Heavy Metals and Crab

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Researchers Burke and Kerton evaluate the levels of trace metal containments in crab processing byproducts and their transfer to selected crab bio-product.

Who should read this paper?

Anyone interested in developing high value marine based bio-extracts from underutilized marine resources, such as crab processing discards, will gain a better understanding of some of the environmental factors affecting heavy metal contaminants and their removal from and/or accumulation in these extracts.

Why is it important?

Evaluating the transfer of trace metal contaminants from crab processing byproducts during the extraction of higher value bio-products will be key to developing safe marketable crab bio-products for natural health products and biomedical applications. To date, such studies have been limited and this has delayed Food and Drug Administration approval, for example, of chitosan as a drug delivery agent.

Currently about 30% of crab resource in Newfoundland and Labrador is discarded as waste yet this discarded material contains valuable components that could be recovered and potentially used as natural health or biopharma products. The results will help the ocean community find methods to fully utilize this raw material which in turn may create new opportunities in coastal communities.

Understanding heavy metal contaminants and how they are removed or accumulated is not well studied in crab-based bio-products and more research is necessary to develop technologies that produce safe, marketable natural health products and biopharma products. This technology is essential for future commercialization success.

About the authors

Heather Burke is the director of the Centre for Aquaculture and Seafood Development at the Fisheries and Marine Institute. She has broad experience in applied research spanning more than two decades with major emphasis on marine bioprocessing and the development of value chains of unutilized marine biomass materials. Most recently, she has worked on several international crustacean bio-extraction and bio-conversion projects for nutraceutical, biomedical, and bioscience applications. She will complete her PhD (environmental science) at Memorial University of Newfoundland in the spring of 2022. Her thesis research focuses on using simple green technologies and an ocean based biorefinery approach for the extraction of higher value bio-products from snow crab processing discards.

Dr. Fran Kerton is a professor in the Department of Chemistry at Memorial University of Newfoundland. She is a member of the Canadian Society for Chemistry, the American Chemical Society, and a Fellow of the Royal Society of Chemistry (U.K.). She obtained her PhD in chemistry at the University of Sussex and was a postdoctoral research associate at the University of British Columbia. In addition to authoring over 70 journal articles, she has contributed several book chapters (*Introduction to Chemicals from Biomass and Sustainable Chemical Processes*). Her current research group is focused on developing environmentally benign transformations of bio-sourced molecules and materials, and “green” polymers. She received the Canadian Green Chemistry and Engineering Award in 2019.

HEAVY METALS IN SNOW CRAB (*CHIONOECETES OPILIO*) BIO-PRODUCTS

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ABSTRACT

Several potential snow crab (*Chionoecetes opilio*) bio-products have been identified having potential applications as feed ingredients (for terrestrial and aquatic animals), natural health products (e.g., nutraceuticals, dietary supplements), bio-medical and pharmaceutical products (e.g., drug delivery systems, wound healing products), and in cosmetics (e.g., shampoo, hair care, creams, lotions). Yet studies regarding the purity and safety of such bio-products remain limited. Due to growing concerns over heavy metal contaminants in the environment (air, soil, drinking water, food), their associated adverse health effects, and their tendency to bioaccumulate in marine crustaceans, we evaluated the levels of trace metal contaminants in crab processing byproducts and their transfer to selected crab bio-products: crab protein hydrolysate and crab chitin. Safety and toxicity concerns of residual heavy metals present in these snow crab processing bio-products are also discussed.

KEYWORDS

Snow crab (*Chionoecetes opilio*); Byproducts; Bio-products; Crab meal; Chitin; Chitosan; Toxicity; Heavy metals; Protein hydrolysate; Aluminum; Arsenic

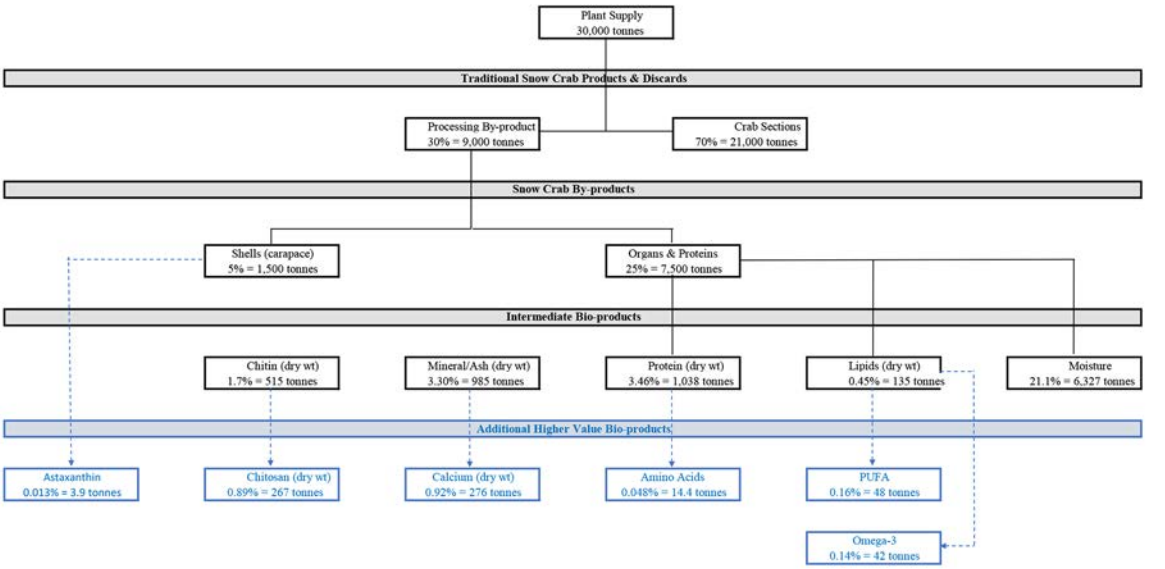

Figure 1: Value chain of N.L. snow crab processing byproducts and bio-products based on an average annual plant supply of 30,000 tonnes of crab [Burke, 2021].

INTRODUCTION

Since the collapse of the Northern cod fishery in 1992, Atlantic snow crab (*Chionoecetes opilio*) has been the most valuable seafood product harvested in Newfoundland and Labrador (N.L.), Canada. In 2019, snow crab landings were 26,894 tonnes of which 16,658 tonnes were exported to the United States (77%), China (8%), Indonesia (6%), and Vietnam (4%), at a value of $415 million [FLR, 2019]. Crab processing plants in N.L. have historically discarded on average about 30% of their total raw material supply in the form of waste and byproducts. In 2019 this amounted to an estimated 8,100 tonnes. Over the last five years, the average annual plant supply of snow crab in N.L. has been approximately 30,000 tonnes.

In N.L., snow crab is primarily processed as individually quick frozen cooked sections which generates waste comprised of carapace (cephalothorax shells), viscera and hepatopancreas, hemolymph [Beaulieu et al., 2009], residual meat, and gills. According to personal communications with industry stakeholders, this material is currently not being utilized commercially but could potentially be recovered from processing plant butchering stations as a byproduct and converted into intermediate bio-products (chitin, crab meal, proteins, lipids) or transformed into higher value bio-products (chitosan, peptides, omega-3, astaxanthin). Potential crab processing byproducts and bio-products that could be produced in N.L. based on an average annual plant supply of 30,000 tonnes are depicted in the crab bio-product value chain in Figure 1.

Many of the identified snow crab bio-products (Figure 1) have potential applications as feed ingredients (for terrestrial and aquatic animals), natural health products (e.g., nutraceuticals, dietary supplements), bio-medical and pharmaceutical products (e.g., drug delivery systems, wound healing products), and in cosmetics (e.g., shampoo, hair care, creams, lotions). Therefore, the purity and safety of the

Table 1: Main heavy metals of concern for seafood and Health Canada maximum allowable levels [Health Canada, 2020].

Heavy Metal	Fish Product	Max Allowable Level (ppm)
Arsenic	Fish Protein	3.5
Lead	Fish Protein	0.5
Mercury	Edible Fish	0.5-1.0

Table 2: Acceptable limits for elemental impurities in natural health products [Health Canada, 2015].

Element	Adult Limit per day	Limit per day per kg body weight
Total Arsenic **or** **Inorganic Arsenic** **Organic Arsenic**	< 10.0 ug/day < 2.1 ug/day < 1.4 mg/day	< 0.14 ug/kg b.w./day < 0.03 ug/kg b.w./day < 20 ug/kg b.w./day
Cadmium	< 6.0 ug/day	< 0.09 ug/kg b.w./day
Lead	< 10.0 ug/day	< 0.14 ug/kg b.w./day
Total Mercury	< 20.0 ug/day	< 0.29 ug/kg b.w./day
Methyl Mercury	< 2.0 ug/day	<0.029 ug/kg b.w./day

Table 3: Industry standard for heavy metal levels in medical grade chitosan [ASTM, 2019; USPC, 2020].

Heavy Metal	Max Allowable Level (ppm)
Lead	<0.5
Mercury	<0.2
Chromium	<1.0
Nickel	<1.0
Cadmium	<0.2
Arsenic	<0.5
Iron	<10
Total Heavy Metals	<40

bio-products developed will be critical for these applications. Due to growing concerns over heavy metal contaminants in the environment (air, soil, drinking water, food), their associated adverse health effects, and their tendency to bioaccumulate in marine crustaceans [Cubadda et al., 2017; Jaishankar et al., 2014; Gupta et al., 2013; Hardisson et al., 2017; Alabi and Adeoluwa, 2020], we evaluated the levels of trace metal contaminants in crab processing byproducts (i.e., crab meal) and their transfer to selected crab bio-products: crab protein hydrolysate and crab chitin.

According to Health Canada, heavy metals including arsenic (As), cadmium, lead, and mercury are considered toxic contaminants in seafood and natural health products (NHPs) if present in certain levels. The main heavy metals of concern (Table 1) for edible seafood and for which Health Canada has established maximum allowable levels include arsenic (3.5 ppm), lead (0.5 ppm), and mercury (0.5-1.0 ppm). The acceptable limits for elemental impurities in natural health products in Canada are presented in Table 2.

For medical grade chitosan, the heavy metals of concern for which industry [ASTM, 2019; USPC, 2020] has established maximum levels (Table 3) include arsenic (<0.5ppm), lead (<0.5ppm), mercury (<0.2 ppm), chromium

(<1.0 ppm), nickel (<1.0 ppm), cadmium (<0.2 ppm), and iron (<10 ppm). The industry standard for medical grade chitosan also recommends that the total heavy metal content should not exceed < 40 ppm [ASTM, 2019; USPC, 2020].

While heavy metals are known to have many adverse health effects (e.g., carcinogenic, occupational asthma, skin lesions, neurotoxic), exposure to heavy metals has been increasing in many parts of the world [Cubadda et al., 2017; Jaishankar et al., 2014]. Metals are naturally present in the environment including soil, water, and air, and, therefore, end up in food [Jaishankar et al., 2014; Gupta et al., 2013; Hardisson et al., 2017; Alabi and Adeoluwa, 2020]. Heavy metals tend to accumulate in the organs and tissues of crustaceans such as crabs and prawns [Sayyad et al., 2020; Olowu et al., 2010; Kim and Yoon, 2011]. Organs and tissues account for 80% of the crab byproducts available from N.L. crab processing plants (Figure 1). Therefore, understanding the levels of heavy metals in snow crab byproducts and how they are transferred throughout the crab bio-product value chain will be key to developing safe marketable crab bio-products for natural health product and biomedical/ pharmaceutical applications.

To date, few studies have been conducted that evaluate the purity or the toxicity of chitin-chitosan polymers, and those studies have focused on molecular weight and degree of deacetylation [Marques et al., 2020; Kean and Thanou, 2010; Matica et al., 2017; Guangyuan et al., 2009]. Therefore, despite the many published studies on chitosan drug delivery products, they are still not approved by the Food and Drug Administration (FDA) as they require studies demonstrating they are safe for human use [Marques et al., 2020; Kean and Thanou, 2010; Matica et al., 2017]. To the authors' knowledge, there have been no studies on the toxicity of chitin/chitosan-based products associated with protein, metals, or other trace contaminants that may be present.

PURPOSE AND SCOPE

The purpose of this study was to determine if heavy metals present in snow crab processing byproducts collected from a local processing plant were effectively removed during extraction of two intermediate bio-products – protein hydrolysate and chitin. Safety and toxicity concerns of residual heavy metals present in these snow crab processing bio-products and how this affects their end use applications are also discussed.

SELECTION OF CRAB BIO-PRODUCTS

Figure 1 identified various bulk intermediate bio-products that could be extracted from snow crab processing byproducts including protein, lipids, chitin, minerals (ash), and astaxanthin. Due to the estimated low yields of lipids and astaxanthin likely to be extracted from the available crab byproducts, these bio-products were not extracted for the purpose of this study. Since chitin and protein are commercially more valuable than the ash, only chitin and protein were extracted and recovered for this study.

METHODS

Collection and Preparation of Crab Byproduct

Figure 2: Snow crab processing byproducts.

Snow crab processing byproducts (Figure 2) collected from a processing plant located in Newfoundland and Labrador (N.L.), Canada, in June 2018 were milled and dried to produce crab meal (Figure 3). The crab meal was kept in frozen storage at -20°C in sealed sanitary plastic containers until the protein hydrolysate and chitin fractions could be extracted. The crab meal, protein hydrolysate, and chitin products were analyzed for proximate composition and trace metals.

Extraction of Snow Crab Bio-products

Raw, fresh, unseparated snow crab processing byproducts were collected in 10 L plastic pails (Figure 2), packed in flake ice, and transported to the Marine Institute's Marine Bioprocessing pilot plant in St. John's, N.L., where the byproduct was immediately frozen at -20°C until it could be further processed. The frozen crab byproduct was later thawed at 4°C and crushed in a Hobart grinder (Figure 4) in a two-step process: (1) Initially the material was milled through a 17 mm plate and (2) subsequently milled through a 13 mm plate. The crushed crab byproduct was then placed on drying trays in a single layer and dried to a constant weight at 105°C in a convection oven at 40% wind speed then ground to a particle size of ~1-2 mm (Figure 5). This dried crab

Figure 3: Snow crab meal.

Figure 4: (L) Hobart Grinder. (R) Snow crab byproduct milled through the 17 mm cutting plate.

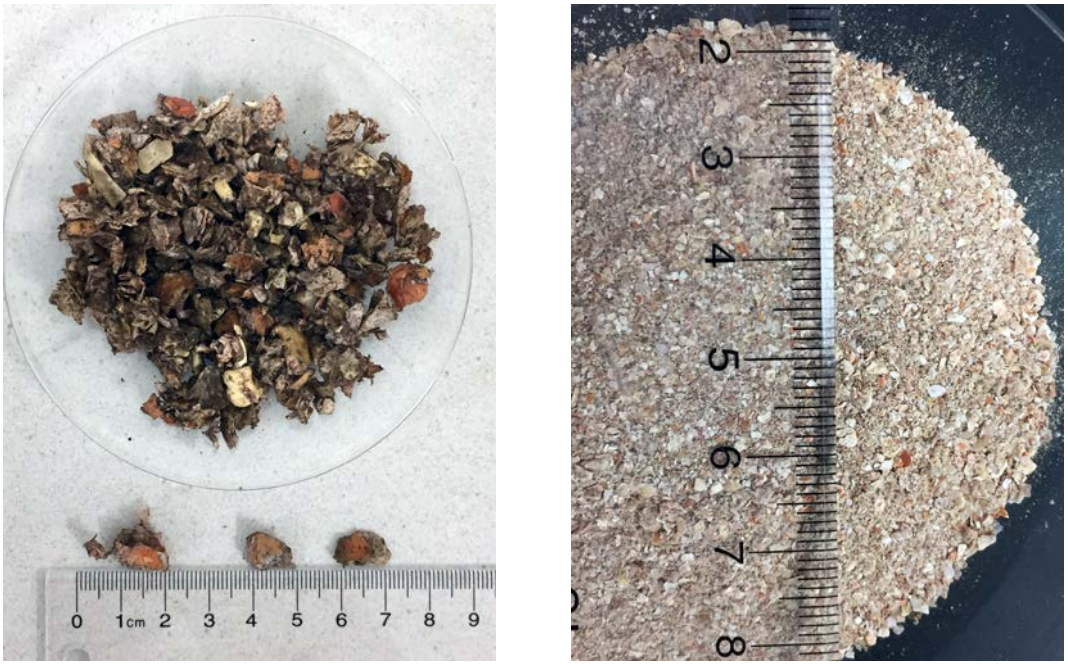

Figure 5: Air dried crab byproduct (L) before milling (13 mm particle size) and (R) after milling (1-2 mm).

meal product was later used for the extraction of additional crab bio-products: protein hydrolysate and chitin.

Protein Hydrolysis

Protein extraction was conducted using the protease enzyme Alcalase 2.4L, since the protein is not considered suitable for use as an animal feed or nutritional supplement if extracted with NaOH [Jo et al., 2011] due to possible chemical contaminants and protein denaturation.

The following protease enzymes were considered: (1) Alcalase, *Bacillus licheniformis*; (2) Protease, *Bacillus subtilis*; and (3) Fungal Acid Protease, *Aspergillus oryzae*. Alcalase 2.4L (*Bacillus licheniformis*) was selected from the above list for the following reasons: (1) It has been reported to be one of the most highly efficient bacterial proteases used to prepare fish and other protein hydrolysates [See et al., 2011]; (2) Gildberg and Stenberg [2001] used Alcalase (2.4 l FG) to deproteinate Northern shrimp (*Pandalus borealis*) waste to obtain a high-quality protein hydrolysate (about 70% of the total amino-N was recovered) without affecting the yield or quality of the chitosan subsequently produced.

Protein hydrolysis was conducted using a modified method based on methods previously reported for salmon [See et al., 2011] and shrimp [Gildberg and Stenberg, 2001]. The hydrolysis was carried out at pH 8-8.55 and 55°C for 120 minutes using a crab byproduct-to-water ratio of 1:10, and 1% (v/w) Alcalase 2.4L. Following hydrolysis, the mixture was heated to 90°C and held at that temperature for 10 minutes to inactivate the protease enzyme [Lindberg et al., 2021]. The protein hydrolysate liquid was centrifuged at 7,000 rpm for 20 minutes, then vacuum filtered through a Whatman No. 41 ashless filter

paper, and the filtrate spray dried using a Buchi mini spray dryer (Figure 6) to collect the protein hydrolysate (Figure 7). The spray drier operating parameters were set at Inlet temperature 180°C; Outlet temperature 40°C; Aspirator 100%; Pump 20%; Q-Flow 30.

Chitin Extraction

Most traditional isolation methods of chitin from crab shells involves three main processing steps following initial particle size reduction which include (1) deproteination – removal of protein using strong alkali and heat treatment (e.g., 1-2% w/v KOH, 90°C for two hours); (2) demineralization – removal of minerals, mainly calcium carbonate, by treatment with strong acid (e.g., 5-7% w/v HCl for two hours at room temperature); and (3) decolouration – removal of pigment using a bleaching/oxidizing agent (e.g., hydrogen peroxide, ethanol, acetone, sodium hypochlorite) to obtain a colourless product [Bruck et al., 2012; Synowiecki and AL-Khateeb, 2000; Duarte de Holanda and Netto, 2006]. This process may be carried out on fresh or dried shells, and the demineralization and deproteination steps may be carried out in reverse order if pigment recovery is not required [Synowiecki and AL-Khateeb, 2000; Duarte de Holanda and Netto, 2006].

In our study, following enzymatic protein hydrolysis and recovery of the soluble protein, the remaining insoluble shell fraction was collected on a Whatman No. 41 ashless filter paper using vacuum filtration and washed a minimum of three times with deionized water to pH 7. Chitin extraction was conducted using a two-step chemical process: (1) Demineralization with 7% HCl (1:10 shells:HCl) for three hours at 25°C; and (2) Deproteination with 10% NaOH (1:10 shells:NaOH) for two hours at 55°C to remove any residual protein not removed by the enzyme treatment. Previous studies have shown that enzymatic deproteination of shrimp using Alcalase did not achieve full deproteination and that the chitin thus obtained contained a residual protein content that was twice as high as chitin obtained via treatment with NaOH [Synowiecki and AL-Khateeb, 2000; Duarte de Holanda and Netto, 2006].

The resulting chitin (Figure 8) was collected on a Whatman No. 41 ashless filter paper using vacuum filtration and washed several times with deionized water to pH 7, followed by low temperature convection drying at 55°C. The chitin sample was not depigmented for this experiment.

A schematic illustration summarizing the extraction, recovery, and purification processes used to prepare crab bio-products for this study is presented in Figure 9.

Proximate Composition

Proximate composition was determined for the dried crab byproduct samples and included determination of Moisture Content-Air Oven Method – AOAC Method 930.14; Kjeldahl Nitrogen – AOAC Method 954.01/988.05; Ash Content – AOAC Method 938.08 Ash of Seafood; and Chitin Content.

Chitin Yield and Chitin Content

Chitin yield was determined following demineralization of 5-10 g of dried crab meal with 50-100 mL of 7% HCl for three hours at 25°C, followed by deproteination

Figure 6: Spray drying snow crab protein hydrolysate using the Buchi mini spray dryer.g5.

with 10% NaOH (1:8 of crab:NaOH) for two hours at 55°C. Chitin was collected on a Whatman No. 41 ashless filter paper using vacuum filtration and washed a minimum of three times with deionized water to pH 7, followed by oven drying at 55-105°C for 24-48 hours. The recovered chitin was analyzed for total nitrogen via the Kjeldahl method (AOAC 954.01/988.05) and ash content (AOAC 938.08).

Chitin yield was calculated for crab meal using the equation:

% Chitin Yield = [weight of chitin (g)/weight of crab meal (g)] x 100 (1)

Chitin content was calculated for crab chitin using the equation:

% Chitin Content = % Nitrogen x 14.5 (2)

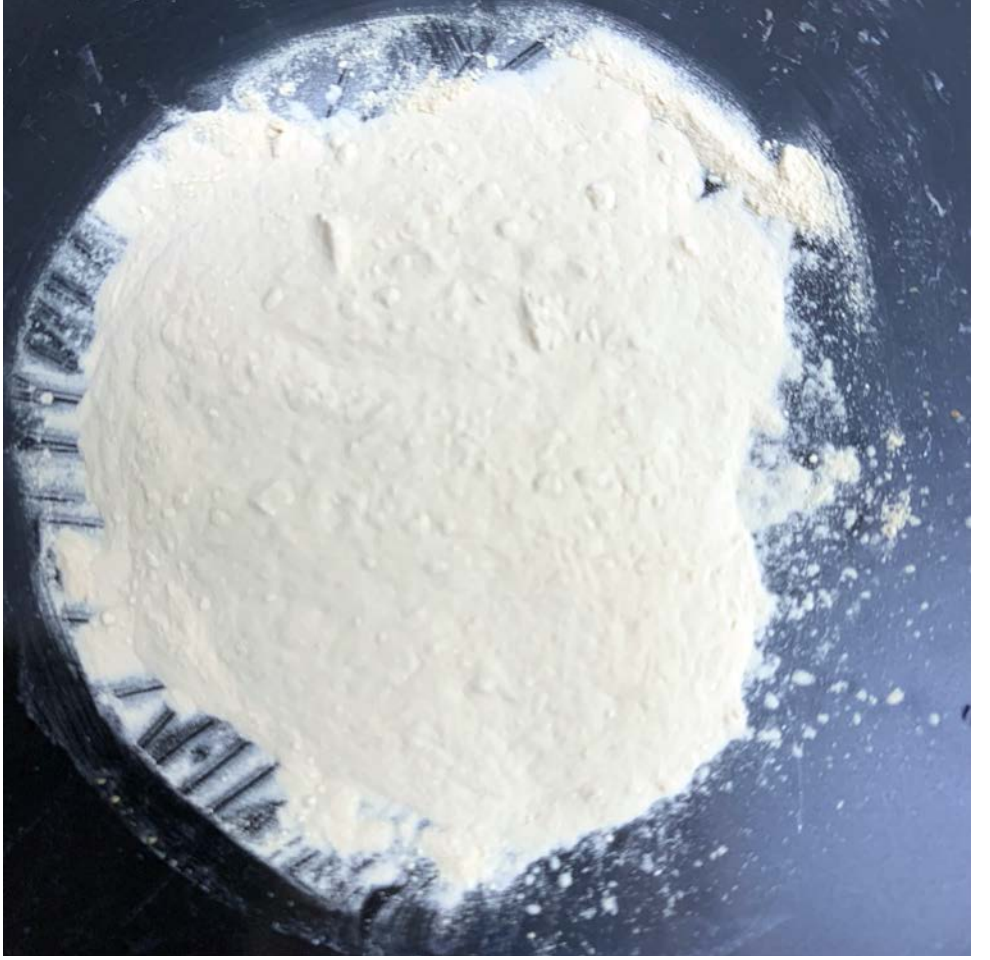

Figure 7: Spray dried snow crab protein hydrolysate powder.

Figure 8: Snow crab chitin.

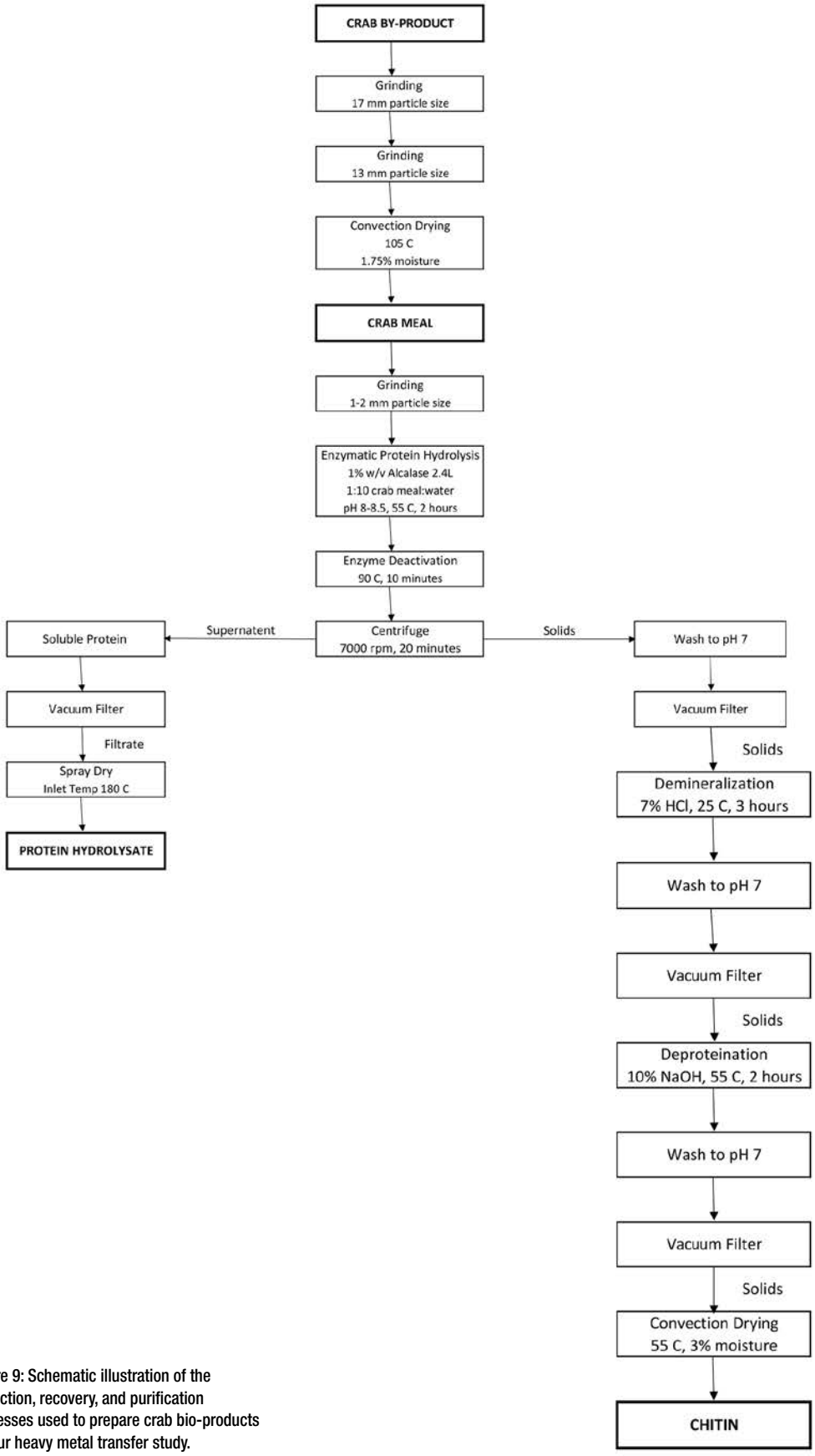

Figure 9: Schematic illustration of the extraction, recovery, and purification processes used to prepare crab bio-products for our heavy metal transfer study.

Table 4: Proximate composition of extracted crab processing bio-products [a].

Sample	% Moisture	% Chitin	% Total Nitrogen	% Protein	% Ash	% Lipid
Crab Meal [b]	1.75	16.74 ± 1.2562	6.12 ± 0.07016	-	36.01 ± 0.85063	8.99 ± 0.4222
Protein Hydrolysate [c]	2.33	-	10.31	64.41	25.20	-
Chitin [c]	3.00	88.07	6.07	-	0.07	-

[a] Results are reported on a dry weight basis, after isolation from the raw (unprocessed) crab byproduct. [b] Results are the mean of three determinations ± standard deviation, except % Moisture for which there was only a single determination. [c] Results represent one determination due to the small sample size available. Assumptions: All nitrogen in protein hydrolysate is due to protein. All nitrogen in chitin fraction is attributed to chitin.

Elemental Analysis (ICP-MS) – Raw Crab Byproducts

Samples of raw (unprocessed) crab byproducts were analyzed by Memorial University's Department of Earth Sciences for elemental analysis. Samples were prepared by ashing for six hours at 550°C. The cooled samples were then acid digested, sonicated, and dried three times prior to diluting in 10 mL of 0.2M HNO_3 in preparation for ICP-MS analysis using a Perkin Elmer Elan DRC II ICP-MS instrument. NIST standard 2977 and USGS T-193 were used as the elemental standards. Procedural blanks were run for each element.

Elemental Analysis (ICP-MS) – Dried Crab Bio-products

Due to a maintenance shutdown of the Memorial University lab that conducted the elemental analysis on the raw (unprocessed) crab byproducts, the subsequently isolated crab bio-products (crab meal, protein hydrolysate, and chitin) were submitted to the Research and Productivity Council (RPC) in New Brunswick, Canada, for analysis of trace metals and mercury. Portions of the samples were prepared by Microwave Assisted Digestion in nitric acid according to RPC's standard operating procedure SOP 4.M26. The resulting solutions were analyzed for trace elements by ICP-MS according to RPC's standard operating procedure SOP 4.M01, while mercury was analyzed by Cold Vapour AAS as per RPC's standard operating procedures SOP 4.M52 and SOP 4.M53. Procedural blanks were run for each element.

RESULTS AND DISCUSSION

Proximate Composition

The proximate compositions of the extracted crab bio-products are presented in Table 4. While we acknowledge that some of the nitrogen in the crab meal is associated with chitin, and that there may be some residual protein nitrogen remaining in the chitin fraction, for ease of calculation and comparison of the results, we assumed that all nitrogen in the protein hydrolysate was due to protein (factor of 6.25 was used to calculate % protein) and that all nitrogen in the chitin fraction was due to chitin (factor of 14.5 was used to calculate % chitin content).

The results of the proximate analyses demonstrate that the extraction methods were effective in separating the protein and the chitin fractions from the crab meal byproduct. The protein hydrolysate contained 64.4%

protein and 25.2% ash. The chitin fraction had an acceptable low ash content below 1% and a high chitin content (88%).

Elemental Composition of Crab Byproducts and Crab Bio-products

The purpose of this analysis was to understand the transfer of heavy metals from snow crab processing byproducts during the extraction of bulk intermediate bio-products – crab meal, protein hydrolysate, and chitin. Elemental compositions of the raw (unprocessed) crab byproducts and the extracted crab bio-products are presented in Table 5. Although the analyses were completed by two different labs, for the purpose of this assessment we assumed that any differences due to lab methods, equipment, or sample preparation were negligible.

The level of heavy metals in the crab bio-products evaluated in this study followed the order of crab meal > crab byproduct > protein hydrolysate > chitin. Heavy metals tend to accumulate in the organs and tissues of crustaceans such as crabs and prawns [Sayyad et al., 2020; Olowu et al., 2010]. Kim and Yoon [2011], for example, demonstrated that copper, arsenic, cadmium, and chromium tend to bioaccumulate in the hepatopancreas and gills of Korean Yeongdeok, crab, and Russian snow crab. The high protein and lipid content in our crab meal byproduct (Table 4) indicates it contained high amounts of meat, hepatopancreas, and gills and may explain the higher total heavy metal content in this sample. In addition, grinding and drying (aluminum drying trays) during the processing of the raw (unprocessed) crab byproduct into crab meal may have contributed to the higher metal content.

Generally, all metals were reduced in the chitin product while some metals (arsenic, sodium, potassium) became more concentrated in the protein hydrolysate. Of particular interest are the high levels of arsenic in the crab meal (21.2 ppm) and protein hydrolysate (54.6 ppm), and the high concentrations of aluminum in the crab meal (185 ppm) and chitin (151 ppm), especially if the intent is to use these bio-products as feed ingredients, natural health products, or for biomedical and pharmaceutical purposes, due to the potential toxic effects of these metals.

Arsenic levels were low in crab chitin (< 0.2 ppm) and raw (unprocessed) crab byproduct (3.64 ppm) but high in crab meal (21.2 ppm) and the protein hydrolysate (54.6 ppm), suggesting that arsenic is associated with the protein fraction of snow crab byproducts, and/or is present in an organic form which would be unable to bind with chitin. Since arsenic was lower in the raw (unprocessed) crab byproduct, it is probable that the grinding steps during processing of the crab meal were an additional source of arsenic which then became more concentrated during isolation and drying of the protein hydrolysate.

Aluminum levels were high in crab meal and chitin but low in the protein hydrolysate sample in the following order: crab meal (185 ppm) > chitin (151 ppm) > protein hydrolysate (5 ppm). An interesting observation is that the aluminum level, while high in the raw (unprocessed) crab byproduct (103 ppm), was higher in the processed crab meal and chitin. This suggests that there are likely two main sources of aluminum in the samples: (1) bioaccumulation from the

Table 5: Elemental composition of raw snow crab byproducts and extracted bio-products on a dry weight basis in parts per million (ppm).

Analytes	Raw Crab Byproduct [a]	Whole Crab Meal [b]	Crab Protein Hydrolysate [b]	Crab Chitin [b]
Aluminum	103 ± 0.0181	185	5	151
Antimony	nd	0.05	0.03	< 0.02
Arsenic	3.64 ± 0.0707	21.2	54.6	< 0.2
Barium	27.7 ± 0.2121	23.9	0.4	< 0.2
Beryllium	nd	< 0.02	< 0.02	< 0.02
Bismuth	0.01 ± 0.0006	< 0.2	< 0.2	< 0.2
Boron	nd	34	76.2	0.8
Cadmium	2.35 ± 0.0495	1.8	0.833	0.004
Calcium	108000 ± 0.1414	117000	16800	< 10
Chromium	0.87 ± 0.0325	1.1	0.3	0.3
Cobalt	0.50 ± 0.0332	0.56	1.29	< 0.02
Copper	40.6 ± 1.768	36.1	17.3	7.5
Iron	159 ± 18.38	179	16	< 4
Lead	0.343 ± 0.00778	0.31	0.06	0.03
Lithium	1.34 ± 0.0306	1.47	2.96	< 0.02
Magnesium	nd	12900	8260	3
Manganese	6.26 ± 0.2734	5.4	0.6	< 0.2
Mercury	<DL	0.16	0.03	< 0.01
Molybdenum	0.39 ± 0.0113	0.36	0.65	< 0.02
Nickel	2.7 ± 0.5021	2.1	4.6	< 0.2
Potassium	nd	7040	19000	13
Rubidium	2.76 ± 0.0075	2.87	7.83	< 0.02
Selenium	nd	2.2	3.2	< 0.2
Silver	2.25 ± 0.0000	1.79	0.07	0.07
Sodium	nd	23500	69700	530
Strontium	2300 ± 34.65	2210	329	< 0.2
Tellurium	nd	< 0.02	< 0.02	< 0.02
Thallium	<DL	< 0.02	< 0.02	< 0.02
Tin	17.4 ± 0.5657	46.2	1.94	0.05
Uranium	0.19 ± 0.00495	0.16	< 0.02	< 0.02
Vanadium	0.66 ± 0.00707	0.6	0.4	< 0.2
Zinc	52.1 ± 1.202	46.8	25.1	< 0.2

[a] Results represent the mean ± standard deviation of two replicates. nd = not determined. <DL = below detection limit. Analysis conducted by Memorial University of Newfoundland, Department of Earth Sciences.
[b] Results represent the determination of one composite sample due to limited sample size available and cost of analysis. Analysis conducted by Research and Productivity Council, New Brunswick.

Table 6: Comparison of heavy metals in crab meal and protein hydrolysate with Health Canada allowable levels for seafood.

Heavy Metal	Maximum Allowable Level (ppm) Seafood [Health Canada, 2020] [H]	Level (ppm) in Crab Meal	Level (ppm) in Protein Hydrolysate
Arsenic	3.5	21.1	54.6
Lead	0.5	0.31	0.06
Mercury	0.5-1.0	0.16	0.03

marine environment and (2) contamination from the grinding and drying steps. While aluminum is not listed as a metal of concern for seafood, natural health products, or chitin-chitosan, it is classified as a neurotoxic agent [Exley, 2014]. This, coupled with reports of increasing concentrations of aluminum in the environment, food, and drink [Hardisson et al., 2017; Pereira et al., 2019; Mirza et al., 2017], is raising health and safety concerns for some consumers. As we currently do not have a good understanding of what constitutes a safe exposure vs. an unsafe exposure [Exley, 2013], limits for aluminum in food and natural health products have not been established.

Protein Hydrolysate

Protein hydrolysates have applications as feed additives for terrestrial and aquatic animals, and as natural health products (e.g., protein supplement) for human consumption. The main heavy metals of concern for edible seafood and for which Health Canada has established maximum allowable levels (Table 1) include arsenic, lead, and mercury. The maximum allowable levels of these metals in Canadian seafood are compared with our crab meal and protein hydrolysate samples in Table 6. Mercury and lead levels were below the Health Canada maximum level of 0.5-1 ppm [Health Canada, 2020] for seafood in the crab meal and protein hydrolysate. Total arsenic levels in the crab meal (21.2 ppm) and protein hydrolysate (54.6 ppm) samples, however, were significantly higher than the Health Canada maximum level of 3.5 ppm (total arsenic) for seafood [Health Canada, 2020] and 8 ppm in livestock feed [Health Canada, 2017]. Arsenic was more concentrated in the protein hydrolysate sample in comparison to the crab meal sample.

The high levels of sodium and potassium (Table 5), while not the focus of our study, may also affect the acceptability of crab meal and protein hydrolysate from a nutritional perspective, in feeds, and natural health products and should be further evaluated.

Arsenic

Arsenic is the twentieth most abundant element on Earth, and in its inorganic forms (e.g., arsenite AsIII, and arsenate AsV) it is lethal to the environment and living organisms being both toxic and carcinogenic [Cubadda et al., 2017; Jaishankar et al., 2014]. Sources of arsenic in the environment come from industrial sources, natural mine deposits, use of pesticides containing arsenic, and inappropriate disposal of arsenic chemicals [Jaishankar et al., 2014].

The type of arsenic determines its toxicity. Organic arsenic has a more complicated chemical structure (bound to carbon atoms) than inorganic arsenic, yet organic arsenic is harmless, whereas inorganic arsenic (iAs) is toxic [Schwarcz, 2018]. Arsenobetaine

($C_5H_{11}AsO_2$) is the most abundant form of arsenic found in seafood but is relatively non-toxic since the arsenic atoms are bound to carbon and, therefore, not available to bond with other biomolecules such as protein [Cubadda et al., 2017; FAO/WHO, 2011; Schwarcz, 2018; Taylor et al., 2017]. Organo-arsenicals, such as arsenobetaine, have low toxicity due to their low biological reactivity and their rapid excretion in urine [WHO, 2000].

Dietary exposure to arsenic is largely influenced by the amount of seafood in the diet [WHO, 2000]. Shellfish and seafood have been identified as a key contributor of iAs exposure in the diet, particularly in countries where large quantities of seafood are consumed (e.g., Japan, United States) and have been categorized as a food that is naturally high in iAs [Cubadda et al., 2017; Taylor et al., 2017; WHO, 2000; EPSA, 2009]. While As in seafood is primarily present in its organic form, some marine species have high iAs levels, with shellfish having higher concentrations than finfish [Taylor et al., 2017; Lorenzana et al., 2009]. Lynch et al. [2014] reported that crustaceans may contain high levels of iAs.

Total arsenic concentrations in some crustaceans have been reported to be > 100 mg/kg [WHO, 2000; Munóz et al., 2000; Ishinishi et al., 1986]. Anacleto et al. [2010] evaluated the total arsenic content in several fish, cephalopods, and Norway lobster and the latter had the highest levels of total arsenic (23.1-51.2 ppm) among the 12 species evaluated. Munóz et al. [2000] reported total arsenic levels of 1.69-137.32 ppm in crustaceans, and Fabris et al. [2006] reported a total arsenic level of 50.7 ppm in Australian lobster. The levels of arsenic found in our snow crab byproduct, crab meal, and protein hydrolysate samples are comparable to these previously reported values.

While arsenic speciation was beyond the scope of this study, it is important to understand which arsenic species are present in our samples and in what proportions to determine potential human toxicity. For illustration, we conducted a theoretical assessment based on previous studies by Cubadda et al. [2017] and Lorenzana et al. [2009]. Cubadda et al. [2017] estimated that of the total arsenic present in shellfish, 5% is attributed to iAs, 50% is due to arsenobetaine, and 45% is due to other organo-arsenic species (other than arsenobataine), which may or may not be toxic. Lorenzana et al. [2009] found that levels of iAs could be as high as 25% in shellfish. Based on the iAs levels reported for shellfish in these previous studies, our protein hydrolysate sample theoretically could contain anywhere from 2.73-13.65 ppm iAs. At this concentration, our crab protein hydrolysate in its current form would not be an acceptable protein supplement when administered at a dosage of 3-4 g/day [Jensen et al., 2019]. At this dosage, based on our theoretical estimate of iAs, our crab protein hydrolysate exceeds Health Canada's daily acceptable limits for NHPs (Table 3) resulting in 164-218 ug/day of total arsenic and 8.19-54.6 ug/day of iAs.

Chitin

Shrimp and crab shell waste are the main commercial sources of chitin. Due to its highly crystalline structure and strong hydrogen

bonds, chitin is not readily dissolved in common solvents which limits its applications. Therefore, it is often converted to its N-deacetylated derivative, chitosan, and/or other modified forms of chitin/chitosan, which are more soluble in dilute organic acids and water [Manuel, 2017]. The control over molecular weight, viscosity, and degree of deacetylation allows the production of a wide range of chitosans which can be used in medical, pharmaceutical, cosmetic, nutraceutical, and industrial fields, and are the main characteristics used to determine quality and price [Manuel, 2017; Jayakumar et al., 2010; France Chitine, n.d.; Roberts, 1992]. Safety is determined by the levels of residual protein, bacterial endotoxins, and heavy metals present [Manuel, 2017; Khor, 2014].

Currently, chitosan is approved in Canada as a NHP for oral administration as a supplement for weight management and maintaining healthy cholesterol levels [Health Canada, 2018]. In the United States, chitosan has been approved by the FDA for wound healing applications [Kumar and Kumar, 2017], and as a Generally Recognized as Safe food additive [Morin-Crini et al., 2019], while its complete approval by the FDA for all biomedical applications is still pending [Kumar and Kumar, 2017]. It is also approved as a food ingredient in Japan and Korea [Morin-Crini et al., 2019].

Morin-Crini et al. [2019] recently conducted a comprehensive review of the many applications of chitosan in several fields. Based on their review of numerous papers and patents reported over the last two decades, they concluded that although therapeutic and biomedical chitosan products are promising, chitosan applications in the biomedical field are still limited due to challenges in accessing biopolymers of sufficient purity and reliability, the high development costs, and the limited number of in vivo studies conducted. Part of this challenge is the lack of a definitive "standard" for either chitin or chitosan [Roberts, 1992], and there are no universally accepted quality standards for the wide array of various chitosans available in the market. However, guidelines and standards have been proposed for chitosan for pharmaceutical and medical applications. Proposed standards by Knapczyk et al. [1989] covered general characteristics, chemical and microbiological purity levels, physiological properties, and biological activity [Roberts, 1992]. More recently, ASTM [2019] and USP-NF [2020] published guidelines for the characterization/evaluation of chitosan/chitosan-salts for use in biomedical and/or pharmaceutical applications. Large chitin-chitosan manufacturers (e.g., Heppe Medical, Primex) produce these biopolymers under some form of quality management system such as ISO 9001, Good Manufacturing Practices, or Good Laboratory Practices and must meet the requirements of the importing countries' health regulations [Khor, 2014].

Our chitin sample meets the USP-NF medical grade chitin-chitosan standard for arsenic, cadmium, chromium, iron, lead, mercury, and nickel, but exceeds the total maximum allowable level of heavy metals when aluminum is considered (Table 7). Our chitin sample also meets the Health Canada requirements for levels of arsenic, lead, and mercury in seafood (Table 7). However,

Table 7: Comparison of heavy metals in chitin with industry standard for medical grade chitosan and Health Canada levels for seafood [Health Canada; 2020; ASTM, 2019; USPC, 2020; CASD, 2014].

Heavy Metal	Maximum Allowable Level (ppm) Medical Chitosan (USP-NF)	Maximum Allowable Level (ppm) Seafood (Health Canada)	Level (ppm) in Chitin Sample
Arsenic	< 0.5	3.5	< 0.02
Cadmium	< 0.2	-	0.004
Chromium	< 1.0	-	0.3
Iron	< 10	-	< 4
Lead	< 0.5	0.5	0.03
Mercury	< 0.2	0.5-1.0	< 0.01
Nickel	< 1.0	-	< 0.2
TOTAL	< 40	-	< 5[a] 156[b]

[a] Does not include aluminum. [b] Including aluminum.

Health Canada has not established limits for levels of total aluminum in food or natural health products.

Aluminum

Varying amounts of aluminum are naturally present in the environment. Aluminum is the third most common element found in the Earth's crust constituting about 8% by weight and is the most abundant metal on Earth [Pereira et al., 2019]. It is one of the most common metals found in the environment and occurs naturally in the air, water, and soil and, therefore, in food [Jaishankar et al., 2014; Gupta et al., 2013; Hardisson et al., 2017; Healthy Canadians, 1998; EPA, 2020]. Mining and processing of aluminum increases its level in the environment [Jaishankar et al., 2014; ATSDR, 2008; EPA, 2000] as does acidification of the soils [Hardisson et al., 2017; EPA, 2020]. This acidification of soils and the transfer of soluble aluminum (Al^{3+}) to the aquatic environment has resulted in increasing concentrations of aluminum in food and drink [Hardisson et al., 2017]. Other sources of aluminum include food additives, aluminum utensils, and tea consumption [Sjögren et al., 2007]. However, aluminum has no known biological role. It is a non-essential toxic metal to microorganisms, animals, fish, aquatic life, and humans [Hardisson et al., 2017; Olaniran et al., 2013]. In humans, it tends to accumulate in the brain and is, therefore, classified as a neurotoxic agent which has been linked to different diseases such as Alzheimer's disease and may interfere with other essential metals [Hardisson et al., 2017; Pereira et al., 2019; Mirza et al., 2017; Exley, 2013; 2014]; however, studies to date have been inconclusive.

Maximum dietary limit intake levels for aluminum have been established by various organizations. The European Food Safety Authority (EFSA) has established a tolerable weekly intake (TWI) of 1 mg Al per kg of body weight [EFSA, 2011]. The Food and Agriculture Association/World Health Organization (FAO/WHO) Expert Committee on Food Additives has set a provisional tolerable weekly intake of 2 mg/kg of body weight/week [Hardisson et al., 2017; FAO/WHO, 2011], stating that a daily aluminum intake of up to 7 mg/kg body weight is tolerable [Bruck et al., 2011]. Dietary limit intake levels have not been established by

Health Canada [2008], and there is currently no established industry standard for aluminum levels in chitin-chitosan.

Aluminum levels in a variety of marine products were reviewed by Jaishankar et al. [2014] for the period 2002-2017. They found that aluminum levels varied widely between areas where products were collected, but overall seafood had the highest reported Al levels ranging from 10.2-204.6 mg/kg, in comparison to other food groups, except for processed cheese which had levels of Al between 270-670 mg/kg attributed to the use of sodium-aluminum phosphate as an emulsifying agent [Soni et al., 2002; Saiyed and Yokel, 2005]. Pereira et al. [2019] reported that in marine samples aluminum levels vary and can range from 0.1 to 19.2 ug/g in a variety of fish to as high as 71.9 ug/g in mussels (*Mytilus edulis*). The Al levels determined for snow crab products in this study are within the range reported by Jaishankar et al. [2014].

Ingestion, inhalation, and dermal contact have all been identified as routes of aluminum exposure [Jaishankar et al., 2014]. Drugs.com reported that in clinical trials the dosage of chitosan administered for glucose control is 1.5 g/day yet could be as high as 15 g/day for weight loss applications [Drugs.com, 2021]. Therefore, our chitin sample could contribute up to 2.265 mg of aluminum daily if used as a weight loss supplement at a dosage of 15 g/day. For a person weighing 80 kg, this is equivalent to 10-20% of the TWI levels established by EFSA and the FAO/WHO Expert Committee on Food Additives. Chitin-chitosan also has various cosmetic applications; aluminum levels in cosmetics has raised concerns due to possible linkages with breast cancer and Alzheimer's disease [Becker et al., 2016; Exley, 2014]. Another proposed use of chitin and chitosan is as a drug delivery agent in inhalation products, and in the manufacture of biodegradable sutures [Matica et al., 2017; Kumar and Kumar, 2017], for which a key consideration is purity. Although the daily aluminum intake through chitin-chitosan products may seem insignificant on its own, the high level of aluminum in our chitin sample may be cause for concern for these types of products when combined with other sources of aluminum exposure by contributing to the body burden of aluminum [Exley, 2013; 2014]. Since aluminum has no biological function [Hardisson et al., 2017; Olaniran et al., 2013], and it is not overtly toxic, it could become covertly toxic because it accumulates in the brain as we age [Exley, 2013; 2014]. Until further scientific data is available regarding safe vs. unsafe exposure levels, a precautionary approach to reduce human exposure to aluminum is advisable [Exley, 2013].

Aluminum was only marginally reduced from 185 ppm in the crab meal sample to 151 ppm in our chitin sample, suggesting that it may bio-adsorb to chitin during the extraction process or that the extraction process was not effective for its removal. Our results indicate that the main source of aluminum is likely bioaccumulation from the marine environment; however, the grinding and drying steps may be an additional source of aluminum contamination. Given the adverse health effects associated with aluminum, it would be prudent to minimize this impurity in chitin-chitosan products intended for natural health products as well as pharmaceutical and biomedical

applications. If the aluminum is in a non-leachable form, the resulting chitin may still be valuable for external applications.

CONCLUSION

Understanding the levels of heavy metals in snow crab byproducts and how they are transferred throughout the crab bio-product value chain will be key to developing safe marketable crab bio-products for natural health product and biomedical/pharmaceutical applications. Two metals of concern were identified in the crab bio-products produced during this study: arsenic which causes acute toxicity and aluminum which may be covertly toxic over time. Two potential sources of these metals were also identified: bioaccumulation from the marine environment and contamination from processing equipment. Arsenic (54.6 ppm) was concentrated in the protein hydrolysate and aluminum (151 ppm) in the chitin fraction.

Speciation of arsenic was beyond the scope of the current study and, therefore, we cannot accurately quantify the concentration of organic and inorganic arsenic in our sample. However, speciation analysis for selective determination of iAs is important to avoid overestimation (or underestimation) of the health risk associated with dietary arsenic exposure [Cubadda et al., 2017]. It is recommended that arsenic speciation be evaluated in future studies to provide a better understanding of the safety and potential toxicity of crab protein hydrolysates for use as a natural health product.

This study has illustrated that care must be taken to remove aluminum and arsenic from the raw (unprocessed) crab byproduct and to ensure the extraction process does not increase the concentration of these metals and inadvertently facilitate their transfer to the final bio-products. The processing steps should be further evaluated with the aim of reducing the arsenic and aluminum content in the bio-products as well as minimizing potential metal contamination from processing equipment. The shell and protein/organs/tissues may need to be separated at the processing plant and processed separately into protein hydrolysate and chitin for more effective removal of metal contaminants. The main limitation of this study was the limited number of samples available. Additional studies using a larger sample size are recommended to better understand levels of heavy metals that are naturally present in raw (unprocessed) snow crab byproducts from N.L. and their final concentrations in extracted crab bio-products.

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