

Occupational exposure to bioaerosols in Norwegian crab processing plants

Marte R Thomassen^{1,2}, Sandip D Kamath³, Andreas L Lopata³, Anne Mette Madsen⁴, Wijnand Eduard⁵, Berit E Bang^{1,6}, Lisbeth Aasmoe^{1,6}

1 Department of Occupational and Environmental Medicine, University Hospital North Norway, Tromsø, Norway

2 Faculty of Health Sciences, Department of Community Medicine, UiT The Arctic University of Norway, Tromsø, Norway

3 Centre for Biodiscovery and Molecular Development of Therapeutics, James Cook University, Townsville, Queensland, Australia

4 National Research Centre for the Working Environment, Copenhagen, Denmark.

5 Department of Chemical and Biological Work Environment, STAMI National Institute of Occupational Health, Oslo, Norway

6 Faculty of Health Sciences, Department of Medical Biology, UiT The Arctic University of Norway, Tromsø, Norway

Corresponding author: Marte Renate Thomassen, postal address: Department of Occupational and Environmental Medicine, PO Box 16, 9038 Tromsø, Norway, marte.renate.thomassen@unn.no, telephone +47 77 75 42 71

Search terms

1. Occupational exposure to bioaerosols
2. Crab processing
3. Occupational health
4. Bioaerosol quantification
5. Crustacean exposure
6. Protein
7. Tropomyosin
8. Endotoxins
9. NAGase
10. Trypsin

Conflicts of interest: none declared

Abstract

Introduction

Aerosolisation of components when processing king crab (*Paralithodes camtschaticus*) and edible crab (*Cancer pagurus*) may cause occupational health problems when inhaled by workers.

Methods

A cross-sectional study was carried out in three king crab plants and one edible crab plant. Personal exposure measurements were performed throughout work shifts. Air was collected for measurement of tropomyosin, total protein, endotoxin, trypsin and N-acetyl- β -D-glucosaminidase (NAGase). T-tests and ANOVAs were used to compare the levels of exposure in the different plants and areas in the plants.

Results

Total protein and tropomyosin levels were highest in the edible crab plant, endotoxin levels were highest in king crab plants. King crab exposure levels were highest during raw processing. Tropomyosin levels were highest during raw king crab processing with geometric mean (GM) 9.6 ng/m³ vs 2.5 ng/m³ during cooked processing. Conversely, edible crab tropomyosin levels were highest during cooked processing with GM 45.4 ng/m³ vs 8.7 ng/m³ during raw processing. Endotoxin levels were higher in king crab plants than in the edible crab plant with GM=6285.5 endotoxin units (EU)/m³ vs 72 EU/m³. In the edible crab plant, NAGase levels were highest during raw processing with GM=853 pmol4-methylumbelliferone (MU)/m³ vs 422 pmol4-MU/m³ during cooked processing. Trypsin activity was found in both king crab and edible crab plants and levels were higher in raw than cooked processing. Differences in exposure levels between plants and worker groups (raw and cooked processing) were identified

Conclusions

Norwegian crab processing workers are exposed to airborne proteins, tropomyosin, endotoxins, trypsin and NAGase in their breathing zone. Levels vary between worker groups and factories.

Introduction

Workers processing seafood are exposed to bioaerosols in their breathing zone that may be inhaled (Bang et al. 2005; Weytjens et al. 1999; Shiryayeva et al. 2013). Bioaerosols are particulate matter or liquid droplets suspended in air, containing agents of biological origin such as endotoxins, microorganisms, and proteins including high molecular weight allergens and enzymes (Jeebhay et al. 2005; Bang et al. 2005) depending on the type of seafood being processed (Jeebhay et al. 2005; Jeebhay et al. 2001). Several steps involved in processing crab, e.g. butchering, de-gilling, cracking, boiling, and washing/scrubbing, are shown to produce aerosols (Jeebhay et al. 2001; Jeebhay and Cartier 2010; Jeebhay 2011). Previous investigations show that a considerable portion of airborne particulate mass produced during crab processing is within respirable range (Jeebhay et al. 2001). Adverse health effects from bioaerosol exposure (Eduard et al. 2012; Shiryayeva et al. 2013; Watanabe et al. 2016; Gautrin et al. 2010; Jeebhay et al. 2008) such as immunological sensitisation, respiratory symptoms, bronchial hyper-responsiveness and occupational asthma have been found in exposed seafood workers. Sensitisation is documented in workers processing fish, mussels, prawns and crabs. Workers in the shellfish industry seem to have more severe symptoms and are more often sensitised than those in the fish industry (Jeebhay et al. 2001; Jeebhay and Cartier 2010; Lopata and Jeebhay 2013; Cartier et al. 2004; Shiryayeva et al. 2010; Thorn 2001).

Total protein levels are often measured in exposure studies. Such data do not describe the individual protein components, but give an indication of the total load of proteins inhaled by the workers.

Adverse health effects or a dose-response relationship between levels of different components of bioaerosols and respiratory symptoms, allergy, and asthma are found in seafood workers, including snow crab workers (Lopata and Jeebhay 2013; Jeebhay 2011; Jeebhay et al. 2001; Gautrin et al. 2010).

In 2013 Lopata and Jeebhay published a review (Lopata and Jeebhay 2013) listing current seafood allergens, showing tropomyosin from crab meat as one of the main allergens causing occupational allergy. Endotoxin, a well-known pro-inflammatory mediator, has been confirmed to be present in crab processing plants (Neis 2004), but exposure levels have not been measured previously. Chitin, a polymer of β -(1-4)-linked N-acetylglucosamine (NAG) is present in the shell of crustaceans (Chen et

al. 2011). Since NAGase is an enzyme known to digest chitin, it is expected to be present in the bioaerosols during crab processing. NAGase can stimulate exposed cells to secrete inflammatory mediators such as interleukin-8(Allermann, Wilkins, and Madsen 2006). Trypsin is a crucial and widespread digestive enzyme, catalysing protein hydrolysis in vertebrates as well as invertebrates. Trypsin exposure is linked to innate inflammatory responses in cell models and is also suspected to play a role in allergy, including seafood allergies(Sun and Lopata 2010; Baur 2005; Larsen et al. 2008). King crab trypsin has been shown to be a potent stimulator of protease-activated receptors linked to inflammatory reactions in airway cell models(Larsen et al. 2011).

Previous studies on crab processing workers have mainly focused on health outcomes and lack data on bioaerosol exposure levels and content. Variations between processing plants such as building parameters and processing technology (plant effect), may contribute to differences in exposure levels. Technologies used in the current plants ranged from mainly manual work with simple tools to modern, highly automated processing lines which will affect bioaerosol production and content(Moody, Roberts, and Huner 1993; Stellman and Office 1998). Important factors affecting the generation and content of bioaerosols are placements of ventilation systems and the proximity of workers to machines. Other important factors such as shielding of work tasks, areas producing high levels of bioaerosols, use of high pressured air or water hoses(Gaddie et al. 1980) along the process lines and during general cleaning play a major role in workers' exposure. .

The aim of this study was to explore the levels of important agents released during the processing of king crab and edible crab in Norwegian crab processing industries. The knowledge gained may form a necessary base for guidelines and advice to plant management to improve workplace design, procedures, and personal protective equipment. This can be used to reduce bioaerosol exposure and health problems among crab processing workers.

Materials and methods

This study is a cross-sectional study with personal air samples taken from workers' breathing zone in three king crab and one edible crab plant on the North Norwegian coastline between November 2009

and October 2011. Written informed consent was obtained from all participants. The study was approved by the Regional Committee for Medical Research Ethics in Northern Norway, Tromsø.

Plants

A graphical illustration of the plant layouts are presented in figure 1. The three king crab plants in 1A-C and the edible crab plant in 1D.

Edible crab (*Cancer pagurus*) in Norway is processed in a few land-based plants. One well established plant built for edible crab processing was included in this study. The plant layout is optimised for crab processing with separate areas for raw and cooked crab. The processing line is streamlined so workers stand close together. Automated brushes rotating at high speed and transportation belts keep workers stationary. Consequently, their exposure should be quite stable through their shift.

The king crab (*Paralithodes camtschaticus*) industry expanded rapidly in the coastal areas of North-Eastern Norway when commercial fishing started in 2002. Many changes in stock migration and regulated capture quotas have resulted in crab processing often being a side-line for land-based fish processing plants. Since king crab plants are often small and not built for crab processing, the processing equipment is placed in the plant during crab season and removed when the season is over. The processing lines have a compact layout and are not optimally placed. Workers often do different work tasks during their shifts.

Study population

Workers were recruited in collaboration with the plant management. At each plant, randomly selected production workers voluntarily carried sampling equipment during their work shifts. In total, 45 people participated in the study; 33 king crab workers (8 female) and 12 edible crab workers (3 female).

Exposure measurements

Personal exposure measurements were performed throughout the work shifts using SKC Sidekick (SKC Ltd., Dorset, UK) sampling pumps. Air flow rates were set to 3.0 L/min for tropomyosin, total

protein/trypsin and NAGase to ensure enough bioaerosols were collected, and 2.0 L/min for endotoxin as it was recommended by the supplier of the sampling head. Each worker randomly carried a backpack containing two or three pumps each connected to a filter cassette. One filter cassette was analysed for total protein and trypsin, one for tropomyosin and the third for either endotoxin or NAGase. The air samples were collected when the shift ended or after 8 hours if the shift lasted longer. The flow rate for each pump was calibrated before and after collection using Bios Defender 520 (SKC Ltd., Dorset, UK) and the sampling times (minutes) were registered. If the flow deviated with more than 10% from the start of shift, the samples were discarded. The mean air volume collected was 872.4L (range 40 – 1440L) for king crab and 1173L (range 97.5 – 1578L) for edible crab. Filter cassettes were placed in the workers' personal breathing zone. For tropomyosin, total protein/trypsin and NAGase, SureSeal Air Monitoring Cassettes (37 mm, 3-pc, styrene SKC Ltd. UK) were used. For endotoxin, PAS6 cassettes (Personal Air Sampler with 6 mm inlet) manufactured at the STAMI National Institute of Occupational Health in Oslo, Norway were used. Protein/trypsin and tropomyosin samples were collected on polytetrafluoroethylene (PTFE/Teflon) filters on polypropylene support (37 mm, 1.0 µm SKC Ltd. UK), endotoxin samples were collected on glass fibre filters (Whatman GF/A, Kent, Maidstone) and NAGase samples on PC (polycarbonate) filters. At break times, the pumps were stored away from the production area to prevent overestimation of exposure, but kept running to represent the mean exposure during the shift, including time away from the production area. Whether workers were processing raw or cooked crab during the shifts was registered. Within an hour after sampling, the external surfaces of the SureSeal Air Monitoring Cassettes were cleaned with 70% ethanol to remove external contaminations before transport and extraction. Tropomyosin, total protein/trypsin and NAGase cassettes were stored at -20°C in the field and at -70°C in the lab. Endotoxin cassettes were stored at +4°C until extraction. Exposure results were calculated as time weighted averages. Field blanks and analytical blanks were included in all analyses.

During sampling, 78 samples of tropomyosin, 67 samples of total protein, 15 samples of endotoxin, 36 samples of trypsin and 13 samples of NAGase were collected. The difference in sample numbers were due to different technical problems.

Total protein analysis

The protein filters were extracted in 1.0 mL PBS with 0.05% Tween20. Samples were transferred to mini eppendorf tubes and stored at -70°C. Manual QuantiPro BCA Assay Kit (Sigma-Aldrich, St. Louis, USA) was used to determine total proteins in filter extracts ($\mu\text{g/mL}$) in the samples by colourimetric reading of Cu^{1+} -BCA complex in a spectrophotometer at 560 nm. Air levels ($\mu\text{g/m}^3$) was calculated from these values by consideration of air flow through the filters and sampling time. Analyses were performed at the Department of Medical Biology at UiT the Arctic University of Norway.

Tropomyosin analysis

Exposure to the major crab allergen, tropomyosin was quantified by a method described by Kamath et al using a capture ELISA method(Kamath et al. 2014). The filters were extracted in 1.0 mL PBS with 0.5% Tween 20 and NaN_3 for conservation, transferred to mini eppendorf tubes with bovine serum albumin (BSA) and kept frozen until analysed. Purified recombinant tropomyosin was used as the allergen standard. A 96 well high binding Costar microtitre plate (Sigma Aldrich, USA) was coated with anti-tropomyosin antibody in carbonate buffer, pH 9.6 and incubated overnight. After blocking the wells with Pierce Superblock buffer (Thermo Fisher, Melbourne, Australia) the standards, blank and diluted or undiluted filter extracts were added to the wells and incubated at room temperature. After washing with phosphate buffered saline, pH 7.2 with 0.05% Tween-20, the wells were incubated with biotinylated detection antibodies and streptavidin-horse radish peroxidase conjugate (Sigma Aldrich, USA). TMB substrate (BD, USA) was used to visualise antibody binding, reaction was stopped using 1N hydrochloric acid, and measured at 450nm.

Endotoxin analysis

The filters were analysed by a quantitative kinetic chromogenic Limulus Amoebocyte Lysate assay(Douwes et al. 1995) and results are expressed in EU/m^3 (EU = endotoxin units, 10 EU \approx 1 ng). Analyses were performed at the National Institute of Occupational Health in Norway.

NAGase analysis

Measurement of NAGase activity was performed on bioaerosol samples from the edible crab industry. NAGase activity was quantified by adding 4-methylumbelliferyl N-acetyl-B-D-glucosaminide (the MUF-substrate, Sigma, USA) to Tris-maleate buffer (pH 5.0)(Frankel et al. 2012). Aerosol samples were suspended by vortex mixing followed by incubation. The enzymatic reaction was stopped and the supernatant was added to Tris buffer 2.5 M. The solution was added to a black microtiter plate and fluorescence was detected at 446 nm and excitation at 377 nm by a fluorescence spectrometer. NAGase activity was calculated by comparing sample fluorescence with that of a standard curve containing 4-MU (0-7095 pmol/mL). Analyses were performed at the National Research Centre for the Working Environment in Denmark.

Trypsin analysis

Protease activity in filter extract was analysed by zymography. Five μL sample extracts were applied on zymographic gels (Novex® no.EC61752, ThermoFisher Scientific) containing a standard curve (0.014 - 0.228 mU/mL) prepared by dilution of a porcine trypsin stock solution with known enzyme activity. Enzyme activity of the stock solution of porcine trypsin was determined by a serine protease assay where the hydrolysis of a chromogenic substrate (DL-BAPNA, Sigma-Aldrich) was measured spectrophotometrically by the increase in absorbance at 405 nm at room temperature for 10 min. Protease activity was calculated by: $\text{Unit} = \text{dA}/\text{dt} \times 1/(\epsilon \times \text{optical path length}) \times 10^6 \times V_{\text{final}}$, where dA/dt = rate of absorbance change and ϵ = extinction coefficient. In our system ϵ was $8800 \text{ M}^{-1}\text{cm}^{-1}$, optical path length 0,709 cm and V_{final} 250 μL . Trypsin standards and aliquots of filter sample extracts were mixed with loading buffer (Novex®, ThermoFisher Scientific) and subjected to electrophoresis at 20 mA/gel for 2h. Thereafter, the gel was washed with washing buffer followed by overnight incubation in developing buffer at 37°C (Novex®, ThermoFisher Scientific). The gels were stained in 0.2 % Coomassie Brilliant Blue R-250 Dye and the activity of gelatin degrading proteases detected as clear zones against the undigested, stained background. The intensity of bands of porcine trypsin (23 kDa) and corresponding size bands in filter extracts, were quantified using UVP Vision Works LS Image Acquisition and Analysis (UVP, LLC, USA) with I-max (point of maximal intensity)

as quantification parameter. The gelatin –degrading activity was abolished by introduction of the serine protease inhibitor aprotinin. Together with the band size and the confirmed trypsin activity in king crab (Rudenskaya et al. 2000) and liquid samples from king crab industry (own unpublished data), this strongly suggests that the protease activity in this region is due to trypsin.

Statistical analyses

The exposure data showed a lognormal distribution so the natural logarithms of the exposure measurements were used. Exposure data are presented as median, geometric mean (GM) and range. Endotoxin samples below detection limit (1 EU/m³) was set to half the value of the detection limit when incorporated in statistical analyses. Single variables were compared using independent-samples *t* test, ANOVAs for more than two groups. A correlation matrix using Pearson's correlation coefficient was computed to measure any linear relationship between bioaerosol components. Due to a low precision of the zymographic analysis of trypsin, the results are presented using a semi quantitative scale based on the 25 and 75 percentile of positive results: Low \leq 25 percentile; 25 percentile $>$ medium \leq 75 percentile; high $>$ 75 percentile. Mann-Whitney U test was used for comparing the semi quantitative results.

The statistical analyses were done using the IBM SPSS software package, version 22. P-values <0.05 were considered statistically significant.

Results

In the edible crab industry, each worker was handling either raw crab or cooked crab and the results are therefore divided into these two groups. In the king crab plants however, the raw and cooked crab areas were only partly separated, and some workers performed less stationary work during processing such as maintenance, cleaning and truck driving (figure 1). Thus, some workers were exposed to bioaerosols from both raw and cooked king crab. The results are therefore divided into; raw crab, cooked crab, or overlapping work tasks (both raw and cooked crab) during the shift.

The difference in exposure levels between the different work areas in king crab plants was borderline significant ($p=0.06$), with highest levels in raw processing (table 1). Exposure levels to protein were not significantly different, but as with tropomyosin, the exposure levels seemed to be highest in raw crab processing.

For edible crab (table 2), the mean tropomyosin exposure level for workers processing raw crab was significantly lower than for those processing cooked crab. Protein levels were not significantly different. Exposure to endotoxin and NAGase was highest when processing raw crab, but the differences in NAGase levels were not significant. However, endotoxin levels during processing raw crab were significantly higher than levels found during processing cooked edible crab.

Trypsin activity was measured in 36 samples, whereof 23 were positive (table 3). In the present data set, the 25th percentile was 0.16 mU/m³ and the 75th 0.72 mU/m³. In the edible crab industry, there was no trypsin activity in the air samples collected from workers handling cooked crab. In the king crab industry the levels of activity were significantly higher when processing raw crab compared to cooked crab.

When comparing exposure levels for workers handling cooked king crab and edible crab, there were significantly higher levels of both protein and allergen in edible crab processing compared to king crab (figure 2A and 2B).

Table 4 shows that levels of tropomyosin and total protein significantly differ between king crab plants. Plant A was the only plant where endotoxin samples were below detection level. Plant A also had the lowest exposure levels to tropomyosin and protein of the king crab processing plants.

Using Pearson's correlation, a significant correlation was found between protein and tropomyosin levels in both king crab ($r = 0.58$, 43 pairs) and edible crab ($r = 0.42$, 24 pairs) separately, as well as in all samples combined ($r = 0.59$, 67 pairs). A significant correlation was found between protein and endotoxin when all samples were included ($r = 0.53$, 15 pairs), but not when king crab and edible crab were analysed separately. No correlation was found between the other components.

Discussion

In this study we measured total proteins, tropomyosin, endotoxin, trypsin and NAGase in bioaerosols collected in workers' breathing zone during processing of king crab and edible crab. Endotoxin and trypsin levels were highest in king crab processing, while total protein and tropomyosin levels were highest when processing edible crab. Processing cooked crab generated higher concentrations of tropomyosin than processing raw crab (figure 2). However, enzyme activity was higher in bioaerosols collected in raw crab processing than in cooked (table 3). Exposure levels varied within raw and cooked processing respectively, but also varied between the different king crab plants.

Most king crab plants are small and originally built for fish processing (figure 1A-C). Workers were subdivided according to whether they were processing mainly raw or cooked crab, or performing work tasks in both raw and cooked king crab during the same shift such as truck driving or cleaning. This subdivision reflects how the work was organised in the crab factories.

Attempts to compare and evaluate bioaerosol exposure levels are hampered by the lack of occupational exposure limits and standardised, reproducible methods for collecting and analysing the samples (Eduard et al. 2012; Douwes et al. 1995). The levels of exposure to total protein and allergens such as tropomyosin have been measured to some extent in other studies in the seafood industry.

Allergen exposure during crab processing has been studied (Griffin P 1994; Malo et al. 1997; Beaudet

et al. 2002; Gill et al. 2009; Weytjens et al. 1999; Abdel Rahman, Gagne, and Helleur 2012) and showed that exposure levels vary within work areas and exposure groups. Comparison of the total protein levels in our study and those in previous reports from seafood industry shows that the range of exposure in our plants (0.3 – 97 $\mu\text{g}/\text{m}^3$) are wider than the range reported in fish processing plants such as the plants studied by Jeebhay et al. in South Africa (Limit of detection - 11.50 $\mu\text{g}/\text{m}^3$)(Jeebhay et al. 2005). Total protein exposure levels ranging from 0.76 - 13 $\mu\text{g}/\text{m}^3$ has been found in the Norwegian salmon industry (Shiryaeva et al. 2013) where an exposure-response relationship was found between protein exposure and self-reported respiratory symptoms and lung function test outcomes. These levels are also lower than the levels found in our study (0.3 – 97.5 $\mu\text{g}/\text{m}^3$). Several studies have found a high prevalence and incidence of allergy and asthma attributed to bioaerosol exposure(Bonlokke et al. 2012; Jeebhay 2011; Gautrin et al. 2010; Howse et al. 2006; Cartier et al. 2004; Neis 2004). However, the levels of bioaerosols and the specific components they contain are important factors for risk assessment. Future studies need to address the question of dose-response and threshold levels in work environments. Because of unpredictable work schedules in this industry, the average exposure over shifts and seasons are difficult to predict. They did not have production every day, and shifts could vary between 2 and 12h in length (normal shift was 8h). This will affect the average level of bioaerosols inhaled by the workers over time. However, we found that workers were exposed to high levels of possible sensitizers and pro-inflammatory agents such as tropomyosin, trypsin and endotoxins (table 1, 2 and 3). In occupational settings, workers are not exposed to a single component, and simultaneous exposure to several components could potentially alter the effect on workers' health. Combined effects of bioaerosol components have been poorly investigated. Bhagwat et al. showed a synergistic effect of endotoxin and seafood proteases, augmenting inflammatory cytokines in an *in vitro* respiratory cell model(Bhagwat 2015).

Inhalation exposure of allergens and subsequent atopic sensitisation and reactivity leading to respiratory problems is a highly prevalent problem in the seafood industry affecting a considerable part of the workforce(Bonlokke et al. 2012; Cartier et al. 2004; Gautrin et al. 2010; Howse et al. 2006; Beaudet et al. 2002). Tropomyosin is the major cross-reactive, heat stable and most abundant allergen

among crustaceans although several other allergenic proteins have been found (Gill et al. 2009; Abdel Rahman, Gagne, and Helleur 2012) and was therefore used as a biomarker to assess inhalation allergen exposure.

The sensitive ELISA method used to measure tropomyosin (Kamath et al. 2014; Malo et al. 1997) has no unspecific binding to other proteins and no loss of binding due to protein modification procedures that may have influenced the levels reported in other studies (Griffin P 1994; Lopata and Jeebhay 2013; Lopata et al. 2005). Previous studies have also used the highly sensitive mass spectrometric based strategies to quantify tropomyosin and arginine kinase (Rosmilah et al. 2012; Abdel Rahman, Gagne, and Helleur 2012; Abdel Rahman et al. 2011; Abdel Rahman et al. 2010). The immunoassay used in this study had a limit of quantification of 100 picograms/m³ of sampled air. Since tropomyosin is heat-stable, both raw and cooked crab are possible sources for aerosolised tropomyosin (Jeebhay et al. 2001; Lopata and Jeebhay 2013). Antibody reactivity of crustacean tropomyosin can also increase after heating, a possible result of protein denaturation and exposure of new epitopes, aggregation and chemical modifications (Kamath et al. 2013; Abramovitch et al. 2013). A study involving snow crab processing found more aerosolised allergen when workers were handling cooked crab than raw (Swanson et al. 2004) which is in line with the results of the present study.

Levels of tropomyosin were significantly higher in the edible crab plant compared to the king crab plants, which may be a result of the way the crabs are handled during processing. Both use high speed rotating cleaning brushes and have poor shielding of workers from the bioaerosols produced.

However, after the king crab is cooked, the clusters are gently packed, glazed (sprayed with water) and frozen with minimal manual handling and no further processing. During edible crab processing most of the handling happens after cooking; sorting, mincing shell to extract all muscle, and packing muscle back into the shell before packaging – tasks that produce bioaerosols. The bioaerosol exposure in king crab plants were not significantly different between raw and cooked processing, apart from trypsin activity, whereas the levels at the edible crab plant were. A reason for this difference may be that workers in the king crab industry were not at a single workstation throughout the shift. Most of the handling involved raw king crab, and raw crab was also the main source of bioaerosol production. In

the edible crab industry, workers were stationary throughout the shift, which may result in greater bioaerosol accumulation as workers may be exposed to bioaerosols from their own workstation, as well as those produced by nearby workers if the ventilation conditions or local exhaust ventilation is not optimal. These differences in how the crabs are processed may partly explain the differences in exposure levels between industries as well as the differences found between handling raw and cooked crab within each industry.

Endotoxin levels in the edible crab plant were lower for workers handling cooked crab than raw crab. Endotoxins are part of Gram negative bacterial cell walls. These may be abundant on the outer shell of the crabs as part of their environment. Levels are therefore expected to be highest when the shell of the crab is cleaned or removed which happens during raw processing. Endotoxin levels of 1350 EU/m³ have been reported in herring industry(Bang et al. 2005). Endotoxins are suggested to be a cause of occupational respiratory symptoms in occupational settings including among seafood processing workers(Sigsgaard and Schlunssen 2004; Sherson, Hansen, and Sigsgaard 1989; Madsen et al. 2015). A “no adverse health effect” of 90 EU/m³ has been suggested(The Nordic Expert Group for Criteria Documentation of Health Risk from Chemicals, 144. Endotoxins 2011), a level exceeded in 2 of 8 endotoxin samples in the edible crab plant, and in 4 of 7 samples in the king crab plants.

Enzyme activity was significantly lower when handling cooked king crab than raw, and there was no enzyme activity in samples taken during cooked edible crab processing. A previous study found that there is trypsin and trypsin-like enzyme activity in extracted tissue samples from crustaceans(Sun and Lopata 2010), but there are no previous publications that have quantified the level of airborne trypsin activity during seafood processing. Trypsin is a common serine protease enzyme and inhaled by workers may have an immunological or non-immunological effect on the lungs(Sun and Lopata 2010; Baur 2005; Florsheim et al. 2015; Madsen et al. 2015). Optimal conditions for several crustacean trypsins are around 40°C and pH6, which is close to the physiological conditions found in human lungs(Sun and Lopata 2010). However, studies have found that heating the enzyme is enough to inactivate the enzyme which would remove the proteolytic activity from enzymes being inhaled by

workers processing cooked crab(Larsen et al. 2011; Sun and Lopata 2010; Bhagwat et al. 2014; Zhang, He, and Guan 1999).

Because chitin is a major component of crustacean shell(Espie and Roff 1995) and NAGase is a widely distributed enzyme that digests chitin(Chen et al. 2011) we would expect NAGase to be present in the processing plants. Our samples were collected in autumn/winter and the levels were higher than levels found in normal Danish homes at that time of year(Frankel et al. 2012) so we expect the crab processing to be a contributor to the NAGase levels. There is a growing body of literature(Muzzarelli 2010) suggesting that NAGase may have a negative impact on health by eliciting an immunological response(Allermann, Wilkins, and Madsen 2006). Exposure to enzymes in occupational settings has been linked to sensitisation and asthma(Green and Beezhold 2011) as well as ODS(Madsen et al. 2015).

We wished to investigate if there was a correlation between the components to explore the possibility of performing simple rounds of measurement where only one or a few components are measured. It would simplify exposure measurements in epidemiological studies. However, correlations such as these were not found to such a degree that we feel it is possible to replace measurements of components with other measurements.

The work task performed by each individual is not necessarily the most important factor for evaluating exposure levels. Jeebhay et al. compared similar exposure groups in two fish plants and found that the department where the subject worked explained most of the variability in exposure levels(Jeebhay et al. 2005). In our study, when comparing the king crab plants (table 4), we also find large differences in the levels of exposure at different plants. This suggests, as did studies on snow crab workers in Canada(Neis 2004; Abdel Rahman, Gagne, and Helleur 2012), that there may be important factors to look at in addition to work tasks and whether the workers are handling raw or cooked crab. It is necessary to look at a complete “plant effect”, the sum effect of the layout of the processing line, ventilation, equipment producing bioaerosols and the technique of each individual worker, in addition to other unmeasured covariates, when evaluating the plant. Plant A had a higher level of technical equipment compared to plant B and C. Plant A was also the only king crab factory not altering

between crab and fish processing, thus the equipment in this plant was permanently placed unlike plant B and C. Plant A also differed by complete separation between raw and cooked processing areas by a solid wall (figure 1A) that prevented cooking fumes dispersing through the plant. Plant B had a separate enclosed room for the cooking vats (figure 1B). However, the door to the room was opened to move the crab in and out. Dispersion of cooking fumes was reduced by the door, but not removed. The ventilation system in the cooking room consisted of a large influx of air into the room, and not removal from the workers and out of the plant. Plant C had no physical barrier between the raw and cooked processing (figure 1C). The difference in bioaerosol levels found between plants may in part be because of these differences. This also illustrates the difficulties of transferring measurements from one plant to generalisation about all crab processing plants and the importance of local variations.

In this study, several important components of bioaerosols have been identified and quantified. The presence of allergens in air samples demonstrate that it is possible for workers to be sensitised by airway exposure. For further assessment of health risks from occupational bioaerosol exposure, dose-response studies with identification of no observed adverse effect levels (NOAELs) for individual components are important for developing occupational exposure threshold limits. Studies of combined effects of bioaerosol components are needed. Identification of specific allergens and in which part of crab processing these allergens are aerosolised would aid the priorities in implementation of preventive measures.

Conclusions

In Norwegian crab processing plants, workers are exposed to bioaerosols containing endotoxins and proteins including tropomyosin, NAGase and trypsin. Higher levels of total protein and tropomyosin were found during processing in the edible crab industry than in the king crab industry, particularly in the cooking area. A significant difference was found between king crab plants in terms of protein, tropomyosin and trypsin levels, suggesting a “plant effect”.

Acknowledgements: This study received financial support from the Norwegian Asthma and Allergy Association with a grant from the Norwegian Extra Foundation for Health and Rehabilitation through EXTRA funds. Author AL Lopata is holder of an ARC Future Fellowship. We thank Eva Kramvik, Merethe Larsen, Marit N Hegseth from the University Hospital North Norway at the Department of Occupational and Environmental Medicine and Lene Madsø from STAMI National Institute of Occupational Health for participation in collecting and analysing samples.

There are no conflicts of interest declared by any of the authors.

References

- Abdel Rahman AM, Gagne S, Helleur RJ. (2012) Simultaneous determination of two major snow crab aeroallergens in processing plants by use of isotopic dilution tandem mass spectrometry. *Anal Bioanal Chem*; 403 821-31.
- Abdel Rahman AM, Kamath SD, Lopata AL, Robinson JJ, Helleur RJ. (2011) Biomolecular characterization of allergenic proteins in snow crab (*Chionoecetes opilio*) and de novo sequencing of the second allergen arginine kinase using tandem mass spectrometry. *J Proteomics*; 74 231-41.
- Abdel Rahman AM, Lopata AL, Randell EW, Helleur RJ. (2010) Absolute quantification method and validation of airborne snow crab allergen tropomyosin using tandem mass spectrometry. *Anal Chim Acta*; 681 49-55.
- Abramovitch JB, Kamath S, Varese N, Zubrinich C, Lopata AL, O'Hehir RE, Rolland JM. (2013) IgE Reactivity of Blue Swimmer Crab (*Paralimnops*) Tropomyosin, Por p 1, and Other Allergens; Cross-Reactivity with Black Tiger Prawn and Effects of Heating. *PLoS One*; 8 e67487.
- Allermann L, Wilkins CK, Madsen AM. (2006) Inflammatory potency of dust from the indoor environment and correlation to content of NAGase and fungi. *Toxicol In Vitro*; 20 1522-31.
- Bang B, Aasmoe L, Aamodt BH, Aardal L, Andorsen GS, Bolle R, Boe R, Van Do T, Evans R, Florvag E, *et al.* (2005) Exposure and airway effects of seafood industry workers in northern Norway. *J Occup Environ Med*; 47 482-92.
- Baur X. (2005) Enzymes as occupational and environmental respiratory sensitizers. *Int Arch Occup Environ Health*; 78 279-86.
- Beudet N, Brodtkin CA, Stover B, Daroowalla F, Flack J, Doherty D. (2002) Crab allergen exposures aboard five crab-processing vessels. *AIHA J (Fairfax, Va)*; 63 605-9.
- Bhagwat SB, Larsen, A.K., Seternes, O.M., Bang, B.E. (2015) Mixed exposure to bacterial lipopolysaccharide and seafood proteases augments inflammatory signalling in an airway epithelial cell model (A549). *Toxicology and Industrial Health*; In print.
- Bhagwat SS, Larsen AK, Winberg J-O, Seternes O-M, Bang BE. (2014) Salmon and king crab trypsin stimulate interleukin-8 and matrix metalloproteinases via protease-activated receptor-2 in the skin keratinocytic HaCaT cell line. *Food and Chemical Toxicology*; 69 303-11.
- Bonlokke JH, Gautrin D, Sigsgaard T, Lehrer SB, Maghni K, Cartier A. (2012) Snow crab allergy and asthma among Greenlandic workers--a pilot study. *Int J Circumpolar Health*; 71 19126.
- Cartier A, Lehrer SB, Horth-Susin L, Swanson M, Neis B, Howse D, Jong M. (2004) Prevalence of crab asthma in crab plant workers in Newfoundland and Labrador. *Int J Circumpolar Health*; 63 Suppl 2 333-6.
- Chen JK, Shen CR, Yeh CH, Fang BS, Huang TL, Liu CL. (2011) N-acetyl glucosamine obtained from chitin by chitin degrading factors in *Chitinibacter tainanensis*. *Int J Mol Sci*; 12 1187-95.
- Douwes J, Versloot P, Hollander A, Heederik D, Doekes G. (1995) Influence of various dust sampling and extraction methods on the measurement of airborne endotoxin. *Appl Environ Microbiol*; 61 1763-9.
- Eduard W, Heederik D, Duchaine C, Green BJ. (2012) Bioaerosol exposure assessment in the workplace: the past, present and recent advances. *J Environ Monit*; 14 334-9.
- Espie PJ, Roff JC. (1995) Characterization of Chitinase from *Daphnia magna* and Its Relation to Chitin Flux. *Physiological Zoology*; 68 727-48.
- Florsheim E, Yu S, Bragatto I, Faustino L, Gomes E, Ramos RN, Barbuto JA, Medzhitov R, Russo M. (2015) Integrated innate mechanisms involved in airway allergic inflammation to the serine protease subtilisin. *J Immunol*; 194 4621-30.
- Frankel M, Beko G, Timm M, Gustavsen S, Hansen EW, Madsen AM. (2012) Seasonal variations of indoor microbial exposures and their relation to temperature, relative humidity, and air exchange rate. *Appl Environ Microbiol*; 78 8289-97.

- Gaddie J, Legge JS, Friend JA, Reid TM. (1980) Pulmonary hypersensitivity in prawn workers. *Lancet*; 2 1350-3.
- Gautrin D, Cartier A, Howse D, Horth-Susin L, Jong M, Swanson M, Lehrer S, Fox G, Neis B. (2010) Occupational asthma and allergy in snow crab processing in Newfoundland and Labrador. *Occup Environ Med*; 67 17-23.
- Gill BV, Rice TR, Cartier A, Gautrin D, Neis B, Horth-Susin L, Jong M, Swanson M, Lehrer SB. (2009) Identification of crab proteins that elicit IgE reactivity in snow crab-processing workers. *J Allergy Clin Immunol*; 124 1055-61.
- Green BJ, Beezhold DH. (2011) Industrial fungal enzymes: an occupational allergen perspective. *J Allergy (Cairo)*; 2011 682574.
- Griffin P RFP, Topping M. D. (1994) Measurement of airborne antigens in a crab processing factory. *Ann Occup Hyg*; 38 4.
- Howse D, Gautrin D, Neis B, Cartier A, Horth-Susin L, Jong M, Swanson MC. (2006) Gender and snow crab occupational asthma in Newfoundland and Labrador, Canada. *Environ Res*; 101 163-74.
- Jeebhay MF. (2011) Occupational allergy and asthma in the seafood industry - emerging issues. *Occupational Health Southern Africa*; 17 10.
- Jeebhay MF, Cartier A. (2010) Seafood workers and respiratory disease: an update. *Curr Opin Allergy Clin Immunol*; 10 104-13.
- Jeebhay MF, Robins TG, Lehrer SB, Lopata AL. (2001) Occupational seafood allergy: a review. *Occup Environ Med*; 58 553-62.
- Jeebhay MF, Robins TG, Miller ME, Bateman E, Smuts M, Baatjies R, Lopata AL. (2008) Occupational allergy and asthma among salt water fish processing workers. *Am J Ind Med*; 51 899-910.
- Jeebhay MF, Robins TG, Seixas N, Baatjies R, George DA, Rusford E, Lehrer SB, Lopata AL. (2005) Environmental exposure characterization of fish processing workers. *Ann Occup Hyg*; 49 423-37.
- Kamath SD, Abdel Rahman AM, Komoda T, Lopata AL. (2013) Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies. *Food Chem*; 141 4031-9.
- Kamath SD, Thomassen MR, Saptarshi SR, Nguyen HM, Aasmoe L, Bang BE, Lopata AL. (2014) Molecular and immunological approaches in quantifying the air-borne food allergen tropomyosin in crab processing facilities. *Int J Hyg Environ Health*; 217 740-50.
- Larsen AK, Seternes O-M, Larsen M, Kishimura H, Rudenskaya GN, Bang B. (2011) Purified sardine and king crab trypsin display individual differences in PAR-2-, NF- κ B-, and IL-8 signaling. *Toxicological & Environmental Chemistry*; 93 1991-2011.
- Larsen AK, Seternes OM, Larsen M, Aasmoe L, Bang B. (2008) Salmon trypsin stimulates the expression of interleukin-8 via protease-activated receptor-2. *Toxicol Appl Pharmacol*; 230 276-82.
- Lopata AL, Jeebhay MF. (2013) Airborne seafood allergens as a cause of occupational allergy and asthma. *Curr Allergy Asthma Rep*; 13 288-97.
- Lopata AL, Jeebhay MF, Reese G, Fernandes J, Swoboda I, Robins TG, Lehrer SB. (2005) Detection of fish antigens aerosolized during fish processing using newly developed immunoassays. *Int Arch Allergy Immunol*; 138 21-8.
- Madsen AM, Zervas A, Tendal K, Nielsen JL. (2015) Microbial diversity in bioaerosol samples causing ODS compared to reference bioaerosol samples as measured using Illumina sequencing and MALDI-TOF. *Environ Res*; 140 255-67.
- Malo JL, Chretien P, McCants M, Lehrer S. (1997) Detection of snow-crab antigens by air sampling of a snow-crab production plant. *Clin Exp Allergy*; 27 75-8.
- Moody MW, Roberts KJ, Huner JV. (1993) Phylogeny of commercially important seafood and description of the seafood industry. *Clin Rev Allergy*; 11 159-81.
- Muzzarelli RA. (2010) Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. *Mar Drugs*; 8 292-312.

- Neis B. (2004) Working Group on Shellfish Asthma Newfoundland Labrador. Workplace Health, Safety Compensation Commission. Report on the Safetynet Snow Crab Occupational Asthma Study for The Working Group on Shellfish Asthma: WHSCC.
- The Nordic Expert Group for Criteria Documentation of Health Risk from Chemicals, 144. Endotoxins. (2011) *Arbete och Hälsa*; 45 53.
- Rosmilah M, Shahnaz M, Zailatul HM, Noormalin A, Normilah I. (2012) Identification of tropomyosin and arginine kinase as major allergens of *Portunus pelagicus* (blue swimming crab). *Trop Biomed*; 29 467-78.
- Rudenskaya GN, Isaev VA, Shmoylov AM, Karabasova MA, Shvets SV, Miroshnikov AI, Brusov AB. (2000) Preparation of proteolytic enzymes from kamchatka crab *Paralithodes camchatica* hepatopancreas and their application. *Applied Biochemistry and Biotechnology*; 88 175-83.
- Sherson D, Hansen I, Sigsgaard T. (1989) Occupationally related respiratory symptoms in trout-processing workers. *Allergy*; 44 336-41.
- Shiryaeva O, Aasmoe L, Straume B, Bang BE. (2010) Respiratory impairment in Norwegian salmon industry workers: a cross-sectional study. *J Occup Environ Med*; 52 1167-72.
- Shiryaeva O, Aasmoe L, Straume B, Olsen AH, Ovrum A, Kramvik E, Larsen M, Renstrom A, Merritt AS, Heldal KK, *et al.* (2013) Respiratory effects of bioaerosols: Exposure-response study among salmon-processing workers. *Am J Ind Med*.
- Sigsgaard T, Schlunssen V. (2004) Occupational asthma diagnosis in workers exposed to organic dust. *Ann Agric Environ Med*; 11 1-7.
- Stellman JM, Office IL. (1998) *Encyclopaedia of Occupational Health and Safety: Industries and occupations*: International Labour Office.
- Sun S, Lopata AL. (2010) The role of shellfish proteases in allergic diseases and inflammation. *Current Allergy & Clinical Immunology*; 23 6.
- Swanson M, Pelley B, Neis B, Lehrer SB, Chrétien P, Carrier A. (2004) Industrial processing of raw crab is associated with less allergen aerosolization. *Journal of Allergy and Clinical Immunology*; 113 S231.
- Thorn J. (2001) The inflammatory response in humans after inhalation of bacterial endotoxin: a review. *Inflamm Res*; 50 254-61.
- Watanabe M, Kurai J, Sano H, Kitano H, Shimizu E. (2016) Prevalence of asthma and wheezes among snow crab workers in western Japan: a cross-sectional study. *J Med Invest*; 63 74-9.
- Weytjens K, Cartier A, Malo JL, Chretien P, Essiembre F, Lehrer S, Swanson M. (1999) Aerosolized snow-crab allergens in a processing facility. *Allergy*; 54 892-3.
- Zhang Z, He Z, Guan G. (1999) Thermal stability and thermodynamic analysis of native and methoxypolyethylene glycol modified trypsin. *Biotechnology Techniques*; 13 781-86.

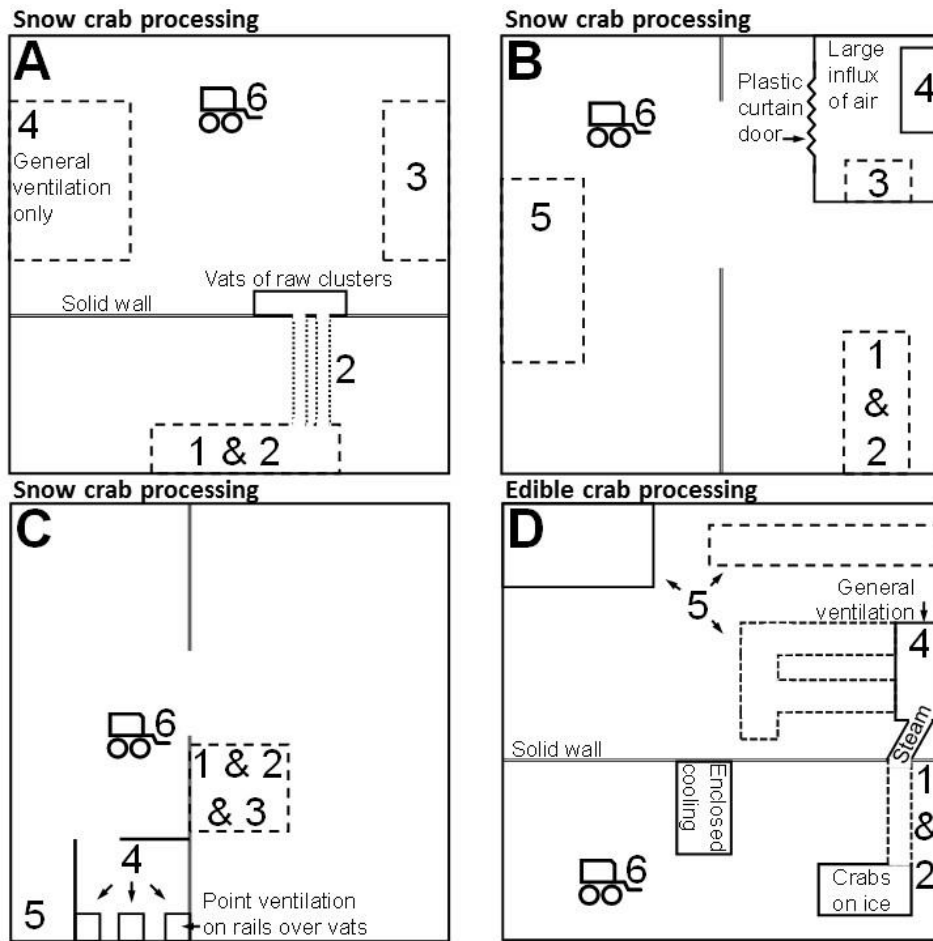


Figure 1: Factory layouts of three different king crab factories (A, B and C) and one edible crab factory (D). Walls and doors separating areas are drawn as solid lines. Rippled lines indicate areas of production. Work tasks are as follows (not all tasks were present in all plants); 1 cracking, 2 de-gilling and cleaning raw crab, 3 packing raw crab for cooking, 4 cooking, 5 processing cooked crab (packing, sorting, mincing shell and muscle), 6 varying work tasks in all areas of the factory (transport of crabs or vats on trucks, cleaning vats or plant, maintenance)

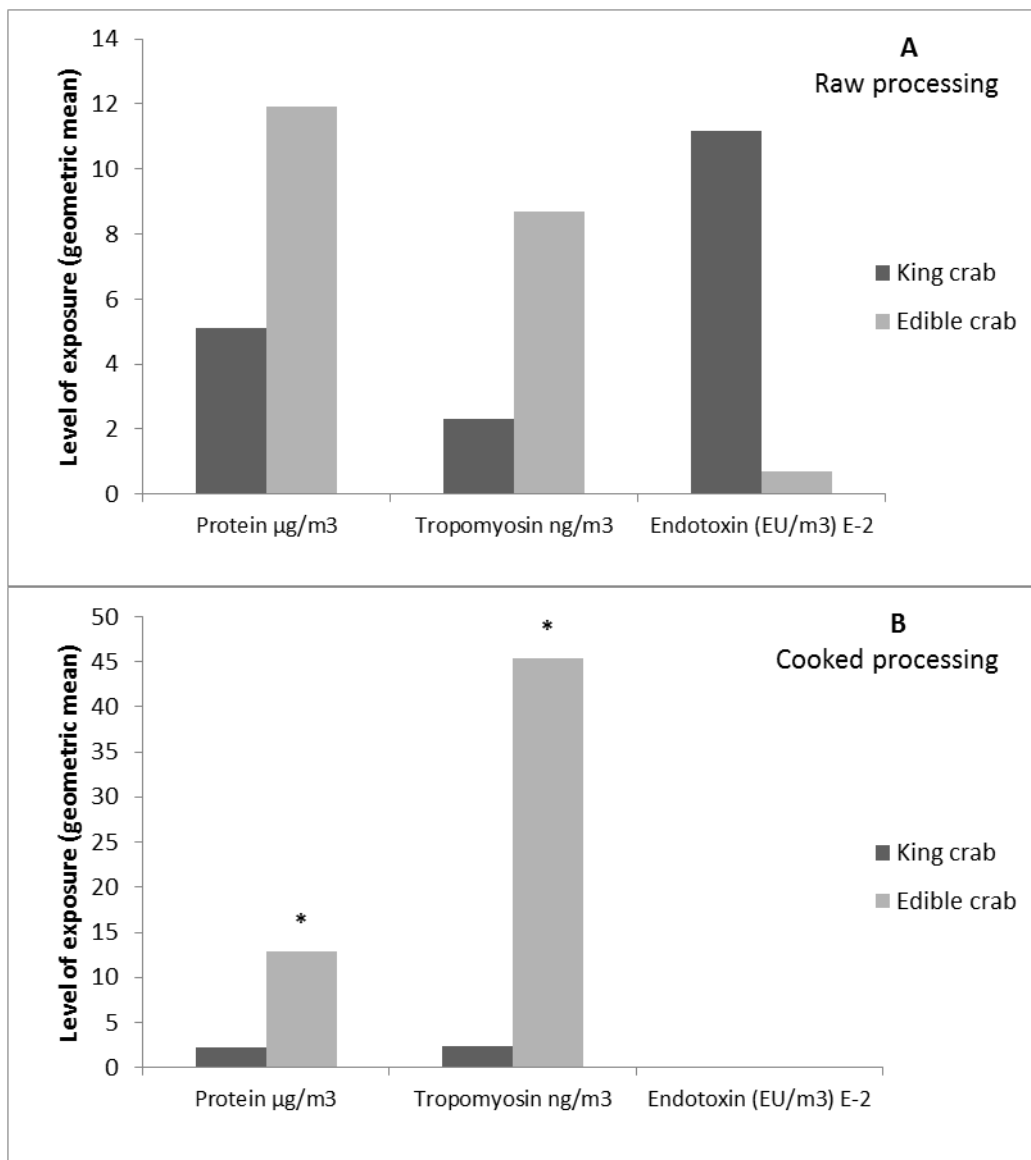


Figure 2: Levels of exposure (geometric mean) of bioaerosol components in processing raw king crab and edible crab (figure 2A) and cooked king crab and edible crab (figure 2B).

* Significant higher level ($p < 0.05$) of bioaerosol component in edible crab processing than in king crab processing.

Table 1: Exposure levels (median, geometric mean (GM), Range = min and max measurements) of bioaerosol components in king crab workers' breathing zone when processing either raw crab, cooked crab or performing work tasks overlapping both processing areas. p-values of ANOVA of processing raw crab, cooked crab or performing work tasks overlapping both areas.

Processing area	Bioaerosol levels in king crab processing										p-values						
	Raw					Cooked						Overlapping work tasks					
Bioaerosol component	Samples (n)	Median	GM	Range	Samples (n)	Median	GM	Range	Samples (n)	Median	GM	Range	Samples (n)	Median	GM	Range	
Tropomyosin (ng/m ³)	27	1.6	2.3	0.1-76.0	21	2.4	2.4	1.3-5.1	6	1.7	1.8	1.5-2.3	6	1.7	1.8	1.5-2.3	0.858
Protein (µg/m ³)	21	3.9	5.1	1.1-48.0	18	2.1	2.3	0.3-26.6	4	3.0	4.1	2.1-16	4	3.0	4.1	2.1-16	0.136
Endotoxin (EU/m ³)	5	630	110	170-24000	2			<1									

Table 2: Exposure levels (median, geometric mean (GM), Range = min and max measurements) of bioaerosol components in edible crab workers' breathing zone when processing raw or cooked crab. p-values of t-tests comparing levels when processing raw and cooked crab.

Processing area	Bioaerosol levels in edible crab processing						p-values		
	Raw			Cooked					
Bioaerosol component	Samples (n)	Median	GM	Range	Samples (n)	Median	GM	Range	
Tropomyosin (ng/m ³)	10	20.1	8.7	0.4 – 72.2	14	59.3	45.4	14.4 – 95.9	0.035
Protein (µg/m ³)	10	11.6	11.9	3.4 – 47.2	14	11.2	12.9	2.4 – 97.5	0.830
Endotoxin (EU/m ³)	5	51	72	17-340	3	8	10	7-20	0.040
NAGase (pmol 4-MU/m ³)	4	1107	853	149-3234	9	491	422	69-2509	0.368

Table 3: Trypsin activity from king crab and edible crab plants: Filter samples were collected from workers' breathing zone

Trypsin activity* in personal samples (number of samples within exposure category)										
King crab					Edible crab					
Exposure level	Zero	Low	Medium	High	Zero	Low	Medium	High	p values king vs edible crab industry	
Production area										
Raw	4	5	4	6	1	0	4	0	0.836	
Cooked	5	2	2	0	3	0	0	0	0.282	
p value raw vs cooked	0.048				0.071					
Plant										
A	4	7	2	0	4	0	4	0	0.668	
B	4	0	3	0						
C	1	0	1	6						
p value between plants	0.005									

* Trypsin activity is presented in a semi quantitative scale based on the 25th percentile (0.16 mU/m³) and 75th percentile (0.72 mU/m³) of positive samples: Low ≤ 25th percentile; 25th percentile > medium ≤ 75th percentile; high > 75th percentile. The table presents p-values for Mann-Whitney U tests comparing processing areas (raw vs cooked), crab type (king crab vs edible crab), and king crab plants (plant A vs B and C).

Table 4: Exposure levels (median, geometric mean (GM), Range = min and max measurements) of bioaerosol components in king crab workers' breathing zone at the three different king crab plants (A, B and C). p-values of ANOVA comparing levels at the three plants.

Bioaerosol level in king crab plants													
Bioaerosol component	Plant A			Plant B			Plant C			p-values			
	Samples (n)	Median	GM	Range	Samples (n)	Median	GM	Range	Samples (n)	Median	GM	Range	
Tropomyosin (ng/m ³)	28	1.7	1.8	0.6-3.8	19	2.0	1.9	0.4-5.1	7	26.9	8.8	0.1-75.9	0.002
Protein (µg/m ³)	20	2.6	2.0	0.3-4.4	16	3.9	3.8	0.4-26.6	7	21.0	18.2	5.6-47.7	< 0.000