	–	Ref.		Ref.		Ref.	
Seasonr	–	–	-	–	-	-	-	-	-	-	-	**0.0009**
Spring	–	–	-	–	-	-	-	-	-	-	−0.47	0.33
Autumn	–	–	-	–	-	-	-	-	-	-	**0.80**	**0.0009**
d Summer	–	–	-	–	-	-	-	-	-	-	Ref	-

If β-coefficients are positive and p-values are smaller than 0.05, there is a significant positive association. Statistically significant values are shown in bold. Grouped work task: 'A' covers 'packing cucumbers', 'planting young plants', 'distributing pesticides', and 'driving the forklift or truck' and combinations of these four tasks, while 'B' covers 'packing cucumbers' for 4 h or more combined with less than 2 h of 'harvesting cucumbers' or 'pruning off leaves from growing plants', and 'C' covers 'harvesting cucumbers', or 'pruning off leaves from growing plants' for 4 h or more, or 'removing old plants, driplines, or strings' or 'severing the plant' maybe combined with each other or with 'packing cucumbers' for less than 3 h.

analyzed together (Table 1).

3.3. Associations between exposure, grouped work tasks, and seasons and serum levels of SAA and CRP

No significant difference was found between Monday morning and Thursday afternoon serum samples concerning content of SAA ($p = 0.71$) and CRP ($p = 0.75$). The Monday-Thursday average levels (called weekly average) of serum SAA ranged between the detection limit (one sample) and 83.1 mg/l (GM = 14.7), and CRP ranged between 0.025 and 8.06 mg/l (GM = 1.01, further data not shown). The weekly average serum levels of SAA correlated significantly with serum levels of weekly average CRP ($r = 0.51$, $p = 0.0028$) and exposure to bacteria ($r = 0.48$, $p = 0.018$), but neither CRP nor SAA correlated significantly with other exposures. Endotoxin exposure correlated significantly with fungal exposure ($r = 0.42$, $p = 0.030$), but not with bacterial exposure ($r = 0.35$, $p = 0.070$), and fungal exposure correlated significantly with bacterial exposure ($r = 0.46$, $p = 0.014$).

When each factor was studied separately, significant associations were found for bacteria, season, and grouped work tasks and SAA-weekly, and for grouped work task and SAA-Thursday. When each factor was studied separately, significant associations were found for grouped work task and CRP-weekly, and for bacteria and grouped work tasks and CRP-Thursday. When all exposures, grouped work tasks, and seasons were studied in one model with random effect of person, significant associations between grouped work tasks vs SAA-weekly, SAA-Thursday, CRP-weekly and CRP-Thursday were found and between season vs CRP-weekly (Table 2).

3.4. Next generation sequencing analysis of exposures

The 16S rRNA gene amplicon sequencing yielded a total of 613,363 reads across 12 samples (average/sample = 32,282 ± 37,169). The ITS region amplicon sequencing yielded a total of 74,443 reads across 9 samples (average/sample = 7444 ± 9386). For fungi the 'planting young plants', 'clearing old plants - stationary' and 'planting young plants' were below the limit of detection. For 16S rRNA gene sequences, cucumber chloroplast sequences were observed in all samples, and for 'planting young plants' and 'distributing pesticides' it was highly abundant (Fig. 1). *Podosphaera fusca* and *Pseudoperonospora* sp. were observed abundantly in a number of samples. Bacterial genera observed ubiquitously across the analyzed samples included *Ralstonia*, *Allo-Neo-Para-Rhizobium*, *Acinetobacter*, *Pseudomonas,* and *Staphylococcus*. The genus *Haemophilus* was found in six air samples, and a representative of the order *Rickettsiales* in eight air samples.

Podosphaera xanthii was abundantly found in four samples: 'harvesting cucumbers' and 'packing cucumbers', as well as in both leaf samples. *Cladosporium sphaerospermum*, the class *Dothideomycetes*, and *Cladosporium* sp., and another representative of the division *Ascomycota* were observed in all samples. Some genera or species were found only on leaves (Fig. 2).

3.5. Bacterial and fungal species – MALDI-TOF MS

MALDI-TOF MS analysis showed a total of 86 different bacterial species in the workers' exposure ($n = 16$), 9 species in the samples collected inside the greenhouse using stationary samplers ($n = 3$), 3 species in the outdoor reference ($n = 9$), and 15 species in the leaf sample (2 leaf samples pooled). The corresponding numbers of detected species for fungi were 15, 5, 3, and 3 species (Table s3 and Table s4, respectively). *Stenotrophomonas maltophilia* and *Bacillus pumilus* were both found in 8 personal samples (Fig. 3) with average exposure levels of 2370 and 534 CFU/m^3, respectively, and *S. maltophilia* was found in very high concentrations on the leaves. *Acinetobacter schindleri*, *Pseudomonas oryzihabitans*, and *Rhizobium radiobacter* were found in 3, 4, and 6 personal samples, respectively, with average exposure levels of 950, 477,

Table 2
β-coefficients for the strength of the effects of log values of exposure levels, season, and task on log values of serum levels of CRP and SAA (mg/l), and significant factors in backward stepwise regression.

Fixed factor		SAA-weekly[x)]		CRP-weekly		SAA-Thursday[o)]		CRP-Thursday	
		β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value
Exposures									
Endotoxin		0.31	0.19	0.34	0.21	0.29	0.24	0.46	0.13
Bacteria		**0.27**	**0.013**	0.19	0.19	0.17	0.20	0.32	0.045
Fungi		0.020	0.69	0.06	0.34	0.048	0.45	0.10	0.18
Grouped work tasks		-	**0.0001**	-	**0.041**	-	**0.050**	–	0.00066
	A	**−0.58**	**<0.0001**	**−0.53**	**0.016**	**−0.51**	**0.019**	−0.63	0.017
	B	**−0.41**	**0.005**	−0.30	0.16	−0.29	0.11	−0.31	0.15
	C	Ref.		Ref.		Ref.		Ref.	
Season		-	**0.040**	-	0.072	–	0.059	–	0.075
	Spring	−0.049	0.85	0.34	0.22	−0.044	0.78	0.34	0.23
	Autumn	0.26	0.19	**0.54**	**0.032**	0.34	0.055	0.54	0.034
	Summer	Ref.		Ref.		Ref.		Ref.	
Significant in stepwise:									
Grouped work tasks		-	**<0.0001**	-	**0.0041**	-	**0.050**	–	0.0066
	A	**−0.58**	**<0.0001**	**−1.12**	**0.0013**	**−0.51**	**0.019**	−0.63	0.0017
	B	**−0.41**	**0.005**	−0.29	0.18	−0.29	0.11	−0.31	0.15
	C	Ref.		Ref.		Ref.		Ref.	
Season		-	-	–	**0.0097**	-	-	–	–
	Spring	-	-	**0.90**	**0.0030**	-	-	–	–
	Autumn	-	-	0.31	0.17	–	–	–	–
	Summer	Ref.		Ref.		Ref.		Ref.	

If β-coefficient are positive and p-values are smaller than 0.05, there is a significant positive association. Statistically significant values are in bold. For grouped work task see also footnote in Table 1 x) Based on average between Monday morning and Thursday afternoon measurement, o) Based on Thursday afternoon measurement.

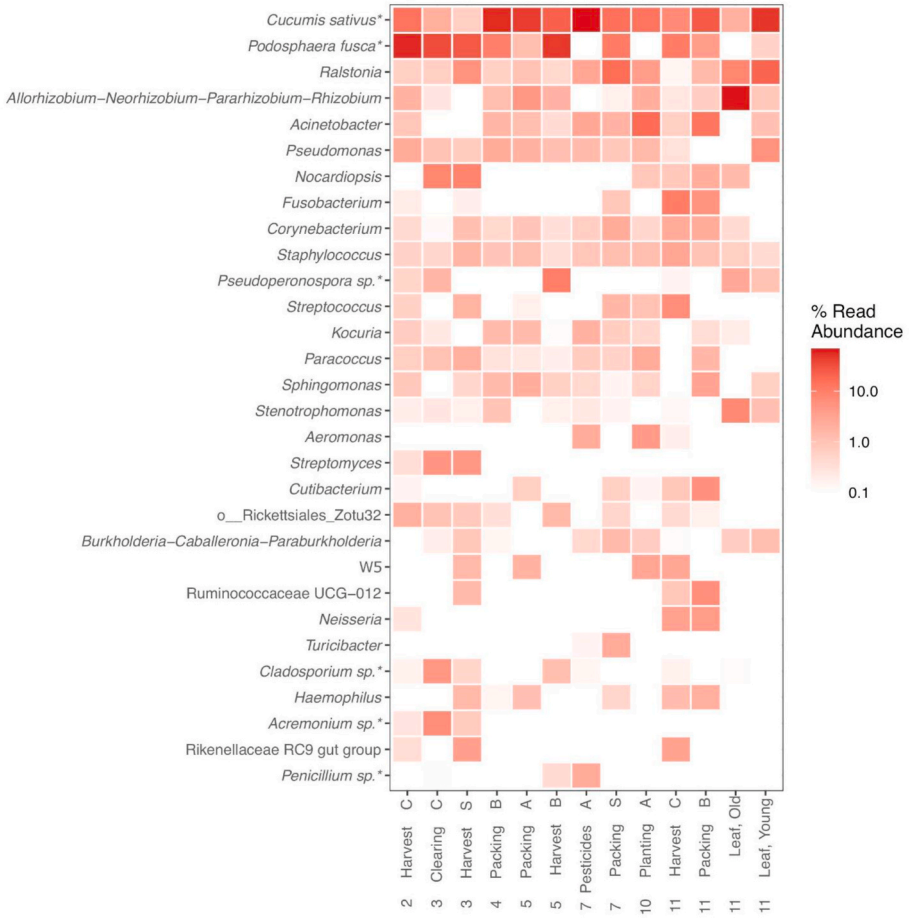

Fig. 1. Heatmap of the 30 most abundant taxa observed in the bacterial community measured using NGS. An asterisk (*) indicates taxonomic groups whose taxonomic assignment was polished using BLAST. The samples are ordered by sampling round from 2 to 11, and letters A, B, and C indicate grouped work task while S indicates samples taken with stationary samplers, see also footnote in Table 1.

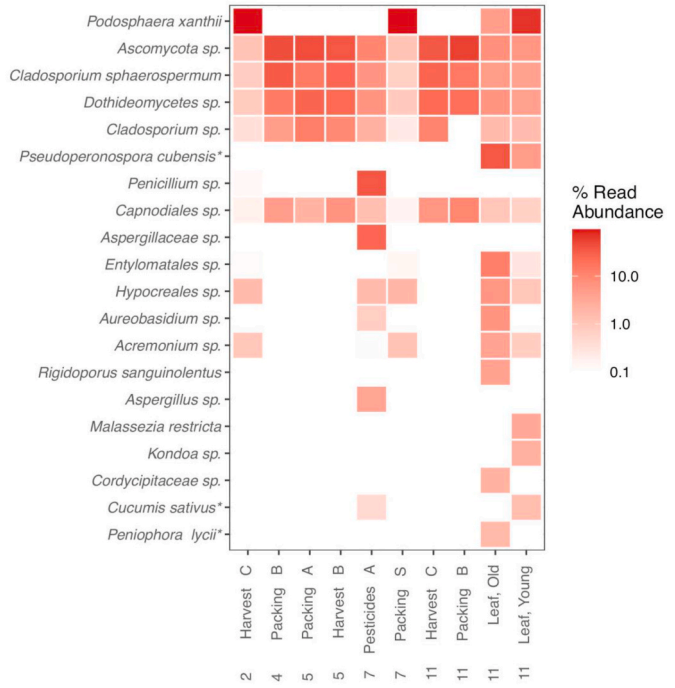

Fig. 2. Heatmap of the 20 most abundant genera (or the highest resolved taxa level) observed in the fungal community measured using NGS. An asterisk (*) indicates groups whose taxonomic assignment was polished using BLAST. The samples are ordered by sampling round from 2 to 11, and the letters A, B, and C indicate grouped work task while S indicates samples taken with stationary samplers, see also footnote in Table 1.

and 1042 CFU/m^3. The exposure to fungi was dominated by *Cladosporium* and *Penicillium* species with *Penicillium brevicompactum* found in most air samples and in high levels. High concentrations of *Wallemia muriae* and *Wallemia sebi* were found on the leaves (Fig. 4).

On one day in the autumn *Cladosporium* sp. and another day in the autumn *Wallemia* sp. were found in concentrations above 3000 CFU/m^3 in the outdoor references.

3.6. Associations between work tasks and NGS data

No significant differences were observed in the richness of the bacterial community, regardless of grouping by work task or grouped work tasks (Fig. s3). The highest overall richness was seen in the samples from 'distributing pesticides' (Fig. s3ac), and grouped work task A (Fig. s3b), respectively. The leaf had a high fungal richness (Fig. s3c). The Shannon – Wiener biodiversity indices for the 16S and ITS regions were not different in the three grouped works tasks (Table 3).

The bacterial community was fully separated by grouped work task in the constrained model ($r^2 = 0.86$, $p <= 0.001$) (Fig. 5a) and the same was found for task ($r^2 = 0.93$, $p = 0.001$). In the fungal community, samples from 'harvesting cucumbers' and 'packing cucumbers' were grouped closely together, while 'distributing pesticides' and 'leaf' samples were grouped individually and far away from the other samples (Fig. 5b). The same tendency was found for grouped work task ($r^2 = 0.64$, $p = 0.078$).

3.7. Associations between work tasks, seasons, and species – MALDI-TOF MS

Microbial (bacterial and fungal data combined) diversity was different for the different work tasks ($r^2 = 0.88$, $p = 0.001$) and grouped work tasks (Fig. 6a, $r^2 = 0.88$, $p < 0.001$), and also when the leaf sample was included in the analysis as a 'task' ($r^2 = 0.93$, $p = 0.001$). The outdoor reference samples were different from the exposures in the greenhouse. Microbial diversity was also significantly different in the different seasons (Fig. 6b).

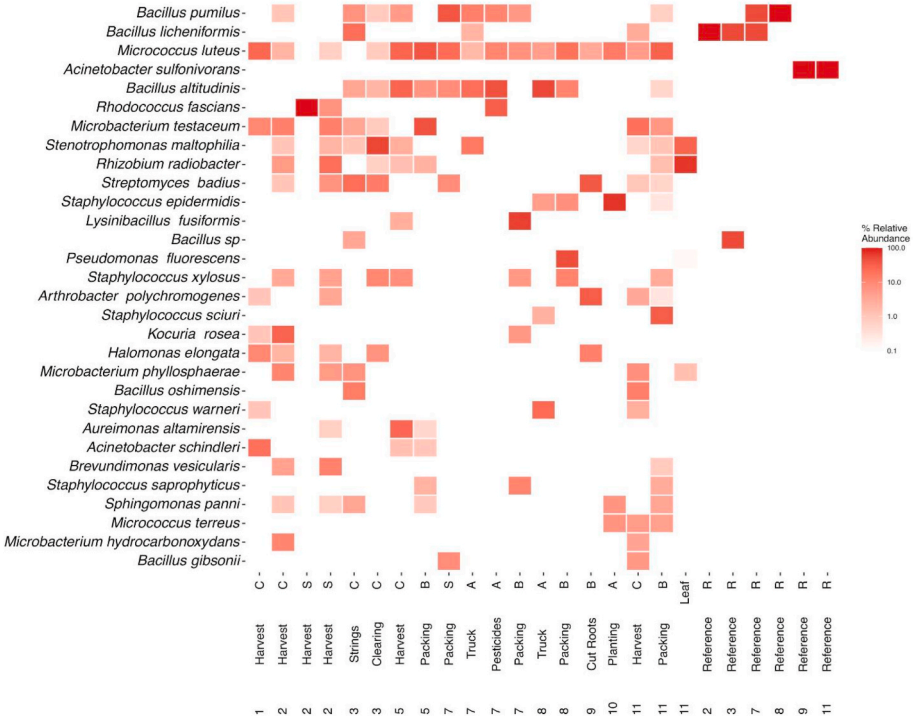

Fig. 3. Heat maps for the Top 30 bacterial species identified by MALDI-TOF MS. The samples are ordered by sampling round from 1 to 11, and the letters A, B, and C indicate grouped work task while S indicates samples taken with stationary samplers, see also footnote in Table 1. The measured exposure level of all species can be found in Table s2.

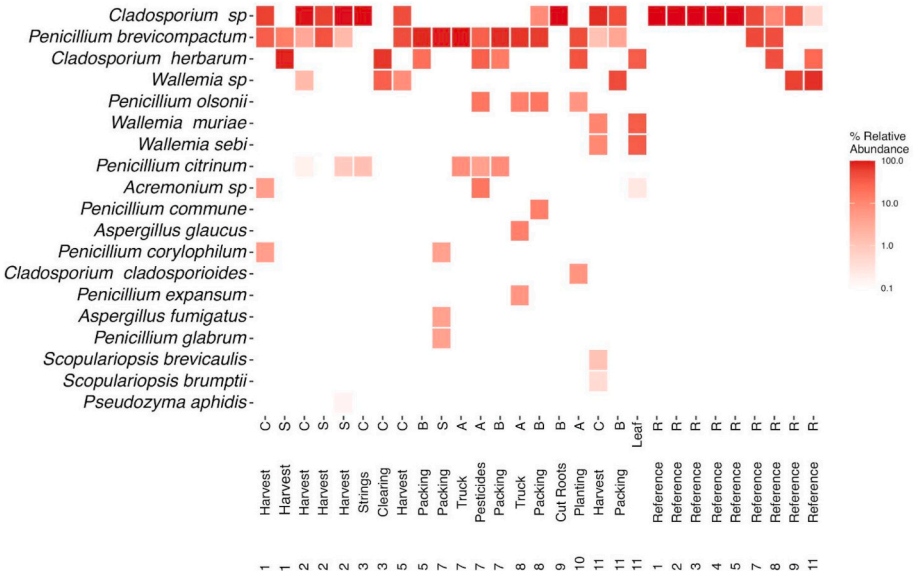

Fig. 4. Heat maps for the 16 fungal species identified by MALDI-TOF MS. The samples are ordered by sampling round from 1 to 11, and the letters A, B, and C indicate grouped work task while S indicates samples taken with stationary samples. The measured exposure level of all species can be found in Table s4.

Table 3
The Shannon–Wiener biodiversity indices of bacteria and fungi (based on ZOTU) in greenhouse workers' exposure divided into grouped work tasks A, B, and C; stationary samples from the packing hall and greenhouse, and two leaf samples.

Grouped work task	A	B	C	Stationary	Old leaf	Young leaf
16S rRNA gene – bacteria						
Shannon-Wiener index (H)	5.95 ± 0.47	5.79 ± 0.4	5.78 ± 0.46	6.37 ± 0.26	5.16	5.81
ITS region fungi						
Shannon-Wiener index (H)	3.18 ± 0.89	2.51 ± 0.18	2.81 ± 0.19	2.93	4.43	3.73

For grouped work task see footnote in Table 1.

4. Discussion

This study aimed at obtaining knowledge about occupational exposure to fungal and bacterial species during work in a cucumber greenhouse in relation to risks associated with daily and working life exposure and to answer two main questions: 1) Is exposure to fungal and bacterial species during work in a cucumber greenhouse affected by the season and grouped work tasks?, and 2) are cucumber workers' serum levels of SAA and CRP associated with the microbial composition of their exposure? The answer to the first question is yes. As for the second question, this study found that the microbial composition in the workers' exposure was associated with grouped work tasks and that CRP and SAA levels were significantly associated with grouped work task. Consequently, it is likely that both exposure level and the species composition of the exposure affect the serum levels of CRP and SAA. In the following, this will be discussed further along with an evaluation of the risks associated with exposure and lifelong exposures.

4.1. Association between microbial composition, work tasks, seasons, and SAA and CRP levels

The microbial composition as measured using MALDI-TOF MS and exposure levels showed seasonality. The measured CRP also showed seasonality, and both CRP levels and exposure to fungi and endotoxin were highest in autumn. SAA also tended to be highest in autumn. However, in general both exposure and inflammatory markers were associated strongest with grouped work task. Thus, exposure levels to endotoxin, bacteria, and fungi were associated with grouped work tasks with the highest levels in grouped work task C and the lowest in grouped work task A. In a previous study, workers were in another greenhouse also exposed to higher endotoxin (Madsen et al., 2009) and fungal (Hansen et al., 2012) levels during removing old cucumber plants than during harvest. In the present study, also the highest levels of SAA and CRP were found for grouped work task C. Grouped work task A included packing of the harvested cucumbers which was done in a hall separated from the greenhouses by a sliding door, but by the same workers who performed all of the other tasks. Grouped work task A also included driving the forklift or truck which was done in the greenhouse and packing hall while other workers harvested cucumbers, packed cucumbers or removed old plants. We have previously shown how exposures in relation to tasks belonging to grouped work tasks C could be reduced i.e. by removing fresh plants instead of dried plants (Madsen et al., 2014). It is possible that such exposure reductions would be paralleled by reduced CRP and SAA levels.

We measured serum levels of SAA and CRP as biomarkers of systemic inflammation, and as elevated levels of CRP and SAA are found for subjects with airway inflammation (e.g. (Heldal et al., 2019; Kilic et al., 2012; Takemura et al., 2006). On one hand the greenhouse workers' serum levels of CRP (GM = 1.01 mg/l) were similar to levels previously reported for adult occupants in a wood smoke impacted community (1.00 ± 0.78 mg CRP/l) in Canada (Allen et al., 2011), for young healthy Danish individuals (GM = 1.2 mg/l) (Bräuner et al., 2008), and young and healthy subjects participating in a course to become firefighters (mean = 1.0 mg/l) (Andersen et al., 2018b), and in the lower end of what has been found for workers in a pulp and paper mill (1.3 and 1.5 mg/l) (Westberg et al., 2019). On the other hand the workers' serum level of SAA (GM = 14.7 mg/l) was higher than median levels of SAA in healthy young (2.29 mg/l), middle-aged (2.47 mg/l), and aged (3.66 mg/l) Swedish adults (Lannergård et al., 2005), and than of workers in a pulp and paper mill (1.7 and 2.3 mg/l) (Westberg et al., 2019). The greenhouse workers were physically active during the whole workday, and regular physical activity has been shown to lower CRP levels (Kasapis et al., 2005), and this may be one of the reasons as to why CRP levels seem to increase with increasing occupational exposure and still being within a normal range.

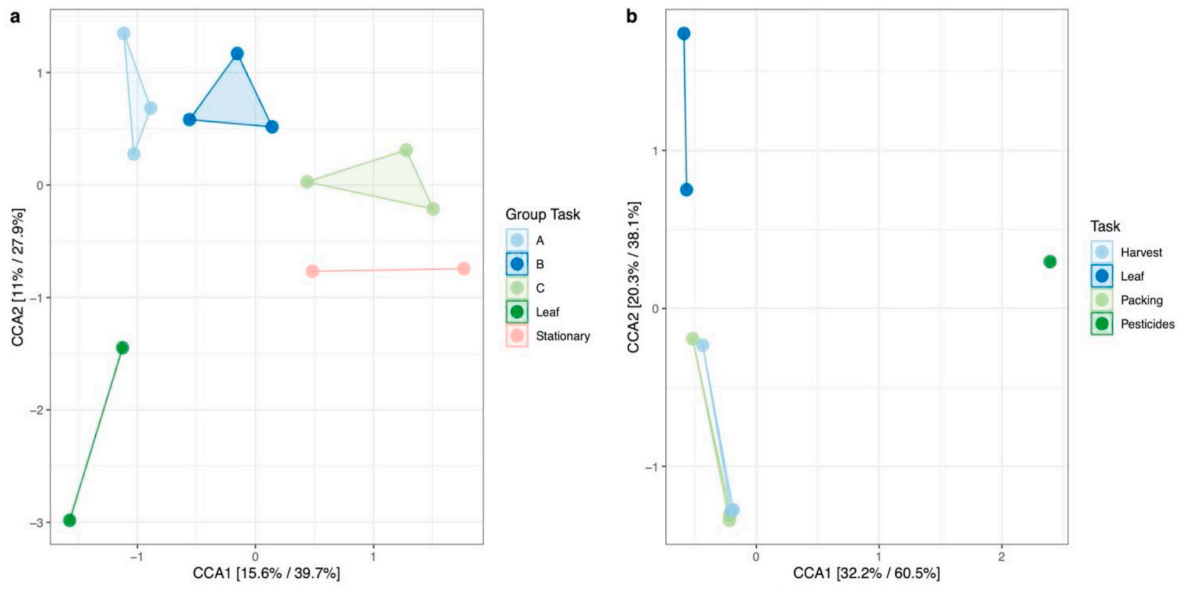

Fig. 5. ab. Canonical correspondence analysis of the bacterial communities ($r^2 = 0.94$, $p < 0.0001$) constrained by grouped work task (a), and fungal communities by task (b) ($r^2 = 0.87$, $p = 0.004$) as measured using NGS. Samples are coloured by grouped work task (a) or task (b) and a polygon is drawn around samples representing the same grouped work task or task.

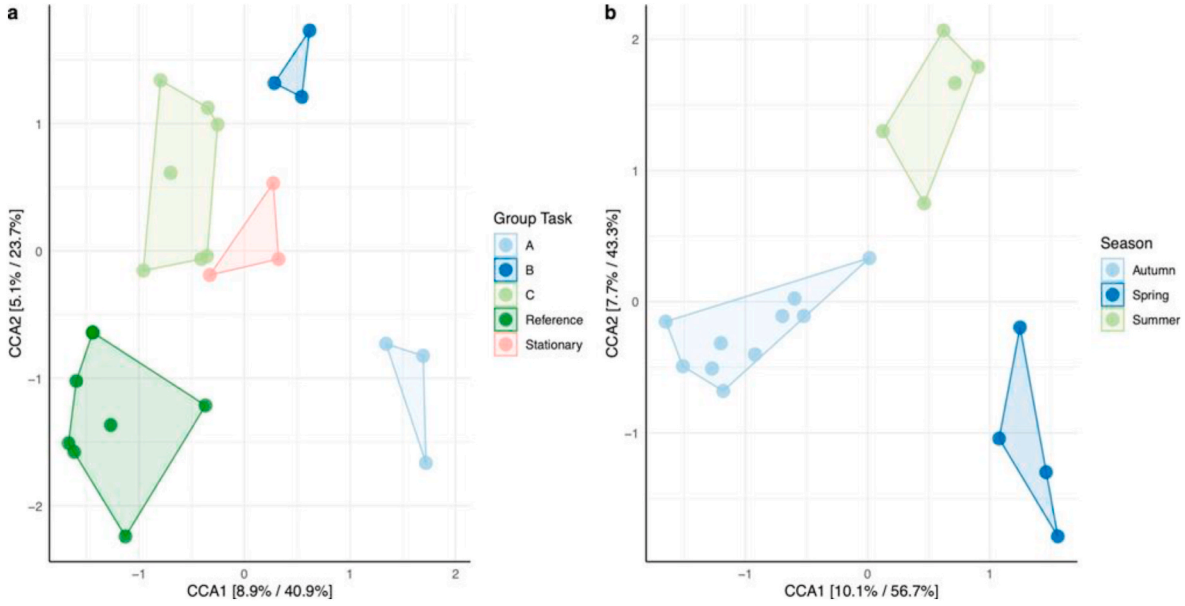

Fig. 6. ab. Canonical Correspondence Analysis (CCA) plot of the combined data for bacteria and fungi identified by MALDI-TOF MS, constrained by the grouped work task (A, B, C see also footnote in Table 1), outdoor references, and stationary samples ($r^2 = 0.86$, $p < 0.0001$ (a), and constrained by season ($r^2 = 0.85$, $p < 0.0001$) (b).

As serum levels of CRP and SAA are affected by several factors (Gabay et al., 1999) we expect that the found association between task vs SAA and CRP could only be revealed because we measured repeatedly on the same workers. Similarly, a study with repeated measurements of exposure to airborne particles in a pulp and paper mill showed significant and positive relations between particle exposure and serum levels of CRP and SAA without workers having elevated levels of CRP and SAA (Westberg et al., 2019). In line with this, a recent review study suggests that if measurements of CRP and SAA are included in the diagnosis of diseases with low grade inflammation, they must be observed over time (Yamada, 2005). The absence of difference between serum levels of SAA and CRP Monday morning and Thursday afternoon found in this study is in accordance with what was found for pulp and paper workers (Westberg et al., 2019), and it may be related to the half-lives of CRP and SAA (Raynes et al., 1983; Takata et al., 2011), and to the fact that the workers sometimes worked in the weekends.

Even though fungi and bacteria in general are inflammogenic, and occupational exposure to high concentrations of bioaerosols are associated with symptoms of the airways (Walser et al., 2015) studies of relations between exposure and self-reported symptoms of the airways or eyes, do not always find positive dose-response relationships for exposure-related symptoms e.g. (Eduard et al., 2004; Schlünssen et al., 2011; Straumfors et al., 2016). In this study, only SAA-weekly and CRP-Thursday correlated directly with bacterial exposure. This may partly be due to the different fungal species in the different exposures. Furthermore a study indicates that fungal and endotoxin exposure may

have a protective effect on atopic asthma and at the same time induce non-atopic asthma in farmers (Eduard et al., 2004). In this study, the fungal and bacterial species compositions differed for the three grouped work tasks. Consequently, the species composition, in addition to the exposure level, seems to affect the serum levels of SAA and CRP in cucumber greenhouse workers. A study with grass seed workers has shown that not only are the exposure levels elevated during the exposure causing development of ODTS, there was also a concurrent shift in the microbial species and genus composition of the exposure (Madsen et al., 2015). The Shannon–Wiener biodiversity as measured on NGS data in this study, did not show differences related to the three grouped work tasks, and it will be relevant to study whether this also holds true if a larger number of exposure samples are analyzed by NGS. Also for grass seed dust causing vs not causing ODTS no differences in the Shannon–Wiener biodiversity were found (Madsen et al., 2015).

The Shannon–Wiener biodiversity indices of the bacteria detected in the exposures using NGS were around 6 which seems to be higher than what has previously been measured for airborne bacteria in pig farms (Kraemer et al., 2018). For fungi (ITS), the Shannon–Wiener biodiversity indices for personal exposure in this study is around 3 which is at the level found in indoor air in homes with visible mold and lower than observed in indoor air in homes without visible mold (Sylvain et al., 2019). We do not yet know whether the indexes are relevant in the evaluation of risks associated with workers' exposure.

4.2. Evaluation of occupational health based on bacterial species and exposure level

The knowledge about occupational exposure to bacterial species, as well as the knowledge needed to evaluate the risk posed by the exposure, is limited. None of the bacteria found in the workers' exposure samples are classified as belonging to a Risk group according to the European Risk Classification system (89/391/EEC, D., 2000). However, the species *Acinetobacter schindleri*, *Acinetobacter parvus, Pseudomonas oryzihabitans*, and *Stenotrophomonas maltophilia* are classified in Risk Group 2 according to a German risk classification system (Affairs, 2010). The workers were exposed to very high concentrations of *S. maltophilia* with an average exposure of 3007 CFU/m^3 in group C tasks. This species can cause infections and is intrinsically resistant to several antibiotics (Looney et al., 2009), and it is identified as an emerging, global, opportunistic pathogen (Brooke, 2012). It has been found in high concentrations in drilling waste water (Daae et al., 2019), but we have found no publications concerning health effects related to occupational exposure nor to infections. Workers were also exposed to high concentrations of *Rhizobium radiobacter*; this bacterium has caused e.g. contact lens-related infections (Fenner et al., 2019). These bacteria are gram negative and may be major contributors to the endotoxin measured in the workers' exposure. Other gram-negative bacteria, e.g. *Aureimonas altamirensis* and *Halomonas elongata*, were also found in the samples of workers' exposure. Infections by *Acinetobacter* species are described as an emerging threat to human health (Visca et al., 2011) – but the species found in this study is not mentioned.

The genus *Fusobacterium* was among the dominating genera in some exposures, and this genus contains a Risk Class 2 species (89/391/EEC, D., 2000). *Haemophilus* was found in six samples, and this genus includes commensal organisms, but also significant pathogenic species. *Halomonas elongata* was found repeatedly in the exposure during group C tasks; we have found no reports of health effects of this gram-negative bacterium. *Microbacterium testaceum* was found in high concentrations during grouped work tasks B and C. Other gram-positive species, *Microbacterium hydrocarbonoxydans* and *Microbacterium phyllosphaerae* and several *Bacillus* species were observed in the workers' exposure. These species have neither been described as a human pathogen nor as causing work-related health effects.

4.3. Evaluation of occupational health based on fungal species and exposure level

Fungal genus and species identification has until recently only been done thoroughly in few studies on occupational exposure as there have been no precise and efficient tools to do this. However, it has now become feasible, and this study shows that the cucumber greenhouse workers were exposed to only few fungal genera and species. The workers were exposed to *Cladosporium* spp. on most workdays, with the highest exposures during tasks belonging to group C. *Cladosporium* spp. has previously been found as the dominating fungus in greenhouses (Cosentino et al., 1991; Rodolfi et al., 2003). Samples analyzed for species showed that the greenhouse workers' average exposure to *Cladosporium* spp. was 4×10^5 CFU/m^3. *Cladosporium* is very common in outdoor and home air, but at lower exposure levels than found during work in the greenhouses. It can cause allergy and trigger asthma attacks (Zureik et al., 2002). For outdoors, a spore-level threshold of 3000 spores/m^3 is suggested as the level at which people who are allergic to *Cladosporium* spp. frequently develop allergy symptoms (Bagni et al., 1977; Twaroch et al., 2015). This level was also exceeded during one day in the autumn in the outdoor references.

The samples analyzed for species by MALDI-TOF MS showed that the greenhouse workers' average exposure to *Penicillium* spp. was 7×10^4 CFU/m^3. The species *P. brevicompactum* constituted 99% of the *Penicillium* exposures, while *P. citrinum* was less commonly present and in lower concentrations. For *Penicillium*, 1×10^4–5×10^7 inhaled spores have elicited asthma attacks in sensitised people with mild asthma (Licorish et al., 1985). The species *P. brevicompactum* (Nakagawa-Yoshida et al., 1997) and *P. citrinum* (Yoshikawa et al., 2006) have caused hypersensitivity pneumonitis in farmers, but *P. brevicompactum* is also often present in normal indoor air in homes (Madsen et al., 2016a). The greenhouse workers were exposed to *P. olsonii* during different work tasks belonging to groups A and B, and this species has previously been shown to have a low inflammatory potential compared to *P. brevicompactum* and *P. citrinum* (Madsen et al., 2020). To our knowledge, *P. olsonii* has not been reported to be associated with occupational health problems.

NGS-data showed that the workers were exposed to the obligate plant parasite *Podosphaera fusca*, except on two workdays with tasks belonging to group A. Whether this and the other obligate plant parasites, *Podosphaera xanthii* and *Pseudoperonospora cubensis*, affect workers' health is to our knowledge unknown.

The workers were repeatedly exposed to *Wallemia* including *W. sebi*, which has been reported to cause subcutaneous infections in humans (Guarro et al., 2008). A study found that twenty percent of children express IgE sensitisation to *W. sebi* (Simon-Nobbe et al., 2008). Exposure to *Wallemia* spp. was often found during work task C. The species *Scopulariopsis brevicaulis* and *S. brumptii* were each found once during 'harvesting cucumbers', and both species are reported to cause e.g. onychomycosis, keratitis, and sinusitis (Cuenca-Estrella et al., 2003). *Aspergillus fumigatus* was found in one sample, and the European risk classification system classifies it within Risk Group 2 (89/391/EEC, D., 2000), which contains 'microorganisms that may cause infectious diseases in humans, but are unlikely to spread in the environment and are possible to prevent or treat'. No other Risk Group 2 fungi were found.

4.4. Risk evaluation based on lifelong exposure

Based on data from four years with personal sampling, this study showed a 'daily exposure' level to 4.8×10^4 CFU of bacteria/m^3. The estimate of working life inhalation of bacteria is 2.2×10^9 CFU, which is 100 times higher than the number estimated to be inhaled in the same period outside working hours. We have found no studies to which we could relate this level. The gram-negative bacteria constituted 11% (GM) of all bacteria in the personal exposure, and in total 27 species were identified using MALDI-TOF. Bacteria in indoor air in homes

(Adhikari et al., 2014) and outdoor air (Fykse et al., 2015) are mainly gram positive, and due to the high fraction of gram-negative bacteria for greenhouse workers it is relevant to consider the endotoxin exposure. Based on data from the four years the 'daily exposure' level to endotoxin was 392 EU/m^3 (~2258 EU inhaled/working day and 1.75 × 10^7 EU/per working life). For measurements on pig farmers in the summer 1991 and the winter 1992, the long-term exposure was 105 ng/m^3 (~1050 EU/m^3), and personal exposure was associated significantly with the yearly decline in FEV1 (Forced Expired Volume) (Vogelzang et al., 1998). Thus the long term exposure of pig farmers to endotoxin in the 1990s was higher than the long term exposure found for cucumber workers found in this study. The farmers' exposure to fungi and bacteria was not measured. Inhalation of endotoxin has previously been shown to dose-dependently increase CRP levels in healthy volunteers (Michel et al., 1997). Thus, inhalation of doses of 5 μg LPS (~5 × 10^4 EU) and 50 μg of LPS (~5 × 10^5 EU) by human volunteers induced an increase in CRP to ~4 μg CRP/mL serum and ~30 μg CRP/mL serum, respectively. The lowest and the highest doses correspond to the average inhalation during 1 work month and almost 1 work year in the cucumber greenhouse.

The four years 'daily exposure' level to fungi was 1.4 × 10^6 CFU/m^3 (corresponding to approximately 3 × as many spores/m^3 (Madsen et al., 2014)). The estimated inhaled dose of fungi during an entire, 40-year working life was on average 6.3 × 10^{10} CFU, which is 3000 times higher than the estimated inhaled dose outside working hours in the same time period. An estimate of 1 year geometric mean exposure of farmers was 2 × 10^6 spores/m^3, and the fungal exposure was associated with non-atopic asthma (Eduard et al., 2004). Hypersensitivity pneumonitis and reduced lung function seem according to a review study to develop after long term exposure to high concentrations fungi (10^7 spores/m^3) – although information on long-term exposure is lacking (Eduard, 2009). Cases of hypersensitivity pneumonitis associated with fungal exposure in greenhouses (Hamaguchi et al., 2009) and a peat moss processing plant (Cormier et al., 1998) have been reported. Due to the 'daily exposure' of cucumber greenhouse workers' it is relevant in the future to study the long term health effect of this.

In addition to the long term effect of occupational exposure on the airways, a prospective epidemiological study shows that elevated SAA level is a risk factors for coronary heart disease (Ridker et al., 2000). Thus, a 5-fold increased SAA level was associated with a 3-fold increased risk of coronary heart disease (Ridker et al., 2000), and SAA is causally implicated in atherosclerosis (Saber et al., 2014; Thompson et al., 2018). Consequently, it would be relevant also to study the long term effect of bioaerosol exposure of cucumber workers.

4.5. Cucumber plants as sources of exposure

We only analyzed bacterial and fungal composition from two leaves in this study, thus they may not be representative of the leaves in the entire greenhouse. The bacteria *Microbacterium* spp. and *Pseudomonas* spp. were common on leaves and in the air, but there were differences between airborne and leaf species. The leaves were taken in the autumn, and *Rhizobium radiobacter* and *Stenotrophomonas maltophilia* were found in very high concentrations both on the leaves and in the air. *Sphingomonas panni* and *Cladosporium* were also found in both leaf and personal air samples. Two species of the xerophilic genus *Wallemia*, *W. sebi* and *W. muriae*, were also found repeatedly and sometimes in very high concentrations in the air samples, and they were also found on the cucumber leaves.

Greenhouse workers' exposure (Hansen et al., 2011) or potential exposure (Adhikari et al., 2011) levels to bioaerosols have previously displayed seasonality. In this study we show that also the microbial species composition in workers' exposure is related to season, but it has not been studied whether there is a shift in the microbial composition on a cucumber life by time.

Several plant- and soil-associated bacteria were found in the majority of air samples: *Rhizobium radiobacter*, *Rhodococcus fascians*, *Microbacterium phyllosphaerae*, and *M. testaceum*. The species *S. maltophilia*, found in the autumn and especially during 'removing old plants', has previously been isolated from cucumber roots; it is an endophyte which may promote plant growth, and it has been tested for its capacity as a biocontrol agent against cucumber diseases (Li et al., 2016). Other microorganisms, which can be used as biopesticides, were also found, e.g. *Bacillus thuringiensis*, *Pseudomonas chlororaphis*, *Pseudozyma aphidis*, and *Pseudomonas synxantha*. Some of the found *Penicillium* species have previously been found on cucumber roots (Menzies et al., 2005). Finally, common cucumber pathogens, such as *Podosphaera*, *Pseudoperonospora cubensis*, and *Stemphylium* were found, and these species were not found in outdoor air. This supports what could be expected – that the plants are the main source of workers' exposure.

4.6. Microorganisms identified with MALDI-TOF MS vs NGS

Using two approaches, MALDI-TOF MS and NGS, to identify microorganisms allowed us to benefit from their different advantages. In general, a high degree of accordance was found between the dominating genera detected using the two methods, and examples are: *Acinetobacter*, *Cladosporium, Pseudomonas*, *Rhizobium*, *Staphylococcus, Sphingomonas*, and *Stenotrophomonas*. Other genera, e.g. *Exiguobacterium*, were found in a few samples using both methods. The genus *Scopulariopsis* was only found using MALDI-TOF MS, and *Penicillium* was found in more samples using MALDI-TOF MS than when using the NGS. In a recent study on fungal aerosols from a pig farm, *Scopulariopsis* was also only identified after culturing and MALDI-TOF MS, but not when using NGS (White and Madsen, 2020). Most isolates identified by MALDI-TOF MS were identified to species level while the NGS approach mainly identified organisms to higher taxa levels. While the found genera *Bacillus*, *Corynebacterium*, *Pseudomonas*, *Rhodococcus*, and *Staphylococcus* all contain species which are classified in Risk Class 2 or 3, MALDI-TOF MS analyses revealed that none of the Risk Class 2 or 3 species were present. On the other hand, some species may have lost their culturability, hence *Acinetobacter* was found in most samples using sequencing, while this genus was only found in leaf samples and a single personal sample using MALDI-TOF MS, indicating that it rarely survived aerosolization. Furthermore, fungi from the genus *Podosphaera* are obligate plant parasite, and were found only using NGS.

The NGS data showed that the airborne dust was dominated by chloroplast and mitochondrial DNA from cucumbers, which may have contributed to the amounts of dust measured in previous studies of cucumber greenhouse workers' exposure (Madsen et al., 2009), and together these findings show that a fraction of the airborne dust derives from the plants.

The DNA extraction kit as well as primer choice have an influence on the microbial communities observed after sequencing (Albertsen et al., 2015). While the selection of DNA extraction kit, primers and database affects which species are found using NGS the selection of agar media is a critical step preceding MALDI-TOF analysis which unavoidably limits the measured microbial composition. We used DG-18 and nutrient agar which support growth of many different fungal and bacterial species. To increase the chance of finding other genera and species, we have also used bird seed agar (Denning et al., 1990) and SSI agar (Uhrbrand et al., 2017). However, we mainly found the same species on bird seed agar as on DG-18 agar, and we only found few species on SSI agar (data not shown separately).

5. Conclusions

It is not yet possible to assess the risk associated with exposure to most fungal and bacterial species in this occupational setting except for the risk classified species. The workers were only exposed to few species which are classified in Risk Group 2 according to the European classification system.

Based on data collected over 5 years this study shows that cucumber greenhouse workers are estimated to inhale approximately 170, 100, and 3000 times more endotoxin, bacteria, and fungi, respectively, during work hours compared to the estimate of the exposures during non-working hours. Therefore, it is important to obtain knowledge about whether this in the long term affects the health of the workers. The exposure levels as well as the microbial composition were associated significantly with grouped work tasks. CRP and SAA levels were also associated with exposure levels, and grouped work tasks with highest levels of SAA and CRP during the highest exposures. Consequently, it is likely that both exposure level and the species composition affect the serum levels of CRP and SAA of exposed workers. The Shannon-Wiener index was not different in the different grouped work tasks. The grouped work task causing the highest exposure and the highest levels of CRP and SAA were exposed frequently or to high concentrations of several species including *Aureimonas altamirensis, Halomonas elongata*, *Stenotrophomonas maltophilia*, *Podosphaera fusca* and *Wallemia* spp. It is relevant to obtain more knowledge about how inhalation of these species affects human health.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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