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Pulmonary illness as a consequence of occupational exposure to shrimp shell powder

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ABSTRACT

Objectives: An employee with no prior history of allergy or asthma, experienced respiratory and flu-like symptoms during production of shrimp shell powder in a seafood savory factory in Norway. We aimed to clarify the diagnosis and to identify the cause of the symptoms by specific inhalation challenge (SIC) and by characterizing the powder's biocontaminants, particle size fractions and inflammatory potential.
Methods: Respiratory and immunological responses were measured the day before and after each of four challenges with 20–150 g shrimp shell powder during three consecutive days. The powder was analyzed for endotoxin, microorganisms and particle size fractions by standardized laboratory methods. Total inflammatory potential was quantified by reactive oxygen species (ROS) production in a granulocyte assay.
Results: The patient had elevated IgG, but not IgE, towards shrimp shell powder. 20 min challenge with 150 g shrimp shell powder induced 15% decrease in FVC, 23% decrease in FEV_1 and increased unspecific bronchial reactivity by methacholine. Neutrophils and monocytes increased 84% and 59%, respectively, and the patient experienced temperature increase and flu-like symptoms. The shrimp shell powder contained 1118 endotoxin units/g and bacteria including *Bacillus cereus*, and 57% respirable size fraction when aerosolized. The ROS production was higher for shrimp shell powder than for endotoxin alone.
Conclusions: Endotoxin and other bacterial components combined with a high fraction of respirable dust might be the cause of the symptoms. The patient's characteristics and response to SIC were best compatible with occupational asthma and organic dust toxic syndrome, while hypersensitivity pneumonitis could not be excluded.

1. Introduction

Occupational exposure to bioaerosols may be associated with increased inflammation of the airways. Occupational exposures are often characterized with peak or continuous exposure to much higher levels of contaminants and dust than usually encountered outside the working environment. Thus, occupational bioaerosol exposure may cause severe health effects that may be either temporary or permanent, depending on host factors and the type and duration of exposure. In addition to nonspecific symptoms of mucous membrane irritation, workers may be at risk of pulmonary illnesses such as occupational asthma and bronchitis, organic dust toxic syndrome (ODTS), and hypersensitivity pneumonitis (HP) (Granslo et al., 2009; Madsen et al., 2012; Morell et al., 2011; Raulf et al., 2014; Sigsgaard and Schlunssen, 2004; Smit et al., 2006; Spaan et al., 2006; Villar et al., 2014). The different disease presentations may depend on the site of the dusts' deposition in the respiratory system; bronchitis and asthma are associated with particle deposition in the bronchi and upper airways, while ODTS and HP are typically associated with particles depositing in the alveoli (Raulf et al., 2014).

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Workers in food production, food processing and other industries involving biological agents are typically exposed to bioaerosols with proteins (allergens) from various sources, as well as a range of contaminants including endotoxins, insect fragments, and microorganisms (Abdel Rahman et al., 2012; Basinas et al., 2012; Baur and Bakehe, 2014; Gautrin et al., 2010; Helaskoski et al., 2014; Kogevinas et al., 2007; Malo et al., 1997; Mapp et al., 2005; Thorne et al., 2004). The bioaerosols may lead to diseases such as asthma (e.g. baker's asthma) and HP (e.g. farmer's lung) (Basinas et al., 2012; Baur and Bakehe, 2014; Gautrin et al., 2010; Madsen et al., 2012; Malmberg et al., 1988; Morell et al., 2011; Rylander et al., 1989; Seifert et al., 2003; Thorne et al., 2005; Villar et al., 2014). Chitin is a major component of crustacean shell. Since lack of the chitin degrading enzyme chitinase is associated with an increased risk for asthma (Brinchmann et al., 2011), chitin has been discussed as a causative agent for asthma specifically for workers involved in crustacean processing. Human experiments indicate that chitin might have an immunomodulatory effect, driving the immune system away from a type 2 response, hence, diminishing the tendency to sensitization in the workforce (Sigsgaard et al., 2015). However, with regard to respiratory illness in the working environment, tropomyosin and not chitin appear to be the most likely offending agent (Bonlokke et al., 2012; Cartier et al., 2004; Malo et al., 1997). High levels of aerosolized allergens such as tropomyosin have been described as important risk factor for the development of occupational asthma, as reported from snow crab processing plants (Abdel Rahman et al., 2012; Bonlokke et al., 2012; Cartier et al., 2004; Gautrin et al., 2010). Endotoxin is a constituent of the cell wall of Gram-negative bacteria and are ubiquitous in the environment, but very high exposure (e.g. $>$ 10,000 Endotoxin units (EU)/m^3) has mainly been reported for specific occupational groups, in particular within agriculture and life-stock farming (Basinas et al., 2012, 2013, 2015; Smit et al., 2006; Spaan et al., 2006), and for very specific production works, such as grass seed and bacterial proteins for animal and fish feed (Madsen et al., 2012; Sikkeland et al., 2008) and in cotton and other textile plant manufacturing (Er et al., 2016; Hinson et al., 2014). Exposure to endotoxin is associated with airway symptoms including nasal symptoms, bronchoconstriction and lung function decline, and high exposure levels may cause HP and ODTS (Madsen et al., 2012; Michel, 2000, 1997; Rylander et al., 1989; Sigsgaard et al., 2005; Thorne, 2000; Timm et al., 2009). Long term occupational exposure to high levels of endotoxin may cause permanent lung damage (Skogstad et al., 2012) and specific illnesses such as byssinosis (Er et al., 2016).

In the present study, we investigated one patient referred to our clinic experiencing fever, chills, and respiratory symptoms while working with shrimp shell powder production in a seafood savory factory. The aim of the study was to clarify the diagnosis and to identify the cause of the symptoms by specific inhalation challenge (SIC) and by characterizing the powder's biocontaminants, particle size fractions and inflammatory potential.

2. Material and methods

2.1. Production facility

The production plant is part of a special marine ingredients company producing 100% natural ingredients to the savory and nutritional markets. The plant produces different marine savory seafood ingredients, like seafood and shellfish powders and granules from codfish, arctic fish, shrimp, and shrimp shell. Shrimp powder is produced by cooking, drying and grinding whole shrimps in the production facility in Norway, whereas shrimp shell powder is produced from pre-made granules (coarse powder). The granules originate from dried and grinded shell removed from cooked shrimps. The production plant receives shrimp shell granules from two Canadian and one Norwegian company, which all use the same shrimp species, *Pandalus borealis*. The shrimp shell granules are shipped in "big-bags" to the production facility where they are grinded into powder and packed in smaller bags. The product specification for seafood savory powder in general states that 95% of the product should be below 200 μm, whereas for the shrimp shell powder 85% is below 100 μm, the latter being mainly inhalable. The raw material and end product is tested for microorganisms (total bacteria count, Enterobacteriaceae, *E. coli*, *Salmonella*, mould/yeast) and approved for distribution only if the concentrations are below pre-defined limits set to avoid food-related health hazards. The quality control does not include tests for microbial byproducts, such as endotoxin.

3. Specific inhalation challenge

3.1. Subject

The patient, a 48 year old never-smoking woman, with no prior medical history of allergy or asthma, was referred to Department of Occupational Medicine at Haukeland University Hospital in Bergen, Norway, due to respiratory (dyspnea, cough with phlegm, occasional wheezing) and flu-like symptoms (fever, joint aches, chills, fatigue) during the workdays and in the evenings and nights following work with shrimp shell powder. The patient did not experience symptoms when involved in the production of other marine savory seafood ingredients including cod powder and powder from whole shrimp. The patient reported to have had respiratory and flu-like symptoms from the very first week the company started producing shrimp shell powder (approximately one year prior to referral). All the symptoms got progressively worse during a work week, with some lessening during weekends, but could last as long as three weeks after exposure. Examination prior to SIC, at which time the patient had been out of exposure for several weeks, showed a healthy individual with no observed or reported respiratory symptoms. Forced Volume Capacity (FVC), Forced Volume in 1 second (FEV_1), carbon monoxide diffusing capacity of the lung (DLCO) and fraction of exhaled NO (FeNO) were within reference values and there was no sign of bronchial hyperresponsiveness with a methacholine provocation test ($PC_{20} > 16$). The patient had a total IgE level of 17 kU/L, negative Phadiatop® and food allergy panel, and no elevated serum IgE towards shrimp. She could consume shrimps without having any symptoms.

3.2. Specific inhalation challenge

Specific inhalation challenge was performed in an inhalation chamber (12.8 m^3). On the first day with challenge, the patient was exposed to lactose powder (Lactose monohydrat GPR RECTAPUR (product number: 24945.360), VWR Chemicals, Leuven, Belgium) by dust tipping from one tray to another 30 cm distance from the face for 10 min as a control (placebo) (E_0). In the absence of any significant change in FEV_1 within the next hour, the patient was exposed to 20 g shrimp shell powder granules mixed with 150 g lactose powder for 10 min (E_1). The challenges continued with increasing exposure (increased concentration of shrimp shell powder and increased duration of challenge) over the next two days (Appendix Table A.1).

Shrimp shell powder produced in the factory was used during SIC. For dosage we took into account the endotoxin concentration in the shrimp shell powder and assumed a similar aerosol concentration in the air as previously measured by a direct reading

dust monitor (Dusttrack II Aerosol Monitor 8532, TSI Incorp., Shoreview, MN, US) in the inhalation chamber for similar agents (powders) mixed with lactose. The maximum concentration of endotoxin exposure was estimated to be 64 EU/m^3 (Appendix Table A.1). Compared to high endotoxin levels reported from various occupational settings (e.g. 5800–11,000 EU/m^3 (Skogstad et al., 2012) and 636–16,300 EU/m^3 (Seifert et al., 2003)) we considered the calculated maximum dosage not to be harmful upon SIC challenge.

3.3. Evaluation of clinical response from SIC

Lung function (FEV_1 and FVC), auscultation results, nasal visual analog scale (VAS) scores, and body temperature was recorded before and immediately after each challenge, then every 10 min for the first 60 min and thereafter every hour the rest of the working day. After the patient left the hospital she measured FEV_1 with a hand-held spirometer (Jaeger AM1$^+$, Visays Healthcare GmbH, Hoechberg, Germany) every hour until going to sleep, during the night if she woke up due to respiratory distress, immediately after waking up in the morning and every hour until arriving at the hospital the next day. For asthma, a positive SIC was defined by a fall in $FEV_1 \geq 15\%$ from baseline, according to the consensus statement by the European Respiratory Society's task force (Vandenplas et al., 2014).

3.4. Evaluation of immunological response

Blood samples were collected in the afternoon on the day before the first challenge, post exposure every day with challenges and approximately 24 h after the last challenge. Leukocyte counts and CRP were assessed daily, and a large panel of immunological markers were assessed the day before and after challenge: ECP (eosinophil cationic protein), tryptase, lymphocyte immunophenotyping: CD3 T-cells, CD3/CD4 T-helper cell ratio, CD19 B-cells, CD56 NK-cells, CD4/CD8-ratio; Immunoglobulines: total IgE, IgA, IgM, IgG, IgG subclasses IgG1, IgG2, IgG3, IgG4, and quantification of complement factor C3 and C4.

3.5. IgE and IgG shrimp shell protein-binding

A DC protein assay (BIO-RAD, Richmond, CA, USA) was used to quantify protein concentrations in shrimp shell granules, shrimp shell powder and codfish powder (produced at the same factory and used as control). Protein was extracted from the shrimp shell powder and tested for IgE- and IgG-binding with serum from the patient who underwent the SIC procedure. The proteins which were isolated from shrimp shell powder include tropomyosin (the main shellfish allergen) as well as other proteins (allergens) from shrimp shell. Binding of anti-shrimp IgE (with IgE isolated from sera from patients with verified shrimp allergy) to proteins isolated from the shrimp shell powder verified the presences of shrimp allergens in the powder.

4. Assessment of biocontamination, particle size distributions and inflammatory potential in the shrimp shell powder

4.1. Endotoxin analyses

The concentration of endotoxin was measured in the shrimp shell powder and in the shrimp shell granules (from two different batches) and in codfish powder (used as control substance) by the Kinetic-QCL™ Kinetic Chromogenic LAL Assay at the School of Public Health, Aarhus University, Denmark. The analyses was performed prior to the SIC, with the intention to verify if endotoxin was present and if so, take into account the concentration when planning the amount of shrimp shell powder to be used during challenges.

4.2. Aerosolisation, particle sampling and extraction of particles

The shrimp shell granules and powder from shrimp shell and codfish were aerosolized in order to determine particle size fractions and to generate aerosol samples to be used in cell assays and for cultivation of fungi and bacteria. Details of the aerosolisation and particle sampling (481, 1993) are described in the Appendix.

The dust collected by polycarbonate filters during aerosolisation was extracted by placing filters in 5 ml sterile water solution with 0.0005% Tween 80 for the cell assays and 0.05% Tween 80 for both the endotoxin assay and cultivation of bacteria and fungi. The filters were shaken orbitally for 60 min (500 rpm) at room temperature. The suspension for endotoxin measurement was centrifuged (1000*g*) for 15 min and the supernatant was used for endotoxin analysis.

4.3. Measurement of the total inflammatory potential

The inflammatory effects induced by the various aerosol fractions were quantified by TIP in a granulocyte assay (Timm et al., 2009) at the National Research Centre for the Working Environment, Copenhagen, Denmark. The assay is based on the differentiated HL-60 cell-line which, upon exposure to microbial compounds, will react by producing reactive oxygen species (ROS), quantifiable by a luminol dependent chemiluminometric assay (Timm et al., 2006) (see Appendix for details).

4.4. Quantification of endotoxin, NAGase, and identification of bacteria and fungi in the various particle size fractions

The supernatants from the 18 aerosol samples were analyzed for endotoxin content in duplicate at the National Research Centre for the Working Environment, Copenhagen, Denmark. The kinetic Limulus Amoebocyte Lysate test (Kinetic-QCL endotoxin kit, BioWhittaker, Walkersville, Maryland, USA) was used. A standard curve obtained from an *Escherichia coli* O55:B5 reference endotoxin was used to determine the concentrations in terms of endotoxin units (EU) (10.0 EU = 1.0 ng). The methods used to quantify the activities of the chitinase NAGase and identification of bacteria and fungi in the various particle size fractions are described in the Appendix.

This study was approved by the Regional Committee for Medical and Health Research Ethics (2015/61). The company and the patient consented to publication of the results.

5. Results

5.1. Clinical

At the start of each challenge (except for placebo challenge), the patient experienced a short period of breathlessness and after challenges E_3 and E_4, she also experienced dyspnea. Several hours after challenges the patient experienced cough with phlegm, chills, headache and joint pains; these symptoms were qualitatively similar to, but milder than the symptoms she experienced during work with shrimp shell powder in the factory. She did not experience wheezing from the chest, and there were no wheezing sounds at auscultation at any time during the week with challenges. On the second day with challenge, the patient had an increase in temperature from 36.3 °C (the patient's normal body temperature) before the challenge to 37.5 °C in the evening after

the challenge. Twenty-four hours after the last challenge the patient had no respiratory or other symptoms, as well as normal spirometry, diffusion capacity and FeNO. She repeated spirometry which was normal as was diffusion capacity and FeNO. A borderline positive methacholine provocation test (PC_{20} 13.7 mg/ml) indicated increased bronchial hyperresponsiveness (Crapo et al., 2000).

5.2. Lung function

The day of the first low-dose challenges, a late reaction was indicated with almost 10% lower FEV_1 values after 10–12 h, coinciding with mild symptoms (Fig. 1). Significant acute effects of the shrimp shell powder challenges were observed for FVC with a 15% fall after E_4 (150 g shrimp shell powder for 20 min) and for FEV_1 with a 19% fall from baseline after E_3 (150 g shrimp shell powder for 10 min) and 23% fall in FEV_1 after E_4 (Fig. 1). On both occasions, FVC and FEV_1 fell within a few minutes after the challenge, started to increase again 10 min after the challenges, and then increased gradually during the first hour after challenge but never reached the baseline level from Day 0; the percentage changes of FEV_1 after challenges is shown in Fig. 1.

5.3. Immunology

The neutrophil count increased from 1.9×10^9/L at baseline to 3.5×10^9/L (84% increase) after the second day with challenge. The monocyte count increased from 0.27×10^9/L to 0.43×10^9/L (59% increase), and the lymphocytes count decreased from 2.0×10^9/L to 1.5×10^9/L (25% decrease) after the second day with challenge. The blood cell counts were still higher on the third and last day (after challenge) as compared to baseline, but not as high as the levels measured on the second day with challenge. CRP increased from 1 mg/L to 5 mg/L, whereas the other immunological markers did not change noticeably from before to after challenge (Appendix Table A.2).

5.4. Protein concentration and specific IgE and IgG

The concentration of proteins in the shrimp shell granules and powder was similar (16.2% and 17.4%, respectively) and slightly lower than the protein concentration in the codfish powder (22.0%). Both the granules and the shrimp shell powder contained shrimp-antigen as verified by anti-shrimp IgE from patient sera with verified shrimp-allergy. However, there was no IgE-binding in serum from the SIC-patient, and the conventional shrimp allergen IgE test was also negative, confirming that the patient was not sensitized to tropomyosin or any other shrimp allergens. On the contrary, the patient had 28 times higher titer of IgG towards shrimp shell powder proteins (1.291, Abs 405 nm) as compared to the IgG titer from pooled sera from patients with verified shrimp allergy (0.046, Abs 405 nm).

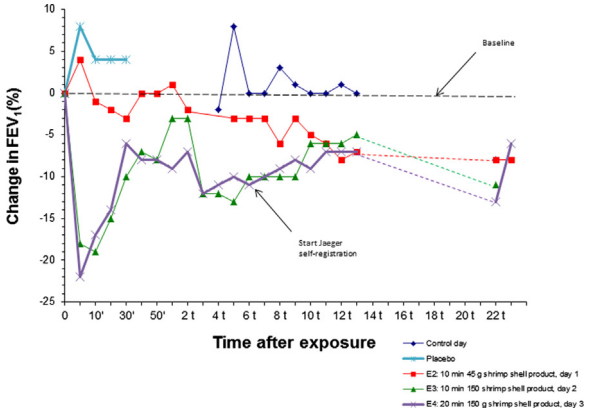

Fig. 1. Change in FEV_1 (% from baseline measured at Day 0) after specific inhalation challenges with placebo and with shrimp shell powder by increasing concentrations and/or increasing challenge duration. "Start Jaeger self-registration" denotes the time when the patient leaves the hospital and continues FEV_1 measures by handhelp device. The first FEV_1 point for the control day starts at 4 h as this first point is measured during the same time of day.

5.5. Particle size distribution

The laboratory-generated aerosols of the shrimp shell powder were measured to have 88.7%, 83.6% and 51.9% particles of inhalable, thoracic and respirable size, respectively. The aerosols from shrimp shell granules consisted of 89.6% inhalable, 86.0% thoracic and 57.0% respirable size particles and for codfish powder 88.8%, 83.3% and 55.3% inhalable, thoracic and respirable size, respectively. The concentration (arbitrary units) as function of aerodynamic diameter (Appendix Fig. A.1) shows that more particles were aerosolised from the two shrimp shell products than from the codfish powder.

5.6. Endotoxin

The endotoxin concentration was higher in the shrimp shell powder (4099 EU/g) than in the shrimp shell granules (1118 EU/g) and the codfish powder (877 EU/g). The concentrations of endotoxin in inhalable dust fractions differed significantly between the three products (shrimp shell granules and powder and codfish powder) (Fig. 2(a)). The concentration of inhalable endotoxin that was aerosolized per minute from 2 g of each product was also significantly different from each other, with the highest release measured from shrimp shell powder (Fig. 2(b)).

5.7. Total inflammatory potential (TIP)

The TIP measured as a cell response to exposure to inhalable, PM_4, and PM_1 dust fractions were significantly different for the three products (all $p < 0.001$ between groups (ANOVA) (Table 1)). The ROS production profile of endotoxin and shrimp shell powder is given in Fig. 3. The inhalable dust fractions showed a dose-response for the two shrimp shell dust samples (data not shown). A large part of the TIP response was caused by the water soluble fraction of the dust. For both the shrimp shell powder and shrimp shell granules, the cell response was higher when stimulated with inhalable compared to PM_4 or PM_1 dust fractions (Table 1). Dilution of the PM_4 fractions of all three products and PM_1 fractions of shrimp shell granules and codfish powder caused increased cell reaction, indicating a cytotoxic response. Thus, the TIP of PM_4 and PM_1 samples increased until the samples were 20 and 10 times diluted, respectively. Spiking with endotoxin caused an increased inflammatory response of both diluted and undiluted samples (data not shown). For the undiluted PM_4 and PM_1 samples the highest TIP was found for shrimp shell powder and the lowest for codfish powder (Table 1).

5.8. Bacteria, NAGase and fungi

A significantly higher concentration ($p < 0.01$) of total bacteria was found in the inhalable dust from shrimp shell granules (135 CFU/mg) compared with inhalable dust from codfish powder (2 CFU/mg) (Table 2). The highest activity of NAGase was found in the PM_4 fractions (Table 2). No significant difference was found in the concentration of total bacteria in the PM_4 fractions of the three products. *Bacillus cereus* constituted the majority of bacteria isolates present in the dust samples and was, with the exception of

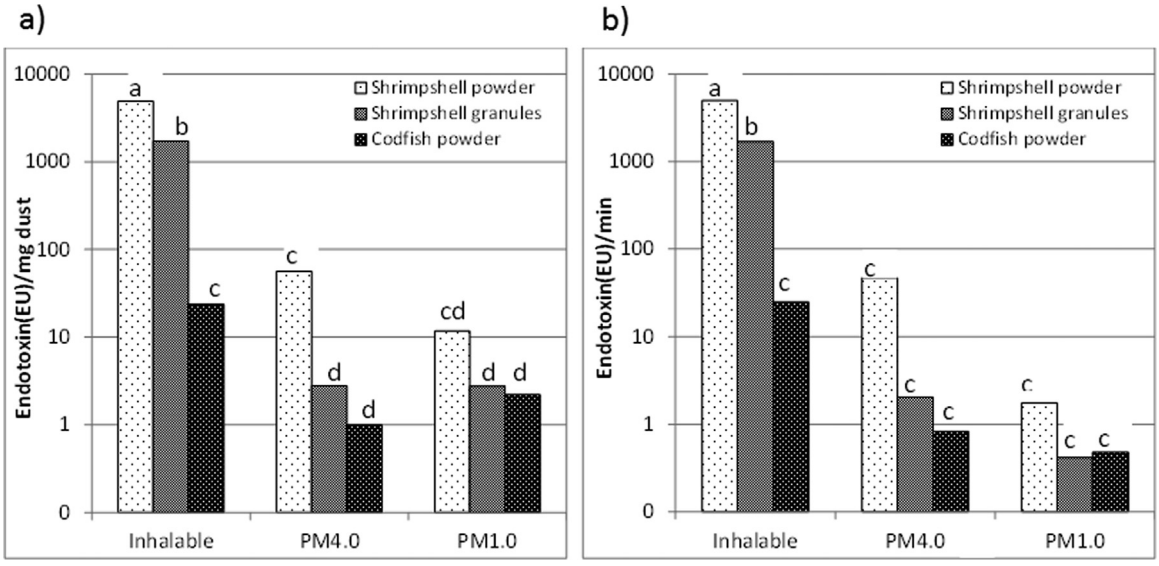

Fig. 2. Average amounts of endotoxin (EU) (a) per mg dust and (b) average amount of endotoxin per minute aerosolized from 2.0 g shrimp shell powder or granules or codfish powder as measured in inhalable, PM_4 or PM_1 dust. Bars followed by the same letter are not statistically significant different.

Table 1
Total Inflammatory Potential (average, *median,* range and n) of dust suspensions and of the water soluble fraction of inhalable, PM_4 and PM_1 dust from shrimp shell and codfish powder.

		Inhalable, undiluted RLU**		PM_4, undiluted RLU		PM_4, diluted 20× RLU	PM_1, undiluted RLU		PM_1, diluted 10× RLU
Shrimp shell powder	Suspension	2.02×10^5 *1.47×10^5* [6.83×10^4–4.40×10^5] 8	a*	4.00×10^4 *4.43×10^4* [2.26×10^4–5.50×10^4] 6	a	[2.65×10^4–3.63×10^4] 2	1.22×10^4 *1.19×10^4* [1.08×10^4–1.41×10^4] 4	a	[3.41×10^4–3.77×10^4] 2
	Soluble	5.76×10^4 *5.79×10^4* [$5.00 \times 10^4 - 6.47 \times 10^4$] 3	b	–	–	–	–	–	–
Shrimp shell granules	Suspension	3.39×10^5 *1.73×10^5* [1.11×10^5–8.93×10^5] 8	a	1.58×10^4 *1.56×10^4* [1.20×10^4–1.94×10^4] 6	b	[3.80×10^4–5.00×10^4] 2	9747 *7524* [5360–1.03×10^4] 4	b	[3.88×10^4–4.53×10^4] 2
	Soluble	1.38×10^5 *1.37×10^5* [9.01×10^4–1.85×10^5] 3	a	–	–	–	–	–	–
Codfish powder	Suspension	5604 *5145* [4.95×10^3–7.05×10^3] 6	c	7649 *8375* [5098–9840] 6	c	[2.39×10^4–2.53×10^4] 2	4379 *4452* [4880–5405] 4	c	[2.05×10^4–3.05×10^4] 2
	Soluble	3722 *3702* [3590–3963] 3	c	–	–	–	–	–	–

* Numbers in the same column followed by the same letter are not significantly different.
** RLU represents accumulated RLU over the 180-min. cell response time. n= total number of samples.

PM_4 fraction of shrimp shell powder, found in all dust samples from the three products. The concentration of *B. cereus* in inhalable dust was found to be significantly higher ($p < 0.05$) in shrimp shell granules than in codfish powder (Table 2). In addition to *B. cereus*, *Micrococcus luteus* was identified in both the inhalable and PM_4 dust fraction of the shrimp shell powder, while *Jeotgalicoccus halotolerans* and *Arthrobacter creatinolyticus* were identified in inhalable dust from shrimp shell granules.

Fungi were detected in the PM_4 fraction of all the 3 products and in the inhalable dust from shrimp shell granules and codfish powder, but not in the inhalable dust from the shrimp shell powder. The concentration of total fungi in the PM_4 fraction was significantly higher ($p < 0.05$) in shrimp shell granules (36 CFU/mg) compared with codfish powder (4 CFU/mg) (Table 2). *Penicillium chrysogenum* was found both in shrimp shell powder (PM_4), shrimp shell granules (PM_4) and codfish powder (inhalable and PM_4), but no significant difference in concentrations were observed (Table 2). *Aspergillus fumigatus* and *Penicillium rugulosum* were identified in the PM_4 fraction of both shrimp shell powder and shrimp shell granules. Moreover, *A. fumigatus* was found in inhalable dust from the shrimp shell granules. Neither *A. fumigatus* nor *P. rugulosum* was found in dust from the codfish powder.

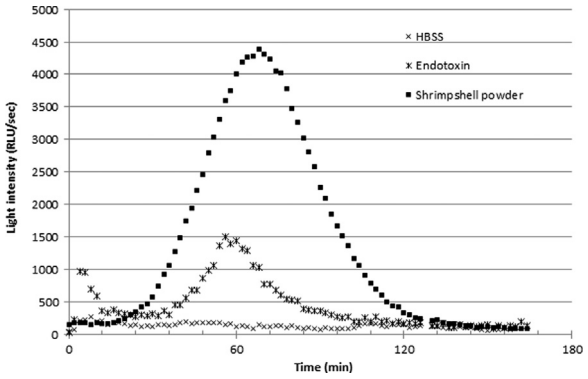

Fig. 3. Example on response profiles in the granulocyte. The granulocyte-like cells were stimulated at time zero with inhalable dust from shrimp shell powder, endotoxin, and a reference (HBSS). Light intensity (in RLU/s) was measured for 1 s every two minutes for 180 min.

6. Discussion

Exposure to shrimp shell powder during SIC caused 15% decrease in FVC, 23% decrease in FEV_1, 1.2 °C rise in body temperature, 84% rise in neutrophils and symptoms of dyspnea, cough with phlegm, chills, headache and joint pains in a women working in a seafood savory factory. The patient had elevated levels of IgG, but not IgE, towards shrimp shell proteins. The shrimp shell powder had high concentrations of endotoxin, and the respirable dust represented more than 50% of the measured inhalable fraction. The patient experienced no symptoms when working with codfish powder; this powder had much lower inflammatory potential than the shrimp shell powder.

Specific inhalation challenge allows for the delivery of defined levels of the suspected agent in a controlled environment to ensure adequate characterization of exposure for a specific duration. The protocol for the present SIC reported in this paper is based on a consensus statement which provides practical recommendations for the use of SIC in the diagnosis of occupational asthma (Vandenplas et al., 2014). With regard to allergic asthma, we did not consider this as a likely diagnosis, as the patient did not have elevated specific IgEs towards tropomyosin or other potential sensitizing allergens isolated from the shrimp shell powder and total IgE of 17 kU/L. Furthermore, the patient had symptoms on the very first week she started working with shrimp shell powder, and thus, no prior allergen sensitization. Also, she did not have any symptoms when working with production of powder based on whole shrimp. Nevertheless, our patient showed acute fall in FEV_1 upon exposure, compatible with a diagnosis of asthma. The

Table 2
Concentrations of microbial species (CFU/mg dust; [Range]) and activity of the enzyme NAGase (pmol/sec/mg dust) in the inhalable and PM_4 fraction of dust from shrimp shell powder, shrimp shell granules and codfish powder.

Microorganism	Geometric mean concentration of aerosolized microorganisms and NAGase					
	Shrimp shell powder		Shrimp shell granules		Codfish powder	
	Inhalable	PM_4	Inhalable	PM_4	Inhalable	PM_4
All bacteria	**1.42×10^1 [2.46×10^0–1.18×10^2] ab[a]**	**3.96×10^0 [BD–1.05×10^1] b**	**1.35×10^2 [4.07×10^1–4.10×10^2] a**	**5.07×10^0 [BD–1.20×10^1] b**	**1.87×10^0 [BD–7.17×10^0] b**	**1.53×10^1 [BD–1.88×10^2] ab**
Arthrobacter creatinolyticus	BD[b]	BD	2.19×10^1 [5.09×10^0–6.36×10^1]	BD	BD	BD
Bacillus cereus	$1.19x10^1$ [BD–1.18×10^2] ab	BD	5.57×10^1 [5.09×10^0–4.05×10^2] a	5.07×10^0 [BD–1.20×10^1] ab	1.87×10^0 [BD–7.17×10^0] b	1.53×10^1 [BD–1.88×10^2] ab
Jeotgalicoccus halotolerans	BD	BD	$4.79x10^0$ [BD–2.55×10^1]	BD	BD	BD
Micrococcus luterus	1.46×10^0 [BD–2.46×10^0] a	3.96×10^0 [BD–1.05×10^1] a	BD	BD	BD	BD
Unidentified bacteria	BD	BD	3.20×10^0 [BD–5.09×10^0]	BD	BD	BD
All Fungi	**BD**	**2.15×10^1 [BD–1.37×10^2] ab**	**3.03×10^0 [BD–2.04×10^1] bc**	**3.63×10^1 [BD–3.61×10^2] a**	**1.42×10^0 [BD–2.39×10^0] c**	**3.94×10^0 [BD–1.05×10^1] bc**
Aspergillus fumigatus	BD	6.73×10^0 [BD–1.90×10^1] ab	$3.03x10^0$ [BD–2.04×10^1] bc	1.93×10^1 [BD–6.01×10^1] a	BD	BD
Penicillium chrysogenum	BD	1.33×10^1 [BD–1.37×10^2] a	BD	1.47×10^1 [BD–3.61×10^2] a	1.42×10^0 [BD–2.39×10^0] a	3.94×10^0 [BD–1.05×10^1] a
Penicillium rugulosum	BD	3.96×10^0 [BD–3.16×10^0] b	BD	1.38×10^1 [BD–7.22×10^0] a	BD	BD
NAGase*	0.062 [0.055–0.067] b	0.23 [0.22–0.24] a	0.056 [0.046–0.069] bc	0.24 [0.24–0.25] a	0.043 [0.41–0.45] c	0.18 [0.17–0.19] a

* A chitinase EC3.2.1.30.

[a] Concentrations in the same row with same letter are not significantly different on a 95% significance level. Where concentrations were below the detect limit a concentration corresponding to half of the detection limit was used for statistical calculation.

[b] BD: Concentration below the detection limit of the method.

methacholine challenge test showed significantly higher bronchial reactivity after SIC, which also could point towards asthma. However, she also showed similar fall in FVC with a FEV_1/FVC ratio >0.7 suggestive of a mild restrictive pattern. Even though she complained of dyspnea upon exposure, she had only rarely experienced wheezing from the chest.

Although there is no general consensus for diagnosing HP caused by occupational exposure with SIC, Munoz and colleagues have published protocols for diagnosing HP by SIC (Munoz et al., 2014). With 15% decrease in FVC, 1.2 °C rise in body temperature, 84% rise in neutrophils and general symptoms, our patient fulfilled the suggested criteria for HP. There was no change in DLCO from before and until 24 h after the last challenge, but DLCO was not measured between these two time points. Further, the patient was not investigated with high resolution CT scan, limiting our ability to verify a diagnosis of HP.

Our patient experienced flu-like symptoms and joint aches, symptoms that have been described repeatedly in young volunteers introduced to farming environment (Malmberg et al., 1988; Sigsgaard et al., 2005; Sigsgaard et al., 2008; Sigsgaard and Schlunssen, 2004). Our investigation of the patient revealed high levels of IgG antibodies, but no IgE, towards shrimp shell proteins. ODTS has a nonimmunologic etiology whereas HP is typically an immunologic disease (Seifert et al., 2003). With HP there is usually prior sensitization to allergens in the organic dust, whereas with ODTS there is no sensitization, and symptoms develop upon first exposure to the organic dust, similar to our patient. Furthermore, neutrophilia which was seen in our patient is typical of ODTS, but not of HP. Bronchoalveolar lavage (BAL) which has been recommended by others (Villar et al., 2014), was not performed. The clinical symptoms, a rise in body temperature, and a decline in FVC and FEV_1 with a restrictive pattern could be compatible with both HP and ODTS.

Diagnostic criteria for separating occupational asthma, ODTS and HP are not clear-cut. It may be difficult to distinguish between asthma and HP as well as between ODTS and acute HP (Seifert et al., 2003). Both ODTS and acute HP are characterized by nonproductive cough and dyspnea (without wheezing), fever, chills and other flu-like symptoms, usually occurring 4–8 h after exposure. The symptoms usually resolve without medication within a few days after removing the exposure. Typically, the patients experience the same symptoms when exposed again to the same agent, possibly with more pronounced symptoms. For HP, patients may upon continued exposure, develop a chronic form of the disease with lung fibrosis. ODTS is described as a self-limiting syndrome, but where some cases may progress to severe acute lung injury (Seifert et al., 2003), and extremely high exposure during ODTS episodes have been attributed to development of asthma (Malmberg et al., 1993; May et al., 1990).

Microbial contaminants, such as endotoxins or bacterial contaminants, or high exposure to respirable particles were considered plausible causes of the condition. Due to the use of these marine savory ingredients in food products, all marine products are routinely tested according to international standards for the presence of microorganisms that serves as indicators of poor hygiene, inadequate processing or post-process contamination of food as well as for the presence of specific pathogens such as Salmonella and *E. coli* which may cause food-borne illnesses. However, the bacterial species, *B. cereus*, were identified in the dust from the shrimp shell products and cod powder, but concentrations were very low and did not exceed the level of 10^5 CFU/g which in most cases have be associated with foodborne emetic and diarrheal illness (Stenfors Arnesen et al., 2008). In Gram-positive bacteria the cell wall consists of peptidoglycans and lipoteichoic acid, which are causes of bacteria-induced inflammation (Laitinen et al., 2001; Moreillon and Majcherczyk, 2003). It has been shown that exposure to non-pathogenic bacteria can cause respiratory symptoms (Haverinen-Shaughnessy et al., 2007; Rastogi et al., 2001), and that spiking of airborne dust with *Bacillus* increase the inflammatory potential of dust samples (Madsen et al., 2014). *B. cereus* is a Gram-positive bacterium that has been found in increased concentrations in dust causing ODTS of grass-seed workers (Madsen et al., 2015). Thus, the presence of *B. cereus* and other Gram-positive bacteria may have contributed to inflammation caused by the shrimp shell products. The presence of *B. cereus* in the marine products is not surprising as this bacteria commonly occurs in foods and can form spores that are extremely resistant to heat, desiccation, disinfectants and irradiation and thus be able to survive in the products even after processing (Eglezos et al., 2010). *A. fumigatus* was also detected in the dust of the shrimp shell products. Spores from *A. fumigatus* may elicit inflammatory responses, and has been shown to cause ODTS among workers in a grass seed plant (Madsen et al., 2012), although in much higher levels than observed in the present study. However, as *A. fumigatus* was only detected in the shrimp shell products and not in the codfish control powder exposure to *A. fumigatus* cannot be excluded as a contributing cause of illness. The chitinase NAGase is a widely distributed enzyme that digests chitin and for microorganisms the presence of chitin induces chitinase production (Chen et al., 2011). Most fungi produce the enzyme and increased exposure to NAGase has been found in an environment where workers developed ODTS (Madsen et al., 2015). However, in the present study, NAGase was not found in increased concentrations in dust from the shrimp shell products compared to the codfish product and thus we do not expect fungi to play an important role in the development of the pulmonary illness.

Endotoxin, which was confirmed in the powder in relatively high levels has been associated with ODTS in occupational settings (Basinas et al., 2012; Madsen et al., 2012). Endotoxin is a lipopolysaccharide from the cell-wall of gram-negative bacteria, while *B. cereus* is a gram-positive bacterium and could therefore not be the source of the endotoxin in the shrimp shell powder. Although endotoxin can probably be released in minute amounts from gram-negative bacteria while they are still growing, endotoxin will for the most part remain associated with the cell wall until disintegration of the organisms. Thus, the endotoxin concentrations that were measured in the shrimp shell powder are likely to be remains of gram-negative bacteria associated with the shrimp before it was boiled and grounded to powder, which would kill the bacteria and causing release of endotoxins, and thereby explaining why the concentration of the endotoxin was high, but not the concentration of bacteria in general. Another potential source which has been suggested to be associated with airway inflammation and respiratory symptoms, are β(1-3)glucans which can be found in fungi, yeast, some bacteria and plant material (Douwes, 2005). We have earlier in a study of fish filleting workers showed how the rinsing water was the component of the work environment with the highest inflammatory potential (IL-8 release) in an ex vivo assay, and significantly higher in filleting workers than in controls there was a release caused by endotoxin and a low effect associated to glucan (Bonlokke et al., 2004), so we expect the effect to be caused by endotoxin in combination with other components in the powder. β(1-3)glucans was not measured in the present study, but given the dryness of the shrimp shell powder and the strict control with bacterial contamination of the products, the biomass of fungi and bacteria, it seems unlikely that β(1-3)glucans are an important contributor to the respiratory problems observed in the present study.

Endotoxin was also detected in the codfish powder; although in lower concentrations compared to the shrimp shell powder and granules. The patient experienced no respiratory or flu-like

symptoms while working with codfish powder or with any of the other marine ingredients produced in the company. The cell assays showed higher TIP in response to exposure to shrimp shell powder and granules compared to codfish powder and also compared to pure endotoxin exposure. Thus, it is possible that endotoxin in combination with the other microbial components (fungi and bacteria) is responsible for the cell responses and the respiratory and clinical symptoms experienced by the patient, or that the high fraction of respirable dust particles and the microbial components and shrimp shell proteins together are responsible for the observed effect.

Taking into consideration that workers are exposed for several hours for several consecutive work shifts during the production of shrimp shell powder, the exposure levels will be much higher than the exposure during the challenges in SIC. This is in line with the milder symptoms experienced with SIC as compared to the symptoms during exposure at work. The patient reported that she had on more than one occasion measured temperatures >39 °C in the evenings after several consecutive days working with shrimp shell powder. This is in contrast to the clinical picture among cotton mill workers, where byssinosis (previously called "Monday Fever") which is caused by endotoxin exposure was first described, and the symptoms in the early stage of the disease were most acute on Mondays (Er et al., 2016). Continued exposure to respirable dust fraction with high concentrations of endotoxin, could in worst case scenario progress to severe acute lung injury (Seifert et al., 2003). Furthermore, long term occupational exposure to high levels of endotoxin have previously been shown to cause reduced lung function even one year after cessation, although those workers had been exposed to much higher levels than we would expect the patient in the present study to experience (Skogstad et al., 2012). Nevertheless, the patient should avoid exposure in order to prevent chronic disease.

In conclusion, exposure to shrimp shell powder during SIC caused a 15% decrease in FVC, a 23% decrease in FEV_1, moderate temperature rise, increase in neutrophils and respiratory and general symptoms similar to those experienced during regular working conditions. IgE mediated mechanisms were not indicated, but high levels of precipitating IgG antibodies were measured in serum from the patient. The ROS production indicated higher inflammatory potential with shrimp shell powder, than with endotoxin alone. The combination of endotoxin and other bacterial components in the shrimp shell powder, the high fraction of respirable dust, flu-like symptoms, and increase in monocytes, suggest a diagnosis of ODTS. However, based on the results from SIC, HP cannot be excluded as a differential diagnosis. Due to the lack of allergic sensitization and sensitization phase, we did not consider allergic asthma as relevant, However, the observed decline in FEV_1 suggest that work related (non-allergic) asthma must be considered and attempted treated. There is a need for better clarification of the diagnostic criteria to help differentiate between asthma, organic dust toxic syndrome and hypersensitivity pneumonitis.

Contributorship statement

RJB, ØS, BEH, JK, TBA, and CS initiated and designed the study, AMM, TS, KU, and TvD contributed with laboratory analysis, RJB drafted the manuscript, and all other authors contributed with interpretation of data for the work and revising the manuscript critically for important intellectual content. All authors approved the final version of the manuscript.

Competing interests

Non declared.

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Ethics

This study was approved by the Regional Committee for Medical and Health Research Ethics (2015/61). The company and the patient consented to publication of the results.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2016.04.033.

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