Green Chemistry and an Ocean Based Biorefinery Approach for the Valorization of Newfoundland and Labrador Snow Crab (*Chionoecetes opilio*) Processing Discards

by © Heather Joy Burke

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ABSTRACT

Snow crab (*Chionoecetes opilio*) is the most important commercial species to the NL fishery and NL's rural economy. According to industry stakeholders, it has replaced cod as "King". In 2020, the Government of Newfoundland and Labrador reported an export value of $648 million from annual landings averaging 30,000 t of snow crab. The NL snow crab industry generates ~30% waste each year (~10,000 t), which typically is landfilled or dumped at sea. These discards contain valuable bioproducts such as pigments, proteins, chitin, and lipids, which could be recovered for use in a wide range of fields from agriculture and aquaculture to biomedical. However, many of the processes used for snow crab valorization require hazardous chemical treatments, such as acids, bases, and flammable solvents, creating environmental concerns such as air and water pollution, and health and safety concerns. In addition, environmental requirements are becoming stricter, making traditional disposal options for crab processing discards more difficult and costly. To address these challenges, I evaluated a combined green chemistry-ocean based biorefinery approach for the valorization of NL's snow crab processing discards.

Four research studies were conducted using a range of methods: semi-structured interviews, analysis of fisheries and aquaculture statistics, evaluation of raw material pre-treatment and collection methods, scientific studies to characterize and stabilize crab discards, as well as comparisons of chemically extracted *vs* "green" extracted crab bioproducts. (1) An inventory assessment of available marine feedstocks showed that crustaceans generate the largest wastes, which in 2015 could theoretically support regional by-product processing facilities on the Northern Peninsula, Northeast Coast, and Avalon

Peninsula. (2) Characterization and stabilization studies showed that seasonality and pre-treatment method had the greatest impact on quality, and that crab by-products have unique intrinsic characteristics that influence quality. (3) Purity and safety of crab bioproducts were evaluated by measuring quantities of trace metal contaminants. Two metals of concern were identified: arsenic, which causes acute toxicity; and aluminum, which may be covertly toxic over time. (4) Sequential extraction of carotenoid pigments, pigmented protein powder, and chitin from crab processing by-products using vegetable oils, citric acid, proteases, and hydrogen peroxide to replace traditional organic (e.g., acetone, ethanol) and inorganic (e.g., HCl, NaOH) reagents was evaluated and demonstrated that these chemical reagents can be replaced with green alternatives.

The findings from these studies were incorporated into a green chemistry-biorefinery model that, with optimizations, could be adopted by industry and the province to address current challenges related to snow crab waste disposal and valorization. The proposed model allows for the extraction of multiple higher value crab bioproducts that are produced using more environmentally friendly and potentially lower cost alternatives, to more traditional chemically intensive and expensive techniques. It is anticipated that this model will provide the groundwork for the development of a provincial crustacean waste disposal and by-product utilization strategy.

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and in preparation of this manuscript. These wonderful individuals and institutions are acknowledged below.

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My sincere thanks also go to several members of the Fisheries and Marine Institute community. Mr. Ron Newhook (retired) former Director of Research, for encouraging me to pursue my own personal goals and professional development through this PhD. I probably would not have started this PhD had it not been for his support and enthusiasm. Mr. Kevin Anderson, Head - School of Fisheries, for providing flexibility with my schedule, financial support towards analytical services and approving access to the Mt. Scio Marine Bioprocessing Research Lab and Pilot Plant Facilities during the Covid-19 pandemic, so that I could complete my research. His understanding, patience, and support during the writing of this manuscript have been invaluable. Dr. Robert Shea, Vice President (Marine Institute) for encouraging me to pursue my PhD as a means of self-improvement

and professional growth, and for his contagious optimism. Mr. Robert Verge (retired) former Managing Director of the Canadian Centre for Fisheries Innovation (CCFI), for his guidance and advice on the challenges related to fish by-product utilization in the NL seafood industry.

The Centre for Aquaculture and Seafood Development of the Fisheries and Marine Institute of Memorial University of Newfoundland was a wonderful resource providing scientific and technical reports, photos, illustrations on current crustacean processing methods, and data sets related to the inventory, quality, characterization, and utilization of marine feedstocks, as well as technical expertise. Special thanks go to Ray Hayter and Wade Murphy in this regard. Mr. Murphy's assistance with sample preparation and pilot plant equipment set-up is also recognized.

Gratitude is extended to Christopher Stamp, former Senior Policy, Planning and Research Analyst with the Licensing, Inspection and Quality Assurance Division of the Department of Fisheries and Lands Resources, Government of Newfoundland and Labrador for providing provincial statistical data and guidance on data analysis and interpretation. Similarly, appreciation is extended to Ms. Leslie Norman, former Statistics Coordinator, Department of Fisheries and Oceans Canada, White Hills – St. John's, NL for the provision of statistical data on landings and discards of Atlantic cod in Newfoundland and Labrador, and guidance on data analysis and interpretation.

I deeply appreciate the support from my industrial collaborators. Dr. Ogmundur Knutsson, former professor of Business and Economics, University of Akureyri, for developing the semi-structured interview template, guiding the interview process with industry stakeholders, and helping me learn this valuable skill. Mr. Robin Quinlan, Vice

President Quinlan Brothers Ltd., for providing access to the crab processing plant in Bay de Verde, NL, and for providing crab processing by-product samples for this study. Appreciation is extended to Mr. Barry Hatch, plant manager, and Trevor, plant supervisor, for collecting the crab by-product samples in 2018 and 2021. Thanks go to Chris Daley, owner of Independent Dockside Graders, for providing guidance and advice on current practices in the snow crab processing industry and providing recommendations on which pre-treatment methods to evaluate. Dr. Pedram Dehdari, Managing Director of Ensymm UG & Co. KG., for providing samples of commercial fungal acid protease for evaluation, and for his input and guidance on enzymatic deproteination protocols and other green technologies for chitin extraction from crustacean shells.

Finally, thanks are extended to many staff of Memorial University including: Mr. Vegnesh Ramakrishnan, Biotechnologist, Centre for Aquaculture and Seafood Development, Marine Institute, for conducting amino acid analyses; Ms. Jeanette Wells, Department of Ocean Sciences, for performing lipid class analysis and fatty acid compositional analysis; Mr. John Allen, Department of Earth Sciences, for performing ICP-MS elemental analysis on the crab by-product samples collected in 2018; and Dr. Wanda Aylward, The Earth Resources Research and Analysis Facility (TERRA), for performing X-ray diffraction scans and providing training on the JADE 2010 software. The assistance from these technical experts was an invaluable contribution to this thesis.

COVID IMPACT STATEMENT

My programme of study was originally scheduled for completion in August 2021. However, due to the Covid-19 Provincial wide shut down in March 2020, which included

Provincial travel restrictions and a university wide suspension of fieldwork, I could not collect crab samples during the 2020 crab fishing season (May to July) which were needed to complete my analytical work. Fieldwork that was planned for May-July 2020 and subsequent experiments were delayed by one full year; therefore, all planned experiments could not be completed.

During the period of May-December 2020, an additional study was included in case I was unable to collect crab samples during the upcoming 2021 crab fishing season. I conducted a study on heavy metals in crab bioproducts using dried crab meal samples prepared during 2018 as the raw material. Although the samples were 2 years old, they had been dried, sealed in sanitary containers, and kept frozen at –20°C, and were deemed suitable for this study. Key challenges however were the limited sample size available and the high cost of metal testing resulting in only 1 or 2 replicates for analysis.

For my final study, the original goal was to demonstrate a proof-of-concept that green technologies could be used to extract multiple bioproducts from snow crab processing by-products, resulting in as little waste as possible. Targeted bioproducts included pigmented oils, pigmented protein (carotenoprotein complex), protein hydrolysate, chitin, and chitosan. While crab samples were obtained in 2021, due to reduced timelines and limited access to lab facilities I was not able to extract and characterize all planned bioproducts, therefore only pigmented oils, carotenoprotein, and chitin were selected for this study.

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ACRONYMS

ANOVA – Analysis of Variance
AOAC – Association of Official Analytical Chemists
ARC – Aquatic Research Cluster
AVG - Average
BOD - Biological Oxygen Demand
CAFID – Canada Newfoundland Cooperation Agreement for Fishing Industry Development
CASD – Centre for Aquaculture and Seafood Development
CDA - Chitosan Deacetylase
CFIA – Canadian Food Inspection Agency
COD - Chemical Oxygen Demand
COS - Chito-Oligosaccharide
DA - Degree of Acetylation
DDA - Degree of Deacetylation
DFA - Department of Fisheries and Aquaculture
DFLR - Department of Fisheries and Land Resources
DP - Degree of Polymerization
DPA – Docosapentaenoic Acid
EFSA - European Food Safety Authority
EHA – Eicosapentaenoic Acid
FAME – Fatty Acid Methyl Esters
FAO - Food and Agriculture Organization of the United Nations
FDA - Food and Drug Administration
FFA - Free Fatty Acid
FFT - Factory Freezer Trawler
FID – Flame Ionization Detector
FOG - Fat-Oil-Grease
GC-MS Gas Chromatography Mass Spectrometry
GlcN - Glucosamine
GlcNAc - N-acetylglucosamine
GLP - Good Laboratory Practices
GMP - Good Manufacturing Practices
GPa - Gigapascals
GRAS - Generally Regarded as Safe
Hc - Hemocyanin
HPLC – High Performance Liquid Chromatography
HPP - High Pressure Processing

iAs - inorganic arsenic
ICP-MS – Inductively Coupled Plasma Mass Spectrometry
IQF – Individually Quick Frozen
ISO - International Organization for Standardization
JOT - Journal of Ocean Technology
LAF - Lactic Acid Fermentation
MI - Fisheries and Marine Institute
MUFA – Monounsaturated Fatty Acids
MUN – Memorial University of Newfoundland
MW - Molecular Weight
NHP - Natural Health Product
NL – Newfoundland and Labrador
NRC - National Research Council of Canada
PA - Pattern of Acetylation
pK_a - the negative base-10 logarithm of the acid dissociation constant (K_a) of a solution
PPE - Personal Protective Equipment
ppm - parts per million
PPO - Polyphenol Oxidases
PTWI - Provisional Tolerable Weekly Intake
PUFA - Polyunsaturated Fatty Acids
QBL - Quinlan Brothers Limited
RSW - Refrigerated Seawater
TAC - Total Allowable Catch
TAML - Tetraamido Macrocyclic Ligands
TSS - Total Suspended Solids
TWI - Tolerable Weekly Intake
WHO - World Health Organization

DEFINITIONS

Bioproducts are products derived from natural renewable biological resources.

Green Chemistry refers to the design of products and processes that minimize or eliminate the use and generation of hazardous substances.

Marine Biomass Waste refers to seafood processing discards, effluents, and fish by-products.

Marine Feedstock Materials are the recovered biomass wastes that are used as inputs in a marine or ocean based biorefinery system.

Ocean-Based Biorefinery refers to a system whereby marine biomass waste streams are recovered and become the input materials for the sustainable conversion into marketable high value bioproducts (e.g., feed, food, nutraceuticals, fine chemicals, pharmaceuticals, cosmetics), bioenergy and biofuels.[1]

Valorization is the process of making something valuable or useful from a current waste stream.

[1] Refer to references 13, 14, 15, 16, 17, and 18 in Chapter 2 for more details on the ocean-based biorefinery.

INTRODUCTION

This thesis seeks to address challenges associated with stabilization and valorization of snow crab processing discards by combining green chemistry and an ocean-based biorefinery approach to minimize environmental hazards and maximize value. Green chemistry refers to the design of products and processes that minimize or eliminate the use and generation of hazardous substances. The ocean-based biorefinery approach, discussed in Chapters 1 and 2, refers to a system whereby marine waste streams are recovered and become the input materials, or feedstock, for sustainable conversion into marketable high value bioproducts (e.g., feed, food, nutraceuticals, fine chemicals, pharmaceuticals, cosmeceuticals), bioenergy and biofuels. Interest in this approach for creating value from crustacean waste streams has been increasing as the seafood industry seeks to maximize value and minimize waste.

The main goal of this thesis is the application of green chemistry within an ocean based biorefinery as a way of reducing environmental impacts while creating value from Newfoundland and Labrador's (NL) snow crab processing discards. Value creation focused on the green extraction of selected snow crab bioproducts (e.g., pigments, protein, chitin), and did not include bioenergy or biofuels.

Objectives of the Research

A key objective of this thesis is redirecting crab processing discards toward extracting valuable bioproducts as an alternative to current at-sea dumping and landfilling practices, thereby reducing environmental pollution, while increasing utilization and

maximizing value. Traditional extraction methods for crustacean bioproducts, however, are chemically intensive processes which generate wastes that cause environmental pollution. This includes the use of hazardous chemicals such as flammable volatile organic solvents (e.g., acetone and ethanol), and highly corrosive reagents such has hydrochloric acid and sodium hydroxide. Many of these chemical reagents are also carcinogenic or toxic. Therefore, a second objective of this thesis is to replace hazardous organic and inorganic chemicals with greener chemistry alternatives that are environmentally friendly. The third objective is to identify simple green processes that are cost effective and easy to implement in coastal areas of the province. The final objective is the culmination of the above into a crab by-product utilization model for the NL snow crab industry.

It is anticipated that this thesis will provide the groundwork for the development of a provincial strategy to address current challenges related to the disposal and valorization of snow crab processing discards, which could be expanded to include other commercially valuable marine species.

Hypothesis

By combining green chemistry with an ocean based biorefinery approach it is hypothesized that environmental health and safety concerns related to the valorization of NL's snow crab processing discards can be mitigated, and valuable crab bioproducts can be obtained cost effectively.

Chapter Outline

This thesis is comprised of seven Chapters which are briefly described below.

Chapter 1 is an introduction to the thesis and provides a review of current and emerging technologies for crustacean processing. A model for value chain optimization of crustacean waste using green chemistry and a general biorefinery approach is proposed for the bio-production of chitin and its derivatives.

Availability of feedstock will be critical to establishing a site to produce bioproducts from crab fishery waste. Chapter 2 describes and evaluates the potential for different sites in Newfoundland.

Chapter 3 describes characterization studies of crab processing by-products and evaluates the effect of collection pre-treatment methods, drying methods, and seasonality on the quality of this biomass material and its suitability for use as a feedstock for the extraction of higher-value crab bioproducts.

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, the levels of trace metal contaminants in crab processing by-products and their transfer to selected crab bioproducts are evaluated in Chapters 4 and 5.

Chapter 6 focuses on evaluating simple green technologies for the extraction of selected crab bioproducts from snow crab processing discards. Sequential extraction of carotenoid pigments, pigmented protein powder and chitin is attempted using simple

extraction methods and green solvents to conceptualize an environmentally friendly, cost-effective crab by-product utilization process.

Chapter 7 is the final Chapter of the thesis. It synthesizes the studies into a green chemistry-ocean based biorefinery model for valorization of snow crab processing discards, discusses the limitations of the research, and provides recommendations for further research to optimize the proposed green chemistry-biorefinery model.

Co-Authorship Statement

I am the major intellectual contributor and principal author of all Chapters presented in this thesis. I contributed to all practical aspects of the research, including design of experiments, data collection and analysis, interpretation of results, and manuscript preparation. Of course, this would not have been possible without the supervision and guidance of my supervisor Dr. Francesca Kerton, the support and direction from my supervisory committee members (Dr. Kelly Hawboldt, Dr. Robert Helleur), and the collaborative contribution of several key individuals. I prepared all manuscripts presented in this thesis which were revised based on the advice and comments from my supervisor and supervisory committee. The contributions and involvement from my supervisor and committee members, and other key individuals are recognized here.

Chief collaborator for Chapter 1 was Francesca Kerton. Dr. Kerton provided comprehensive editorial reviews of the manuscript.

Chief collaborators for Chapter 2 included Francesca Kerton, Kelly Hawboldt, Robert Helleur, Ogmundur Knutsson, Robert Verge, Christopher Stamp, and Leslie Norman. Dr. Kerton, Dr. Hawboldt and Dr. Helleur provided guidance on the scope and

experimental design and conducted comprehensive editorial reviews of the manuscript. Dr. Ogmundur Knutsson developed the semi-structured interview template and guided the interview process. Christopher Stamp provided provincial fisheries and aquaculture statistical data and guidance on data analysis and interpretation specifically in relation to the calculation of processing discards. Ms. Leslie Norman provided statistical data on landings and discards of Atlantic cod in Newfoundland and Labrador, and guidance on data analysis and interpretation specifically in relation to the calculation of processing discards. Mr. Robert Verge provided guidance and advice on the challenges related to fish by-product utilization in the NL seafood industry.

Chief collaborators for Chapter 3 included Chris Daley, Wade Murphy, Vegnesh Ramakrishnan, Jeanette Wells, John Allen, Francesca Kerton, Kelly Hawboldt, and Robert Helleur. Mr. Daley provided guidance and advice on current practices in the snow crab processing industry and provided recommendations on pre-treatment methods. Mr. Murphy assisted with sample preparation and pilot scale equipment set-up. Mr. Ramakrishnan performed amino acid analyses. Ms. Wells conducted lipid class analysis and fatty acid compositional analysis. Mr. Allen performed ICP-MS elemental analysis. Dr. Kerton, Dr. Hawboldt and Dr. Helleur provided comprehensive editorial reviews of the manuscript. Dr. Kerton also provided financial support towards analytical services.

Chief collaborators for Chapters 4 & 5 included Francesca Kerton, Peter Crowhurst and Kevin Anderson. Dr. Francesca Kerton provided support and guidance during the preparation of Chapter 4 and is a co-author of this Chapter which has been published in the Journal of Ocean Technology (JOT). While 95% of the JOT manuscript (Chapter 4) is my original work, Dr. Kerton provided key contributions related to experimental design,

interpretation of the results, formulating the conclusions, and providing a critical review of the work. Mr. Crowhurst provided analytical services for the analysis of trace metals and mercury. Mr. Anderson provided financial support for analytical services and access to the Marine Institute's Marine Bioprocessing Research Lab and Pilot Plant Facilities during the pandemic.

Chief Collaborators for Chapter 6 included Pedram Dehdari, Francesca Kerton, Wanda Aylward, and Kevin Anderson. Dr. Dehdari provided samples of commercial fungal acid protease for evaluation, and guidance on enzymatic deproteination protocols and other green technologies for chitin extraction from crustacean shells. Dr. Kerton provided input on the experimental design and comprehensive editorial reviews of the manuscripts. Dr. Aylward performed X-ray diffraction scans and provided training on the JADE 2010 software. Mr. Anderson provided financial support for analytical services and access to the Marine Institute's Marine Bioprocessing Research Lab and Pilot Plant Facilities during the pandemic.

Dissemination of Research

- Chapter 1 was published in 2017 in the book "Fuels, Chemicals and Materials from the Oceans and Aquatic Resources" © John Wiley & Sons Ltd. I am the sole author of this publication.
- Chapter 2 was presented as a seminar in 2018 as part of the Fisheries and Marine Institute's "School of Fisheries Seminar Series".
- Chapter 3 was presented as a poster at the 2021 Atlantic BIOCON Student Poster Competition winning first place in the graduate student category. Dr. Francesca Kerton provided editorial review of the poster submission.

- Chapter 4 was published in the Spring 2022 edition of the *Journal of Ocean Technology,* Vol, 17, No,1. I am the primary author (95% authorship). Dr. Francesca Kerton is the second author (5% authorship).
- Chapter 6 is currently in preparation for submission to either the journal *Biotechnology Reports* or *Nature Sustainability*.

CHAPTER 1. Innovations in Crustacean Processing: Bio-production of Chitin and its Derivatives

1.1 Introduction

The global seafood sector is perhaps the most complex of all food sectors. It is based on more species (about 1000 commercial species) and a wider range of processing technologies than any other food sector [1]. Total world fisheries production from wild capture and aquaculture sources was 179 million tonnes (Mt) in 2018, with wild capture fisheries accounting for 97 Mt and aquaculture contributing 82 Mt [2]. Wild capture fisheries production has been relatively stable for the past 30+ years at approximately 90Mt, whereas aquaculture production has increased by 8-9% in the same time-period [2, 3]. Twenty countries produce 74% of the world capture fisheries production with the top 5 producers being China (15%), Indonesia (8%), Peru (8%), Russian Federation (6%) and the United States (6%) [2]. The FAO has reported that 34.2% of fish stocks in 2017 were overfished meaning they were fished at biologically unsustainable levels, compared with 28.8% in 2011 and require strict management plans to rebuild stocks to levels where they are biologically sustainable [2, 4]. In 2018 more than 156 Mt (87%) of the world fish production was utilized for direct human consumption, 18 Mt (10%) was reduced to

fishmeal and fish oil, while the remaining 5 Mt (3%) went to other non-food uses such as bait, aquaculture feed, animal feed and pharmaceutical uses [2].

While the seafood sector contributes significantly to the world requirement for protein, it also has a reputation as being the most wasteful food sector. Approximately 70 Mt of fish are processed by filleting, freezing, canning, or curing and these activities generate 30-50% waste [4]. In 2009 approximately 46% of the landed volume (by weight) of all seafood produced in Canada was discarded as waste [5]. Similarly, in 2007 it was estimated that about 47% of South Korea's seafood harvest by weight was by-product. According to the US Department of Agriculture, over half of the world production of fish ends up as by-product (or waste) [1]. The tuna canning industry generates up to 65% solid waste, and fillet production of farmed salmon generates 45-50% waste, including belly flaps, heads, bones, frames, viscera, skin, gills, and dark muscle [4]. Of relevance to this chapter, crustacean processing generates anywhere from 30-70% waste. In NL, shrimp destined for cooked and peeled products result in 60-70% waste, and snow crab produces about 30% waste when processed into IQF (individually quick frozen) cooked sections. Fish processing discards contain high quality proteins, lipids, omega-3 fatty acids, micronutrients (e.g., Vitamin A, D, riboflavin, niacin) and minerals (e.g., iron, zinc, selenium, and iodine) [4]. Crustacean processing discards contain valuable products including proteins, lipids, astaxanthin, organic acids, essential amino acids, chitin, and calcium [6].

Today, consumers are much more aware and better educated about the health and environmental impacts of the use of chemicals in aquaculture and seafood production. This increased awareness, coupled with a desire for environmental sustainability, has led many

consumers to search for more natural products produced without chemical additives and harvested using sustainable practices. With respect to value added products such as nutraceutical and natural health products which can be extracted from marine processing by-products, consumers want high quality products with high biological activity [1,7,8]. In this regard, processing efficiencies are of particular importance. Seafood processors must be responsive and flexible to industry and consumer needs. They must be able to enhance the quality of and add value to their products while adhering to buyer and market specifications for environmentally sustainable products. As global demand for fish protein and sustainable seafood increases, the seafood sector can no longer afford the processing inefficiencies of the past which generated more than 50% waste (by-product). Proper biowaste management will be critical to meet regulatory requirements and the growing ecological and environmental concerns of the local and international markets [7].

The large volumes of crustacean biomass waste generated from the seafood processing sector often end up in landfills or are dumped at sea. While the processing of such biomass waste does produce high value products, this often requires more processing steps with extra costs [8,9,10], and the use of harmful chemicals such as acids, bases, and flammable solvents. These conventional approaches also result in overheating, high energy use, and loss of end-product functionality [8,9,10], and therefore lack the green principles of chemistry and engineering. However, these materials can be repurposed with a focus on the extraction of high value specialty products (e.g., nutraceuticals, pharmaceuticals, specialty chemicals) using green chemistry and an ocean-based biorefinery approach, while attempting to convert all input materials into useful products with minimum processing steps. This will require the development and use of innovative technologies.

1.2 Scope & Purpose

The scope of this chapter is to highlight and review some of these innovative technologies and how they can be implemented through a biorefinery approach to optimize the value chain of crustacean biomass with a particular focus on bioproduction of chitin, chitosan and chito-oligosaccharides from shrimp and crab.

1.3 Innovations in Crustacean Processing

1.3.1 Conventional Processing Technologies

Conventional processing of cooked shrimp and crab in NL have traditionally been labour intensive, and use inefficient cooking methods that result in overcooking, high energy use, yield and quality losses. For example, traditional cooking methods for snow crab use large immersion cookers requiring heating large volumes of cook water to 98°C. This requires the use of large boilers and typically takes 4 hours for the cook water to reach temperature (personal communication with industry stakeholders).

1.3.1.1 Cold Water Shrimp (Pandalus borealis)

Atlantic Canada, Greenland, Norway, Denmark, Iceland, Faroe Islands and Russia are major producers of cold water shrimp (*Pandalus borealis)* - the primary cold-water shrimp resource in the North Atlantic (Figure 1.1). Northern shrimps are typically harvested from near shore or offshore harvest sites using commercial fishing trawlers. Offshore shrimps are processed at sea onboard factory freezer trawlers (FFT). The shrimp are washed in seawater and then they are blast frozen at – 40ºC, shell-on. Offshore shrimp processed in this manner are referred to as “industrial shrimp” and are sold shell-on raw frozen to the

consumer. Alternatively, the shrimp may be cooked shell-on prior to freezing and sold as shell-on cooked frozen [11].

Figure 1. 1 Northern cold-water shrimp, Pandalus borealis (Photo Courtesy of CASD, Marine Institute, MUN)

Inshore shrimps are processed fresh onshore. In Atlantic Canada, shrimp are harvested using trawl technology. When the trawl is retrieved from the ocean floor, it is hoisted high above the deck with the cod end positioned above the holding pound (Figure 1.2). The cod end is opened, and the shrimp are released onto the deck (Figure 1.3) and transferred to bagging stations either above deck or below in the fish hold. Shrimp are loaded by hand into buckets lined with mesh bags and filled to a weight of 26 lbs. The shrimp bags are placed in the fish hold where they are direct iced until they reach shore.

Figure 1. 2 Northern Shrimp in trawl positioned above holding pound

(Photo Courtesy of CASD, Marine Institute, MUN)

Upon landing the shrimp are unloaded at the processing plant's receiving dock where they are placed in a maturation solution for 24-36 hours prior to processing. Maturation solutions are often proprietary blends of one or more processing aids that typically containing phosphates, sulfites and enzymes (personal communication with industry stakeholders). Inshore shrimps are typically processed into a cooked and peeled product and sold as frozen meats. The maturation process aids peeling (i.e., removal of shell and head material), improves water retention and texture, and helps prevent melanosis (an oxidative enzymatic discolouration reaction which is discussed in Chapter 3 - section 3.4.1.4). Following maturation, the shrimp undergo immersion cooking at 98 °C for 1-2 minutes (total cooking time may be 10-30 minutes depending on the cooking process), or continuous steam cooking at 100 °C, followed by cooling (immersion bath or cold-water spray), automatic peeling and grading, glazing, individual quick freezing (IQF), and packaging. Shrimp processed in this manner are referred to as “cooked & peeled” (Figure 1.4, Figure 1.5).

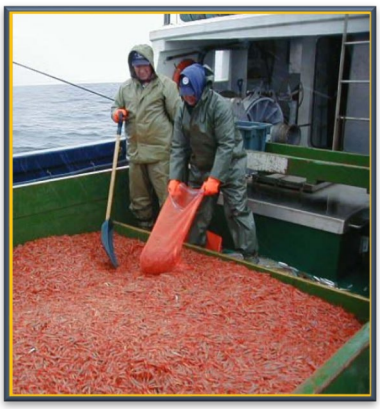

Figure 1. 3 Northern Shrimp held in pound onboard vessel

(Photo Courtesy of CASD, Marine Institute, MUN)

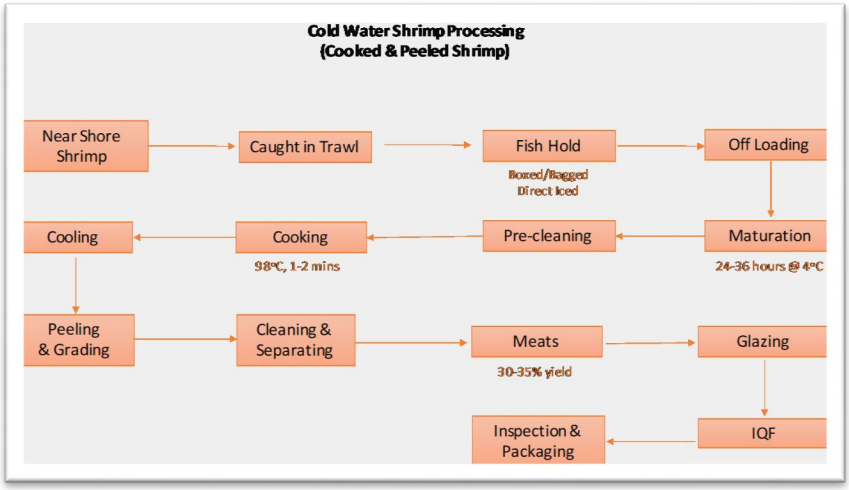

Figure 1. 4 Process flow for cooked and peeled shrimp

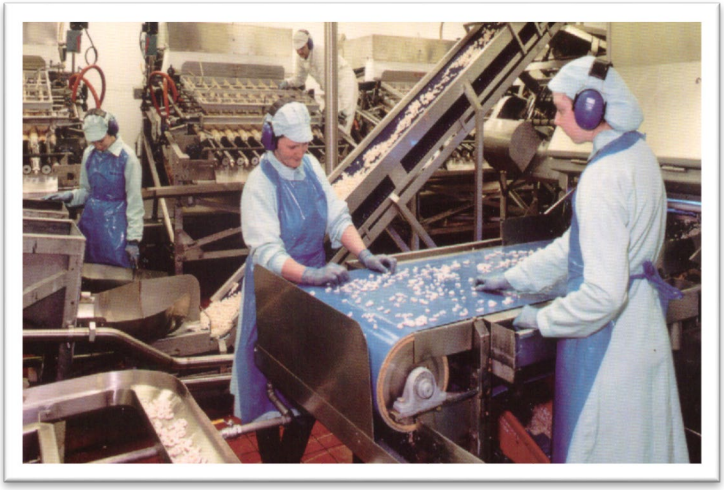

Figure 1. 5 Commercial processing of cooked and peeled shrimp

(Photo Courtesy of School of Fisheries, Marine Institute, MUN)

1.3.1.1.1 Onboard Handling

In the shrimp fishery in Atlantic Canada, the catch has traditionally been stored and handled in polyethylene mesh bags filled to a weight of 26 lbs [12]. This practice can be detrimental to the quality of landed shrimp when you consider that the shrimp in the bottom of the fish hold get damaged from the weight of the shrimp and ice on the top, and an

aluminum pound board is placed on top of the last layer of shrimp. This causes crushing of the shrimp in the lower layers and results in greater yield loss due to breakage and drip loss, as well as autolytic deterioration due to the release of digestive enzymes. In addition, inshore vessels are at sea fishing up to 3 days. Therefore, the first day's catch is already 3 days old before it is landed and offloaded at the processing plant. The 3-day old shrimp will then be placed in the maturation solution for up to 2 days prior to production. Thus, shrimp may be 5-days old by the time it is processed into the final cooked and peeled product. The range in age of the landed shrimp coupled with crushing damage due to storage and handling practices, has resulted in inconsistent product quality [12].

Boxing of shrimp is a method that has traditionally been used by the Scandinavian shrimp fishing fleet to hold their catch while at sea. Boxing involves mixing shrimp and ice in a specific ratio and storing in 380L insulated tubs/boxes. The vessels used in the Scandinavian fishery are larger than those in Atlantic Canada and they are designed with an open hold concept to facilitate boxing the product. The shrimp products exported by these countries are seen by the marketplace as superior products to Atlantic Canadian products. The higher quality is thought to be linked to handling procedures that include boxing of the raw material instead of bagging [12].

A study comparing boxing vs bagging of shrimp catches conducted in 2003-2004 concluded that boxing of shrimp catches provides improvements over bagging in terms of the landed quality of shrimp [12]. Improvements were noted in the finished product colour (Figure 1. 6). There was also a reduction in the amount of broken shrimp and freshness was maintained over a longer period. Other advantages of boxing were related to reductions in handling issues, less time to store the product, and reduced time to offload the vessel.

However, for the Atlantic Canadian fleet to switch from mesh bags to insulated tubs will require a high initial investment. Mesh bags cost about $0.35 each whereas the insulated boxes cost about $300 each, and each vessel would need 160-200 boxes. Vessels under 55' will have difficulty carrying more than 100 boxes. Therefore, modifications to the fish hold would be required to accommodate the insulated boxes, or smaller vessels will have to limit the amount of product they will be able to carry per trip which would not be economically feasible. Anecdotal information suggests that since the release of the study results, about 50% of the NL shrimp fishing fleet has adopted a modified boxing method using fish tote pans, the other 50% are still using the mesh bag system.

Figure 1. 6 Boxed shrimp after processing (left); and bagged shrimp after processing (right)

(Photos Courtesy of CASD, Marine Institute, MUN)

1.3.1.1.2 Maturation Process

After landing and offloading, shrimp are placed in a maturation solution for a period of 24-36 hours. The maturation solution is maintained at 0-4ºC and consists of a 1-10% solution of polyphosphates, and may contain salt, sulfites and enzymes, in which the shrimp

are immerged. The solution is usually recycled and periodically refreshed with polyphosphate addition, and eventually discarded every 24-36 hours. This technique increases the meat yield from 20-30% as it makes the shells easier to remove during the mechanical peeling process and aids in water retention [13,14,15]. KATCH 150, for example, is a specialty blend of polyphosphates designed for use in the process of deshelling shrimp and is reported to increase yields while improving quality, color, and texture [16]. In addition to providing a better yield, phosphates have been shown to increase consumer acceptability of cooked and peeled shrimp due to improvements in colour, texture, and flavour [13].

1.3.1.1.3 Cooking and Blanching Equipment

Commercial shrimp cooking is an important processing step which has a direct impact on product yield and quality (i.e., colour, flavour, and texture). There are 2 main types of commercial shrimp cooking equipment currently in use: continuous belt cookers and batch immersion cookers. KM Fish Machinery offers both continuous and batch cookers in a variety of sizes [17]. Mepaco offers continuous cookers designed to use direct/indirect steam, oil/water immersion or thermal screw configuration [18]. Innotec's Impingement Flash Cooker uses a patented water cooking technique to cook shrimp at relatively low temperatures. The process can be controlled using multiple adjustable temperature zones and a specially designed cooking belt. Innotec[2] describes the technology as a gentle way of cooking resulting in improved quality, better yield, and optimum food safety [19].

[2] Innotec was acquired by OctoFrost Group in 2017. https://octofrost.com/news-room/octofrost-group-innotec-systems

1.3.1.1.4 Automatic Peelers and Graders

A typical shrimp peeling system for cold water shrimp consists of peelers that are automatic bulk fed. The peelers are a series of inclined rollers arranged such that a smaller roller is placed between two adjacent larger rollers to form a peeling "nip". The peeling rollers operate via a drive mechanism which rotate the larger rollers in alternating rotational directions. The larger rollers nip the shrimp shell thus pulling it away from the meat. From there the shrimp meat is cleaned and any remaining shell and unwanted material is separated as a waste material from the cooked and peeled meat. The final product is then IQF (individually quick frozen), inspected and graded prior to final packaging.

1.3.1.2 Snow Crab (Chionoecetes opilio)

Canada, the world's largest producer of snow crab, *Chionoecetes opilio* (Figure 1.7), accounts for approximately 2/3 of the global supply. In 2015, about 75% (70,139 t) of Canada's snow crab exports went to the United States with the rest (23,380 t) destined for China and Japan [20].

Figure 1. 7 Snow crab in fish pans for stowage in fish hold

(Photo Courtesy of CASD, Marine Institute, MUN)

Snow crabs are harvested using conical pots (Figure 1.8). After the pots are hauled, the crab is placed in the vessel's fish hold and are either direct iced or placed in a refrigerated sea water (RSW) system. When the vessel lands, the crab are offloaded, iced, and transported to the processing plant where they are sorted and graded according to size and liveliness condition. Crab is then placed in a warm water bath prior to butchering. During butchering the carapace, gut, viscera, liver, and gills are removed. The remaining crab sections are cleaned and bled in an ice water bath or slurry system. The cleaned sections are then either cooked by immersion in boiling water at 98°C for ~12 minutes, or via continuous steam cooking to an internal temperature of 71°C. These temperatures are required to kill microorganisms and inactivate spoilage enzymes, mainly polyphenol oxidases (PPOs) which cause a phenomenon known as "crab bluing" (blue discolouration of the meat) and melanosis (black spot). Crab discolouration reactions are discussed in more detail in Chapter 3 section 3.4.1.4. Higher cooking temperatures result in yield losses due to solubilization of protein.

Figure 1. 8 Conical crab pot used by harvesters

(Photo Courtesy of CASD, Marine Institute, MUN)

Following the cooking process, the sections are cooled to 4 °C by cold water immersion or a cold-water spray. From there the sections are brine dipped and frozen at –30 °C. The frozen sections are then packaged and sold as shell-on clusters/sections or may be further processed and sold as meat combo packs, snap and eat clusters/sections, claws (cap-off), or split clusters/sections [21] (Figure 1.9).

Figure 1. 9 Cooked snow crab sections in master carton

(Photo Courtesy of CASD, Marine Institute, MUN)

1.3.1.4 Crustacean Biomass Waste

In 2014, total commercial fish landings in Canada were reported to be 832,414 Mt, which included 329,821 Mt of crustaceans (i.e., lobster, shrimp, crab) [22]. In 2011 the CASD estimated that 46% of total Canadian landings are unutilized (or wasted), and that 25% of the national fish waste is comprised of crustacean processing discards [5]. Therefore, an estimated 382,910 Mt fish waste was generated in Canada in 2014 resulting in ~ 95,728 Mt of crustacean discards representing 29-30% of total crustacean landings. A similar situation likely exists in other regions of the world with fishing industries. Most of this unutilized raw material is dumped at sea or in landfills with significant disposal costs to industry, lost economic opportunity, and has a negative impact on the environment.

Crustacean shells take a long time to decompose due to their high chitin and mineral contents [117], and their high degree of crystallinity making them resistant to enzymatic degradation [118], so they can remain in the environment for a long time. Additionally, crustacean processing discards and effluents typically have high BOD (biological oxygen demand), COD (chemical oxygen demand), TSS (total suspended solids), and FOG (fat-oil-grease) levels [119] which can lead to eutrophication and anoxia of the marine environment. This condition slows the breakdown of organic material by aerobic microorganisms and can lead to fish kills due to the depletion of dissolved oxygen [120].

During the production of cooked and peeled cold water shrimp, up to 70% of the landed weight will end up as processing waste consisting of the shell, head, viscera, and protein which is removed during the peeling, washing, and separating steps (Figure 1.10) The production of IQF cooked snow crab sections generates ~30% waste comprised of the carapace (shell), gut, viscera, and protein (Figure 1.11).

Shrimp and crab processing waste contains high value products including chitin, astaxanthin, calcium, and protein which could be recovered to produce high-end nutraceutical and pharmaceutical products. This will be further explored in section 1.6 Utilization of Marine By-products.

Figure 1. 10 Shrimp shell waste collected from shrimp peelers

(Photo Courtesy of CASD, Marine Institute, MUN)

Figure 1. 11 Crab shell waste from butchering process

(Photo Courtesy of CASD, Marine Institute, MUN)

1.3.2 Innovations in Crustacean Processing

The role of technology in seafood processing has evolved rapidly over the last decade to support innovation, productivity, waste reduction, waste recovery and utilization, increase shelf-life, improve food safety, and facilitate exports [1]. In recent years there have been many advances made with respect to processing technologies specifically for crustaceans. These innovations have been developed with the goal of obtaining quality and

yield improvements, processing efficiencies and cost reductions, and to offset decreases in the labour force.

In developed countries the aging workforce together with low cost competing products from Asia and the difficulty of attracting and keeping skilled processing line workers, further complicates the seafood processing industry. This has sparked a trend in developed countries towards improving processing technologies and processing automation in a traditionally labour-intensive sector. To be competitive in today's market seafood processors must produce high value low-cost products [1].

1.3.2.1 Ice Slurry Systems

For crustacean processing, chilling technologies are critical throughout the production process to ensure product quality and minimize deterioration. Traditionally, flake-ice and refrigerated sea water (RSW) systems have been employed for rapid chilling to decrease the final product temperature to just below 0°C. However, due to the highly perishable nature of crustaceans they must be rapidly chilled to sub-zero temperatures immediately after harvesting/butchering to prevent spoilage. Newer chilling systems have recently enabled the storage of seafood at sub-zero temperatures through the addition of salts or other compounds to ice-water mixtures which are usually referred to as "ice slurry systems". Such systems have been receiving increasing attention for the storage and preservation of aquatic foods due to their faster chilling rate in comparison with traditional flake-ice or RSW and reduced physical damage to the product. The spherical ice particles of an ice slurry mixture do less damage to aquatic food tissue than traditional flake-ice

particles [23]. Other major benefits of ice slurry systems are covered in detail by Pineiro *et al.* [23].

Ice slurry is a homogenous mixture of small ice particles and carrier liquid which can be pumped. The liquid can be pure freshwater or a binary solution consisting of water and a freezing point depressant (e.g., sodium chloride, ethylene glycol, ethanol, or propylene glycol). The size, shape and smoothness of the ice particles are important characteristics for the slurry to be an effective coolant. Kauffeld *et al.* [24] provide a detailed discussion on the characteristics of ice particles in ice slurry and their effect on cooling and suggest that globular ice particles make better ice slurry than dendritic ice particles.

Most applications of ice slurry are for indirect contact cooling. However, it is used for direct contact cooling in fish processing applications. The use of ice slurry systems for shellfish was first reported by Chinivasagam *et al.* [25] who studied the spoilage patterns of five Australian prawn species. Their results indicated that storage in slurry ice increased the shelf-life of prawns from 10-17 days to more than 20 days and decreased the development of volatile compounds (e.g., amines, sulphides, ketones, and esters) in comparison to prawns stored in flake-ice. In 2002, Huidobro *et al.* [26] evaluated the effect of slurry ice and flake ice on the quality of shrimp stored onboard, with the focus on shell appearance (i.e., brightness). This study indicated that storage in ice slurry caused the development of dull colour in shrimp shell and should only be used if the final product is sold shell-off. This dull colour may indicate that the water soluble carotenoproteins normally present in shrimp is leached into the ice slurry mixture. This may have an impact

on quality and yield of potential bioactive compounds that could be extracted from the shells.

The choice of ice slurry system must be chosen carefully based on the intended cooling application as different systems produce different types of ice particles which ultimately affects the effectiveness of cooling. There are more than 700 ice slurry systems in use in the fishing industry worldwide with Iceland, Japan, and Norway among the top 3 users of the technology [24]. In Canada, ice slurry systems have been customized for installation onboard shrimp and crab vessels to improve quality and yield of the catch, and to decrease operating costs.

1.3.2.2 Automated Crab Butchering Machines

Crab butchering is a labour-intensive process that requires skilled workers who remove the mandibles and the carapace from the crab prior to separating it into two sections from which the gills and viscera are subsequently removed. Due to a decreasing labour supply, snow crab processors have been seeking more effective, automated mechanical processes to complete this step in their production process. In 2010 a Newfoundland based company, Quinlan Brothers Ltd. (QBL), with support for the Department of Fisheries and Aquaculture (DFA), the Canadian Centre for Fisheries Innovation (CCFI), National Research Council (NRC) and Centre for Aquaculture and Seafood Development (CASD), developed a viable prototype of an automated crab butchering machine [27]. QBL has since commercialized this technology and have incorporated it into its crab processing line.

In 2014 the Baader Group launched its new and revised Automated Crab Butchering machine called the BAADER 2801 (replaces the CB801) [28]. After 2 years of research

and development and working closely with Snow Crab processors to automate the manual butchering process, Baader now offers an automated solution that incorporates new electronics, new butchering methods, a smaller footprint and more emphasis on hygiene and safety [28].

1.3.2.3 CoolSteam® Cooking

The CoolSteam® Technology developed by Laitram Machinery (Figure 1.12) uses a forced convection method in which a low-temperature mixture of air and steam is constantly circulated inside the cooking chamber with uniform and efficient heat distribution. This provides a more consistent cook. Shrimp and crab processors in North America have been replacing older immersion cookers with the CoolSteam® cooking technology due to several advantages this technology provides over immersion cooking, namely: Improved quality, improved yield (due to lower cooking losses), cost, and energy savings imparted by lower cooking temperatures and more efficient heat distribution.

Figure 1. 12 Laitram CoolSteam® Demo Cooker

(Photo Courtesy of CASD, Marine Institute, MUN)

1.3.2.4 High Pressure Processing

High pressure processing (HPP) of foods was first commercialized in Japan in 1992 as a means of microbial inactivation in jams and fruit juices. According to Smelt *et al*. [29], high pressure induces effects that result in vegetative cell death including: 1. Unfolding of globular proteins [30]; 2. Membrane damage (e.g., detachment and inactivation of membrane proteins) [31]; 3. Disintegration of ribosomes [32]; 4. Intracellular pH changes [33].

The technology has since been applied to a range of foods such as deli meats, bacon, guacamole, salsa, fish, and shellfish [34]. Unlike thermally processed foods, HPP treated foods retain the appearance, flavor, texture, and nutritional qualities of the unprocessed product [29,34,35]. More recently, the technology has been applied commercially to bivalves (oysters) and crustaceans (lobster) to aid in raw meat removal from the shell.

Industrial high-pressure processing (HPP) systems (Figure 1. 13) consist of either a vertical or horizontal HP vessel and an external pressure generating device such as a single acting hydraulically driven pump [36]. HPP involves the application of high hydrostatic pressure to packaged foods, or whole raw shellfish (i.e., in the shell). The pre-packaged food or whole raw shellfish is placed into a carrier which is automatically loaded into the HP vessel, and the vessel plugs are closed. Water is pumped into the vessel until the desired maximum pressure is reached (pressurization). Most HP vessels operate up to a maximum pressure of 50,000 – 87,000 PSI. The pressure is maintained for the desired dwell time (usually 1-2 minutes). Following the HPP cycle, the pressure is gradually released (decompression) and the carrier is automatically ejected from the vessel. With respect to processing shellfish there are 2 main objectives of applying HPP technology: 1)

Inactivation of *Vibrio* species (gram-negative bacteria) in oysters; 2) to achieve clean separation of meat from the shell to facilitate shucking (oysters, mussels) and meat extraction (lobster, crab). In the case of oysters, bands are placed around the shells prior to HPP to avoid loss of meats and prevent recontamination of the meats post HPP due to opening of the shells. Because the food product is surrounded by water during HPP, all molecules are subjected to the same amount of pressure at the same time due to the isostatic principle of pressure transmission [37, 38], consequently the product form/shape is maintained.

Figure 1. 13 NC Hiperbaric Wave 6600 55L Horizontal High Pressure Processing System

(Photos Courtesy of CASD, Marine Institute, MUN)

HPP technology provides clean separation of meat from the shell and facilitates a new approach to crab meat extraction (Figure 1.14). This offers potential to open new markets and dramatically increase the value of crab products. Commercializing HPP technology involves developing new technology for crab meat extraction and incorporating both the HPP and meat extraction technologies into a highly automated production system. Meat extraction is now done manually around the world, despite many efforts to find a better solution. It is for that reason it is done mostly in low-wage countries. The use of HPP enables easier meat extraction and potentially a greater degree of automation for the extraction process. Some research with respect to meat extraction automation for high

pressure processed snow crab has been conducted (2012-2017) at Memorial University of Newfoundland through the Canadian Centre for Fisheries Innovation and the Centre for Aquaculture and Seafood Development, however, the results have yet to be commercialized.

Figure 1. 14 Raw HPP snow crab meat extracted from shell

(Photo Courtesy of CASD, Marine Institute, MUN)

1.4 Utilization of Marine By-products

Many failed attempts have been made to commercialize high value specialty products from crustacean by-products mainly due to the lack of a suitable strategy to optimize the biomass value chain. Most efforts have focused only on the recovery technologies without identifying the specific targeted applications of the final product. Other problems arise because: the raw materials are treated as waste rather than input materials; the extraction process is very costly with too many processing steps; the processing methodology has no flexibility and cannot be easily adapted to account for biological variations in the biomass waste; traditional manufacturers are not ready to be the early adopters of the new technologies [8, 9, 10, 39].

Rustad *et al.* [39] proposed a 3-step strategy for the utilization of marine by-products which if properly implemented would address the limitations identified above. The proposed strategy includes:

1. Development of technologies to take care of the by-products.
2. Development of simple technologies to produce bulk products for further refining.
3. Development of technologies to take care of the valuable components.

As previously mentioned, one of the main limitations in using marine biomass waste as input feedstock for the extraction of additional value chain products is how they are treated and handled. For marine biomass waste to be further utilized to extract high value products suitable for human consumption, or use in biomedical applications, several criteria must be considered. First and foremost, high quality by-products are required. Secondly, the yield of the desired products must also be high. Controlled and standardized processes must be developed that are flexible enough to handle biological variations, produce stable, safe, and high-quality products, and are cost effective. The end products must have documented proof of bioactive, nutritional, and functional properties [8, 9, 10, 39].

In this section, we will explore processing technologies that can be applied to implement the proposed strategy with a focus on value chain optimization of crustacean biomass waste using a biorefinery approach. In this scenario the biomass waste becomes the input feedstock while the end-use products and their applications form the value chain [9]. By extending the value chain in this manner all input materials can be converted to useful products with minimum processing.

1.4.1 Processing Technologies for Crustacean By-products

As discussed in section 1.5.2 Innovations in Crustacean Processing, several processing innovations have been developed to improve yield and quality of crustacean products. These technologies, while initially designed and implemented to improve yield and quality of traditional value chain products (e.g., cooked and peeled shrimp, cooked snow crab sections), may also help improve the quality of the leftover unutilized materials (i.e., shell - chitin, heads - oil and protein, viscera - enzymes, liver - oil and protein) which could be used as the input materials for the development of additional value chain products (e.g., nutraceuticals, pharmaceuticals). For example, shells could be used to produce chitosan-based drug delivery agents, or glucosamine as a nutraceutical. These applications are already under development but have yet to be put into practice on a commercial scale. For this strategy to succeed, however, these unutilized materials must be treated and handled as input materials rather than as waste streams. This will require modifications to existing processing lines and educating processors about how to take care of the unutilized raw materials so that the value chain can be extended beyond traditional products.

Ice Slurry – may not be best choice for shrimp if recovery of the pigment is the goal, however, for chitin extraction where pigment recovery is not the main objective, ice slurry may aid in decolourization of the shell thereby minimizing the need for a chemical bleaching step in the process. For crab shell, ice slurry may delay oxidation of the shells and adhering meat and prevent the black/blue discolouration which often develops in crab shell waste due to presence of polyphenol oxidase [40].

Automated Crab Butchering Machines – currently are not well designed to collect the carapace, viscera, gills, and liver that are removed during this step. These

machines could be redesigned with a collection chute and conveyor system to rapidly flume these nutrient rich materials into an appropriate holding/storage container until they can be further processed into higher value products such as chitin, bioactive peptides, and protein hydrolysates. The collected crab raw materials would need to be processed immediately, or stabilized quickly (e.g., by freezing) to maintain quality.

Cookers and Blanchers – New cooking technologies have been designed to cook crustaceans at lower temperatures and shorter time periods with less water. The impact of milder cooking could translate to higher quality shell and protein materials which could help transform these traditional waste streams into input materials for the recovery for value added products.

Peelers - The remaining shrimp shell waste that accumulates at the peeling step represents a valuable unutilized resource for the extraction of high value bioactive compounds. Typical shrimp plants are not designed to properly collect and store this raw material for further utilization and hence it ends up in landfills or dumped at sea. As with crab butchering technology, shrimp peelers could be redesigned with a collection chute and conveyor system to rapidly flume the shell materials into an appropriate holding/storage container until they can be further processed.

HPP - The shell material resulting from HPP of snow crab and lobster represent unutilized raw materials that could potentially be used for value chain optimization for the extraction of high value nutraceuticals, pharmaceuticals, etc. HPP rarely affects the primary structure of low molecular weight molecules such as peptides, lipids, vitamins due to the very low compressibility of covalent bonds at pressures below 2 GPa (~290,000 PSI) [38, 41, 42, 43], and therefore the same may be true for other bioactive compounds contained

in the shell such as astaxanthin and chitin. It is also possible that the quality of these compounds may be higher in HPP shell than in traditional thermally processed shells resulting in higher value products with potentially better yields. Finally, HPP could be applied as an additional safety measure for value added products extracted from crustacean waste streams to inactivate spoilage/pathogenic microorganisms and enzymes and extend shelf-life.

1.4.2 A Biorefinery Approach for Value Chain Optimization of Crustacean Biomass Waste

The term biorefinery first appeared in scientific literature in 2001, but it wasn't until 2007 when the biorefinery concept began to achieve more significance in scientific publications [44]. According to the International Energy Association Bioenergy [45], biorefining involves the processing of biomass in a sustainable manner to obtain marketable bio-based products (food, feed, chemicals) and bioenergy (biofuels, power, heat). Similarly, Cherubini [46] describes biorefining as the integration of biomass transformation processes and equipment to produce fuels, energy, and chemicals. Gonzalez-Delgado & Kafarov [44] define biorefining as the processing of sustainable biomass to obtain energy, biofuels, and high value products.

The general biorefining concept uses a wide range of technologies to separate biomass into its principal constituents (e.g., carbohydrates, proteins, fats) which can be further transformed into value-added products and biofuels. The concept is like that of oil refineries which fractionate complex mixtures of crude oil feedstock to obtain multiple products (e.g., petroleum, diesel, gasoline, kerosene, lubricants, tar, etc.) [44]. Most biorefinery applications have focused on the production of biofuels from plant-based

feedstock from both food (e.g., barley, corn, soybean wheat) and non-food crops (e.g., wood fuel, Camelina [45, 46, 47, 48, 49, 121].

In recent years' significant research efforts have focused on the development of biorefineries for land-based feedstocks from agricultural and forestry wastes, yet little attention has been placed on ocean-based feedstocks [44, 47, 48, 49]. Kerton *et al.* [48, 49] have suggested that a biorefinery could be developed using ocean-sourced feedstocks from finfish and shellfish waste particularly in rural, coastal areas from Southeast Asia to the Eastern Seaboard of the United States and Atlantic Canada.

A proposed biorefinery approach for value chain optimization of cold-water shrimp and snow crab is presented in Figure 1.15. In this model, the waste from traditional processing of cooked and peeled shrimp or cooked snow crab sections becomes the feedstock for the biorefinery which separates the unutilized shell components into their main constituents: chitin, proteins, pigments (astaxanthin), and lipids. These additional value chain products can be further processed into higher value products such as chitosans, calcium carbonate, bioactive peptides, amino acids, and fatty acids. Traditional transformation processes, however, require the application of hazardous chemical treatments which pose a threat to the environment and in the case of biomedical applications, could render these bioproducts unsuitable. According to Kerton *et al.* [48], the application of green chemistry may be a means of reducing the environmental impact in the valorization of crustacean waste. New extraction, oxidation, deproteination and demineralization methods, such as biological methods, will be necessary, for example, to mitigate the negative environmental impacts of more traditional chitin/chitosan production

processes which use strong inorganic acids (HCl) and bases (NaOH, KOH), as well as flammable and often toxic organic solvents (hexane, acetone, ethanol) [57, 67].

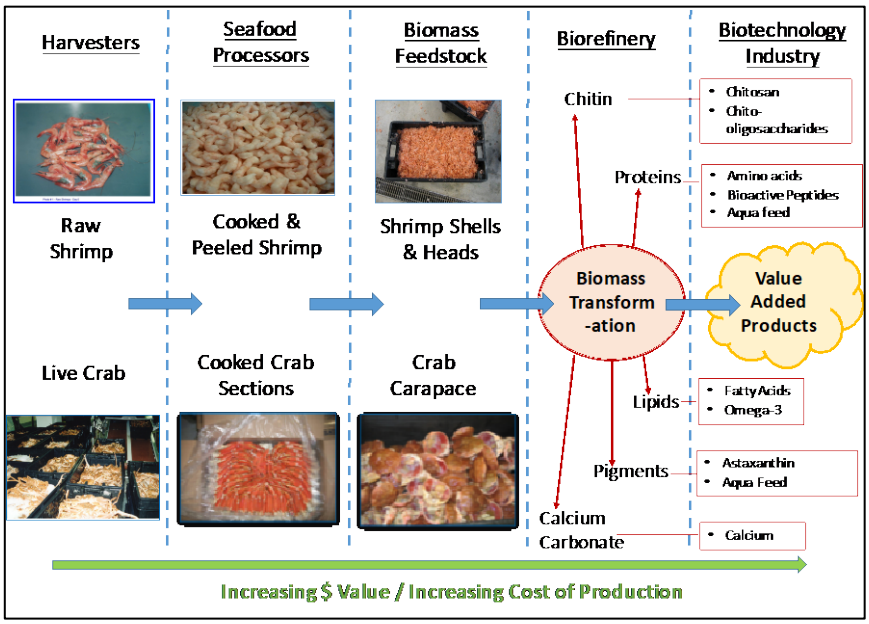

Figure 1. 15 Biorefinery approach for value chain optimization of cold-water shrimp and snow crab

While more value can theoretically be extracted from shrimp and crab biomass under this model, the cost of innovative and multiple extraction processes could be prohibitive in comparison to current disposal options. Green technologies are not necessarily innovative, but they are important as alternatives to the more chemically hazardous methods often used by industry. By combining green chemistry with a biorefinery approach several principals of green extraction can be met such as: use of renewable resources; use of alternative "greener" solvents; production of co-products instead of waste; targeting non-denatured, biodegradable extracts without contaminants [122]. In addition, sustainable cost-effective production methodologies must be developed for the model to be economically feasible.

1.5 Bio-production of Chitin and its Derivatives

1.5.1 Background

Chitin was discovered in 1811 by French scientist Henri Braconnot who named the material fungine [50]. Odier found the same substance in 1823 and called it chitine [51]. However, it wasn't until the 1950s that a sustained interest in chitin research, including its derivatives, developed. Since then, four broad areas of chitin research have developed including: (1) Isolation from shellfish and other sources; (2) Structural studies and properties; (3) Chemical derivatization; (4) Applications for chitin, chitosan, and their derivatives [52].

Chitin (Figure 1.16) is a linear amino polysaccharide composed of β-(1-4) linked N-acetyl-D-glucosamine units which may be de-N-acetylated up to 60-80% [53, 54, 55] (to produce chitosan (Figure 1.17). Chitin contains 6-7% nitrogen [53, 56]. The source of chitin affects its crystallinity, purity, polymer chain arrangement and its properties [55]. There are three polymorphic forms of chitin: α, β, and γ chitin. The α-form is the most abundant and is found in crab and shrimp shells. β-chitin is found in molluscs such as squid, and γ-chitin has been isolated from the stomach lining of squid and cuttlefish [56, 57]. In α-chitin the chains are arranged in an antiparallel fashion which promotes strong hydrogen binding between the chains resulting in a very tight and compact crystalline structure which requires harsh methods for extraction 56, 57, 58]. β-chitin is arranged in parallel sheets and is held together by weaker intermolecular forces and can be easily, but irreversibly, transformed into α-chitin by steam. The γ-form is a combination of α and β-chitin containing two

parallel (β) and one antiparallel (α) strands [56, 57], which can also be converted to α-chitin by treatment with lithium thiocyanate [59].

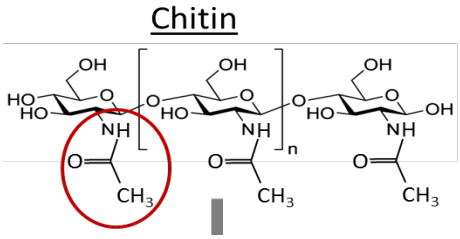

Figure 1. 16 Chemical structure of chitin with acetyl group

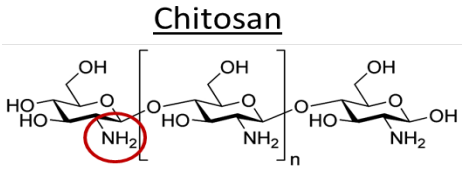

Figure 1. 17 Chemical structure of chitosan with amine group

Together chitin and chitosan have gained an outstanding reputation with numerous applications in the fields of water engineering, cosmetics, paper engineering, textile engineering, food engineering, agriculture, photography, chromatographic separations, medical and pharmaceutical in recent decades [55, 60, 61]. Chitin and chitosan are generally non-toxic, non-soluble in water and most organic solvents.

Chitin is the second most abundant natural polymeric form of N-acetyl-D-glucosamine and is the structural component of crustaceans, insects, arthropods, fungi, and yeast. Shrimp and crab shell waste are the main commercial sources of α-chitin. Due to its highly crystalline structure and strong hydrogen bonds, chitin is not readily dissolved in common solvents. Therefore, it is often converted to its more N-deacetylated derivative, chitosan, which is soluble aqueous acids [57]. Traditional methods of chitin extraction are chemically intensive processes using large volumes and high concentrations of HCl, NaOH

and ethanol. Long exposure of chitin to HCl can significantly modify its physicochemical properties; cleavage reactions can decrease the molecular weight and significant deacetylation of the chitin can occur, both of which negatively affect the fundamental properties of the chitin isolated [62, 63].

Chitosans are characterized mainly by viscosity, which is a measure of its molecular weight (MW), and degree of deacetylation (DDA). Both MW and DDA affect the functional properties of chitosan. The control over these two parameters allows the production of a wide range of chitosans which can be used in medical, pharmaceutical, cosmetic, nutraceutical and industrial fields [61, 64]. The traditional method of chitosan production uses a high concentration and large volumes of base (up to 70% w/v) to which chitin is added followed by heating at 95-110°C for up to 2 hours [48]. This is a chemically intensive and environmentally hazardous process. Chemical extraction methods may also introduce toxic contaminants (e.g., metals) rendering the chitosan unsuitable for biomedical applications, and is a source of environmental pollution [57, 65].

Due to the limitations associated with traditional chemical extraction methods for chitin and chitosan, there have been significant efforts directed towards the use of more eco-friendly methods such as enzymatic methods, lactic acid fermentation (LAF), combined biological and chemical methods, as well as combined use of waste materials. These methods can prevent waste, use safer solvents, produce innocuous degradation products, and minimize health and safety concerns. In this section we will explore selected bio-production methods for chitin, chitosan, and their derivatives.

1.5.2 Isolation and Extraction of Chitin and Chitosan

Most traditional isolation methods of chitin from shrimp and 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 2 hours); (2) demineralization - removal of minerals, mainly calcium carbonate, by treatment with strong acid (e.g., 5-7% w/v HCl for 2 hours); and (3) decolouration - removal of pigment using a bleaching/oxidizing agent (e.g., hydrogen peroxide, ethanol, sodium hypochlorite) to obtain a colourless product [57, 66, 67]. 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 a concern [67, 68].

Chitosan is produced by the deacetylation of chitin (Figure 1.18) using highly concentrated NaOH or KOH solution (40-50% w/v) at high temperature (>100 °C) for 2 hours. Chitosan is differentiated from chitin based on the degree of deacetylation (DDA). The term chitosan is preferred when chitin reaches a DDA above 60%, [69, 70].

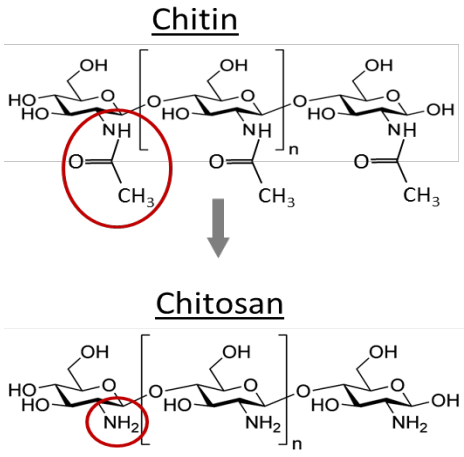

Figure 1. 18 Deacetylation of chitin

1.5.2.1 Enzymatic Extraction

There has been limited industrial use of enzymatic methods for chitin extraction due to the high costs associated with such methods on the industrial scale [57]. However, enzymatic hydrolysis has generated significant research interest because it produces a higher-quality chitin and subsequently higher-quality chitosan than chemical methods.

Commercially available proteolytic enzymes (Table 1.1) such as Alcalase (EC 3.4.21.62), chymotrypsin (EC 3.4.21.2), and papain (EC 4.3.22.2) have been used remove protein and extract chitin from shellfish waste [71, 72]. Synowiecki and Al-Khateeb [73] extracted chitin from shrimp (*Crangon crangon*) waste using Alcalase to achieve 89.0% deproteination. The chitin thus obtained contained a residual protein content of 4.4-7.9%, which is about twice as high compared to commercial product treated with NaOH [74]. Bacterial release of chitin was also achieved using proteases isolated from *Pseudomonas maltophilia* or *Bacillus sp*. TKU004 for deproteination [75, 76]. Gagne and Simpson [77] showed that residual protein levels in shrimp waste following deproteination with chymotrypsin and papain were 1.3% and 2.8% respectively. However, a high enzyme to waste ratio (E/W) of 0.7% for chymotrypsin and 1% for papain was required for maximum deproteination. A study conducted by Jo *et al.* (2008) comparing the effectiveness of deproteination of various commercial proteases found that Delvolase exhibited the highest DP for crab shell waste which, when treated with 1% Delvolase, reached 85% DP within 1 day. However, complete removal of the residual protein associated with the chitin was not achieved using Delvolase. Gildberg and Stenberg [78] used Alcalase (2.4 1 FG) to deproteinate Northern shrimp (*Pandalus borealis*) waste with the goal of obtaining a high-

quality protein hydrolysate and producing chitosan from the resulting press cake. Their process showed that Alcalase treatment allowed about 70% of the total amino-N to be

Table 1. 1 Sources of protease and chitin deacetylase enzymes for deproteination and deacetylation of chitin

Protease Enzymes	Source	Reference
Alcalase (EC 3.4.21.62),	Commercial	[71, 72, 78]
Chymotrypsin (EC 3.4.21.2)	Commercial	[71, 72, 77]
Papain (EC 4.3.22.2	Commercial	[71, 72, 77]
Protease	*Pseudomonas maltophilia* or *Bacillus sp*. TKU004	[75, 76]
Delvolase	Commercial	[83]
A21 crude protease	*Bacillus mojavensis*	[79]
Chitin Deacetylase Enzymes	**Source**	**Reference**
CDA (EC 3.5.1.41)	*Mucor rouxii* *Absidia coerulea* *Vibrio cholera* *Absidia corymbifera DY-9* *Mortierella sp. DY-52* *Saccharomyces cerevisiae* *Colletotrichum lindemuthianum ATCC 56676*	[84] [6] [85] [80, 82] [86] [87] [88]

recovered without affecting the yield or quality of the chitosan subsequently produced. Younes *et al.* [79] used non-commercial *Bacillus mojavensis* A21 crude protease to obtain 88% deproteinization of shrimp (*Metapenaeus Monoceros*) shells. The optimum process conditions included: an enzyme/substrate ratio of 7.75 units/mg; temperature of 60°C; incubation time of 6h. The solid fraction was chemically demineralized and subsequently converted to chitosan by alkali treatment yielding a chitosan with a low degree of acetylation (4%), and high antimicrobial activities.

While enzymatic hydrolysis has been shown to produce a higher-grade chitin than chemical methods, at least on a lab scale, enzymatic conversion to chitosan remains a challenge. For example, the conversion of chitin to chitosan has been studied on a laboratory scale using various sources of chitin deacetylase (CDA, E.C.3.5.1.41) (Table 1.

1). Chitin deacetylases (CDAs) result in a chitosan that has a more regular pattern of acetylation (PA) than a chitosan produced using hot NaOH. CDAs recognize a specific pattern of four GlcNAc units in chitin of which one undergoes deacetylation [80]. CDA was first found in extracts from the fungus *Mucor rouxii* (Araki & Ito, 1975), but since then several different fungal CDAs have been discovered (Zhao et al, 2010). However, the CDAs studied thus far have been ineffective in deacetylating natural insoluble crystalline chitin and only results in a 1% increase in the degree of deacetylation [80, 81, 82]. Prakash *et al.* [6] used fungal CDA isolated from *Absidia coerulea* to deacetylate chitin. Their results suggested that even with surplus chitin substrate and high enzyme activity only a small percentage of the substrate is transformed to chitosan. Win and Stevens (2001) used various physical (heating, sonication, grinding) and chemical (derivatization, interaction with saccharides) pre-treatments to try and increase the accessibility of the acetyl groups of crystalline chitins to fungal CDA, however, these treatments were ineffective.

1.5.2.2 Lactic Acid Fermentation

Chitin extraction using lactic acid fermentation (LAF) has been performed on a lab scale, however, it is not yet used commercially due to the cost of lactic acid production [99]. An advantage of LAF over traditional chemical extraction methods is that it allows for the recovery of chitin, proteins and astaxanthin [67, 73, 89, 90, 91].

Lactic acid bacteria (LAB) typically grow between 10 and 50°C and requires a carbon and nitrogen source. At the beginning of the growth cycle, lactic acid is produced by the breakdown of a carbon substrate such as glucose or sucrose. The lactic acid results in an acidic pH and this environment has been shown to affect the demineralization of

shrimp shells, due to solubilization of calcium, but to varying degrees depending on the fermentation conditions. Deproteination is achieved by the enzymatic action of LAB on shell proteins, free ammonia released during shell solubilization, and eventually catabolism of amino acids [89, 92].

Cira *et al.* [89] reported that the optimum LAF parameters using a Lactobacillus spp. strain B2 (isolated form shellfish waste) were 10% (w/w) sucrose with an inoculum level of 5% (v/w) which promoted high acidification (i.e., pH decreased from 7.5 to 4.6 and 0.53 mmol/g TTA) at 36°C achieving 85% demineralization and 87.6% deproteinization after 6 days of incubation. In comparison, however, Adour *et al*. [92], reported low levels of demineralization (60%) and deproteinization (20.6%) of white shrimp shell when fermented with *L. helveticus* at 30°C using 300g/L glucose substrate and an inoculation level of 10% (v/v). Reasons cited for the low levels of demineralization and deproteinization, respectively, included: the initial alkalinity of the of the culture medium (8.5-9.0) likely due to the ammonia release from shells during their preparation (1.5-2g/L) may have interfered with acidification of the culture medium; the presence of free ammonia as a second nitrogen source.

Greene *et al.* [62] recently evaluated lactic acid demineralization of green crab shells. They found that contrary to their expectation, the rate of demineralization did not increase on a linear scale with increasing concentration of lactic acid. Instead, the rate of demineralization had a positive correlation with the conductivity of the solution, which measures mobility of the H+ ion in solution. Because lactic acid is very viscous, the more concentrated the solution, the lower the mobility of the H+ ion, therefore, the lower the rate

of demineralization as the H+ ion has limited ability to attack the shell. The use of undiluted (11.4 M) lactic acid resulted in only 5% removal of calcium carbonate after 180 minutes at room temperature, compared with 61% removal with 1.14 M and 53% removal with 2.28 M lactic acid. Other factors affecting the rate of lactic acid demineralization include temperature, shell to acid ratio and reaction time [62, 93, 94]. Green *et al.* [62] reported that under ideal conditions 90-95% of the calcium carbonate and other minerals can be removed from green crab shells in 90 minutes, but the remaining 5-10% took more than 350-400 minutes. This was attributed to the "shrinking core model" in which the demineralization reaction slows over time because the reactive sites of the calcium carbonate (and other minerals) move deeper into the interior of the shell [62, 93].

1.5.2.3 Integrated Extraction Methods

Neither enzymatic, nor lactic acid fermentation, alone has proven to be effective for the deproteination and demineralization of crustacean shells. To improve the recovery of chitin and reduce the amount of alkali and acid required, enzyme treatments combined with chemical treatments, as well as LAF in combination with chemical treatments, have both been studied as an alternative to chemical extraction for chitin recovery [6, 62, 78, 89, 95, 96, 97]. Greene et al [62] for example, did not obtain high rates of demineralization using lactic acid alone. However, by combining lactic acid (1:4 acid to water ratio) in a 1:1 molar ratio with 5.00 M HCl, demineralization was improved, and the amount of HCl required was reduced in comparison to using only HCl. The combined acid mixture resulted in 77.8% weight loss compared with only 37.5% using lactic acid alone. Gildberg & Sternberg [78] combined the use of a commercial protease (Alcalase 2.4 I FG) to remove proteins

from northern shrimp shells (*Pandalus borealis*) followed by chemical demineralization, deproteination and deacetylation of the press cake to produce chitosan. Using this method, 70% of the total amino-N was recovered as protein hydrolysate compared with less than 15% in the conventional chitosan production process. It was also possible to recover the astaxanthin pigment using this method. In another experiment, chitin obtained from LAF was further purified after pigment extraction, using acid and alkali treatments [89]. This method reduced the amount of chemicals required for chitin purification by 50-77% with respect to conditions previously reported in the literature [67, 98].

Other innovative approaches have included combining waste materials such as green crab shells and lactic acid from milk processing [62], and snow crab shells with lactic acid from rye grass fermentation [99]. The intent of this approach is improving the economics of both chitin extraction and lactic acid production by combining them in a single biorefinery process. In this method, lactic acid dissolves shell calcium carbonate which buffers the fermentation reaction and increases the lactic acid yield. Adour *et al* [92] investigated the feasibility of recovering chitin from white shrimp shells by LAF using date juice waste as a substrate. Date juice has a high calcium content (75.1 mg/L) which seems to have interfered with demineralization but improved the proteolytic activity of *L. helveticus* resulting in nearly complete deproteination of the white shrimp shells.

1.5.2.4 Physical Chemo-Enzymatic Chitin Extraction

Physical methods using mechanical pre-processing to breakdown crustacean discards into smaller particles are promising as alternative means of chitin extraction, however obtaining high yields of purified chitin at low cost has proved challenging. It has

been suggested that a combined approach using physical, chemical, and enzymatic methods may provide a cost effective and environmentally friendly compromise to the traditional chemically intensive chitin extraction process applied to crustacean shells [57]. In 2012-14, the author and her research team at the Centre for Aquaculture and Seafood Development (CASD) tested this hypothesis by developing a 240 kg batch pilot scale demonstration system (Figure 1. 19) based on a modified chemo-enzymatic process with novel mechanical pre-treatment to produce chitin from shrimp and crab shell waste. The physical-chemo-enzymatic process included a series of mechanical pre-treatment steps for particle size reduction, enzymatic removal of protein using commercially available protease enzymes, followed by mild acid treatment for demineralization which required 80% less HCl over the traditional process [107].

Although the pilot scale physical-chemo-enzymatic chitin extraction process developed by the CASD effectively eliminated the use of chemicals in the deproteination step and significantly reduced the chemical usage in the demineralization step, the depigmentation step remains a chemically intensive process. In addition, the CASD chitin pilot processing line generated large volumes of wastewater laden with astaxanthin and protein [107]. Ideally, pigment and protein recovery would be incorporated into the extraction process, or green oxidation catalysts could be used for pigment removal [48].

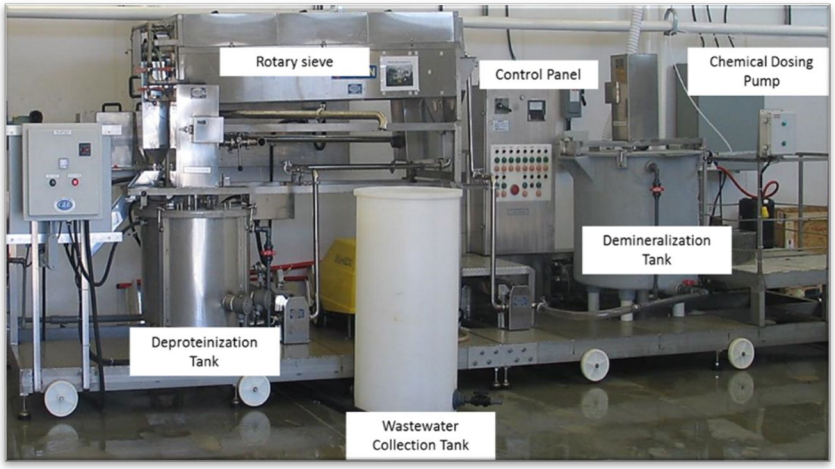

Figure 1. 19 Chitin pilot processing line (Photo Courtesy of CASD, Marine Institute, MUN)

1.5.3 Non-chemical Structural Modifications of Chitin and Chitosan

Biomedical, pharmaceutical, and biotechnological applications (Table 1.2) are the strongest high-value growth markets for chitin, chitosan, and their derivatives. The main developments in this field have included their use in wound dressings, controlled drug release, hair care and as a bone filling agent produced from hydroxyapatite-chitin-chitosan composite [100].

Chitosan has unique physical, chemical, and biological properties that make it commercially attractive, however commercial processing to produce a consistent high-quality product has proven to be difficult and expensive [9]. Superior quality chitosan should have a DDA of 70-90% [6, 105], with a minimum of 78% DDA required for biomedical applications [107]. Chitin has a very stable crystalline structure and to achieve the high DDA necessary to produce chitosans suitable for biomedical uses requires a long incubation period, or a multi-stage process, in a harsh chemical environment at high temperature. This type of heterogeneous process generates chitosan products of inconsistent quality, with variations in molecular weight (MW), degree of deacetylation

(DDA), solubility and viscosity, and a random pattern of acetylation (PA) [82, 106]. Consequently, chitosans produced in this manner vary in their biological activities and have been difficult to characterize resulting in their limited use in biomedical, pharmaceutical, and biotechnological applications [82]. In addition, chitin, chitosan, and their derivatives intended for use in biomedical applications must meet high quality standards and must be produced under some form of quality management system such as ISO 9001, GMP (good manufacturing practices) or GLP (good laboratory practices) and meet the requirements of the importing countries health regulations [52]. Chitosan quality will vary depending on the intended application; however, some general quality parameters are provided in Table 1.3 for biomedical, pharmaceutical, and biotechnological uses.

Table 1. 2 Biomedical, pharmaceutical, and biotechnological applications of chitosan and chitosan derivatives

Potential Applications	Principle Properties/Characteristics	Reference
Surgical sutures	Biocompatible	[101]
Dental implants	Biodegradable	[101]
Artificial skin	Renewable	[101]
Rebuilding of bone, bone filling agent	Film forming	[100, 101]
Corneal contact lenses	Hydrating agent	[101]
Time release drugs for animals and humans	Nontoxic, biological tolerance	[100, 101]
Encapsulating material	Hydrolyzed by lyzosyme Wound healing properties Efficient against bacteria, viruses, fungi	[101]
Wound dressing	Biocompatible, antimicrobial, blood clotting, wound healing	[100]
Gene delivery	Nontoxic, biocompatibility, catioinic, chelating ability with DNA	[102, 103]
Tissue engineering & wound healing	Antimicrobial, low toxicity, biodegradable, catioinic-electrostatic interactions, porous structure, gel forming properties, high affinity for in vivo macromolecules	[103, 104]

Table 1. 3 Quality specifications for biomedical/pharmaceutical chitosan applications [107]

Parameter	Quality Specification
Appearance	white to off-white/beige powder
Protein (%)	<0.2
Total ash (%)	<0.2
Moisture (%)	<10.50
Viscosity (mPA*s)	100-1000
DDA (%)	78.0-84.0
Heavy metals	< 40ppm
Insolubles	<0.5% (w/w)
Bacterial Endotoxins	<500EU/g
Microbial Enumeration	TAMC: <2000 CFU/g TYMC: <200 CFU/g

Chitin and chitosan have high molecular weight and therefore high viscosity which limits their use in vivo due to poor solubility. However, modified forms of chitin and chitosan have low molecular weight, hence low viscosity, and short chain lengths which improve their solubility in aqueous acidic solutions making them readily absorbable in vivo [108]. Both chitin and chitosan can be modified by acid hydrolysis which cleaves the glycosidic linkages resulting in a lower molecular weight, shorter chains of β(1-4) linked homo- or hetero-oligomers of GLcNAc and/or GlcN. These shorter chains are called chito-oligomers (COS), have chain lengths up to n=15 and MW up to 10 kDa, with lower viscosity and greater solubility in water at neutral pH if the DP is less than 10 [57, 117]. Other derivatization techniques include chemical/enzymatic substitutions, chain elongation and depolymerization reactions [108]. For biomedical applications, the main form for application is as a "gel" made with chitosan oligomers. The main methods for gel formation include solvent evaporation; neutralization; cross linking; ionotropic gelation; or freeze drying [108]. Chitosan is a cationic amino-polysaccharide, is non-toxic, anti-bacterial and biodegradable, which makes it attractive for biomedical uses. However, chitosan does not dissolve in neutral or basic aqueous solutions which limits its usage in biomedical

applications, therefore derivatization is necessary. Derivatives of chitosan can be obtained by chemical modifications which substitute the amino groups either at or appended to chitosan primary amines, which also enhances its antibacterial properties, thus extending its application in the field of biotechnology [6, 9, 55]. However, non-chemical modifications using micro-organisms/enzymes are more desirable to reduce possible contamination of the end products with toxic substances, minimize production costs, and to reduce impacts on the environment.

While eco-friendly methods of chitin extraction on a pilot scale show promise and may soon be a commercial reality, similar green technologies for chitin to chitosan conversion, and bioproduction of COS from chitin and chitosan require more intensive research. For example, conversion processes using chitinolytic enzymes and lactic acid bacteria have been evaluated on a laboratory scale, however, these processes remain too costly to implement on an industrial scale [109]. While an enzymatic method using CDAs, in comparison to the chemical method, of chitin to chitosan conversion has potential to produce high quality chitosan with well controlled DA and PA, as discussed in section 1.7.2.1 Enzymatic Extraction, this method remains a challenge due to the highly crystalline nature and insolubility of chitin, lack of well characterized CDAs, and limited data explaining the mode of action and catalytic mechanism of CDAs on chitin [82, 110, 111]. The high cost of fungal and bacterial sources of chitinases and chitosanases for the bioproduction of COS has limited their industrial applications [112, 113, 114].

Despite the limitations and challenges, several researchers have proposed various bioproduction models for chitin, chitosan and their oligosaccharides which use a combination of lactic acid fermentation and enzyme treatments [48, 57, 115, 116].

However, under the proposed biorefinery model presented earlier (1.6.2 A Biorefinery Approach for Value Chain Optimization of Crustacean Biomass Waste), the ideal process (Figure 1.20) would primarily use biological methods (perhaps in combination with physical and mild chemical treatments) to separate and recover all the unutilized shell components including chitin, proteins, and pigments (astaxanthin) which could then be further processed into higher value products such as chitosan, COS, calcium carbonate, bioactive peptides, and amino acids. While in principle, such an approach seems feasible, biological extraction and conversion methods that are effective, efficient, and cheap must be further developed for the proposed model to be sustainable.

1.6 Conclusions

A comparison of the advantages and disadvantages of chemical conversion and bioconversion methods to produce high quality chitin, chitosan and COSs has revealed that existing technologies are currently too expensive for commercial production. Therefore, further research and investment to generate functionalized biomedical chitosan products at the right cost and of sufficient quality and purity is needed. This will likely require a combined approach using optimized physical, chemical and/or biological methods which include recovery of pigments, proteins, and other value chain products so that quality and yields are maximized while keeping production costs low.

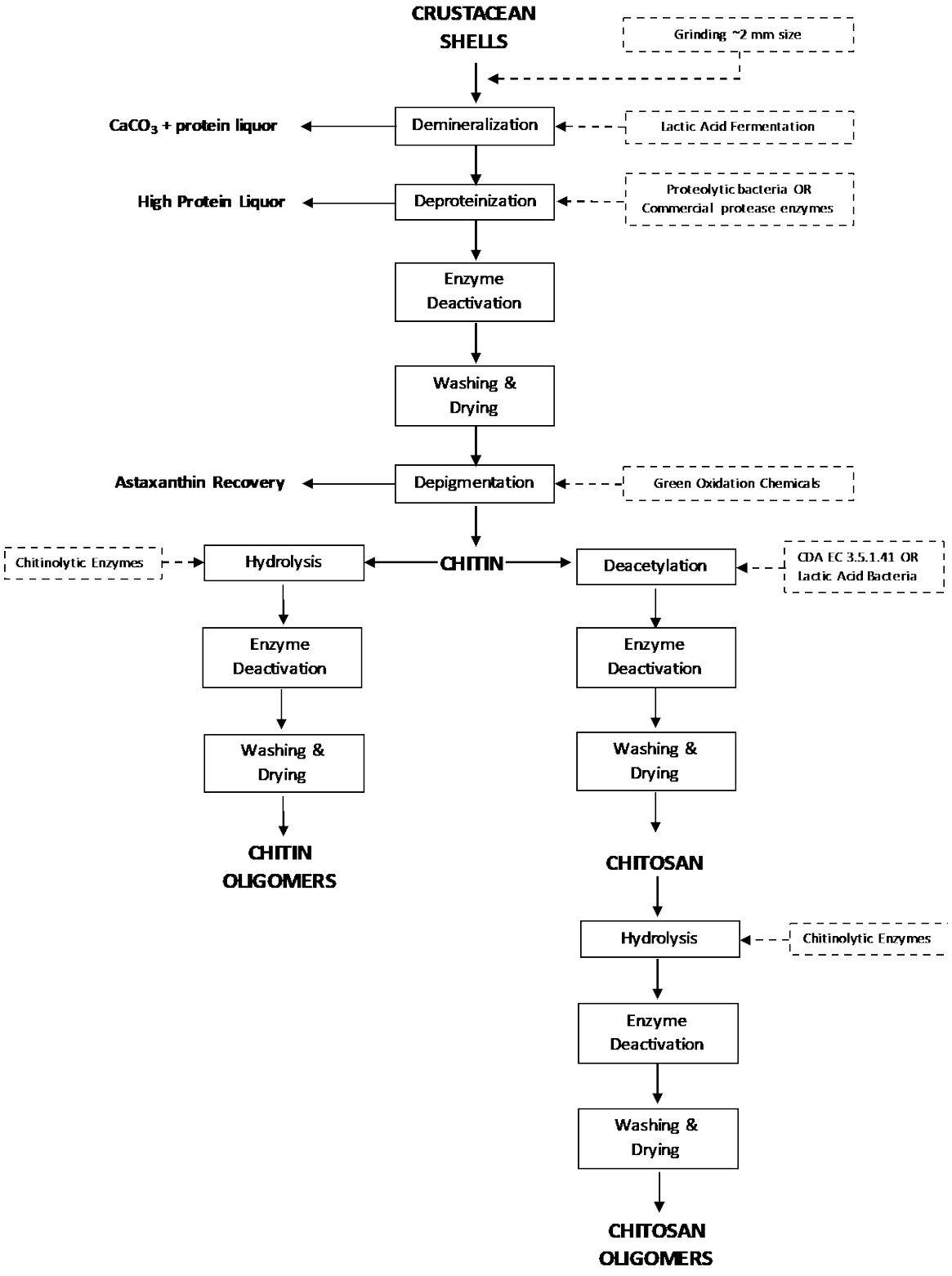

Figure 1. 20 Proposed bioproduction of chitin, chitosan and COS with recovery of protein, pigment and $CaCO_3$

Note to Reader:

- Chapter 2 begins on page 61

1.7 References

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CHAPTER 2. Inventory Assessment of Unutilized Marine Biomass Feedstocks Generated by the Newfoundland and Labrador Seafood Processing Industry

2.1 Background

This Chapter was initially written between 2016-2018. When this study began the most current fisheries statistics available were for the 2015 fishing season. The study period included a 10-year history spanning 2006-2015. The provincial fisheries data set was provided by the Department of Fisheries and Lands Resources, Government of Newfoundland and Labrador and may contain a 1-2% margin of error due to a transition during the study period to a new database management system.

Although the focus of this thesis is valorization of Snow crab (*Chionoecetes opilio*) processing discards generated by the Newfoundland and Labrador seafood industry, it was necessary to conduct a detailed inventory assessment of the main commercial species processed in NL to fully comprehend the by-product opportunities and challenges facing our seafood processing industry. Most of our seafood processing plants are multi-species plants and only produce crab for 2-3 months of the year. Therefore, processors will need to be equipped to handle many different types of processing by-products. Until now, a detailed multi-species inventory assessment of NL's seafood processing by-products, as presented in this chapter, has not been undertaken.

2.2 Definitions

Marine Feedstock Materials recovered marine biomass wastes that are used as input materials in a marine biorefinery system.

License (Processing) is a written authorization from the Minister allowing a processing facility to process one or more species categories [92].

Marine Biomass Waste refers to seafood processing discards, effluents, and fish by-products.

Minimum Processing Requirement is a provincial regulatory requirement for Newfoundland and Labrador. It refers to the minimum amount of processing that is needed to transform a species from its live and/or landed states before the product may be shipped out of the province [92].

Marine Biorefinery is a system whereby marine biomass waste streams are recovered and become the feedstock materials for the sustainable conversion into marketable high value bioproducts and biofuels [13, 14, 15, 16, 17, 18].

Primary Processing refers to washing, cleaning, icing, skinning, shucking, filleting, portioning, pickling, cooking, salting, curing, drying, freezing, or canning of fish in preparation for market [92].

Secondary Processing means the processing of fish for market beyond the primary processing stage (e.g., breading, battering, marinating) to add value [92].

Semi-structured Interview is a research method frequently used in the social sciences. The interviewer prepares an interview guide comprised of an informal grouping of topics and questions that must be covered during the interview [90, 91].

2.3 Introduction

A Canadian study conducted in 2011 estimated that approximately 46% of total wild capture landings end up as waste [1]. Canadian landings, (excluding marine plants, lumpfish roe and miscellaneous categories) were 823,274 t live weight in 2015 contributing approximately 378,706 t of waste (based on the estimated 46% waste generated annually) [2]. Atlantic Canada generates 80% of all wild capture landings with the provinces of Nova Scotia and Newfoundland and Labrador each contributing 30%. These two provinces account for 60% (493,964 t in 2015) of total national landings and generate 60% (227,224 t in 2015) of the total national biomass waste from fisheries [2].

The by-products generated by a fish farm can be as high as 50% of the biomass production [3, 4]. On average, between 2006 and 2015, the NL salmon aquaculture industry lost 12-15% of its production each year due to disease (e.g., infections due to Salmon Anemia virus (ISAv) on the south coast) and other factors (e.g., super chill) which induce mortality [5, 6]. On average, an additional 18-20% is discarded as processing waste/offal. In 2013, just under 10,000 t of waste was generated by the NL salmon aquaculture industry [7, 8]. In 2015-16, 8 million kg of unutilized salmonid biomass were exported to the Maritimes for rendering [9].

Other reports have cited varying estimates of the quantity of fish processing wastes generated in Newfoundland and Labrador ranging from 48,245 t in 2009, up to 146,960 t in 2001 [10, 11, 12]. The volume and type of waste generated annually will vary depending on species harvested, volume of landings, the type of processing operation, product forms, and market demands. A limiting factor in the studies conducted thus far is that the data used

is from different years, and typically have focused on a single year. Also, there has not been an accurate account of the quantity of waste materials diverted to other uses such as animal feed, fish meal, and bait rather than ocean dumping or landfilling.

While these unutilized marine resources represent a tremendous opportunity as feedstock materials for value creation (e.g., biofuels, nutraceuticals, functional foods, cosmetics, pharmaceuticals, specialty chemicals), we have not yet been able to determine with any degree of certainty the volumes that are generated and available for value addition within the province. Knowledge of the volume and type of unutilized marine biomass materials available within the province are critical factors which will direct the kinds of products that should be extracted, and the processes required to extract them.

2.4 Scope & Purpose

To advance the opportunity to convert unutilized marine biomass materials (e.g., seafood processing discards, effluents, and by-products) into higher value products, an inventory assessment of these potential marine biomass feedstocks generated in Newfoundland and Labrador (NL) is required. The purpose of this study was to identify sources, locations, and quantities of unutilized marine biomass materials that are available within the province, and to identify opportunities for improving the industry's utilization of these materials. The inventory assessment focused on unutilized marine biomass materials generated by the seafood processing sector from landings of crustaceans, groundfish, pelagics, and farmed salmon, and did not include at-sea discards from the harvesting sector or hatchery waste from the aquaculture sector. The study period was limited to the 10-year time-period spanning 2006-2015. While the associated costs of

transporting the potential marine biomass feedstocks to a processing facility, and the potential net revenues that could be generated from currently unutilized marine biomass materials are important factors to consider, these factors are outside the scope of this chapter.

The specific objectives of this chapter include:

1. Identify potential sources and types of marine biomass feedstocks available in Newfoundland and Labrador for value addition.
2. Create an inventory of potential marine biomass feedstocks available and their location in the province, by species and economic zone[3].
3. Divide the inventory list into categories based on the type, composition, and quantity of the feedstock and the products it may be suitable for (e.g., bulk intermediate products or higher value bioproducts).
4. Determine the availability of potential marine biomass feedstocks based on criteria such as time of year, allocation to other uses, and ease of collection for processing.
5. Identify the barriers and limitations to better utilization of marine biomass materials and suggest ways to overcome these barriers and limitations.
6. Provide strategic recommendations for utilization of selected marine biomass materials as feedstocks, and possible locations for future development of a central or regional marine biorefinery facility in NL.

[3] Economic zones are geographic areas defined by the NL Task Force on Community Economic Development. There are 20 economic zones in the province which are used to facilitate economic planning and development.

2.5 Methods

2.5.1 Literature Review

A review of the scientific literature was undertaken in relation to the current state of world fisheries and aquaculture; global, national, and provincial fish utilization; potential uses of unutilized biomass feedstocks from crustacean, groundfish and salmonid processing; structure of NL fisheries and aquaculture sectors and its impact on fish utilization rates.

2.5.2 Types of Marine Biomass Feedstocks

Consultations were held with representatives from the Department of Fisheries and Land Resources (DFLR), the Canadian Centre for Fisheries Innovation (CCFI), the Centre for Aquaculture and Seafood Development (CASD), the Association of Seafood Producers (ASP), and the Newfoundland Aquaculture Industry Association (NAIA), to identify potential sources of marine feedstocks that could be utilized for the extraction of higher value bioproducts and improve our fish utilization rate.

2.5.3 Inventory of Marine Biomass Feedstock

Fish utilization rates for the major species or species groups identified from the stakeholder consultations were calculated over a 10-year period (2006-2015) from statistical data sets available from public sector sources such as Fisheries and Oceans Canada (DFO), Fisheries and Land Resources (DFLR), and Statistics Canada. The data sets were used to calculate the quantities of unutilized marine biomass materials (i.e., processing discards) produced in NL for each species (or species group) of interest. Weighted averages (refer to Appendix 1 for sample calculation) were calculated for supply to plant, production

output and processing discards generated within a 10-year period from 2006 to 2015. Weighted averages were used to provide an average value somewhere between the values reported for 2006 and 2015 since landings and supply-to-plant vary considerably from year to year due to quota allocations, total allowable catch, weather conditions, etc. This makes it challenging to assign a normal average to landings and supply-to-plant. Similarly, production output and processing discards also vary considerably from year to year due to for example, landed volumes, type of products produced, and markets for by-products. Therefore, a weighted average was thought to be a more practical measure for average landings and supply-to-plant, production output, and processing discards.

2.5.4 Geographical Distribution of Marine Biomass Feedstocks

Geographical distribution of processing plants by species and NL economic zone were evaluated to identify the location of marine biomass feedstocks within the province. This information was also used to provide recommendations on the possible location(s) for a marine biorefinery processing plant which theoretically would include a feedstock collection/stabilization facility and a bioprocessing facility.

2.5.5 Semi-structured Interviews

A semi-structured interview is a research method frequently used in the social sciences. The interviewer prepares an interview guide comprised of an informal grouping of topics and questions that must be covered during the interview. It allows the interviewer to ask questions in different ways for different interviewees, and for respondents to express their views in their own terms. It is best used when the interviewer has only one opportunity to interview someone and when several interviewers are sent out to collect data. The

interviewer and respondent engage in a conversation which may deviate from the guide if felt appropriate by the interviewer and therefore, it is recommended to record the interview and transcribe it later [89, 90].

Semi-structured interviews were conducted August-November 2016 with fifteen stakeholders (harvesters, processors, industry associations and government) using an interview guide based on the Fisheries and Aquaculture Competitive Index (FACI) questionnaire originally developed by the University of Iceland and later modified by the EU Horizon 2020 PrimeFish Project Consortium[4]. The interviews were conducted to fill data gaps and to assess factors affecting industry's ability to utilize marine biomass feedstocks; current use and availability of marine biomass feedstocks; and the impact of the current industry structure and other factors (e.g., technology, certification, regulations) on the utilization of marine biomass feedstocks. Ethics approval was acquired prior to conducting the interviews.

2.6 Literature Review

2.6.1 Overview of World Fisheries and Aquaculture

A brief overview of the state of world fisheries and aquaculture was discussed in Chapter 1, section 1.1 Introduction. While wild capture fisheries production has been relatively stable at approximately 93-97 Mt per year, aquaculture production has increased on average by 8-9% annually contributing 82 Mt to the global seafood supply in 2018 [19,

[4] PrimeFish was an EU Horizon 2020 project comprised of 16 consortium members with 14 EU members, 1 Vietnamese member and 1 Canadian member. The goal of PrimeFish was to develop a web-based software decision tool for the seafood industry which will allow users to compare their competitiveness against other countries and/or companies across several factors. www.primefish.eu

20, 23, 24, 93]. Most of the growth in the aquaculture sector is concentrated in developing countries, particularly Asia and South America [21]. About 90% of world aquaculture production by weight is contributed by Asia with 67% produced by China [21, 22].

While global aquaculture production has increased, it is showing signs of slowing down. Global aquaculture production decreased slightly from 77 Mt in 2010 to 73 Mt in 2014 [24, 25]. Between 2005-2014 fish culture grew by 5.8% annually, down from 7.2% growth achieved between 1995-2004 [26]. Since 1984, the global growth rate in total finfish aquaculture has decreased by 0.34% per year, whereas for salmon this decline has averaged 1.2% per year [27]. This observed decrease in the growth rate of aquaculture has been attributed to several factors, such as: lack of suitable space for grow out operations; changing market conditions (e.g., decrease in price of salmon due to increase in supply); limited access to feed and the increasing costs of feed inputs; environmental challenges resulting in more stringent regulations; and increasing consumer awareness of food safety and quality of farmed fish products [27, 28].

Although the growth of the global aquaculture industry is slowly declining, between 2012-2016 it contributed ~45% of the global seafood supply for human consumption [29, 30, 25]. Production is expected to increase in the coming years due to the increasing world population, a growing demand for fish protein, and stagnating seafood supply coming from wild capture fisheries which are already 75% fully exploited or overexploited [25, 26, 28]. Demand for seafood is predicted to increase rapidly as the world population increases by an estimated 2.5 billion by 2056 [31]. According to the FAO [26] the world per capita consumption of seafood is about 19.7 kg/person/year, and this will exceed 20

kg/person/year beyond 2015, a significant increase over the 1960's consumption of only 9.9 kg/person/year.

2.6.2 Utilization of Global Fisheries and Aquaculture Waste

The seafood industry generates about 45-50% waste [1, 3, 4, 23, 32]. Based on this estimate, the seafood industry generated 75-83.5 Mt of waste in 2014 from its 167 Mt of production [23]. This value will vary depending on the species and products produced (refer to Chapter 1 section 1.1 Introduction, for more information on volumes of waste produced by species and product forms). These traditional waste streams contain valuable components such as proteins and peptides, lipids and omega-3 fatty acids, micronutrients, minerals (e.g., calcium), pigments (e.g., astaxanthin), and other bioactive compounds such as chitin [23, 33]. If recoverable, these bioproducts represent a tremendous opportunity for the seafood industry to minimize processing inefficiencies (by reducing waste and maximizing production), reduce environmental impacts, improve productivity and profitability, and contribute to the sustainability of coastal communities. This has yet to be realized in most seafood producing countries as evidenced by the tremendous volume of seafood waste reported each year [1, 3, 4, 23, 32].

In recent years, concerns over human health issues and the ocean environment have resulted in consumers seeking cost effective natural seafood products, produced without chemical additives (e.g., antibiotics), and harvested/grown using sustainable practices. As the global demand for seafood, natural health products and nutraceuticals continues to rise, this presents a dilemma for the seafood industry as it must learn how to increase fish production and minimize impacts on the environment in a cost-effective manner. Industry,

governments and academia have responded to this situation by moving towards more sustainable harvesting practices (e.g. potting technology for cod and turbot, seabed friendly trawls, escapement mechanisms in crab pots for undersized animals), implementing biomass conservation efforts such as fishery improvement projects (e.g. 3Ps[5] cod, MSC[6] certification of NL shrimp), adopting sustainable aquaculture practices (e.g., RAS[7]), incorporating advanced processing technologies (e.g., robotics, HPP[8] for better yield recovery and sushi grade quality from shellfish), and focusing considerable effort on the extraction of high value specialty products (e.g., biomedical chitosan, omega-3 concentrates for nutraceutical applications, bioactive peptides for treatment of diabetes, collagen for cosmetics and skin creams) from unutilized biomass using biotechnology.

While there are numerous research studies published [3, 13, 15, 16, 32-50] related to the utilization of marine biomass feedstock for the extraction/isolation of high value bioproducts such as those mentioned above, commercially there has been limited success in this area. Some researchers have attributed this lack of commercial success to several factors including: the high cost of production due to too many processing steps; limitations of conventional food processing technologies which can lead to high energy use and loss of end product functionality; failure to identify specific applications of the end products; too much focus on the recovery technologies; raw materials being treated as waste; lack of flexibility in the processing technology to account for biological variations in the biomass

[5] 3Ps is a NAFO fishing zone located off the southwest coast of NL
[6] Marine Stewardship Council
[7] Recirculating Aquaculture System
[8] High Pressure Processing

feedstock; traditional seafood manufacturers not being ready to incorporate biotechnology processes into their production lines [13, 35, 36, 37].

A few criteria have been proposed that must be met if marine biomass feedstocks are to be further utilized to produce marketable high value bioproducts intended for animal/human use. These criteria include: (1) high quality feedstocks are required; (2) yield of the desired products must be high; (3) controlled and standardized collection, preservation and transformation processes must be developed; (4) processes must be flexible enough to account for biological variations; (5) processes must be cost effective and relatively simple to implement; (6) end products must be stable (i.e. maintain their bioactivity), safe, high quality, and cost effective; (7) end products must have proof of bioactivity, nutritional value, and functional properties; (8) there must be suitable markets for the end products [13, 35, 36, 37, 54].

2.6.3 National Fisheries Landings and Aquaculture Production

2.6.3.1 Capture Fisheries

National fish landings in Canada are reported as landings by commercial seafisheries, landings by commercial freshwater fisheries, and commercial aquaculture production. From 2007 to 2015, on average, Canadian seafisheries accounted for 82% (880,995 t) of national fish landings, aquaculture production accounted for 15% (163,598 t), and freshwater fisheries contributed the remaining 3% (28,923 t), (Figure 2.1). Total national landings averaged 1,073,515 t between 2007-2015. Total national landings decreased by 13% from 1,189,397 t in 2007 to 1,032,976 t in 2015. Landings of marine fish decreased by 17% from 986,922 t in 2007 to 817,637 t in 2015. Commercial freshwater

fisheries showed a 13% decline reaching only 27,965 t in 2015. However, aquaculture production increased by 10% with 2015 production reported to be 187,274 t representing an increase of 17,202 t over 2007 production levels.

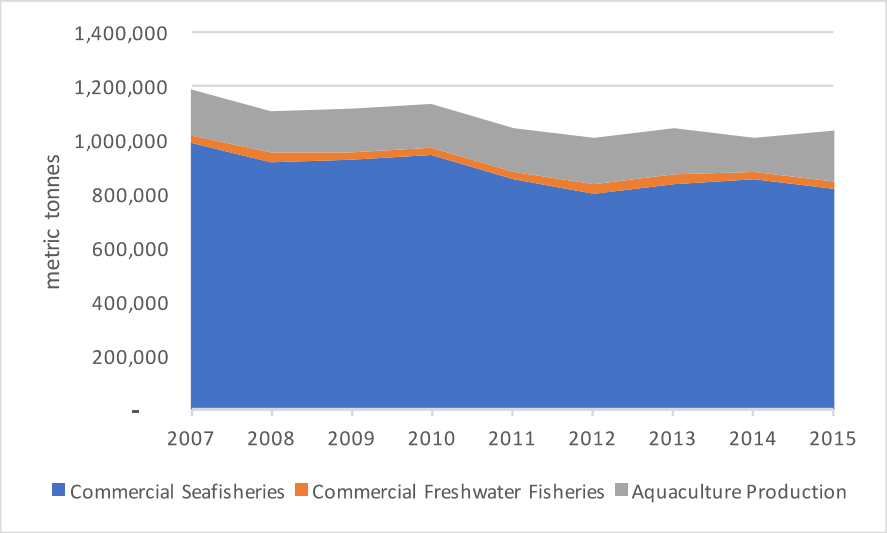

Figure 2. 1 National fisheries landings and aquaculture production 2007-2015 [57]

Historically, the mainstay of the Canadian fishery was Atlantic cod (*Gadus morhua*). Landings of Atlantic cod in Canada by Canadian vessels were at levels above 300,000 t, peaking at over 500,000 t in 1982 and 1983 [26, 55] (Figure 2.2). About 70% of Atlantic cod landed in Canada is harvested in Newfoundland and Labrador. In 1982, the TAC[9] for NL (i.e., NAFO regions 2J3KL + 3Ps + 3NO) was 280,000 t. The peak landings for Atlantic cod, however, was set in 1968 at 810,000 t of which >60% was harvested by foreign fleets [26, 55, 56]. By 1992 the Atlantic cod stocks were in decline and on July 2 that year, Fisheries minister John Crosbie announced a moratorium on the commercial northern cod fishery. The collapse of the northern cod fishery in the late 1980s and early

[9] Total allowable catch

1990s has been attributed to a combination of overfishing and an ecosystem regime shift, particularly in northern regions which also affected capelin stocks (i.e., the main food source for northern cod) [56]. In addition, “cod off Labrador and eastern NL grow slowly and are less productive” compared with other cod populations (e.g., the Flemish Cap) [56].

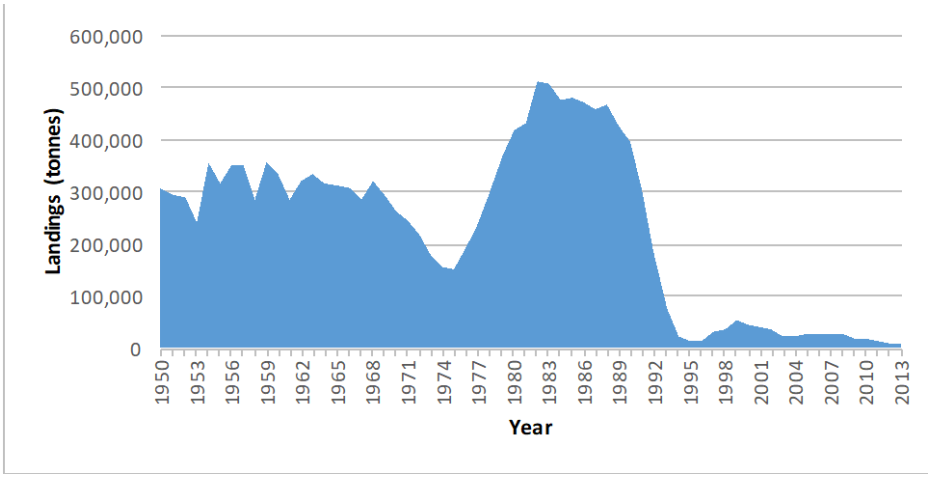

Figure 2. 2 Atlantic cod landings in Canada [24]

It was not until the cod moratorium of 1992 that other species, particularly northern shrimp (*Pandalus borealis*) and snow crab (*Chionoecetes opilio*), began to dominate the Canadian fishing industry. By 1994, the Canadian seafood industry had transitioned to shellfish, with northern shrimp and snow crab becoming the major commercial species. Landings of northern shrimp increased from 43,163 t in 1992 to 139,312 t in 2002, peaking at 188,216 t in 2007 [57]. Landings have been declining since 2007. Snow crab landings increased from 37,255 t in 1992 peaking at 106,812 t in 2002 [57]. Since 2002 snow crab landings have remained just under 100,000 t averaging around 93,588 t between 2002 and 2015 [57]. The value of shrimp has continually increased since the moratorium from $93.8

million to $588.9 million in 2015 [57]. Snow crab prices have seen more fluctuation ranging from $61 million in 1992, peaking at $613 million in 2004, dropping to $215 million in 2006 and rising to $522 million in 2014 [57]. In NL, the major species caught by the inshore fleet (i.e., < 35') in 2010 included cod ($8M), lobster ($19M) and snow crab ($31M). The major species of the near shore fleet (35-65') included northern shrimp ($59M) and snow crab ($121M), while the offshore vessels' (> 65') major species are surf clams ($35M) and northern shrimp ($120M) [58].

A review of total landings and landed value of Canadian capture fisheries over the last 3 decades illustrate that while landings have decreased by 30% since 1990, value has increased by 56%. In 1990 total landings and landed value were reported to be 1.6 Mt and $1.4 billion, respectively [57]. By 2001, total landings had decreased to 1.05 Mt, but the value increased by 33% to $2.1 billion CDN [57]. By 2015, landings declined to less than 1 Mt however the landed value was the highest recorded at $3.2 billion [57]. The decrease in landings was mainly due to reduced catches of groundfish and pelagics. The increase in landed value has primarily been attributed to the increased value of Canadian shellfish, mainly lobster, crab, and shrimp [57]. However, inflation rates have also increased which may cause the observed increase in seafood value to be higher than it is. For example, in Canada prices in 2018 were 35.4% higher than prices in 2000. During this period, the Canadian dollar experienced an average inflation rate of 1.70% per year [59].

With the transition to shellfish, the industry was drastically restructured resulting in a decrease in fleet capacity, fewer processing plants, and a shrinking workforce due to out migration initially, and more recently an aging workforce coupled with few new entrants to the fishing sector.

2.6.3.2 Aquaculture

While more than 580 species (including finfish and shellfish) are farmed around the world [26] globally, the most important farmed fish species are carp, salmon, tilapia, and catfish [30, 60]. While farmed Atlantic salmon (*Salmo salar*) represents only ~3.12% of the total global aquaculture production [24, 26], it is the most important commercially farmed species in Norway, Chile, Scotland, and Canada [61, 62, 63].

Total global production of farmed Atlantic salmon has increased significantly in the last 40 years from 38,797 t in 1985 to more than 2.3 Mt in 2014 [61] valued at $10 billion USD [60]. While 95% of the world's cultured Atlantic salmon come from large producers in Norway, Chile, Scotland and Canada, there is also modest production in the U.S., Faroe Islands, Iceland, Ireland, New Zealand, France, Spain, and Tasmania [61].

From a Canadian perspective, the most important farmed species is Atlantic salmon which accounts for more than 80% of the country's farmed finfish production [60] with 58% grown in British Columbia, 24% in New Brunswick, 13% in Newfoundland and Labrador and 5% in Nova Scotia [61]. Canada is the fourth largest producer worldwide with a total production volume of 121,926t in 2015 worth $668 million [62].

Modern commercial finfish aquaculture began in Canada in the 1970s, with the main species produced being Pacific salmon in British Columbia and Atlantic salmon in New Brunswick and Nova Scotia. While the industry expanded in the 1980's with the production of Atlantic salmon it was dominated by numerous small companies. However, in recent years the industry has been consolidated and now consists of a few large, vertically integrated companies which encompass all phases of the aquaculture value chain [63]. In British Columbia, for example, there were approximately 100 companies participating in

the industry in the 1980's compared with only 11 remaining in 2015 [63]. Table 2.1 lists the major salmon aquaculture companies operating in Canada in 2015 by province.

Table 2. 1 Largest active salmon aquaculture companies in Canada, by Province 2015 [63]

Province	Largest Salmon Aquaculture Companies
Newfoundland and Labrador[10]	Cooke Aquaculture & Northern Harvest Sea Farms[11]
New Brunswick	Cooke Aquaculture, Northern Harvest Sea Farms & Benson Aquaculture
Nova Scotia	Cooke Aquaculture
British Columbia	Marine Harvest, Cermaq & Grieg Seafood

2.6.4 National Fisheries and Aquaculture Discards

There are different types of discards generated from commercial fisheries and aquaculture activities. In fisheries (Figure 2.3) there are discards that occur at sea which include by-catch, undersized animals, unmarketable product, damaged or dead fish. These discards represent 0-20% of the total allowable catch (TAC) but are not landed and are not reported as part of the national landings [64]. From those fish that are landed and further processed there are processing discards such as heads, frames, gut, liver, gonads, trimmings, skin, and shells (where applicable). Some of these discards, however, do not get processed but rather are discarded at sea by harvesters who bleed and gut fish onboard.

Discards from aquaculture (Figure 2.4) include: (1) mortalities which are either ensiled or landfilled in an approved organic disposal site; and (2) processing discards which

[10] Greig NL opened its headquarters in NL in 2015 and is now operating as Greig Seafood Newfoundland since 2020. The company officially opened its NL based smoltification facility April 21, 2022.
[11] Northern Harvest Sea Farms was acquired by Marine Harvest, now MOWI Canada East, in 2017.

include heads, frames, gut, liver, gonads, skin and trimmings, shells and undersized meats (from mussels).

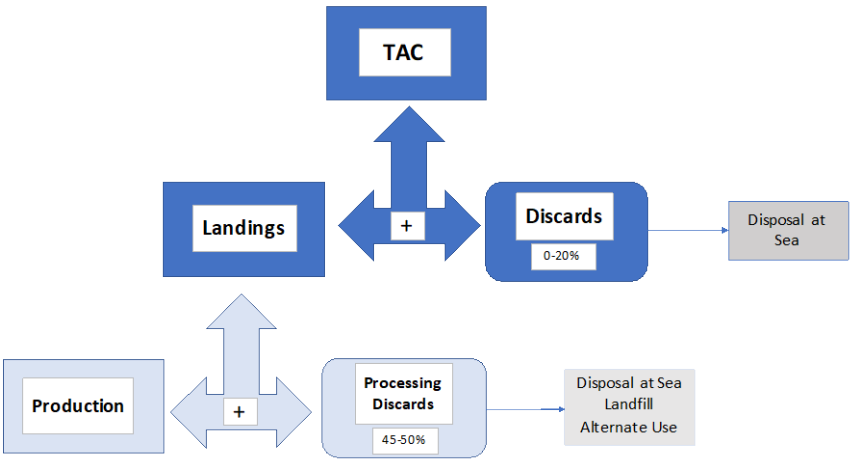

Figure 2. 3 Schematic representation of fisheries discards

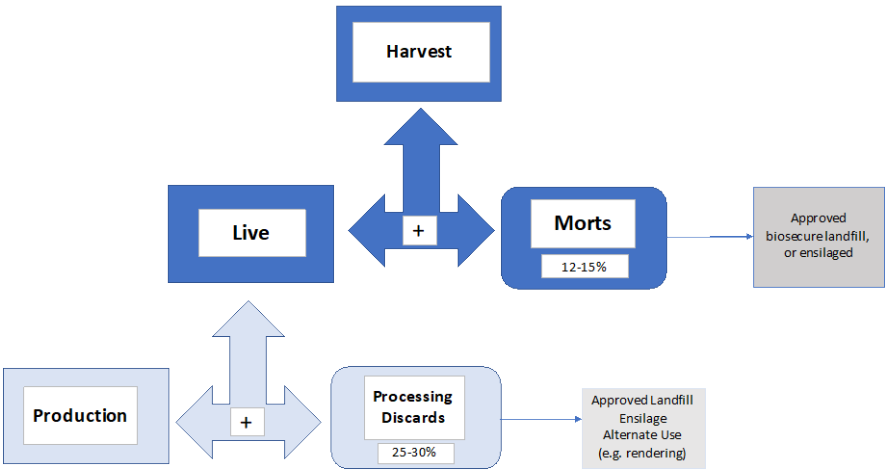

Figure 2. 4 Schematic representation of aquaculture discards

A summary of the volume of utilized and unutilized resources from the 2009 Canadian wild capture fishing industry by species and region is presented in Table 2.2. These data were compiled for the 2009 production season as part of a study sponsored by DFO's Aquaculture Management Directorate in 2011 [1]. The 2009 data provides an indication of the major trends and significance of the fisheries and aquaculture resource utilization problem. The 2009 data illustrates that only 52% of the total national landings from capture fisheries were utilized and that the remaining 46% amounted to 413,549 t of wasted resources at the national level. This waste material was estimated to contain 26% groundfish, 32% pelagics, 25% crustaceans, 15% molluscs, and 1% from wild salmon. The data also indicate that NL and the Maritimes generated the most waste accounting for 98,789 t (24%) and 169,414 t (41%), respectively. Of the 98,789 t of fisheries waste produced by NL, more than 50% of the waste was generated from crustacean processing, mainly northern shrimp, and snow crab [1]. The study also reported that national aquaculture discards represented 19% of the national production volume [1].

The 2009-2010 study included the 6 DFO management regions. The study determined that 78% of national fisheries and aquaculture waste is generated in the Atlantic provinces with the Maritime region contributing 37%, Newfoundland and Labrador 30%, and the Gulf region 11% [1].

Assuming the processing discard rate reported in the 2009-2010 study is applicable to other time periods, the estimated average total volume of annual seafood processing discards generated in Canada between 2007 and 2015 was 450,000 t (Figure 2.5). The composition of these unutilized resources included 90% discards from marine fisheries, 7%

from aquaculture, and 3% from freshwater fisheries, based on the results of the 2009-2010 study [1].

Table 2. 2 Summary of Canadian commercial fisheries resource utilization (in tonnes) by region for 2009[12],[13] [1]

Summary of Canadian Commercial Fisheries Resource Utilization by Region for 2009						% of National Total	
Region	Total Landings (t)	Total Processed (t)	% Utilized	Total Unutilized (t)	% Unutilized	Utilized	Unutilized
NL	311,312	213,052	68%	98,790	32%	24%	11%
Gulf							
NS	17,173	1,535	9%	15,002	87%	0.2%	2%
NB	57,200	50,918	89%	13,762	24%	6%	2%
PE	32,856	10,358	32%	26,089	79%	1%	3%
Maritimies	271,203	89,692	33%	169,415	62%	10%	19%
Quebec	59,154	35,595	60%	24,153	41%	4%	3%
Central and Arctic							
Yukon	n/a	n/a	n/a	n/a	n/a	n/a	n/a
NWT	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Alberta	1,457	n/a	n/a	n/a	n/a	n/a	n/a
Saskatchewan	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Manitoba	12.13	n/a	n/a	n/a	n/a	n/a	n/a
Ontario	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Pacific							
British Columbia	131,500	65,161	50%	66,339	50%	7%	7%
National Total	893,985	466,311	52%	413,550	46%	52%	46%

Using 2014 as an example, and assuming the 46% discard rate applies, Canadian wild capture landings (excluding marine plants, lumpfish roe and miscellaneous categories) of 832,414 t contributed approximately 382,910 t of waste. Atlantic Canada generated 80% of all wild capture landings with the provinces of Nova Scotia and Newfoundland and Labrador each contributing 30%. These two provinces accounted for 60% (499,448 t) of total national landings and generated 60% (229,746 t) of the total national biomass waste [65].

[12] Total Processed + Total Unutilized does not equal Total Landings because (1) some regions imported landings from other regions for processing, therefore the amount of unutilized product could not be determined; (2) process data was not available for all regions.

[13] % Utilized + % Unutilized does not equal 100% due to (1) rounding; (2) data gaps which affected calculations of the volume of unutilized resources.

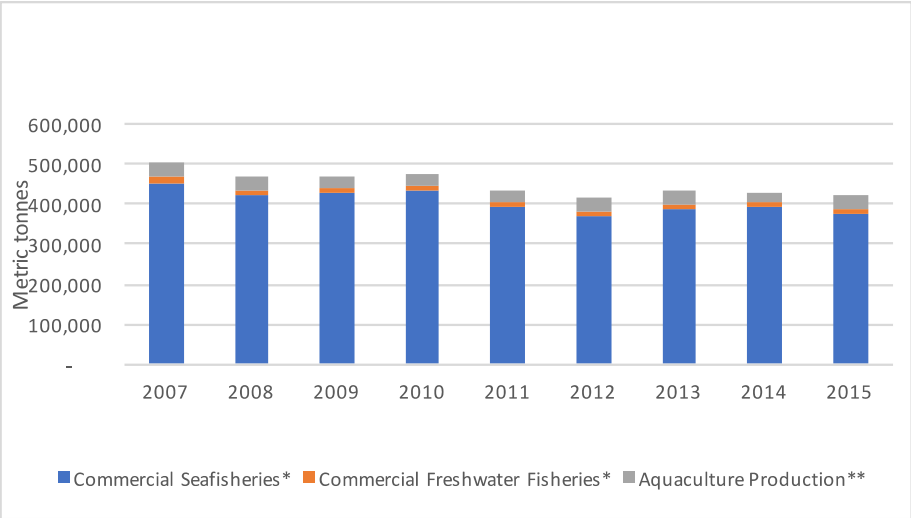

Figure 2. 5 Estimation of national fisheries and aquaculture discards for 2007-2015[14]

2.6.5 Disposal and Utilization of National Fisheries and Aquaculture Discards

In Canada, there are limited options for disposal of fisheries and aquaculture discards. For wild capture fisheries, disposal options for processing discards include rendering into fishmeal/oil; compost or fertilizer; disposal at sea; or landfill. Processors must demonstrate that all other options for disposal are either unavailable or economically prohibitive before obtaining authorization to dispose of their discards in a landfill [66].

Environment Canada issued 500 permits for the disposal at sea of fish processing waste between April 2000 and March 2011 [67]. Most ocean dumping of fish processing waste occurs in NL followed by Quebec. For example, in 2015 Environment Canada issued 31 permits for at-sea disposal of fish processing waste. Of these, 27 permits were issued in NL, 3 were issued in Quebec, and I was issued in NS [94]. In the Maritimes, a waste audit is required identifying environmentally friendly disposal options before an ocean dumping

[14] * = Estimate is based on 46% discard rate and ** = Estimate is based on 19% discard rate [1].

permit is issued. As a result, in NB, NS, and PE all fish waste is now either composted or recycled in fishmeal plants and the number of permits issued for at sea disposal has decreased to 1 per year [67].

In 2012, the results of a project for 100% utilization of fish farm waste were released by DFO [68]. The study was funded by DFO's Aquaculture Innovation and Market Access Program and West Coast Fishculture (WCF). The results of this study are briefly summarized here to illustrate alternatives to landfilling and at-sea dumping for fish by-products. In 2012, the province of British Columbia produced 15,206 t of fish waste and 25,344 t of fish offal from aquaculture. This waste material was mainly disposed of through composting at a cost of > $2 million/year. West Coast Fishculture (WCF), however, have been using an enzymatic digestion process to utilize 100% of aquaculture mortalities and processing discards to produce oil (biofuel and nutraceutical) and an organic certified soil amendment. Cuttings/trimmings are compressed to extract oil, and all remaining materials are combined to produce a high nutrient soil amendment. WCF has the capacity to utilize 324.5 t of fish production and process waste per year. Based on 2010 production levels, WCF have capacity to produce 289.7 t of soil amendment annually [68]. In 2018, WCF[15] was producing a fish-based soil amendment for Simply Fish Soil Amendment [91].

2.7 Results

Discussion of the results presented below begins on page 98 of this thesis Chapter.

[15] WCF was acquired by AgriMarine Holdings in 2013. It is not clear if Simply Fish Soil Amendment is still in operation.

2.7.1 Industry Structure

This section summarizes the number and type of fishing vessels registered in NL (refer to Appendix 2 for more information), the number of harvesters, number of harvesting licenses by species, plant processing capacity and capacity of the farmed salmon industry up to 2015. These statistics influence both the quantity and quality of fish products and by-products available in NL.

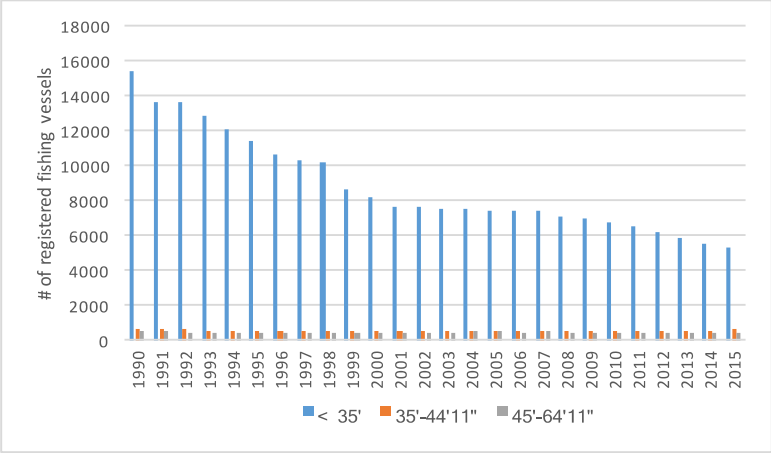

Figure 2. 6 NL region commercial fishing vessels registered by size between 1990 and 2015 [16]

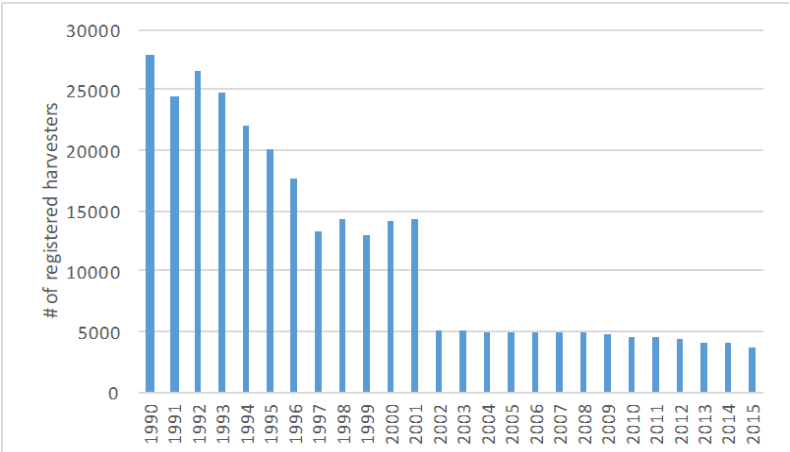

Figure 2. 7 Number of commercial harvesters registered in NL between 1990-2015 [17]

[16] Data retrieved and compiled from http://www.dfo-mpo.gc.ca/stats/commercial/licences-permis/licences-permis-atl-eng.htm (July 10, 2017).
[17] Data retrieved and compiled from http://www.dfo-mpo.gc.ca/stats/commercial/licences-permis/licences-permis-atl-eng.htm (July 10, 2017).

Table 2. 3 Number of harvesting licenses issued for NL by species of interest [18]

Year	# of Licences by Species					
	Groundfish	Herring	Mackerel	Capelin	Shrimp	Crab
1985	7316	20	461	937	0	126
1986	7199	818	656	1847	0	275
1987	7352	3091	716	2180	0	320
1988	7521	2704	752	2434	8	626
1989	7624	2446	759	2692	8	688
1990	7720	2309	752	2568	0	706
1991	7322	2190	754	2583	0	730
1992	7078	2222	799	2570	0	744
1993	5588	2220	976	2437	0	735
1994	8449	3344	1603	2852	57	736
1995	8213	3292	1918	2765	51	751
1996	7925	3223	2103	2687	46	758
1997	6682	3228	2258	2685	45	776
1998	6680	3228	2352	2670	21	777
1999	5323	2639	2020	2207	412	778
2000	5039	2517	1991	2105	420	779
2001	4714	2365	1925	1968	431	779
2002	4693	2362	2032	1960	438	781
2003	4671	2354	2064	1953	437	3340
2004	4653	2360	2148	1949	439	3356
2005	4657	2377	2516	1939	450	3364
2006	4609	2372	2609	1941	437	3389
2007	4591	2365	2738	1937	446	3407
2008	4499	2341	2703	1919	462	3449
2009	4398	2372	2670	1934	461	3455
2010	4306	2350	2618	1920	461	3445
2011	4183	2322	2574	1881	372	3266
2012	4008	2307	2476	1865	363	3156
2013	3796	2242	2352	1824	345	2809
2014	3786	2074	2265	0	331	2796
2015	3742	2032	2227	1733	451	2625
Total Change	**-3574**	**2012**	**1766**	**796**	**451**	**2499**
% Change	**-49%**	**10060%**	**383%**	**85%**	**5638%**	**1983%**

[18] Data retrieved and compiled from http://www.dfo-mpo.gc.ca/stats/commercial/licences-permis/licences-permis-atl-eng.htm (July 10, 2017).

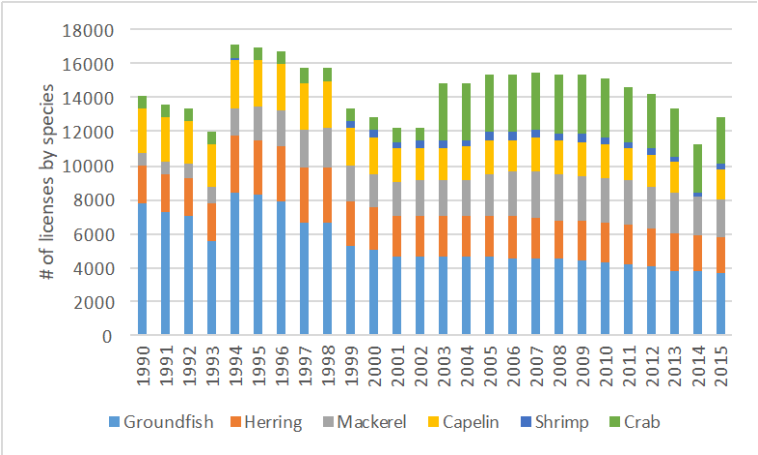

Figure 2. 8 Number of licensed harvesters in NL by species of interest [19]

Table 2. 4 Licensed fish processing plants in NL [20]

YEAR	NL LICENSED PROCESSING PLANTS				
	PRIMARY	SECONDARY	RETAIL	AQUACULTURE	TOTAL
2001	122	7	14	5	148
2002	125	7	14	5	151
2003	123	4	14	4	145
2004	116	5	14	3	138
2005	117	4	12	4	137
2006	119	5	12	4	140
2007	116	5	12	5	138
2008	113	5	13	5	136
2009	101	4	8	5	118
2010	102	4	10	5	121
2011	98	4	14	5	121
2012	89	3	14	4	110
2013	73	3	13	6	94
2014	73	2	13	5	94
2015	73	2	13	6	94

[19] Data retrieved and compiled from http://www.dfo-mpo.gc.ca/stats/commercial/licences-permis/licences-permis-atl-eng.htm (July 10, 2017).
[20] Data retrieved and compiled from FFA Seafood Industry Year in Review reports and List of Licensed Processors available at https://www.gov.nl.ca/ffa/publications/fisheries-and-aquaculture/archived-seafood-industry-year-in-review/

Table 2. 5 NL Atlantic salmon aquaculture data 2005-2015 [21]

Year	NL Salmonid Production			
	# of Licensed Farms	**Total Hectares**	**# of Hatcheries**	**Production (t)**
2005	46	731	4	4,991
2006	45	1,047	2	7,300
2007	53	1,698	1	4,857
2008	68	1,698	1	8,877
2009	75	1,965	3	11,551
2010	81	2,056	3	12,899
2011	84	2,205	3	14,264
2012	87	2,376	3	16,831
2013	87	2,402	4	22,196
2014	87	2,402	4	5,980
2015	87	2,402	4	19,684

2.7.2 Types of Marine Biomass Feedstocks Generated in NL 2006-2015

Table 2.6, and Figures 1.10 and 1.11 (Chapter 1) describe the type of marine biomass feedstocks which are generated in NL and potentially available for use to produce bulk intermediate or higher value bioproducts.

[21] Data was compiled from the Newfoundland and Labrador Department of Fisheries and Aquaculture Annual Reports, Seafood Industry Year in Review reports, and Annual List of Aquaculture Sites available at https://www.gov.nl.ca/ffa/

Table 2. 6 Description of marine biomass feedstocks generated in NL and their current uses

Source of Raw Material	Description of Feedstock	Current Uses
Northern Shrimp (*Pandalus borealis*)	Shells, heads, viscera and protein from cooked & peeled processing plants	Dumped at sea or in landfill
Snow Crab (*Chionoecetes opilio*)	Carapace (shell), gut, viscera, protein from cooked section processing	Dumped at sea or in landfill
Farmed Salmonids (*Salmo salar*)	Gut, roe, liver, trimmings, heads, frames, skins	Silage, rendering plant in NB, mink feed; trimmings and off-cuts used for value added products such as smoked salmon pub snacks
Groundfish (various)	Gut, roe, liver, trimmings, heads, frames, skins	Dumped at sea or in landfill; Cod - gut, roe, liver dumped at sea; tongues & cheeks removed and sold, head & bones ground into pet food or sold to mink farmers, frozen heads sold as bait, skins sold for leather, napes & v-bones minced
Pelagics (various)	Gut, roe, liver, trimmings, heads, frames, skins	Compost, mink feed, bait, dumped at sea or in landfill

2.7.3 Inventory of Marine Biomass Feedstocks Generated in NL 2006-2015

An inventory of all seafood, by species or species group, with respect to the amounts of raw material supplied to all processing plants, production output and processing discards generated was prepared. The availability, current and potential applications of marine biomass feedstocks generated by the NL seafood processing industry are also identified. These data are tabulated and available in Appendix 3. The data is also summarized in Tables 2.7, 2.8 and 2.9.

Table 2. 7 Volume of NL seafood supply, production output and processing discards for all species 2006-2015 [22]

All Species	Volume (t)			Yield	
YEAR	**Supply to Plant**	**Production Output**	**Discards**	**% Yield**	**% Discards**
2006	355,537	201,420	154,117	57%	43%
2007	340,505	189,640	150,865	56%	44%
2008	328,705	181,471	147,234	55%	45%
2009	301,496	175,270	126,226	58%	42%
2010	304,885	168,415	136,470	55%	45%
2011	268,721	140,720	128,001	52%	48%
2012	256,093	135,924	120,169	53%	47%
2013	266,679	147,493	119,186	55%	45%
2014	244,787	122,076	122,711	50%	50%
2015	240,785	133,460	107,325	55%	45%
TOTAL	2,908,193	1,595,889	1,312,304		
Weighted Ave	**296,082**	**163,729**	**132,861**	**55%**	**45%**

Table 2. 8 Average annual volume of NL processing discards by species per processing facility 2015 [23]

Average annual volume of processing discards by species per processing facility 2015			
Species	**# Processing Plants**	**Total Volume of Discards (t)**	**Ave Discards per processing plant (t)**
Shrimp	10	29627	2963
Crab	27	15013	556
Farmed Salmon	8	3490	436
Groundfish	57	22102	388
Pelagics	59	22415	380
Other	135	14678	109

[22] Data for "supply to plant" and "production output" were provided by the Licensing and Policy Division of the Department of Fisheries and Land Resources, Government of Newfoundland and Labrador. Processing Discards were calculated as the difference between supply to plant and production output. Supply to plant includes NL landings, as well as raw materials purchased from other provinces and countries. Supply to plant is reported as live round weight.

[23] Production data and discard data were not available for individual processing plants due to privacy requirements. Therefore, this model assumes that all plants processed similar volumes of raw materials, however this would not be the case as some plants have higher processing capacity than others and the supply of raw material to each plant will vary.

Table 2. 9 Availability and applications of feedstocks generated by the NL seafood processing industry [24]

Feedstock type, availablilty, and potential applications of feedstocks generated from the NL seafood processing industry (2006-2015)					Potential Feedstock Applications	
Source of Raw Material	**Seasonal Availability***	**Average Annual Processing Discards (tonnes)**	**Availability for Alternate Use**	**Comments**	**Intermediate Bulk Products**	**Higher Value Bio-Products**
Northern Shrimp (*Pandalus borealis*)	June - November	41,702	High	Could be recovered at the plant from peelers	Dried shells, chitin, proteins, lipids, pigments, calcium carbonate	chitosan, chito-oligosaccharides, amino acids, bioactive peptides, aqua feeds, fatty acids, omega-3, astaxanthin, calcium
Snow Crab (*Chinocetes opilio*)	April - July	15,164	High	Could be recovered at the plant from butchering stations	Dried shells, chitin, proteins, lipids, pigments, calcium carbonate	chitosan, chito-oligosaccharides, amino acids, bioactive peptides, aqua feeds, fatty acids, omega-3, astaxanthin, calcium
Farmed Salmonids (*Salmo salar*)	Year Round	3,061	Low	Industry currently has little processing discards. Mortalities & fish health are main concern. Biosecurity requirements determine how discards are utilized.	Fishmeal, oil, protein, bones, dried heads & frames, skin, silage, mink feed	biodiesel, omega-3, bioactive peptides, fish leather, collagen, calcium
Groundfish (various)	**March - December**	18,560	Low	Much of the discards are not landed but are discarded at sea (~25%), and therefore not currently collected. Other processing discards are directed to alternate uses and not currently available.	Oil, protein, bones, dried heads & frames, fresh/frozen/canned livers, skin, cheeks & tongues	biodiesel, cod liver oil, bioactive peptides, fish leather, collagen, calcium
Atlantic Halibut	March - Setpember					
Cod	May - December					
Greenland Halibut	May - November					
Redfish	March - October					
Pelagics (various)	**Jan - December**	16,886	Low	Most of the discards are diverted to alterante uses. Only 10% estimated to be discarded.	Fishmeal, oil, protein, bones, dried heads & frames, fresh/frozen/canned livers, skin	biodiesel, omega-3, bioactive peptides, fish leather, collagen, calcium
Capelin	June - August					
Atlantic Herring	Jan - December					
Mackerel	August - November					

** DFO Fisheries Notices 2017 NL - http://www.nfl.dfo-mpo.gc.ca/NL/CP/Orders/2017/Notices-list*

[24] Theoretical discards for Atlantic cod between 1998 and 2016 were calculated as an example to illustrate discard rates based on selected cod by-products and are available in Appendix 4.

2.7.4 Geographical Distribution of Licensed Fish Processing Plants by Species and Economic Zone

Figure 2.9 below shows the 20 economic zones and the location of licensed fish processing plants in the province. Table 2.10 cross-references the number of processing plants by species and economic zone.

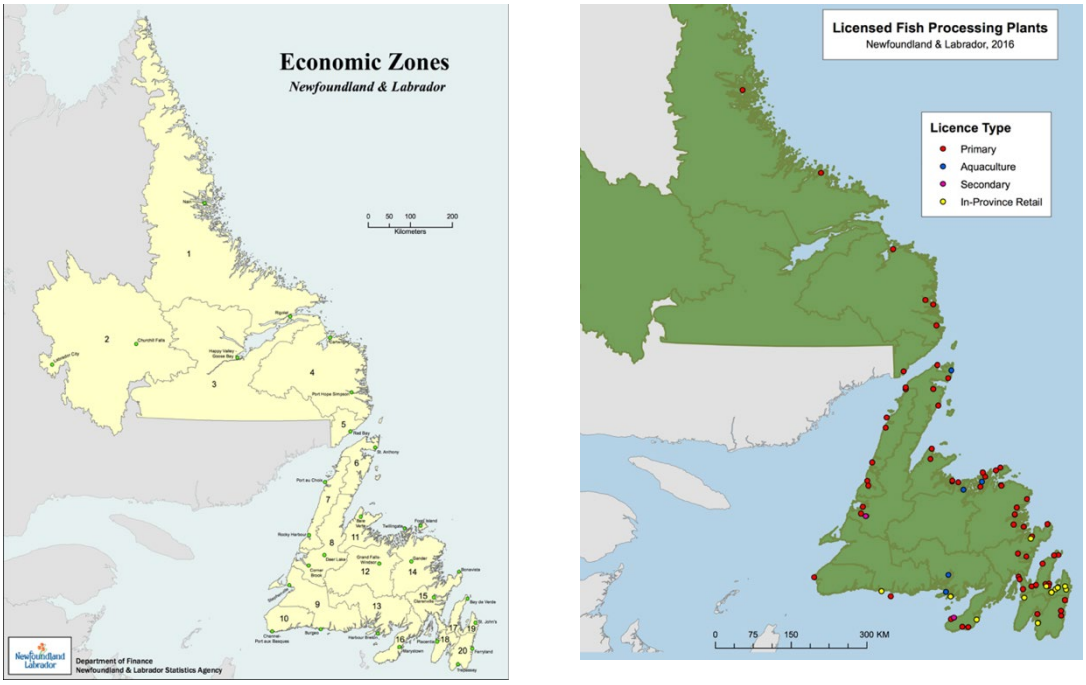

Figure 2. 9 NL Economic zones (left) and distribution of licensed fish processing plants [25]

2.7.5 Semi-structured Interviews

This section summarizes the key factors as identified by industry stakeholders that affect marine biomass feedstock utilization in Newfoundland and Labrador (Table 2.11). Semi-structured interviews were conducted with 15 stakeholders during the months of August-November 2016.

[25] Maps are courtesy of the Department of Finance NL Statistics Agency and the Department of Fisheries, Forestry and Agriculture, Government of Newfoundland and Labrador www.gov.nl.ca.

Table 2. 10 Summary of the number of processing plants in NL by species/group and NL economic zone in 2015 [26,27,28,29]

NL Economic Zone	# of Plants by Species or Species Group for 2015					
	Northern Shrimp	**Snow Crab**	**Salmonids**	**Groundfish**	**Pelagics**	**Other**
1	0	1	0	1	0	2
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	1	2	0	2	2	4
5	0	0	0	0	0	0
6	2	2	0	3	3	8
7	2	2	0	6	4	17
8	0	1	0	2	4	6
9	0	0	0	1	1	2
10	0	0	1	1	1	4
11	0	1	0	2	4	7
12	0	0	0	1	0	3
13	0	0	3	1	1	1
14	2	4	3	6	8	22
15	1	2	0	6	5	12
16	0	2	0	4	3	13
17	2	6	1	11	11	19
18	0	0	0	2	2	5
19	0	0	0	6	6	7
20	0	4	0	2	4	3
TOTAL	**10**	**27**	**8**	**57**	**59**	**135**

[26] The total number of processing plants by species exceed the total number of licensed processing plants in the province because most of the processing plants are multi-species facilities meaning they are licensed to process more than one species.

[27] Since 2016 there are only 7 licensed shrimp plants in the province. Two plants closed due to quota cuts, and another was destroyed by fire.

[28] Distribution of processing plants by species and economic zone was also evaluated for the 10-year time-period 2006-2015. Results are available in Appendix 4. Only the most recent data is considered relevant for this assessment and is presented here.

[29] Data were provided by the Licensing and Policy Division of the Department of Fisheries and Land Resources, Government of Newfoundland and Labrador

Table 2. 11 Factors affecting marine biomass feedstock utilization in NL according to industry stakeholders

Factors/Stakeholder Groups	Harvesters	Processors	Industry Associations	Government
Current Utilization of Marine Biomass Feedstocks (i.e. by-products)	No use for cod livers & bones so it is thrown overboard; Livers not collected	Capelin used for fishmeal and mink farms	We don't collect livers or by-products - since moratorium focus has been on higher value species. Pre-moratorium focus was on volume not value or full utilization	There is one major processor focused on herring; little development and much of our herring has gone into bait; a little has gone into value added such as smoked or wet cured
	Cod heads, cheeks tongues and britches collected & utilized	Biggest obstacle towards more value-added products and use of by-products is policy and government		
	Some fishermen sell discards to mink farms	Would like to do more research on by-products - we put 60% of shrimp back in the water and 35% of crab	Don't focus on by-products and full utilization because we have such low volumes; no history or culture of using by-products; high costs and lack of resources to focus on utilization of by-products	
	Not economical to transport fish discards to mink farms or for them to pick it up, volumes too low	We don't do anything with crab shells; we do a little bit with flavourants	We have the largest cold water shrimp and snow crab fishery, but yet we could not figure out how to utilize the shell by-products	
		Cod by-products go back into bait, it's not being collected or utilized		
	Capelin is harvested for animal feed (zoos), may be worth more today than what the food market was 25 years ago	Currently there is no reason for us to use the liver if we don't know what quantity or quality we are getting	Majority of salmon is fillet and HOG with almost full utilization of by-products - we have markets for swim bladders - full utilization is paramount for our industry	
		Salmon processors are making high value products from parts of the fillet that were historically by-products	For salmon, there are huge markets in Asia for heads, salmon frames, swim bladders - so we are near to 100% utilization	
	Processors buy HOG product, then sell heads back to fisherman for bait	Focus on how to take our traditional salmon products and maximize its value - trying to find alternate uses for by-product streams - pet foods, dog treats, pub snacks		
	By-catch is not wanted by processors, so harvesters throw it back			

Factors/Stakeholder Groups	Harvesters	Processors	Industry Associations	Government
Industry Structure	Harvesting by gillnets destroys smaller fish	Landings difficult to manage, buy from 150 different harvesters	We land fish in relatively small volumes spread over a wide area - causes inconsistencies in quality and how the grading system is implemented	Cod is still a challenge, it is still in development - indsutry is a bunch of smaller players and one major operation that does half the cod that is caught in the province
	Gillnets no good for catching cod in July, so we fish in August - fishing it later in the year would require bigger boats	Union (FFAW) is a big influence on policy		
	70-80% of fisherman do not want to fish in Sept, Oct, Nov, but because have small boats that can't handle weather	Fishery is too ineffecient, boats are too small, too many landing sites, MPR is a limitation to growth		The largest cod processor is only operating at 40% capacity and requires a massive injection of funding to improve processing capacity
	Current price setting mecahnism and grading system is a discentive for quality			Current focus is on the change from shellfish to ground fish
	Problem with small boats - fish sit in the bottom, not gutted or bled with no ice at 26C in the summer	No one will invest in a large plant without security of raw material	Legislation restricts ability for industry to change - MPRs, Price setting board, restrictions on foreign company investment, should ban gillnetsn - Iceland only uses 8% gillnets, NL uses 80% gillnets	in snow crab, a lot of the product is landed in a very short time so you must have enough capacity in the plant to push that product through. So, for three or four months of the year, and a lot of it is in 2 months, that big peak. You have all of this capacity but all of a sudden it's not needed for the rest of the year

Factors/Stakeholder Groups	Harvesters	Processors	Industry Associations	Government
Industry Structure	Not the fisherman's fault, as DFO issues licenses without requiring harvesters to have training	Plants are multi-species and follow the cycles of landings from harvesters - causes challenges due to variations in markets, supply and quality of the raw materials		There are issues of seasonality and the number of landing sites. All of those issues need to be solved. The federal government and the number of licenses that are given out, the provincial government issues of licenses that are giving out.
	Biggest problem is not the IQ system, it's the price - its too low			
	Processors buy our HOG product, then sell heads back to us for bait	Main business is crab so the plant will not buy cod from a fisherman unless he has something else to offer - cod is low value		
	Processors are benefiting from the fisheries management system, not the harvesters			Good prices for crab and shrimp hide the issues in that industry
	Too many harvesters and not enough product to be sustainable	Burn all gillnets; use bigger boats; use longline, pots or traps; have to fish differently to get better quality	Lack of trust between harvesters and processors/buyers	The main business is crab, so a lot of stuff is connected to the crab industry – cod, sea cucumber – processors buy cod and sea cucumber to maintain access to crab
				The FFAW has a lot of pull with regards to policy decisions on the provincial and federal level. On the processing side it's a little splintered. Whatever their stance is on something I mean 9 chances out of 10 they've gotten their way.

Factors/Stakeholder Groups	Harvesters	Processors	Industry Associations	Government
Geographical Distribution and Logistics	Not economical to transport fish discards to mink farms or for them to pick it up, volumes too low	Too many landing sites	We land fish in relatively small volumes spread over a wide area - inconsistencies in quality and how the grading system is implemented	Major challenge is getting product across the Gulf on the Marine Atlantic Ferry Service. If there is a storm delay or the ferry is not operating, you lose freshness and shelf-life on your product. May do better with HOG in this case as the product would not lose its chill as fast as smaller fillets and portions
		Transportation is 3400 miles or more from landing sites to the processing plant; Adds cost - off loading boats, trucking, off loading again	We must deal with issues such as geographical isolation - makes our province costly	Major challenges include: getting product form the wharf to the plant due to long trucking distances; gluts at the plant when large volumes are landed in peak season - fish held for extended periods; time of year when landed (warm temps); too many landing sites and low volumes

Factors/Stakeholder Groups	Harvesters	Processors	Industry Associations	Government
Quality of Raw Materials	Quality of cod is suffering due to lack of feed (i.e. capelin)	If you want to use fish by-products as a raw matieral for value added products it must be very fresh. This starts with the harvester and when and how he takes the fish out of the water	Seasonality, harvest times and handling affect quality	If we get back into cod, the entire industry must be focused on quality and consistency throughout the entire vlaue chain, starting with harvesting
	Industry knows how to handle crab, but not cod	Plants are multi-species and follow the cycles of landings from harvesters - causes challenges due to variations in markets, supply and quality of the raw materials		
	Quality issues with cod fished in summer			
	Problem with small boats - fish sit in the bottom, not gutted or bled with no ice at 26C in the summer			
	Crab - concerns with mortalities and soft shell and handling		To fully utilize by-products we need quality, capital and volume	Personal attitudes, education, training, investment in better handling technologies are needed to make improvements in quality of cod.
	Problem with NL fishery is that its volume driven, not quality driven			
	Gillnets should be banned, but they are cheap and easy to use			
	Transition from shellfish to groundfish is challenging - harvesters do not know what the processors need/want (quality) or the markets	Quality must be consistent and starts with the harvesting; must fish differenlty to improve quality (i.e. time of year, type of gear, onboard handling, etc)		
	Big difference in quality of landings between inshore and offshore		Traceability, freshness and quality are more important than the type of fish	
	We work closely with processors to improve onboard handling and quality of shellfish			

Factors/Stakeholder Groups	Harvesters	Processors	Industry Associations	Government
Markets	Crab is high value, cod is not	Plants are multi-species and follow the cycles of landings from harvesters - causes challenges due to variations in markets, supply and quality of the raw materials	Majority of salmon is fillet and HOG with almost full utilization of by-products - we have markets for swim bladders - full utilization is paramount for our industry	2 major salmon players on the south coast; all fresh and sold to Canada and the US; about 50-50 fillets and HOG
	Shrimp is also important but crab is key species for plants			
	Snow crab is now King in NL, but showing signs of trouble			Good prices for crab and shrimp hide the issues in that industry
	Transition from shellfish to groundfish is challenging - harvesters do not know what the processors need/want (quality) or the markets		Certification and traceability programs are critical for market access	The main business is crab, so a lot of stuff is connected to the crab industry – cod, sea cucumber – processors buy cod and sea cucumber to maintain access to crab
	Biggest problem is not the IQ system, it's the price - its too low		NL does a poor job of marketing its seafood	Salmon is sold fresh: 70-80% as HOG and 20-30% as fillets
			For salmon, there are huge markets in Asia for heads, salmon frames, swim bladders - so we are near to 100% utization	
Fisheries Resources	Quality of cod is suffering due to lack of feed (i.e. capelin)			Shrimp resource is down; haven't seen a big decline in crab but science says recruitment is low
	Fish are starving in many areas			

2.8 Discussion

2.8.1 Industry Structure

The NL fishing fleet has been predominantly composed of vessels in the <35' category. In 1985, the <35' vessel segment accounted for 91% (14,184 vessels) of the total provincial fishing fleet (Figure 2.6). Since 1985, the NL fishing fleet has decreased by ~59% (9178 vessels). The reduction in the NL fleet has mainly occurred in the <35' category accounting for a reduction of 8857 vessels. Since 1985 all fleet categories have declined, except for the 65'-99'11" category which has seen a modest increase from 9 vessels in 1985 to 28 vessels in 2015. Currently, the fleet is dominated by vessels <35' (5327 vessels in 2015) followed by vessels in the 35'-44'11" (625 in 2015) and 45'-64'11" (408 vessels in 2015) categories, which together make-up 99% of the provincial fishing fleet [69, 70]. This creates challenges for the harvesting and processing sectors since much of the fishing fleet is composed of small inshore and nearshore vessels which have limited capacity to properly handle the catch and the potential biomass feedstock destined for further processing into higher value bioproducts. For example, these smaller vessels will have less cooling, processing and storage capacity compared with vessels that are > 64'11" (semi-structured interviews).

In addition to the major fishing fleet reduction, NL has also seen a major reduction in employment and processing capacity in the seafood sector. The number of harvesters decreased from 26,564 in 1985 to 3,787 in 2015 (Figure 2.7). The number of licensed processing plants has declined from 148 in 2001 to 94 in 2015 [51] (Table 2.4). Most plants are multi-species processing plants and have dedicated processing lines per species. Most

of the processing capacity exists in the primary processing sector which accounts for 77% of the total licensed seafood plants in NL.

As the industry transitioned from the collapse of the northern cod stocks in the 1980s and 1990s, there was a shift in the number of harvesting and processing licenses issued by species (Figure 2.8, Table 2.4). There was a decline in groundfish licenses and increases in pelagic and shellfish licenses. Between 1985 and 2015 the number of groundfish licenses decreased by 49% from 7316 to 3742, herring saw a 10,000% increase from 20 to 2032 licenses, mackerel licenses increased from 461 to 1766 (383% increase), capelin increased by 85% from 937 to 1733 licenses, shrimp licenses increased from none to 451, and the number of crab licenses increased from 126 to 2625.

In Newfoundland and Labrador (NL), aquaculture production is currently focused on salmon (86% by volume and 92% by value) and mussels (14% by volume and 8% by value) [5]. Total annual production has increased significantly since 2000 from a total of 2,718 t worth $13.6 million, to 26,551 t with a market value of $197 million in 2013 [6], making NL the second largest producer of aquaculture products in Canada with the second highest production value. Table 2.5 provides a profile of the NL salmon aquaculture industry for the period 2005-2015. Commercial farmed salmonid production has increased significantly since 2005 (Table 2.5) [5, 6]. However, in 2014 NL experienced a significant drop in salmonid production (Table 2.5) and value due to ISAv (infectious salmon anemia virus) and super chill events which resulted in significant salmon mortalities [5, 6]. Total salmonid production in 2014 dropped by 73% to 5,980 t [5, 6]. Production levels rebounded in 2015 to 19,684 t valued at $149 million [5, 6]. In 2015, there were 87 licensed salmonid sites spanning 2,402 hectares, representing a ~90% increase in licensed farm sites and a

228% increase in total hectares since 2005 [5, 6]. There are currently 4 hatcheries operating in the province. Most salmon aquaculture production occurs on the south coast of Newfoundland on the Connaigre Peninsula, mainly the Coast of Bays region in Bay D'Espoir. There are no licensed aquaculture sites (finfish or shellfish) in Labrador [5, 6].

2.8.2 Types of Marine Biomass Feedstock Generated in NL 2006-2015

The types of marine biomass feedstocks generated in the province have been categorized by species or species group (Table 2.6). The main crustacean species harvested and processed in NL between 2006 and 2015 include inshore northern shrimp (*Pandalus borealis*) and snow crab (*Chionoecetes opilio*). Inshore shrimps are typically processed as a cooked and peeled product. This process removes the heads, shell, viscera, and protein during the peeling, washing, and separating steps and ends up as waste. Snow crab is primarily processed as IQF cooked sections which generates waste comprised of carapace (shell), gut, viscera, and protein.

2.8.2.1 Crustaceans (Shrimp and Crab)

A detailed discussion of the harvesting methods, onboard handling practices, and processing methods used for Northern shrimps (*Pandalus borealis*) and Snow crabs (*Chionoecetes opilio*) was presented earlier in Chapter 1, sections 1.5.1.1 Cold Water Shrimp (*Pandalus borealis*) and 1.5.1.2 Snow Crab (*Chionoecetes opilio*).

2.8.2.2 Groundfish

The main groundfish species harvested in NL by volume include Atlantic cod, Greenland turbot and flounders (i.e., American Plaice, Yellowtail flounder, Grey sole/witch flounder, Winter flounder). Flounders are mainly sold whole frozen to Asian markets

without further processing. The main product forms of cod include fresh/frozen fillets and portions (UK), wet salted (Spain), and head-on-gutted (HOG) (US). Cod are predominantly harvested by the inshore fleet and are bled and gutted onboard. In 2015, 68.7% of total cod landings in NL were harvested by the under 35' vessel size class, and approximately 84% of total cod landings were harvested using gillnets [71]. The gut, gonads and livers are discarded at sea. HOG, or dressed, fish are iced and brought to shore for further processing. The dressed weight represents, on average 85% of the live weight. From the dressed weight, according to Gardner Pinfold Consultants Inc. [72], the utilization rate for the main cod products would be 100% for fresh HOG; ~45% to wet salted, 40% to fillet. Currently, additional utilization is already realized from fillet and salted product. For example, tongues and cheeks are removed and sold separately, heads and bones are ground into pet food or sold to mink farmers, heads are also frozen and sold back to harvesters for bait (Interviews), napes and v-bones are minced, while skins are sold for leather products [72]. Greenland turbot (halibut) (*Reinhardtius hippoglossoides*) is mainly sold to markets in the US, Asia, and Europe as frozen HOG, HGT (head, gut tail removed), fillets or steaks, and heads [73, 74]. Ocean Choice International owns 91% of the Yellowtail flounder quota which they harvest year-round and process as frozen at-sea in various product forms including fresh/frozen fillets skin-on/skinless; shatter pack skinless/skin-on fillets; fillet block; whole round; H&G (headed and gutted); and by-products, depending on markets [74].

2.8.2.3 Pelagics

The major pelagic species harvested in NL include capelin, herring, and mackerel. Traditionally, only female capelin was sold commercially, mainly to Japanese markets while the males were discarded. However, antidumping regulations were implemented in 2006 [75] resulting in male capelin primarily being sold to the US for zoo food or used as bait [76]. Herring is typically sold whole, frozen, with only a few processors producing other product forms (e.g., fresh/frozen fillets, smoked, salted dried, pickled/cured, livers, roes, and oil). Mackerel is usually sold whole, frozen into the food market, while broken product is sold as bait. According to local processors, there are few discards or waste materials available from herring, mackerel, and capelin processing. Provincial fisheries authorities, however, estimate that roughly 10% of landings are being discarded due to insufficient markets.

2.8.3 Inventory of Marine Biomass Feedstocks Generate in NL 2006-2015[30]

On average, between 2006-2015, NL generated 45% processing discards from total landings which amounted to ~132,861 t of waste annually (Table 2.7). This is on par with the 46% national average discard rate reported in the 2011 study. Groundfish accounted for 18,560 t (14%) of the average annual discards, pelagics 16,886 t (13%), salmonids 3,061 t (2%), shrimp 41,702 t (31%), and crab 15,164 t (11%), other species (e.g., whelk, sea cucumber, sea urchin, etc.) contributed the remaining 42,822 t (32%).

Within the groundfish category, cod accounted for 34% of total landings (Table 2.17), followed by turbot at 30% (Table 2.18), flounder at 19% (Table 2.19) and redfish at

[30] Tables 2.11 - 2.26 are available in Appendix 2.

8% (Table 2.20). Capelin accounted for 44% (Table 2.25) of the annual average pelagic landings, followed by herring at 35% (Table 2.22) and mackerel at 20% (Table 2.24).

The major crustacean species harvested in NL are northern shrimp and snow crab. Together, these two-species contributed more than 42% of the total annual average discards from all species within the study period (Table 2.13 and Table 2.14). At 2%, farmed salmon processing discards are a relatively small component of the total provincial processing discards (Table 2.15). However, it should be noted that this 2% does not include losses at the farm level due to mortalities.

Processing discards for shrimp (Table 2.13), crab (Table 2.14) and salmonids (Table 2.15) have been relatively consistent at 65%, 30%, and 19%, respectively, as a percentage of total supply per each species. In NL, shrimp, crab, and salmon are dominated by one main product form per species: cooked and peeled shrimp (100%), cooked crab sections (>80%), and HOG salmon (>80%). Therefore, this unutilized marine biomass material is consistent in terms of yield and composition. For shrimp and crab, volumes may vary annually due to changes in quotas, TAC (total allowable catch), and landings. Production levels for salmon are better controlled but may be affected by mass mortality events such as ISA (infectious salmon anemia) and super-chill as seen in 2014. However, the supply to the plant for each of these three species has been relatively consistent since 2012, with shrimp and crab each averaging about 49,000 t, and salmon production (except for 2014) averaging around 20,000 t thereby generating on average 32,000 t shrimp discards, 17,000 t crab discards and 3,600 t salmon discards. In 2015, 10 shrimp plants produced 19,627 t of discards, and 27 crab plants generated 15,013 t of discards for a combined total of 44,640

t of discards. On average, assuming plant production volumes are similar, each shrimp plant generated 2,963 t and each crab plant produced 556 t of discards (Table 2.8).

Groundfish processing discard rates have been less predictable and inconsistent, ranging from 38% to 67% throughout the study period (Table 2.16). Similarly, processing discards from pelagics have also been inconsistent ranging from 14% to 35% during the study period (Table 2.21). This is not surprising given the wide range of product forms in which groundfish and pelagics may be marketed as described in sections 2.8.2.2 Groundfish and 2.8.2.3 Pelagics. This creates inconsistency in the volume and composition of the supply of processing discards from groundfish and pelagic species. Consequently, these processing discards are difficult to utilize as a feedstock material.

2.8.3.1 Current Utilization of Marine Biomass Feedstocks

Table 2.9 summarizes the availability, current and potential applications of marine biomass feedstocks generated by the NL seafood processing industry. Currently, there is no reported use of shrimp and crab processing discards although this material is the most abundant and readily available source for use as marine biomass feedstocks for value addition. A major challenge with utilization of shrimp and crab processing discards is the short harvesting season, particularly for crab (April-July), making stabilization of the raw material a key consideration for value addition.

In 2016, DFLR reported that only 5,022 t of processing discards had been utilized with salmonid offal from NL's farmed salmon industry utilizing the bulk of this material (i.e., 5,000 t) for rendering and silage (Table 2.26). The remaining 22 t included male capelin diverted to compost and mink feed, and a small quantity (265 t) of HOG cod by-

products diverted to unspecified uses. This represents a utilization rate of about 5% of total annual processing discards generated in NL.

2.8.4 Geographical Distribution of Licensed Fish Processing Plants by Species and Economic Zone

Since 2006 the total number of licensed processing plants have decreased from 140 to 94 (36%) (Table 2.4). Shrimp processing plants have decreased from 13 to 7 (46%), crab plants have decreased from 44 to 27 (37%), salmon processing plants have decreased from 12 to 8 (33%), groundfish plants have decreased from 86 to 57 (34%), and pelagic plants have declined from 90 to 59 (34%) (Table 2.4, Appendix 5).

The main economic zones for licensed shrimp processing plants during the study period included zones 4, 6, 7, 8, 14, 15, 17 and 18 (Figure 2.9). Since 2012, however, there have been no licensed shrimp processing plants in zones 8 and 18. In 2015, shrimp plants were concentrated in zones 6 (2 plants) and 7 (2 plants) on the Northern Peninsula (Port aux Choix - OCI, Black Duck Cove - Gulf Shrimp/Quinlan Brothers), zone 14 (2 plants) on the North-East Coast (Notre Dame Seafoods, Twillingate); and zone 17 (2 plants) on the Avalon Peninsula (QuinSea, Quinlan Brothers). However, in 2016, the Quinlan Brothers shrimp plant was destroyed by fire leaving only 1 shrimp plant on the Avalon. The shrimp plant in Twillingate also closed in 2016 due to reductions in shrimp quotas.

The main economic zones for licensed crab plants during the study period were zones 1, 4, 6, 7, 8, 11, 14, 15, 16, 17, 18, 19, and 20 (Figure 2.9). There have been no licensed crab plants in zone 19 since 2008, and in 2015 there were no licensed crab plants in zone 18. In 2015, the highest concentration of crab plants was in zone 14 (4 plants) on the North-East Coast, and zones 17 (6 plants) and 20 (4 plants) on the Avalon Peninsula.

For salmonid processing, the location of licensed processing plants included zones 10, 13, 14, 15, 16, and 17 (Figure 2.9). There have been no licensed plants in zone 15 since 2012 and none in zone 16 since 2008. In 2015 salmon processing has occurred mainly in zone 13 (3 plants) on the south coast and zone 14 (3 plants) on the North-East Coast.

During the study period, licensed groundfish and pelagic plants were present in all economic zones except zones 2 and 3. In 2015 there were no licensed groundfish or pelagic plants in zone 5. Between 2006 and 2014 there were no licensed groundfish or pelagic plants in zone 4. During the study period, there was only 1 licensed pelagic plant in zone 12 and that was in 2008. The highest concentration of groundfish and pelagic plants have been in zones 14, 15 and 17 during the study period. In 2015 groundfish plants were concentrated in zones 7 (6 plants), 14 (6 plants), 15 (6 plants), 17 (11 plants) and 19 (6 plants) while pelagic plants were more concentrated in zones 14 (8 plants), 17 (11 plants) and 19 (6 plants).

While seafood processing plants are scattered along Newfoundland and Labrador's long coastline (Figure 2.9) creating logistical challenges to transporting processing discards to a central location, there are pockets of high concentrations of processing plants, generating large volumes of processing discards, where regional by-product processing facilities could be located. Presumably, the required support infrastructure (e.g., wharfs, roads, transportation services, power, water supply, etc.) are already established in these areas.

In 2015, 10 shrimp plants produced 29,627 t of discards, and 27 crab plants generated 15,013 t of discards for a combined total of 44,640 t of discards representing ~42% of the total provincial discards. On average, assuming the processing capacity of

each plant is similar, each shrimp plant generated 2,963 t and each crab plant produced 556 t of discards. The highest concentration of shrimp and crab plants in 2015 occurred in the following regions:

- Northern Peninsula (zones 6 & 7) – 4 shrimp plants = average 11,842 t discards
- Northeast Coast (zone 14) - 2 shrimp plants, 4 crab plants = average 8150 t discards
- Avalon Peninsula (zones 17 and 20) – 2 shrimp plants, 10 crab plants = average 11,486 t discards

It should be noted that since 2016 there are only 7 licensed shrimp plants in the province. Two plants closed due to shrimp quota cuts (Twillingate zone 14, and Clarenville zone 15), and another was destroyed by fire (Bay de Verde zone 17) and was not re-built.

2.8.5 Semi-structured Interviews

Interviews were conducted with harvesters, processors, industry associations, and government representatives to assess factors affecting industry's ability to utilize marine biomass feedstocks. Authorization by the interviewees was granted to the author to use the findings on the condition that the identities of the interviewees, and any proprietary information provided during the interview, were not disclosed. The results of the interviews are summarized in Table 2.11.

2.8.5.1 Current Utilization of Marine Biomass Feedstocks

On the wild capture side, there is very little use of marine by-products as feedstock materials. Cod are gutted and bled at sea with the livers and gut materials thrown overboard. There is limited use of cod by-products due to the low volumes of raw materials available which make it uneconomical. There is, however, a market for male capelin which was traditionally a by-product. It is now being caught as food for zoos and sold to the United States. One harvester estimated that the value of capelin as an animal feed today is more valuable than it was as a food 25 years ago. Capelin is also used locally for fishmeal production and mink feed. Although there is one large herring processor (The Barry Group Inc.) in the province doing some value-added product (e.g., first-stage marinated herring), most of the herring harvested in NL is sold as bait. Some processors indicated that they would like to do more with their fish by-products, but they need help with the research. Processors also stated that one of the major barriers to utilization is the quality of the by-products available.

According to industry stakeholders from the aquaculture sector, unlike the wild capture seafood industry, the farmed salmon industry is nearing 100% utilization of its by-products. While most of the salmon by-products are currently diverted to mink feed, silage and rendering applications (Table 2.26), there are markets for the heads, frames, and swim bladders in Asia. Salmon processors are also producing high value products such as pub snacks, from what were once by-products of fillet production.

2.8.5.2 Industry Structure

All stakeholders agreed that a key issue impacting growth, innovation and by-product utilization in NL is the structure of the fishing and seafood industry. Factors identified included: there are too many harvesters; there are too many small boats; there are too many landing sites; the use of gillnets – gillnets are easy and inexpensive so fisherman prefer to use this method of catching fish (cod) even though it produces a lower quality fish; small boats limit when fish is harvested and decrease product quality – small boats cannot handle the weather in the fall and winter; seasonality – fisherman harvest in the summer when fishing is easy; regulations inhibit innovation – minimum processing requirements, price setting mechanism, restrictions on foreign company investment were all cited as examples of regulatory restrictions; union (FFAW[31]) has a lot of influence on policy – harvesters, processors, associations and regulators viewed this as a barrier to growth in the industry; main species is crab and this is currently the key driver in the industry – processors will buy cod, a low value product, but only as a means to maintain access to crab; there is also a lot of overcapacity on the processing side to handle large volumes of crab that are landed in a very short time (April – July).

2.8.5.3 Geographical Distribution and Logistics

Geographical distribution and logistics are major challenges for maintaining freshness, access to markets, and can be cost prohibitive. A major challenge to utilization of marine biomass feedstocks is the geographical distribution of landing sites and processing plants around the province. There are hundreds of landing sites, and

[31] Fish Food and Allied Workers

transportation can be 300-400 miles (400-600 km) or more from the landing sites to the processing plant. This adds significant cost due to off-loading vessels, trucking, and off-loading trucks. According to some stakeholders, it is not economical to transport fish discards around the province because the volumes are too low. Newfoundland's geographical isolation also makes it costly to operate in this province. There are numerous logistical challenges other than getting fish from the wharf to the plant, such as: gluts at the plant when large volumes are landed in peak season which may result in fish being held for extended periods in warm temperatures thus decreasing quality; the number of landing sites and low volumes of fish landings which are spread out over a wide area – this can cause inconsistencies in quality; and time of year when landed (warmer temperatures). It is often difficult getting product across the Gulf on the Marine Atlantic Ferry if there is storm delay, or the ferry is not operating which may cause fish in transit to lose freshness and shelf-life particularly on fresh product (e.g., Atlantic salmon). This impacts market access and NL's ability to supply the market with fresh seafood products whether it's fresh fillets, livers, or swim bladders.

2.8.5.4 Quality of Raw Materials

Stakeholders cited several factors affecting quality of raw materials including seasonality; vessel size; onboard handling methods; and gear-type used (e.g., gillnet *vs* longline or pots). Harvesters stated that the quality of cod is suffering due to a lack of food citing low capelin stocks as an example. Harvesters also said that they know how to handle crab, but not cod, which is causing quality problems. Harvesters indicated that small boats cannot properly handle fish onboard and that fish sit in the bottom of the boat, not gutted,

not bled, and not iced in the summer. They also noted there is a big difference in the quality of landings between inshore and offshore vessels.

Processors and industry associations agreed that to fully utilize fishery by-products, quality must be maintained throughout the value chain, starting with harvesting. Quality starts with the harvester and is dependent on when (time of year) and how (gear-type) the product is harvested, and how it is handled onboard (iced or not). Government stakeholders agreed that quality is a major challenge, particularly for cod, and that the entire industry must be focused on quality and consistency throughout the entire value chain, starting with harvesting. Government stakeholders suggested that personal attitudes, education, training, and investment in better handling technologies are needed to make improvements in quality.

However, because the NL capture fishery operates under the independent harvester model (i.e., harvesters operate independently of processors), the processing sector is challenged due to uncertainty of supply, seasonal availability, and variability in quality of the raw materials available to them. Consequently, most of NL's wild capture seafood is sold unprocessed or semi-processed [88], resulting in low availability of by-products for additional value creation.

2.8.5.5 Markets

Crab is much more valuable than cod and has replaced cod as "King" in NL. Shrimp is also important, but the main species is crab. Because prices for crab and shrimp are high regulators believe it masks the problems in the industry (e.g., overcapacity, lack of by-product utilization). In terms of by-products, stakeholders stated that there are huge markets

in Asia for fish heads, frames, and swim bladders. The salmon industry has done a better job of accessing these markets, stating nearly 100% utilization of by-products, than the wild capture fishery. Unlike the farmed salmon sector, processors of wild capture species must follow the cycles of the landings which causes challenges due to variations in markets, supply, and quality of the raw materials. Associations and processors see certification (e.g., MSC, BRC, organic) as critical for market access, but state it does nothing to improve product quality or price in the market, so it is an added cost of doing business, but does provide a competitive advantage. Stakeholders also stated that NL does a poor job of marketing its seafood and see this as an area where improvements can be made in terms of market access. A challenge for by-products is the lack of understanding of market opportunities and how to access these markets once identified.

2.8.5.6 Fisheries Resources

In 2016 DFO scientists indicated that they expected Northern cod stocks in NL to double by 2019. However, on March 23, 2018, it was announced that DFO's most recent survey indicated that the Northern cod spawning stock biomass has decreased by 29% since 2016. Contributing factors to the decline include low levels of younger cod, a changing environment, declining food supply (i.e., capelin and shrimp), and increases in the TAC in recent years [78]. In fact, in some areas (e.g., 3Ps) harvesters believe that cod are starving due to lack of capelin (cod's main food source). The most recent DFO capelin stock assessment supports this theory. Since 2014 capelin are smaller, younger, and less abundant and have declined by 70% over the last two years [79, 80]. The WWF[32] indicate that the

[32] World Wildlife Fund

decline is largely due to environmental factors, but fishing cannot be ruled out as a contributing factor [80, 81]. In the last 3 years, there have also been major decreases in northern shrimp resources with landings reduced by 27% from 84,965t in 2015 to 61,916t in 2016 [57], and while crab seems stable for now, there are signs that the stock may be in trouble due to low recruitment. Therefore, industry stakeholders are more interested than ever before in maximizing utilization and increasing the value of our limited fisheries resources.

2.8.6 Barriers and Limitations to Feedstock Utilization in NL

Many failed attempts have been made to utilize marine by-products generated from the NL seafood industry, including several attempts to produce carotenoids, chitin and chitosan from shrimp and crab shells (APT Inc., Quinlan Brothers Ltd., Blue Ocean Products, Shell-Ex), protein hydrolysates from groundfish heads and frames (Fishery Products International), and more recently fish silage from salmonid frames and mortalities (Newfoundland Aquaculture Industry Association, Dept. Fisheries and Land Resources, Cooke Aquaculture, Northern Harvest). The major contributing factors inhibiting commercial success have been: (1) relatively low volumes of unutilized marine biomass available for further conversion into higher value products; (2) remoteness of most fish processing plants and aquaculture sites [1]; (3) heterogeneity of the biomass feedstock [1]; (4) poor quality of the available unutilized marine biomass feedstock due to high susceptibility to spoilage [1] and improper handling; (5) lack of a suitable provincial strategy and limited investment to encourage maximum utilization; (6) limited regulations to prevent dumping of unutilized marine biomass into landfills or at sea; (7) lack of

appropriate conversion technologies; (8) lack of market development; (9) and other barriers to market entry (e.g., certifications, trade tariffs).

The low volumes of fish by-products that are utilized typically go into low end uses such as pet food, mink feed, fishmeal, and silage [1]. Higher value products that have been attempted have seen little commercial success due to low yields and high production costs (e.g., < 4% yield bioactive peptide from salmonid frames, ~ 2.5% yield for high DDA chitosan from shrimp shells). Another major shortcoming has been that local processing companies have tried to implement technologies developed in other regions without modifying those technologies for use in NL.

In 2009 a local fish processor, Quinlan Brothers Ltd. (QBL), partnered with US based company Chitin Works to transfer chitin processing technology to NL. The intent was to build a multimillion-dollar chitin and chitosan processing plant in Old Perlican, NL. However, this venture did not go as anticipated due to differences in the raw material, differences in environmental regulations in the US and Canada, and lack of acceptance by the local community where the processing plant was to be located [82]. More recently, a local biotechnology start-up company, Shell-ex, received seed funding of $81,600 from the Provincial Government in 2014 through the Fisheries Technology New Opportunities Program (FTNOP) to assist with the purchase of equipment needed for the company's planned chitin extraction plant in Twillingate, NL [83]. Problems with the supply of shells, the bioconversion technology, and market access have prevented the company from establishing the proposed chitin plant.

Industry stakeholders have identified several factors that inhibit better utilization of by-products in NL such as: inadequate information on volumes and availability at the

species level and regional level; lack of understanding of by-product opportunities, technical and financial feasibility; difficult logistics due to geographical distance to a central processing facility (raw materials) as well as markets (finished products); regulations; and lack of coordination/cooperation among processors [72; section 2.5.5 Semi-structured Interviews]. However, the real barriers preventing better utilization of NL's capture fisheries resources are the structure of the industry, and the noticeably absent provincial strategy to deal with fish by-products and fish waste produced by capture fisheries.

2.8.6.1 Industry Structure

In the capture fishery in Newfoundland, availability of fish, its quality and prices are highly variable. This is largely due to the independent harvester model under which the capture fisheries are operated. In this model harvesters operate independently of processors. Historically, these two sectors have not agreed on objectives, their efforts have not been coordinated, and consequently the seafood value chain has not been optimized.

Independent harvesters' fish when it is best for them, not when the markets want the products, and not necessarily when the fish are in the best condition [88]. Most of the NL fleet is comprised of small vessels (< 35') with inadequate onboard handling facilities. There are 400 landing sites which in most cases are hundreds of kilometers away from the processing plants resulting in deterioration in the quality of the raw materials during transport and before they can be processed. This limits value creation as processors have no choice but to buy fish when it is available regardless of quality and market demands. Prices are set by a price setting board, not by the market, and are largely influenced by the

FFAW. Therefore, processors cannot set prices based on market values and/or market acceptance. Additional challenges arise because processors will often buy other species (e.g., cod) at top price, regardless of quality, simply to maintain access to their supply of crab.

In Iceland, vertical integration throughout the value chain is one of the factors that has led to a more valuable cod fishery with all players focused on quality and full utilization. In NL, however, processors are not permitted to own or control quotas or fishing enterprises under existing federal government policies. There are some exceptions for established previously existing arrangements such as seen with Clearwater and Ocean Choice International [88]. Both are large processing companies which own their vessels and have offshore quota allocations. However, while there is no ban on vertical integration arrangements for harvesters (i.e., harvesters could integrate forward owning their own processing operations), this type of vertical integration is not prevalent in the NL wild capture fishing industry. The NL fishery is dominated by numerous small fishing enterprises that operate for only 3-4 months of the year. Therefore, these companies cannot invest in the capital required to become vertically integrated [88].

2.8.6.2 Strategy for Utilization of Fish By-products

Currently there is insufficient capacity and lack of a suitable strategy to properly manage the large volumes of marine biomass feedstocks which are generated from capture fisheries in a very short time frame, so that it can be readily transformed into marketable higher value products. For example, the snow crab season in NL is very short, spanning a period of 3-4 months (April-July). In 2014 NL landings of snow crab approached 50,000 t

and generated approximately 16,000 t of unutilized biomass feedstock [51]. The industry is currently not prepared to collect, stabilize, store, and convert this large volume of biomass feedstock into higher value products. Consequently, it is typically collected in offal bins as a waste material and towed by barge for ocean dumping or sent to a local landfill. For example, in 2016, Beothic Fish Processors Ltd. located in Valleyfield, NL, was issued a disposal at sea permit (permit no. 4543-2-06929) by Environment Canada for the disposal of up to 1600 t of fish waste and other organic matter from industrial fish processing operations [52]. In 2017, 3 T's Limited in Woody Point, NL was issued a disposal at sea permit (permit no. 4543-2-06934) by Environment Canada for the disposal of up to 24 t of crab waste consisting of shells and associated organic wastes [53]. Personal communication (October 2017) with a local crab processor in Bay de Verde, NL identified that crab shell waste in that region is currently sent to the local landfill for disposal.

2.8.6.2.1 Geographical Distribution and Logistics

Seafood processing plants are scattered along Newfoundland and Labrador's long coastline (Figure 2.9). This creates logistical challenges to transporting processing discards to a central location. Depending on the location of the by-product processing facility, the transportation costs could be significant. However, knowledge of the quantity of discards available at each location would be needed to determine the cost effectiveness of transporting the raw materials to a central processing facility. Also, stabilization of the processing discards on-site may be required if logistics make maintaining freshness difficult. However, there are currently no provincial or regional strategies, programs, or facilities in place to utilize fish processing discards.

2.8.6.2.2 Stabilization Technologies

One option for stabilization of fish processing discards and fish waste has been investigated as a pilot study by the Newfoundland Aquaculture Industry Association (NAIA) for salmon by-products and mortalities. NAIA and two of their member companies (Cooke Aquaculture-Cold Ocean Salmon and Northern Harvest) invested in the purchase and installation of two ensilaging systems which have been in use since June 2016. The ensilage pilot project was funded under the premise that the end-product silage would be bio-secure and could be transported to a commercial anaerobic digester in western NL for bio-secure disposal. While the ensilaging process does address the issue of biosecurity, there are additional costs to the growers for transportation and disposal. Further complications arose due to the inconsistency of the silage material, and the amounts that could be effectively added to the anaerobic digester due to the high oil and ammonia content of the silage. When this chapter was written the NL aquaculture industry did not have a potential buyer market for the ensiled fish material, nor the infrastructure, to recover their investment cost. Thus, while the ensilage pilot project has addressed some immediate short-term biosecurity and environmental concerns, it has inadvertently created other unforeseen complications.

In 2017, local company Shell-ex entered an arrangement through NAIA to collect salmon silage from Cooke Aquaculture-Cold Ocean Salmon and Northern Harvest. This arrangement requires that the silage be processed with phosphoric acid rather than formic acid, as Shell-ex is interested in using the silage as a soil amendment. The feasibility of this arrangement had not been determined at the time this chapter was written. In 2016 the Fisheries and Marine Institute's Centre for Aquaculture and Seafood Development

undertook a separate study to evaluate and improve the quality and consistency of silage produced by the NL farmed salmon industry. The proprietary results of this study were presented to NAIA and its members in 2018.

2.8.7 Opportunities for Better Utilization of Marine Biomass Feedstocks

NL seafood processing plants currently discard, on average, 45% (Table 2.7) of their total raw material supply in the form of waste and by-products. The discard rate is highest for shrimp (67%) and lowest for farmed Atlantic salmon (18%) (Table 2.8). To improve our fish utilization rate, the goal must be 100% utilization of the raw material with a coordinated effort among regulators, harvesters and processors focused on achieving that target. While 100% utilization may not be attainable, a goal of anything less will result in sub-optimal utilization rates.

Other countries (e.g., Norway and Iceland) have implemented models whereby fish discards are prohibited and nearly all parts of the fish are utilized. For example, Norway produced ~815,000 t of fish "waste" in 2011 representing 30% of total fish production but utilized over 75% of this material as feedstock for feed production, resulting in only 195,000 t being dumped at sea, mainly by the fishing fleet [84]. RUBIN, a Norwegian based foundation operating for the period 1992-2012, was funded by the Ministry of Fisheries and Coastal Affairs and the Fishery and Aquaculture Industry Research Fund. Its mandate was to promote full utilization of fish and value adding of marine by-products [84]. Codland, an Icelandic collaboration founded in 2012, consists of seven fishing and ocean related companies that together strive to maximize value from every part of the fish. Products include fish oil, collagen, dried products, calcium, fillets, fishmeal [85]. Codland

was established from the Iceland Ocean Cluster whose mission is "to create value by connecting together entrepreneurs, businesses and knowledge in the marine industries" [86].

Iceland's success in creating better utilization of its fisheries resources, particularly cod, is driven by two main factors: (1) its economic dependence on cod; and (2) its culture. Other factors contributing to Iceland's superior cod value chain include a year-round fishery, vertical integration, auction pricing system, significant focus on ensuring high quality raw materials, and focus on 100% utilization of raw materials to maximize output value [88]. Stakeholders throughout the entire Icelandic cod value chain are dedicated to maximizing the value extracted from the cod resource. Iceland has successfully maximized the value of its cod resource by utilizing all parts of the fish. Products such as canned liver, fish liver oil, fish leather, collagen for cosmetic applications, and pharmaceutical products make the list of higher value products extracted from Icelandic cod by-products [87].

While the structure of the NL, Norwegian and Icelandic fisheries are quite different, the Norwegian and Icelandic models offer examples of how value creation through improved utilization of fisheries resources can be achieved.

2.9 Conclusions

This study (including data collection, statistical evaluation, and stakeholder interviews) aimed to improve the understanding of the volumes and availability of processing by-products at the species level and regional level, recommend opportunities for better utilization of by-products, and to suggest possible locations for future development of a central or regional marine biorefinery facility in NL.

An inventory assessment of unutilized marine biomass feedstocks generated in NL from the processing of crustaceans, groundfish, pelagics, and farmed salmon was conducted for the 10-year period spanning 2006-2015. This assessment revealed that although the annual volume of available feedstocks for the selected species has declined by 46,792 t, the average annual processing discard rate remains at ~45% of the supply to plant. The decline in the volume of discards is a result of the decline in the supply to plant of the selected species which decreased by 114,752 t and is not attributed to better utilization of fisheries resources. In fact, in 2016, based on plant production reports, NL utilized only ~5% of its total seafood processing discards, the majority (99.5%) of which were farmed salmon processing discards that had been diverted to silage and rendering applications.

During the study period, processing discard rates for shrimp, crab and salmonids were relatively consistent at 65%, 30%, and 18%, respectively, as a percentage of total supply per each species. Shrimp, crab, and salmon are dominated by one main product form per species. Therefore, the unutilized by-product material from these species is consistent in terms of yield and composition. Groundfish processing discard rates have been less predictable and inconsistent, ranging from 38% to 67% throughout the study period. Similarly, processing discards from pelagics have also been inconsistent ranging from 14% to 35%. This can be attributed to the wide range of product forms in which groundfish and pelagics may be marketed, which creates inconsistency in the yield and composition of unutilized groundfish and pelagic by-products.

The current inventory assessment highlighted that of the species evaluated, shrimp and crab generate the largest volumes of discards (42% combined total) which currently have no other use and are therefore dumped in landfills or towed by barge and dumped at

sea. Shrimp and crab processing wastes contain high value products, including chitin, astaxanthin, calcium, protein and ω3 fatty acids, which could be recovered at the processing plant from shrimp peelers and crab butchering machines and utilized to produce high end nutraceutical and pharmaceutical products (Table 2.9).

At 2%, farmed salmon processing discards are a relatively small component of the total provincial processing discards. The salmon industry has also suggested that it is nearing 100% utilization and has minimal processing discards. Discards that are produced are currently diverted to mink feed, silage, or rendering. As the farmed salmon industry expands, industry is focusing on higher value uses of processing discards and by-products such as pub snacks and Omega-3 oils for North American markets, and swim bladders for Asian markets.

While the volume of groundfish and pelagic discards are high (~ 22,000 t each in 2015), their availability is low (Table 2.9). Cod livers and roes, for example, are dumped overboard by harvesters, while other processing by-products such as heads and frames, are diverted to low value uses such as pet food, mink feed, fishmeal, and bait.

While seafood processing plants are scattered along Newfoundland and Labrador's long coastline creating logistical challenges to transporting processing discards to a central location, there are pockets of high concentrations of processing plants, generating sufficient volumes of processing discards (e.g., Northern Peninsula, 2015 - 2 shrimp plants and 10 crab plants generated about 11,486 t of discards), where regional by-product processing facilities could be located.

Barriers to successful utilization of unutilized marine biomass materials stem from the lack of a suitable strategy to deal with fish by-products. A strategy has not emerged due to lack of industry cooperation, complex industry structure, and lack of capital to process by-products. In 2014, the DFLR released its Sustainable Aquaculture Strategy. A component of that strategy was an Aquaculture Waste Management Action Plan [77] to enable bio-secure methods to dispose, reduce, recycle, reuse and/or transform the various types of aquaculture waste generated on farms and from processing plants. However, a comparable strategy has yet to emerge to deal with fish waste from wild capture fisheries. A key objective of this thesis is to lay the groundwork for the development of such a strategy for NL.

2.10 Recommendations

This study has identified several challenges that must be addressed before NL can improve its fish utilization rate. To address these challenges, it is recommended that the province and industry work together to develop a suitable strategy to deal with fish by-products generated from wild capture fisheries. This will require cooperation among harvesters, processors, and regulators, with support from academia to conduct research on by-product opportunities. It is also recommended that the framework already developed for the Aquaculture Waste Management Action Plan be reviewed and where feasible, adopted/adapted and applied to wild capture fisheries.

Based on volume, consistency, and availability of unutilized crustacean by-products it is recommended that the province and industry consider establishing regional processing facilities to collect, stabilize and produce intermediate bulk products, such as dried shell,

chitin, or protein hydrolysates, from shrimp and crab processing discards including shells, viscera, and protein. Regional processing facilities could be established in regions where there are pockets of shrimp and crab plants generating large quantities of processing discards, such as:

- Northern Peninsula (Economic Zones 6 & 7)
- Northeast Coast (Economic Zone 14)
- Avalon Peninsula (Economic Zones 17 & 20)

Before such facilities are established, however, a feasibility study should be undertaken to: (1) quantify by-product feedstock volumes available from shrimp and crab in each region; (2) identify by-product opportunities and assess technical and financial feasibility of each option; (3) make a recommendation regarding the feasibility of implementing a marine biorefinery facility in each region. However, such a feasibility study is beyond the scope of this thesis and should likely be undertaken jointly by the Government of NL, the FFAW[33] and the NL seafood industry associations (ASP[34], SPANL[35], GEAC[36]).

Note to Reader:

- The Appendix for Chapter 2 begins on page 131
- Chapter 3 begins on page 146

[33] Fish Food and Allied Workers Union
[34] Association of Seafood Producers
[35] Seafood Processors Association of Newfoundland and Labrador
[36] Groundfish Enterprise Allocation Council

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2.12 Appendix

Appendix 1 - Weighted Average Sample Calculation for Crab Supply to Plant 2006-2015

Snow Crab	Supply to Plant		
YEAR	Total Volume (t)	% Weight	Weighted Volume (t)
2006	46,286	9.3%	4,314
2007	48,766	9.8%	4,788
2008	50,943	10.3%	5,225
2009	51,755	10.4%	5,393
2010	49,411	9.9%	4,916
2011	51,266	10.3%	5,292
2012	50,217	10.1%	5,077
2013	49,439	10.0%	4,921
2014	50,861	10.2%	5,208
2015	47,726	9.6%	4,586
TOTAL	**496,670**	**100%**	**49,720**

Step 1

% Weight = (Annual Volume ÷ Total Volume) × 100

Example for Year 2006: % Weight = (46,286 ÷ 496,670) × 100 = 9.3%

Step 2

Weighted Volume = % Weight × Annual Volume

Example for Year 2006: Weighted Volume = 9.3% × 46,286 = 4,314 t

Step 3

Total Weighted Average for the Study Period = Sum of the Weighted Volumes = 49,720 t

Appendix 2 - NL Fishing Fleet Structure by Vessel Size 1985-2015

Table 2. 12 NL fishing fleet structure by vessel 1985-2015 [37]

Year	NL Region Commercial Fishing Vessels Registered by Size					
	< 35'	35'-44'11"	45'-64'11"	65'-99'11"	> 100'	Total
1985	14184	799	502	9	89	15583
1986	14438	768	493	9	84	15792
1987	15326	770	481	8	78	16663
1988	15828	662	494	10	85	17079
1989	15730	715	488	9	85	17027
1990	15360	694	502	7	75	16638
1991	13678	646	497	8	76	14905
1992	13587	638	485	8	75	14793
1993	12848	601	470	8	74	14001
1994	12075	561	445	9	40	13130
1995	11403	536	438	8	13	12398
1996	10659	521	420	8	28	11636
1997	10357	522	430	10	23	11342
1998	10245	528	434	9	21	11237
1999	8605	534	443	9	22	9613
2000	8219	527	449	8	24	9227
2001	7693	519	463	11	26	8712
2002	7640	552	483	10	24	8709
2003	7564	579	492	12	30	8677
2004	7489	587	504	14	31	8625
2005	7454	586	501	11	25	8577
2006	7388	584	485	11	25	8493
2007	7392	588	494	9	28	8511
2008	7077	601	480	11	23	8192
2009	6967	601	468	13	22	8071
2010	6780	602	465	14	23	7884
2011	6540	592	450	22	25	7629
2012	6225	583	450	20	23	7301
2013	5850	591	429	24	22	6916
2014	5546	603	429	24	15	6617
2015	5327	625	408	28	17	6405
Total Change	**-8857**	**-174**	**-94**	**19**	**-72**	**-9178**
% Change	**-62%**	**-22%**	**-19%**	**211%**	**-81%**	**-59%**

Appendix 3 - Inventory of NL Fish Processing Discards NL 2006-2015

[37] Data retrieved from http://www.dfo-mpo.gc.ca/stats/commercial/licences-permis/licences-permis-atl-eng.htm (July 10, 2017).

Table 2. 13 Volume of NL shrimp (Pandalus borealis) supply, production output and processing discards 2006-2015[38]

Northern Shrimp	Volume in metric tonnes			Yield (%)	
YEAR	Supply to Plant	Production Output	Discards	% Yield	% Discards
2006	71,779	26,026	45,753	36.3%	63.7%
2007	78,558	26,331	52,227	33.5%	66.5%
2008	85,728	28,461	57,267	33.2%	66.8%
2009	48,088	15,664	32,424	32.6%	67.4%
2010	58,919	18,355	40,564	31.2%	68.8%
2011	53,603	18,988	34,615	35.4%	64.6%
2012	56,416	17,942	38,474	31.8%	68.2%
2013	52,047	16,814	35,233	32.3%	67.7%
2014	45,324	13,958	31,366	30.8%	69.2%
2015	44,129	14,502	29,627	32.9%	67.1%
Weighted Ave	**62,603**	**20,977**	**41,702**	**33.5%**	**66.6%**

Table 2. 14 Volume of NL Snow crab supply, production output and processing discards 2006-2015[39]

Snow Crab	Volume in metric tonnes			Yield (%)	
YEAR	Supply to Plant	Production Output	Discards	% Yield	% Discards
2006	46,286	33,089	13,197	71.5%	28.5%
2007	48,766	34,335	14,431	70.4%	29.6%
2008	50,943	35,917	15,026	70.5%	29.5%
2009	51,755	36,239	15,516	70.0%	30.0%
2010	49,411	34,896	14,515	70.6%	29.4%
2011	51,266	35,668	15,598	69.6%	30.4%
2012	50,217	34,415	15,802	68.5%	31.5%
2013	49,439	34,396	15,043	69.6%	30.4%
2014	50,861	33,938	16,923	66.7%	33.3%
2015	47,726	32,713	15,013	68.5%	31.5%
Weighted Ave	**49,720**	**34,596**	**15,164**	**69.6%**	**30.5%**

[38] Data for "supply to plant" and "production output" were provided by the Licensing and Policy Division of the Department of Fisheries and Land Resources, Government of Newfoundland and Labrador. Processing Discards were calculated as the difference between supply to plant and production output. Supply to plant includes NL landings, as well as raw materials purchased from other provinces and countries. Supply to plant is reported as live round weight.

[39] Data for "supply to plant" and "production output" were provided by the Licensing and Policy Division of the Department of Fisheries and Land Resources, Government of Newfoundland and Labrador. Processing Discards were calculated as the difference between supply to plant and production output. Supply to plant includes NL landings, as well as raw materials purchased from other provinces and countries. Supply to plant is reported as live round weight.

Table 2. 15 Volume of NL farmed salmonid production and processing discards 2006-2015[40,41]

Salmonid Production	Volume in metric tonnes			Yield (%)	
Year	Supply to Plant	Production Output	Processing Discards	% Yield	% Discards
2006	8,295	5,512	2,783	66.4%	33.6%
2007	5,519	4,614	905	83.6%	16.4%
2008	10,090	7,516	2,574	74.5%	25.5%
2009	13,126	10,389	2,737	79.1%	20.9%
2010	14,658	11,583	3,075	79.0%	21.0%
2011	16,209	13,159	3,050	81.2%	18.8%
2012	19,126	16,197	2,929	84.7%	15.3%
2013	25,223	20,984	4,239	83.2%	16.8%
2014	6,795	6,312	483	92.9%	7.1%
2015	22,368	18,878	3,490	84.4%	15.6%
Weighted Ave	**16,968**	**14,089**	**3,061**	**83.0%**	**18.0%**

Table 2. 16 NL groundfish (all species) supply, production output and processing discards 2006-2015[42]

Groundfish (All)	Volume in metric tonnes			Yield (%)	
Year	Supply to Plant	Production Output	Discards	% Yield	% Discards
2006	43,761	26,755	17,006	61%	39%
2007	41,672	25,861	15,811	62%	38%
2008	45,217	26,494	18,723	59%	41%
2009	39,281	23,783	15,498	61%	39%
2010	39,666	20,898	18,768	53%	47%
2011	31,798	16,232	15,566	51%	49%
2012	26,999	11,645	15,354	43%	57%
2013	33,536	10,992	22,544	33%	67%
2014	34,222	13,794	20,428	40%	60%
2015	34,952	12,850	22,102	37%	63%
Weighted Ave	**37,914**	**20,941**	**18,560**	**55%**	**49%**

[40] Data for "supply to plant" and "production output" were provided by the Licensing and Policy Division of the Department of Fisheries and Land Resources, Government of Newfoundland and Labrador. Processing Discards were calculated as the difference between supply to plant and production output.

[41] Includes farmed Atlantic salmon, Arctic char and trout production. Production output types include head-off-gutted, head-on-gutted, dressed, smoked, fillets, steak, and battered/in pastry, with head-on-gutted being the major product type produced.

[42] Data for "supply to plant" and "production output" were provided by the Licensing and Policy Division of the Department of Fisheries and Land Resources, Government of Newfoundland and Labrador. Data set includes Atlantic cod. Processing Discards were calculated as the difference between supply to plant and production output. Supply to plant includes NL landings, as well as raw materials purchased from other provinces and countries. Supply to plant is reported as live round weight.

Table 2. 17 Atlantic cod as % of total NL groundfish landings[43]

Year	Cod (te)	Groundfish (te)	% Cod
2006	17,050	43,761	39%
2007	17,845	41,672	43%
2008	17,599	45,217	39%
2009	14,472	39,281	37%
2010	12,229	39,666	31%
2011	9,746	31,798	31%
2012	8,334	26,999	31%
2013	8,225	33,536	25%
2014	10,324	34,222	30%
2015	10,723	34,952	31%
AVE	12,655	37,110	34%

Table 2. 18 Turbot as % of total NL groundfish landings[44]

Year	Turtbot (te)	Groundfish (te)	% Turbot
2006	11,265	43,761	26%
2007	9,835	41,672	24%
2008	8,833	45,217	20%
2009	10,417	39,281	27%
2010	11,579	39,666	29%
2011	11,050	31,798	35%
2012	10,512	26,999	39%
2013	10,777	33,536	32%
2014	11,351	34,222	33%
2015	11,051	34,952	32%
AVE	10,667	37,110	30%

[43] Data retrieved and compiled Dec 20, 2017 from DFLR Landings and Landed Value Reports 2006-2015: http://www.fishaq.gov.nl.ca/stats/landings/index.html
[44] Data retrieved and compiled Dec 20, 2017 from DFLR Landings and Landed Value Reports 2006-2015: http://www.fishaq.gov.nl.ca/stats/landings/index.html

Table 2. 19 Flounder as % of total NL groundfish landings[45],[46]

Year	Flounder(te)	Groundfish (te)	% Flounder
2006	1,910	43,761	4%
2007	5,328	41,672	13%
2008	12,392	45,217	27%
2009	7,958	39,281	20%
2010	10,511	39,666	26%
2011	5,431	31,798	17%
2012	2,972	26,999	11%
2013	9,902	33,536	30%
2014	8,257	34,222	24%
2015	7,008	34,952	20%
AVE	7,167	37,110	19%

Table 2. 20 Redfish as % of total NL groundfish landings[47]

Year	Redfish (te)	Groundfish (te)	% Redfish
2006	6,476	43,761	15%
2007	2,496	41,672	6%
2008	1,493	45,217	3%
2009	2,662	39,281	7%
2010	2,584	39,666	7%
2011	3,069	31,798	10%
2012	1,330	26,999	5%
2013	2,993	33,536	9%
2014	2,096	34,222	6%
2015	4,262	34,952	12%
AVE	2,946	37,110	8%

[45] Flounder includes American Plaice, Yellowtail Folunder, Greysole/Witch, and Winter Flounder.
[46] Data retrieved and compiled Dec 20, 2017 from DFLR Landings and Landed Value Reports 2006-2015: http://www.fishaq.gov.nl.ca/stats/landings/index.html
[47] Data retrieved and compiled Dec 20, 2017 from DFLR Landings and Landed Value Reports 2006-2015: http://www.fishaq.gov.nl.ca/stats/landings/index.html

Table 2. 21 Volume of NL pelagics (all species) supply, production output and processing discards 2006-2015[48,49]

Pelagics (All)	Volume in metric tonnes			Yield (%)	
Year	Supply to Plant	Production Output	Discards	% Yield	% Discards
2006	109,504	94,387	15,117	86%	14%
2007	103,430	86,354	17,076	83%	17%
2008	89,024	70,303	18,721	79%	21%
2009	95,443	79,013	16,430	83%	17%
2010	83,604	71,345	12,259	85%	15%
2011	62,569	44,804	17,765	72%	28%
2012	58,821	42,923	15,898	73%	27%
2013	64,873	51,252	13,621	79%	21%
2014	58,141	42,966	15,175	74%	26%
2015	63,945	41,530	22,415	65%	35%
Weighted Ave	**83,316**	**68,316**	**16,886**	**82%**	**20%**

Table 2. 22 Herring as % of total NL pelagic landings[50]

Year	Herring (te)	Pelagics (te)	% Herring
2006	24,927	109,504	23%
2007	22,660	103,430	22%
2008	28,323	89,024	32%
2009	27,872	95,443	29%
2010	26,032	83,604	31%
2011	24,863	62,569	40%
2012	24,919	58,821	42%
2013	29,569	64,873	46%
2014	25,731	58,141	44%
2015	26,859	63,945	42%
2016	27,179	68,527	40%
Ave	26,267	77,989	35%

[48] Data for "supply to plant" and "production output" were provided by the Licensing and Policy Division of the Department of Fisheries and Land Resources, Government of Newfoundland and Labrador. Processing Discards were calculated as the difference between supply to plant and production output. Supply to plant includes NL landings, as well as raw materials purchased from other provinces and countries. Supply to plant is reported as live round weight.

[49] Data set includes pelagic by-products.

[50]Data retrieved Dec 20, 2017 from DFLR Landings and Landed Value Reports 2006-2015: http://www.fishaq.gov.nl.ca/stats/landings/index.html

Table 2. 23 Volume NL "other" supply, production output and processing discards 2006-2015[51,52]

Other Species	Volume in metric tonnes			Yield (%)	
Year	Supply to Plant	Production Output	Discards	% Yield	% Discards
2006	75,912	15,651	60,261	21%	79%
2007	62,560	12,145	50,415	19%	81%
2008	47,703	12,780	34,923	27%	73%
2009	53,803	10,182	43,621	19%	81%
2010	58,627	11,338	47,289	19%	81%
2011	53,276	11,869	41,407	22%	78%
2012	44,514	12,802	31,712	29%	71%
2013	41,561	13,055	28,506	31%	69%
2014	49,444	11,108	38,336	22%	78%
2015	27,665	12,987	14,678	47%	53%
Weighted Ave	**54,442**	**12,551**	**42,822**	**23%**	**79%**

Table 2. 24 Mackerel as % of total NL pelagic landings[53]

Year	Mackerel (te)	Pelagics (te)	% Mackerel
2006	44,927	109,504	41%
2007	44,584	103,430	43%
2008	23,036	89,024	26%
2009	34,238	95,443	36%
2010	33,195	83,604	40%
2011	7,337	62,569	12%
2012	2,619	58,821	4%
2013	5,170	64,873	8%
2014	3,432	58,141	6%
2015	701	63,945	1%
2016	4,513	68,527	7%
Ave	18,523	77,989	20%

[51] Data for "supply to plant" and "production output" were provided by the Licensing and Policy Division of the Department of Fisheries and Land Resources, Government of Newfoundland and Labrador. Processing Discards were calculated as the difference between supply to plant and production output. Supply to plant includes NL landings, as well as raw materials purchased from other provinces and countries. Supply to plant is reported as live round weight.

[52] Other species include Atlantic king crab, porcupine crab, rock crab, toad crab, eel, clams, scallops, lobster, lumpfish, periwinkles, sea cucumber, sea urchin, whelk.

[53]Data retrieved Dec 20, 2017 from DFLR Landings and Landed Value Reports 2006-2015: http://www.fishaq.gov.nl.ca/stats/landings/index.html

Table 2. 25 Capelin as % of total NL pelagic landings[54]

Year	Capelin(te)	Pelagics (te)	% Capelin
2006	39,946	109,504	36%
2007	36,052	103,430	35%
2008	37,561	89,024	42%
2009	33,230	95,443	35%
2010	24,240	83,604	29%
2011	30,166	62,569	48%
2012	31,153	58,821	53%
2013	30,019	64,873	46%
2014	28,861	58,141	50%
2015	36,501	63,945	57%
2016	36,722	68,527	54%
AVE	33,132	77,989	44%

Table 2. 26 Volume of NL fish discards diverted to alternate uses 2016[55]

Volume (Kg) of Discards Diverted to Alternate Uses in 2016*					
Source of Discards	**Other (Unknown)**	**Compost**	**Rendering/ Silage**	**Mink Feed**	**Total**
Male Capelin		13,000		9,000	22,000
Salmonid Offal			5,000,000		5,000,000
HOG Cod	264,860				264,860
Total	264860	13,000	5,000,000	9,000	5,286,860
*Personal communication with Christopher Stamp, DFLR , June 20, 2017					

[54]Data retrieved Dec 20, 2017 from DFLR Landings and Landed Value Reports 2006-2015: http://www.fishaq.gov.nl.ca/stats/landings/index.html

[55] Data provided by Licensing and Policy Division of the Department of Fisheries and Land Resources, Government of Newfoundland and Labrador.

Appendix 4 - Theoretical Discards from NL Atlantic Cod Landed Between 1998-2016

NL Atlantic cod landings and theoretical discards 1998 - 2016[56,57]

			Estimated Discards (metric tonnes)								
Cod Landings, NL	**Volume in Metric Tonnes**	**Estimated Fillet Yield, Skinned (tonnes)**	**Head**	**Guts**	**Liver**	**Roe**	**Bones**	**Fins & Lugs**	**Skin**	**TOTAL DISCARDS**	**% Discards**
YEAR	Landed RW	36%	21%	7%	5%	4%	14%	10%	3%		
1998	19,239	6,926	4,040	1,347	962	770	2,693	1,924	577	12,313	64%
1999	34,778	12,520	7,303	2,434	1,739	1,391	4,869	3,478	1,043	22,258	64%
2000	25,115	9,041	5,274	1,758	1,256	1,005	3,516	2,512	753	16,074	64%
2001	19,847	7,145	4,168	1,389	992	794	2,779	1,985	595	12,702	64%
2002	18,039	6,494	3,788	1,263	902	722	2,525	1,804	541	11,545	64%
2003	11,191	4,029	2,350	783	560	448	1,567	1,119	336	7,162	64%
2004	11,501	4,140	2,415	805	575	460	1,610	1,150	345	7,361	64%
2005	12,586	4,531	2,643	881	629	503	1,762	1,259	378	8,055	64%
2006	15,080	5,429	3,167	1,056	754	603	2,111	1,508	452	9,651	64%
2007	15,064	5,423	3,163	1,054	753	603	2,109	1,506	452	9,641	64%
2008	15,454	5,564	3,245	1,082	773	618	2,164	1,545	464	9,891	64%
2009	10,960	3,946	2,302	767	548	438	1,534	1,096	329	7,015	64%
2010	9,385	3,379	1,971	657	469	375	1,314	938	282	6,006	64%
2011	7,446	2,681	1,564	521	372	298	1,042	745	223	4,766	64%
2012	7,132	2,567	1,498	499	357	285	998	713	214	4,564	64%
2013	7,104	2,558	1,492	497	355	284	995	710	213	4,547	64%
2014	8,356	3,008	1,755	585	418	334	1,170	836	251	5,348	64%
2015	8,420	3,031	1,768	589	421	337	1,179	842	253	5,389	64%
2016	13,243	4,767	2,781	927	662	530	1,854	1,324	397	8,476	64%
TOTAL	269,939	97,178	56,687	18,896	13,497	10,798	37,791	26,994	8,098	172,761	64%

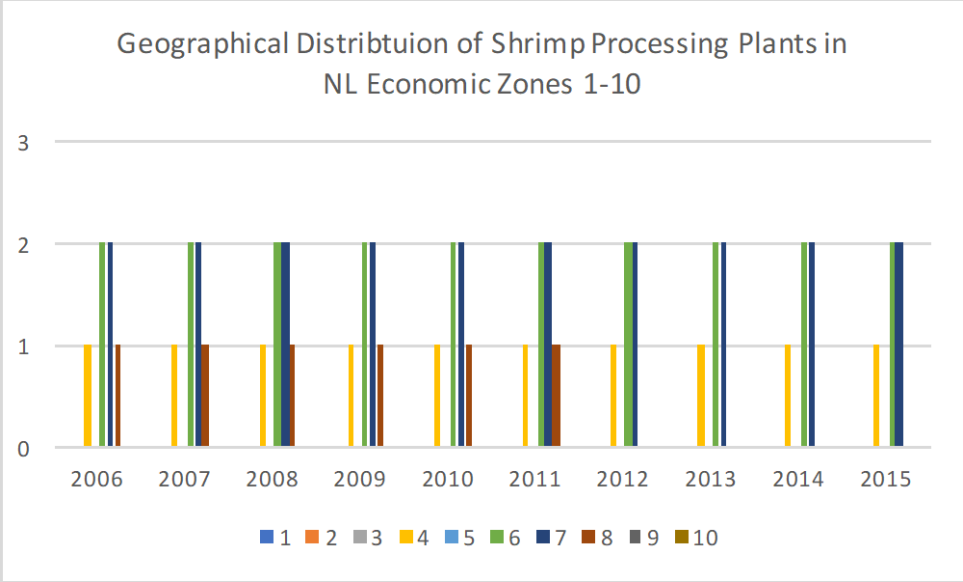
[56] Landings are reported as live round weight (RW) and data were provided by Fisheries and Oceans Canada, Whitehills, NL.

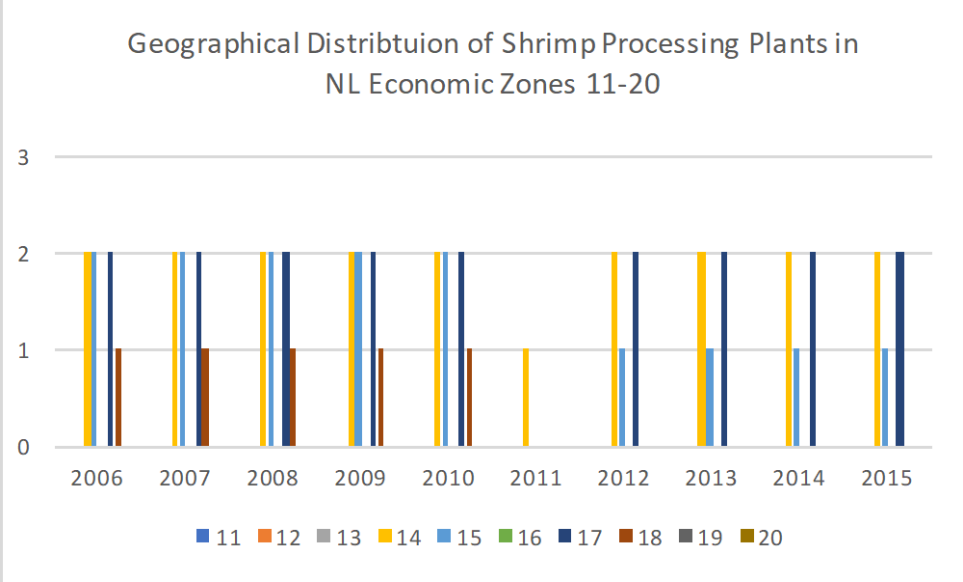
[57] Fillet yields and processing discards were estimated from Landed RW using FAO estimates of fillet yield and discard yields obtained by hand filleting. It is important to note that the weights of guts, liver and roe vary significantly with season, fishing ground and condition of the fish. Fillet yield will vary depending on the experience of the filleter and whether cod is hand filleted or machine filleted. Hand filleting yields tend to be higher than machine filleted depending on how much meat is removed with the head.
http://www.fao.org/wairdocs/tan/x5911e/x5911e01.htm#Physical%20composition%20and%20yields

Appendix 5 - Geographical Distribution of Processing Plants by Economic Zone 2006-2015

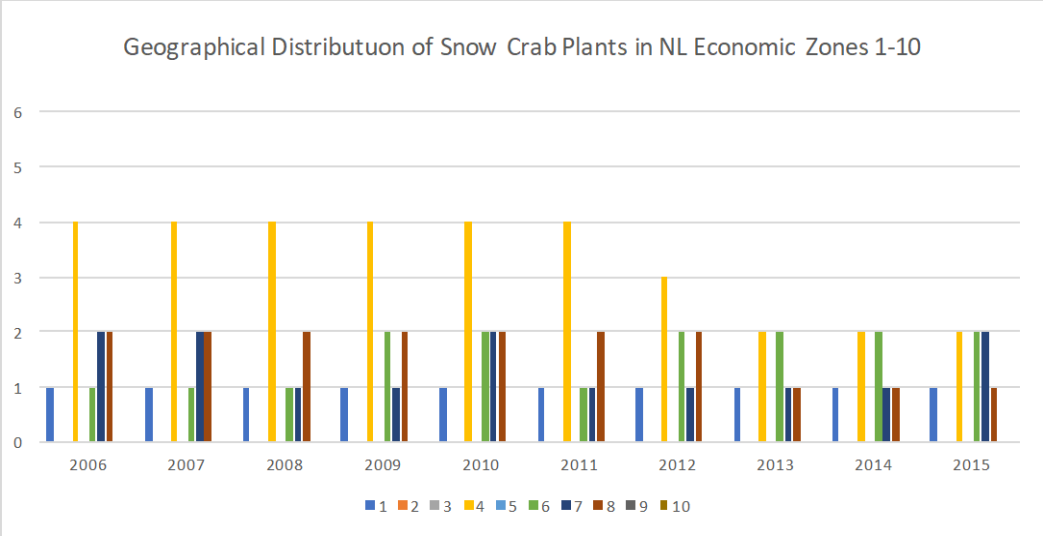
Data related to the geographical distribution of processing plants and economic zones were provided in 2016 by the Licensing and Policy Division of the Department of Fisheries and Land Resources, Government of Newfoundland and Labrador.

Shrimp





Snow Crab


Geographical Distributuon of Snow Crab Plants in NL Economic Zones 1-10
6
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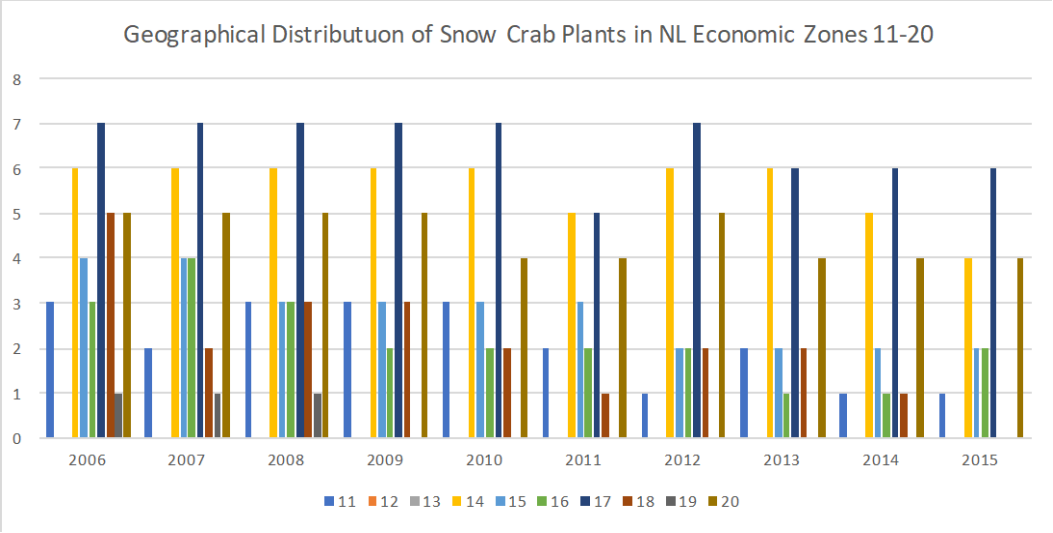
2011
2012
2013
2014
2015
1 2 3 4 5 6 7 8 9 10


Geographical Distributuon of Snow Crab Plants in NL Economic Zones 11-20
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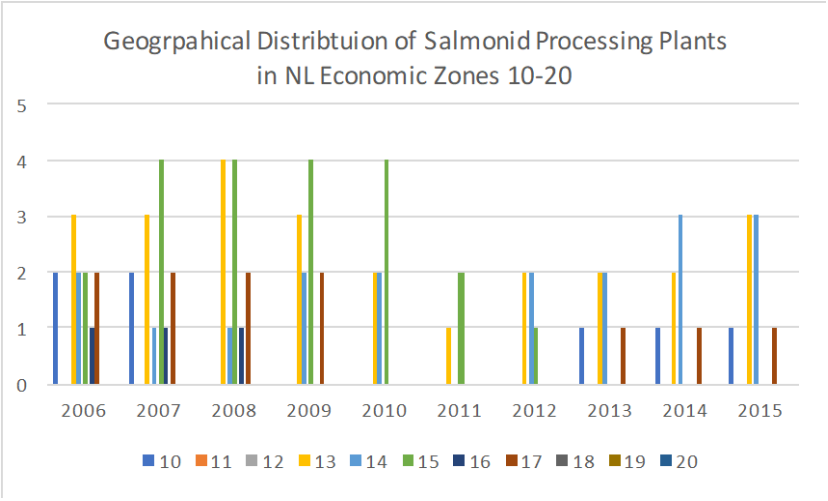
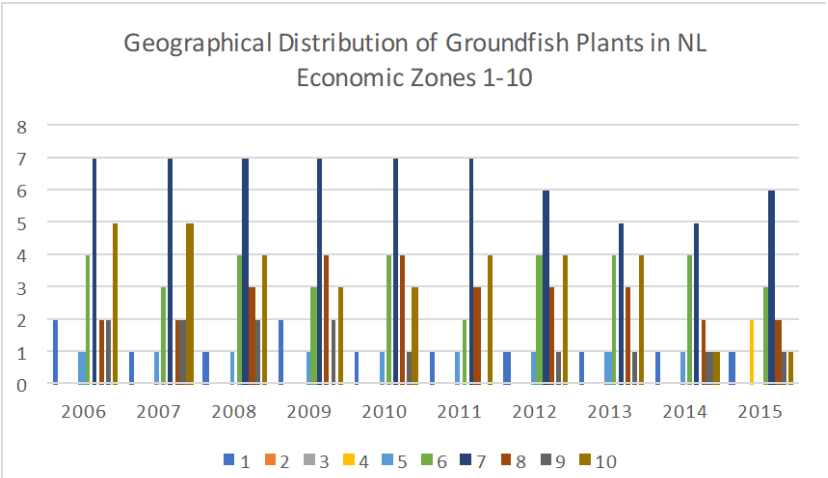
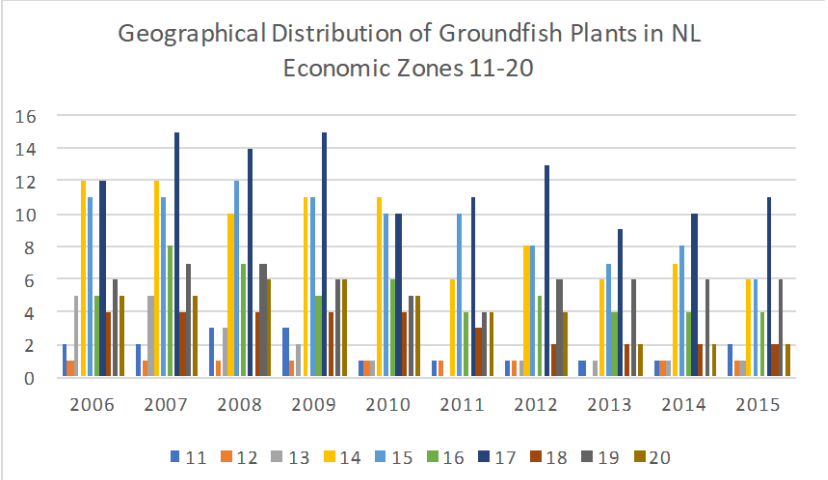
11 12 13 14 15 16 17 18 19 20

Farmed Salmonids

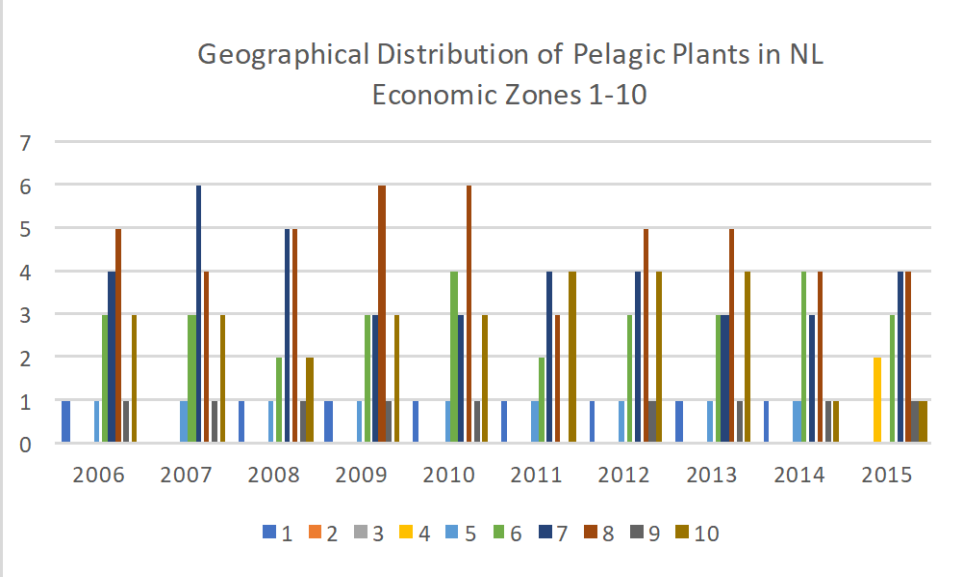


Groundfish





Pelagics


Geographical Distribution of Pelagic Plants in NL
Economic Zones 1-10
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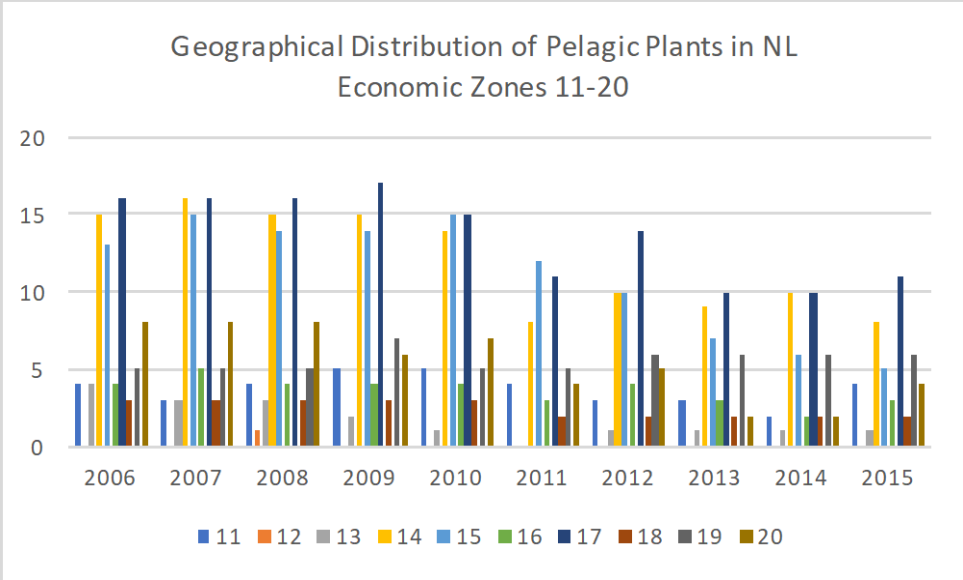
Geographical Distribution of Pelagic Plants in NL
Economic Zones 11-20
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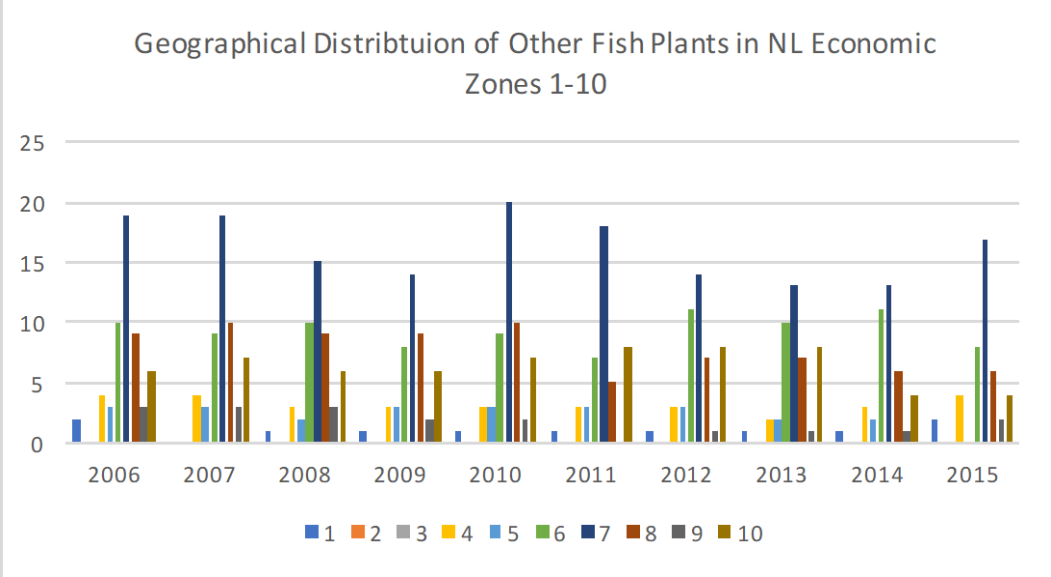
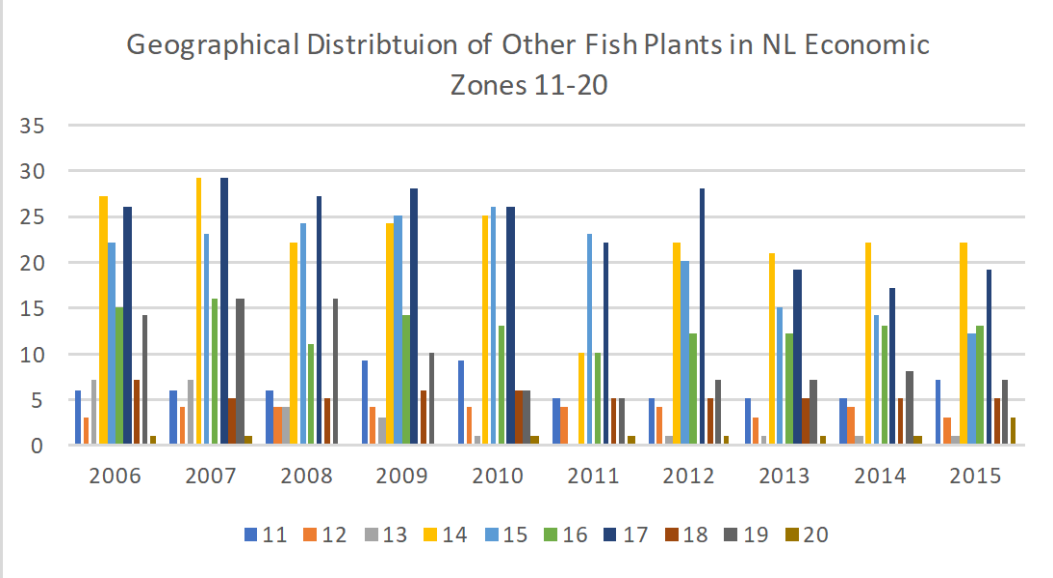
Other Fish Species





CHAPTER 3. Characterization and Stabilization of Marine Biomass Feedstock from Snow Crab (*Chionoecetes opilio*) Processing

3.1 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. In 2019, snow crab landings were 26,894 t of which 16,658 t were exported to the United States (77%), China (8%), Indonesia (6%) and Vietnam (4%), at a value of $415 million [1]. Crab processing plants in Newfoundland and Labrador have historically discarded on average about 30% of their total raw material supply in the form of waste and by-products (refer to Chapter 2 - Table 2.14). In 2019 this amounted to an estimated 8,100 t of discards based on landings of about 27,000 t [1]. Snow crab (*Chionoecetes opilio*) is primarily processed as IQF cooked sections, which generates waste comprised of carapace (cephalothorax shells), viscera and hepatopancreas, haemolymph [2], residual meat and gills. This material is currently not being utilized commercially but could potentially be recovered from processing plant butchering stations and converted into intermediate bioproducts (chitin, crab meal, proteins, lipids) or transformed into higher value bioproducts (chitosan, bioactive peptides, omega-3 PUFAs) (refer to Chapter 2 - Table 2.9).

Because snow crab processing in Newfoundland and Labrador is dominated by one main product - cooked crab sections which make-up more than 80% of crab products produced in the province - this processing by-product material should be consistent in terms of its yield and composition making it an attractive source of a marine biomass feedstock.

Furthermore, using this by-product material will provide an alternative to current at-sea dumping and landfilling practices, thereby reducing environmental pollution and impacts from this sector of the food industry.

In 1997, P. Janes and Sons Ltd. located in Hants Harbour, NL, initiated a project to evaluate commercial products that could be produced from their crab processing operations [3]. Specifically, P. Janes and Sons Ltd. were interested in dehydrating crab waste to produce crab flake and crab meal. They were also interested in establishing a chitin/chitosan production facility that would use the dehydrated crab flake as an input material. The project identified minimum production volumes of finished product (300 t) and by-product (> 3000 t) required for the dehydration facility to be economically viable at that time and indicated that access to sufficient raw material was uncertain and would require cooperation among the various crab processors. The project also identified other challenges that would need to be overcome to make such a venture viable including (i) access to chitin and chitosan markets (Japan); (ii) rapid deterioration of the by-products within 48 hours thereby limiting raw material access to a 300 km radius of the proposed crab by-product processing facility. Currently, there are no major commercial uses of crab processing discards in the province of Newfoundland and Labrador. There has been sporadic production of dried shell for sale to chitin/chitosan producers. This material is still one of the most abundant and readily available sources for use as a feedstock to produce higher value marine based bioproducts, even though crab quotas and the number of crab processing plants have decreased significantly since 1997.

In 2015 there were 27 crab processing plants in operation in NL which generated a combined total of 15,013 t of discards from 47,726 t of raw material (refer to Chapter 2 -

Table 2.8 and Table 2.14). However, crab landings and discards have declined in recent years, as has the number of crab processing plants. In 2019 there were 22 licensed crab processing plants operating in NL which generated a combined total of 8,100 t of processing discards [1]. The highest concentration of crab plants in 2019 occurred in the following regions and generated 86% of the total discards: (1) Northeast Coast - 7 crab plants = 3,675 t discards = 45.5% of total discards; and (2) Avalon Peninsula - 7 crab plants = 3,300 t discards = 40.7% of total discards.

The need for full utilization and the production of higher value bioproducts is even more urgent now for the economic viability and survival of the crab plants that remain. Currently, however, there is insufficient capacity and lack of a suitable strategy to properly manage the large volumes of discards which are generated from crab processing plants in a short time frame (April-July) so that it can be readily utilized as a feedstock for conversion into marketable higher value products. The industry is not equipped to collect, stabilize, store, and convert this raw material into higher value products. Consequently, crab processing by-products are typically collected in offal bins as a waste material and towed by barge for ocean dumping or sent to a local landfill for disposal (refer to 2.7.2 Types of Marine Biomass Feedstocks Generated in NL 2006-2015).

It is well known that snow crab shells contain valuable components such as calcium, carotenoid pigments (e.g., astaxanthin), and chitin [2, 4, 5]. Less is known about the protein and lipid composition of snow crab processing by-products. While these materials can be a valuable source of nutritional components [2, 6, 7] due to their high contents of proteins, lipids, and carotenoids [7, 8, 9, 10, 11, 12], few studies have been conducted on the amino acid, fatty acid, and carotenoid compositions of snow crab processing by-products as a

function of seasonality and treatment method. Even fewer studies have focused on the collection and stabilization methods required to ensure the quality of snow crab by-products is maintained so they can be used as feedstock to produce higher value marine bioproducts (e.g., nutraceutical and pharmaceutical products).

3.2 Scope & Purpose

Snow Crab (*Chionoecetes opilio*) is an important commercial species in Newfoundland and Labrador [13]. According to stakeholders (refer to section 2.8.5 Semi-structured Interviews), crab is the main commercial species and is the key driver in the NL seafood industry. Stakeholders stated that one of the major barriers to full utilization is the quality of the by-products available. Due to the short harvesting season (April-July), utilization of crab processing discards is quite challenging since this material spoils rapidly if it is not stabilized in a timely manner. Therefore, collection and stabilization methods of crab processing by-products are key considerations for their potential use as a feedstock for value addition.

This chapter evaluates the effect of collection pre-treatment and stabilization methods on the quality of snow crab (*Chionoecetes opilio*) processing by-products generated from a Newfoundland and Labrador snow crab processing plant and characterizes the pre-treated stabilized crab by-products for use as a feedstock for intermediate and higher value marine bioproducts. The collection pre-treatment methods are methods employed to help preserve the crab by-products until the main stabilized form of the by-products could be prepared. It was decided that the collection pre-treatment and stabilization methods selected should be simple, and easy to implement for the NL snow

crab processing industry. Therefore, the pre-treatment methods were selected based on current industry practice and consultation with Independent Dockside Graders, who recommended a control treatment and a seawater treatment. These pre-treatment methods are described in section 3.3.1. The stabilization methods were selected based on existing capabilities within the NL seafood processing industry (air drying) and common stabilization technologies (freeze drying) used globally for high value food products and bioproducts. These stabilization methods are described in section 3.3.3.4.

3.2.1 Objectives

The specific objectives of this study include:

1. Collection of crab processing by-products (feedstock) from a local crab processing plant throughout the processing season using two simple pre-treatment methods (control method and a seawater[58] treatment method) as described in section 3.3.1.
2. Evaluation of the effect of each pre-treatment method on crab processing by-product quality.
3. Evaluation of the effect of seasonality on crab processing by-product quality.
4. Conducting an elemental analysis via ICP-MS (refer to section 3.3.3.2) on a subsample of the pre-treated crab processing by-products.
5. Collecting subsamples from each pre-treatment method and evaluating the effect of two different drying methods (air-drying and freeze-drying - refer to section 3.3.3.4),

[58] Seawater treatment was included based on recommendations from crab processors as a method to prevent rapid deterioration of the crab by-products due to oxidative discolouration reactions (bluing/melanosis). Since all plants have access to an adequate supply of seawater, this was considered as a possible simple pre-treatment and stabilization method.

as chosen stabilization methods, on the quality of the crab processing by-product material.

6. Characterization of the air-dried and freeze-dried crab processing by-product material including moisture, ash, protein, lipid, salt, astaxanthin, chitin, lipid class profile, fatty acid composition, and amino acid composition.
7. Identifying potential intermediate and higher value bioproducts that could be produced from crab biomass feedstock samples based on the compositional analysis.
8. Identifying best method(s) for collections and stabilization of crab processing by-products to obtain a high-quality feedstock to produce intermediate and higher value bioproducts identified under objective 7.

3.3 Methods

3.3.1 Sample Collection of Raw Crab Biomass Feedstock

Samples of crab processing by-products (crab biomass feedstock) were collected from Quinlan Brothers Ltd. processing facility in Bay de Verde, NL at three sampling intervals during the 2018 crab fishing season: (1) May 2, 2018; (2) June 30, 2018; and (3) July 25, 2018. Feedstocks were collected as composite samples from the plant butchering line post grinding[59] and contained shells (carapace), mandibles, belly flap (abdomen), gut, hepatopancreas, gills, gonads, and residual meat. Samples were collected in 10 L - 20 L

[59] During processing of IQF snow crab sections as described in section 1.5.1.2 (page 16), the unused material (e.g., carapace, internal organs) is separated from the sections (comprised of shoulders, legs, and claws) and conveyed to a large industrial grinder in preparation for disposal. This was the collection point for the crab biomass feedstock used in this study.

plastic pails. Two treatments were employed to collect the crab biomass feedstocks as illustrated in Figure 3.1.

1. Control Treatment – 3 x 10 L – 20 L pails were filled with the crab feedstock material (Figure 3.1A), lids were placed on the pails, which were then placed in fish tubs and covered with flake ice (Figure 3.1B) for transport to the Marine Institute, in St. John's, NL.
2. Seawater Treatment – 3 x 10 L - 20 L pails were filled with the crab feedstock material; seawater was then added to the pails until the crab material was covered (Figure 3.1C), lids were placed on the pails which were then placed in fish tubs and covered with flake ice (Figure 3.1B) for transport to the Marine Institute, in St. John's, NL.

Upon arrival at the Marine Institute, the May 2018 samples were placed in a chill room overnight at 4°C. The next day the samples were repackaged in 1 Kg plastic trays (Figure 3.1D) and placed in frozen storage at –20°C. However, significant black discolouration (Figure 3.1E) was observed in the control treatment samples. Therefore, it was decided that the June and July samples be placed in frozen storage at –20°C immediately upon arrival at the Marine Institute.

A brief description of the samples collected for this study are identified in Table 3.1. The mean air temperatures and average sea surface temperatures were also noted at the time of sample collection and are presented in Table 3.2.

Table 3. 1 Description of crab feedstock samples collected in 2018

Sample #	Sample Description		
	Collection Date	Treatment Group	Storage Temp (°C)
1	May 2, 2018	Control	– 20
3	May 2, 2018	Seawater	– 20
5	June 30, 2018	Control	– 20
6	June 30, 2018	Seawater	– 20
7	July 25, 2018	Control	– 20
8	July 25, 2018	Seawater	– 20

Table 3. 2 Mean air temperatures and average sea surface temperatures at time of sample collection

Collection Date	Mean Air Temp St. John's[a] (°C)	Avg Sea Surface Temp Carbonear[b] (°C)
May 2, 2018	8.6	2.7
June 30, 2018	15.0	6.6
July 25, 2018	21.3	11.2

[a] http://climate.weather.gc.ca/climate_data/daily_data_e.html?StationID=50089&timeframe=2&StartYear=1840&EndYear=2018&Day=1&Year=2018&Month=5

[b] https://www.seatemperature.org/north-america/canada/carbonear-may.htm

A. Collection of crab control samples in 10-20L pails.

D. May 2018 crab control sample repacked in plastic tray.

B. Pails containing crab feedstock packed in flake ice for transport.

E. Black discoloration in crab control sample May 2018.

C. Collection of sea water treated crab samples.

F. Hobart grinder used for shell milling/particle size reduction.

Figure 3. 1 Sample collection and pre-treatment of snow crab processing by-products collected in 2018

3.3.2 Sample Preparation of Raw Crab Biomass Feedstock

3.3.2.1 Control Treatment Group

1. Crab feedstock samples were removed from frozen storage and placed in a 4°C chill room for 1-4 hours to thaw.
2. Crab feedstock samples were weighed prior to milling.
3. Crab feedstock materials were crushed in a Hobart grinder (Figure 3.1F) in a 2-step process:
    a. Step 1- Crab feedstock was milled through a 17 mm plate.
    b. Step 2- Crab feedstock was then milled through a 13 mm plate.
4. Milled samples were collected in polybags, weighed, then vacuum packaged.
5. The vacuum packaged samples were frozen and stored at –20°C until further analysis could be performed. All analyses were completed within 12 months.

3.3.2.2 Seawater Treatment Group

1. Crab feedstock samples were removed from frozen storage and placed in a 4°C chill room for 16-36 hours to thaw.
2. Crab feedstock samples were drained (Figure 3.2) for 2-3 minutes and weighed prior to milling.
3. The drained crab feedstock samples were then prepared according to steps 3-5 as outlined in section 3.3.2.1 above.

Figure 3. 2 Draining of seawater treated crab sample.

3.3.3 Quality Evaluation and Characterization of Crab Biomass Feedstock Samples

A quality evaluation and characterization studies of all control and seawater treatment samples were undertaken. The following analyses were conducted.

3.3.3.1 Sensory Assessment

Initial visual and odour assessments were conducted on the raw, thawed feedstock samples prior to grinding, primarily to determine if there was any discolouration or off-odours in the feedstock samples prior to freezing.

Additional odour and visual assessments were conducted using the 100 mm line scaling method (Appendix 1) on the frozen, raw, ground crab samples after thawing. Since there are no sensory standards currently developed for crab processing by-products, it was decided to use the 100 mm line scaling method which is a standardized method used by CFIA (Canadian Food Inspection Agency) inspectors for conducting seafood quality assessments for regulatory compliance[60]. If crab processing by-products are intended to be used as feedstock for valorization into high quality bioproducts, the CFIA standard should be an appropriate indicator of crab by-product quality.

3.3.3.2 Elemental Analysis (ICP-MS)

Subsamples of the raw ground crab feedstock samples were submitted to MUN's Department of Earth Sciences for elemental analysis (including heavy metals). Samples were prepared by ashing for 6 hours at 550°C. The cooled samples were then acid digested,

[60] This method is regarded as an accurate and objective method when assessors are properly trained and calibrated. I have been trained by CFIA on this method and have been a former sensory trainer for Provincial Fisheries Inspectors.

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. The step-by-step sample preparation method is described in detail in Appendix 2 (page 229).

3.3.3.3 Moisture Analysis

Moisture content of the raw ground crab feedstock samples was determined using the air oven method according to AOAC method 930.14.

3.3.3.4 Evaluation of Drying Methods

Each of the six raw crab feedstock samples were further subdivided into two different groups for evaluation of drying methods.

Air Oven Drying: Samples were thawed overnight at 4°C, placed on aluminum drying trays and dried at 105°C in a convection oven to constant weight then milled to a particle size of ~1-2 mm using a Ninja high speed blender prior to determination of proximate composition. Due to the variations in sample size, samples were dried in lots ranging from 300 g to approximately 1 kg. Drying time varied depending on sample size, therefore, drying was continued until there was no change in sample weight.

Freeze Drying: Samples were thawed overnight at 4°C, placed in silicon cupcake trays (Figure 3.3A) and re-frozen at -80^{o}C. Each tray contained approximately 300 g of raw material (12 cupcakes x 25 g each). Frozen samples (cupcakes) were removed from the trays and placed directly into glass freeze drying jars (approximately 12 cupcakes per jar) which were then connected to a Labcono laboratory freeze drier (Figure 3.3B) and dried under vacuum for a minimum of 48 hours to ensure adequate moisture removal. The dried samples were milled to a particle size of ~1 mm using a Ninja high speed blender prior to

determination of % moisture (AOAC method 930.14) and proximate composition (section 3.3.3.5).

A B

Figure 3. 3 Crab feedstock samples: A - in silicon trays prior to freezing; B - in Labcono freeze drier

3.3.3.5 Proximate Analysis

Proximate composition was determined for the air-dried and freeze-dried samples and included determination of: Kjeldahl nitrogen AOAC Method 954.01/988.05; Fat by Soxhlet AOAC method 920.39; Ash via AOAC method 938.08 Ash of Seafood; Salt content, as % NaCl, was determined using DFO's recommended laboratory methods for assessment of fish quality for salt content as reported in Canadian Technical Report of Fisheries and Aquatic Sciences No. 1448. Detailed procedures are described in Appendix 3.

3.3.3.6 Total Astaxanthin Content

Astaxanthin was extracted from the air-dried and freeze-dried samples using a simplified methanol extraction procedure reported by Lopez-Cervantes *et al.* [14]. The detailed procedure is reported in Appendix 4. Immediately following extraction, the

samples were placed in a cuvette and the absorption was measured at λ_{max} (476 nm) in a HACH DR600 Spectrophotometer. The pigment concentration was calculated using Equation 3.1 and reported as total astaxanthin.

$$Astaxanthin\ \left[\frac{\mu g}{g}\right] = \frac{A*D*10^6}{100*G*d*E} \qquad \text{(Eqn. 3.1)}$$

Where: A = Absorption at λ_{max}, D = Volume of extract [ml], G = Sample weight [g], d = Cuvette distance (10 mm), and E = Extinction coefficient (2100 for Astaxanthin) [15].

3.3.3.7 Chitin Content

Chitin content was determined following demineralization of 5-10 g of dried crab feedstock with 50-100 mL of 5-7% HCl w/v for 3 hours at 25°C, followed by deproteination with 10% NaOH w/v (1:8 of crab:NaOH) for 2 hours at 55°C [4]. The chitin was collected on a Whatman No. 4 filter paper using a Buchner funnel and washed a minimum of three times with deionized water to pH 7, followed by oven drying at 105°C for 24-48 hours. The recovered chitin was analyzed for total nitrogen via the Kjeldahl method (AOAC 954.01/988.05), residual protein nitrogen via a Modified Lowry method (Appendix 5) and ash content (AOAC 938.08). Chitin nitrogen was calculated using equations (3.2) and (3.3).

% Chitin Nitrogen = % Total (Kjeldahl) Nitrogen – % Protein Nitrogen (Eqn. 3.2)

% Protein Nitrogen = % Residual (Lowry) Protein ÷ 4.94 (Eqn. 3.3)

Where: 4.94 is the nitrogen-to-net protein conversion factor for fish and fish products [46].

3.3.3.8 Analysis of Fatty Acids and Lipid Classes

Fatty acid profiles and lipid class composition were determined on lipid fractions which were extracted according to the method of Parrish (1999) [16] from the air-dried and freeze-dried crab feedstock samples. Lipid class composition was determined using an Iatroscan Mark VI TLC-FID, silica coated Chromarods and a three-step development method [17]. For all samples, lipid extracts were transesterified using sulfuric acid and methanol for 1 hour at 100°C. The FAME were analysed on a HP 6890 GC FID equipped with a 7683 autosampler. This analysis was performed by ACC of Memorial University of Newfoundland. The detailed methodology is described in Appendix 6.

3.3.3.9 Determination of Amino Acid Profiles

This analysis was performed by the Marine Institute's Centre for Aquaculture and Seafood Development (CASD) using the procedure outlined below.

The amino acid hydrolysis was carried using ~10 mg dried sample treated with 6 N HCl at 110°C for 24h under nitrogen. After hydrolysis, the samples were cooled to room temperature and filtered using a 0.2 µm PTFE syringe filter (Whatman GD/X 13). The filtrate was collected and stored for analysis after derivatization.

The amino acid analysis was carried out using a gas chromatograph-mass spectrometer (ThermoFisher Trace 1300 GC/ISQ-LT MS). The amino acid standards were purchased from Sigma-Aldrich. All amino acids were weighed separately prepared in 0.1 N HCl to prepare a stock solution at a concentration of 0.5 mg/ml. Five different concentrations of the stock solution were used to build a calibration curve. DL-Norleucine was used as an internal standard at a concentration of 0.5 mg/ml. The internal standard was

added to both standards and samples. Both standards and samples were subjected to derivatization using *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA). During the derivatization process, 50 µl aliquots of standards/samples were taken in a 10 ml test tube and completely dried at 70°C under nitrogen for five min. After drying, 100 µl of neat MTBSTFA was added, followed by 100 µl of acetonitrile were added. The test tubes were tightly capped and were heated at 100°C for two hours. The sample was then allowed to cool at room temperature, and 200 µl of acetonitrile was again added to the tubes. The samples were then transferred into GC vials for analysis.

The GC analysis was carried out using SLB-5ms, 20 m x 0.18 mm column with an internal diameter of 0.18 µm. The inlet temperature was 280°C and was operated in splitless mode. The split flow was maintained at 100 ml/min, and the splitless time was 0.3 min. The column flow was 0.5 ml/min. The GC oven was maintained at 60°C for 0 min, ramped up to 100°C at 20°C/min and held for 1 min, ramped up to 290°C at 10°C/min and held for 3 min and ramped up to 340°C and held for 2 min. The MS transfer line temperature was 320°, and the ion source temperature was 280°C. The standards and samples were scanned using MS in the range of 40-639 (*m/z*).

3.3.3.10 Statistical Analysis

Results were compared either using a two-tailed paired T-test (e.g., to identify differences due to pre-treatment methods and drying methods), or analysis of variance (ANOVA) and Tukey's test (e.g., to determine effects of seasonality) to identify variances in compositional analysis of the samples. All analyses were performed using the Data Analysis ToolPak in Microsoft Excel for Mac, Version 16.44. Alpha level 0.05 was selected

as the threshold of significance to test the null hypothesis that all sample means are the same.

3.4 Results and Discussion

3.4.1 Sample Preparation and Sensory Assessment of Raw Crab Biomass Feedstock

This section summarizes the sensory quality observations noted during preparation of the crab feedstock samples (Figure 3.4) included in this study. Also in this section, is a summary (Tables 3.3 and 3.4) of some of the challenges encountered, particularly during storage of the May 2018 samples, and milling/grinding of the seawater treated samples, which may have affected the sample characteristics, and/or may account for some of the variability between the samples.

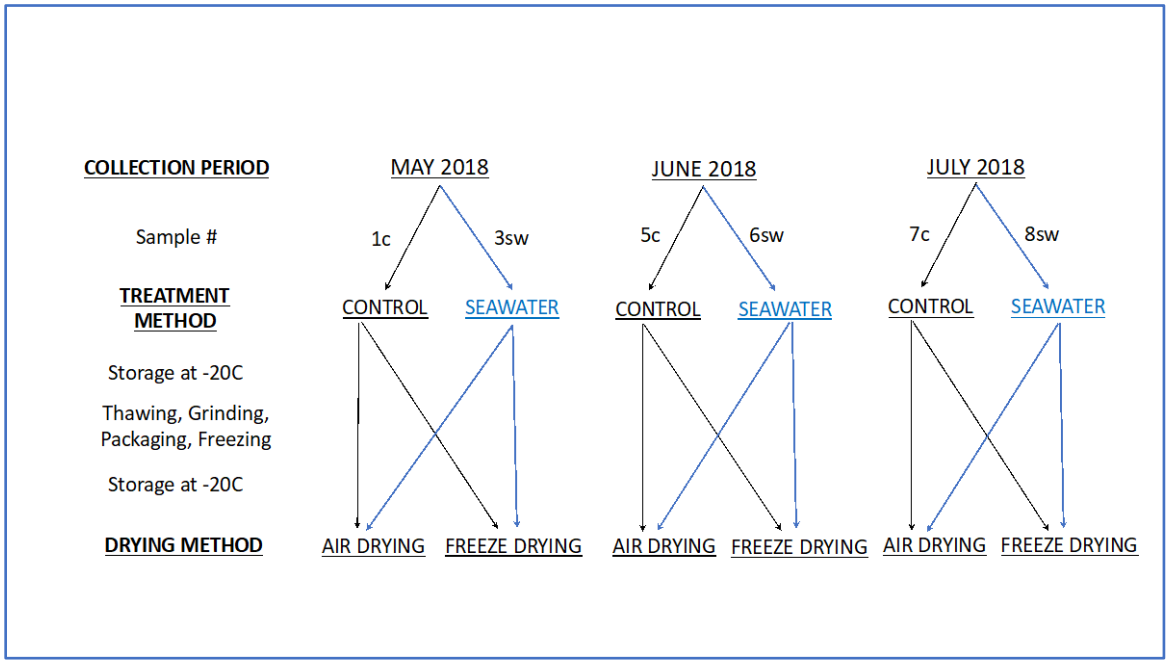


Figure 3. 4 Schematic representation of the preparation of crab biomass feedstock samples

The initial sensory quality is particularly important if the intent is conversion of crab feedstock materials into higher value bioproducts such as aqua feeds, nutritional

additives, nutraceuticals, or pharmaceuticals, all of which have high standards for product quality and purity. Sensory quality can be an early indicator of raw material acceptability for its intended end use.

Sensory assessments of the raw crab feedstock samples were conducted prior to grinding and freezing and included visual and odour assessments. The purpose of this initial assessment was to determine if there were any obvious quality issues such as discolouration, or spoilage odours, that could negatively impact the suitability of the crab feedstock for higher value bioproducts. These assessments should indicate whether raw material handling practices were appropriate and effective for maintaining sensory quality.

3.4.1.1 Control Treatment Group

The May 2018 control sample (Sample 1c) exhibited significant black discolouration in comparison to the June (Sample 5c) and July (Sample 7c) control samples (Table 3.3 and Figure 3.5). This was attributed to the collection and storage method employed for the May control sample. The May control sample was stored in 10L pails and placed in a 4ºC chill room overnight and then repackaged into 1kg trays for freezing and storage. During overnight storage at 4ºC the crab feedstock became discoloured likely due to biochemical reactions catalyzed by polyphenol oxidase (PPO) enzymes which cause bluing and melanosis in crab [18, 19, 47]. This reaction takes place within a few hours after harvest when crustaceans are exposed to atmospheric oxygen [47]. Consequently, it was decided to freeze the June and July control samples in the collection pails immediately upon return to the Marine Institute. This resulted in little to no discolouration of the June and July feedstock samples (Figure 3. 5). This preliminary result suggests that freezing by-

products upon their production will be critical to future valorization of crab processing discards.

Table 3. 3 Initial yield and sensory observations of control samples during sample preparation

Sample # [a]	Collection Date	Weight of Feedstock (Kg)		% Recovery [b]	Sensory Observations
		Before Milling	After Milling		
1c	May 2, 2018	0.60	0.545	90.8	Stored in trays; severe black discolouration of meat and shells
5c	June 30, 2018	9.453	9.39	99.3	Stored in pails; no discolouration of shells; meat had slight grey discolouration
7c	July 25, 2018	5.665	5.660	99.9	Stored in pails; no discolouration of meat or shells

[a] *c = control treatment.* [b] *% Recovery = [weight after milling ÷ weight before milling] x 100*

A B C

Figure 3. 5 Visual observations of 2018 crab control samples after frozen storage: A - May; B - June; C - July

3.4.1.2 Seawater Treatment Group

Visual observations of the seawater treated crab feedstock samples are described in Table 3.4 and Figure 3.6. The May 2018 seawater treated sample (Sample 3sw) exhibited a blue-grey discolouration of the feedstock material, discoloured seawater, and had a slight fishy odour. The June seawater treated sample (Sample 6sw) had no discolouration of the

shell or meat, had a fresh seaweed odour, but did have brown coloured seawater containing visible solids. The July seawater treated sample (Sample 8sw) had some black discolouration of the shells and meat near the surface of the pail, but the feedstock material below the surface was completely covered with seawater and displayed no discolouration. The seawater however was a reddish brown colour and had a sour off-odor. Both the June and July seawater treated samples were very difficult to grind as the shells were very soft and pliable and blocked the grinder barrel. The July seawater treated sample had to be refrozen and subsequently ground from a partially frozen state.

Table 3. 4 Initial yield and sensory observations of seawater treated samples during sample preparation

Sample # [a]	Collection Date	Weight of Feedstock (Kg)		% Recovery [b]	Sensory Observations
		Before Milling	After Milling		
3sw	May 2, 2018	1.975	1.070	54.2	Small sample size, frozen in trays, had to mill from frozen; slight fishy odour; feedstock colour blue grey near surface; seawater dark grey
6sw	June 30, 2018	6.525	5.535	84.8	Frozen in pails; no discolouration of shell or meat; fresh seaweed odour; seawater dark brown with visible solids; shells very soft and pliable, difficult to grind; blocked the grinder barrel
8sw	July 25, 2018	5.72	4.445	77.7	Frozen in pails; seawater slight sour odour with dark reddish-brown color; shells near surface had some black discolouration; shells below surface pink in color, no off odour; difficult to grind, shells pliable and soft; had to refreeze drained material and grind from frozen to avoid significant yield loss

[a] sw = seawater treatment. [b] *% Recovery = [weight after milling ÷ weight before milling] x 100*

The discolouration on the surface of the crab feedstock suggests oxygen in the headspace of the collection pails may have triggered a series of oxidative enzymatic reactions (bluing and melanosis) catalyzed by PPO and oxygen that contribute to crab meat and shell discolouration [18, 19, 47]. Discolouration of the seawater suggests that similar biochemical reactions occurred during sample preparation, chilled storage, and thawing due to loose meat/protein particles and other soluble components present in the storage water. The sour odour noted in the July sample may be a result of the warmer seawater and air temperatures experienced during the collection period (Table 3.2). The July samples were collected in 20L pails likely resulting in longer cooling and freezing times in comparison with the May and June samples which were both collected in 10L pails. The larger headspace in the 20L pails likely contributed to the surface discolouration observed in the July seawater sample.

3.4.1.3 Sensory Assessment (100 mm Line Scaling Method)

Following initial grinding of the feedstock samples, the material was vacuum packaged and frozen until further analysis could be performed. When needed for analysis, the frozen ground samples were thawed and again evaluated for sensory quality. Sensory quality was evaluated based on the 100 mm line scaling method (Appendix 1). When this method is employed, the lower the score on the 100 mm line scale, the higher the quality of the raw material. Scores below 50 are a “pass”, while scores above 50 are a “fail”.

The results of this sensory assessment are presented in Table 3.5 and Figure 3.7. Sample 1c (May control) had the highest score, while samples 6sw and 8sw (June seawater

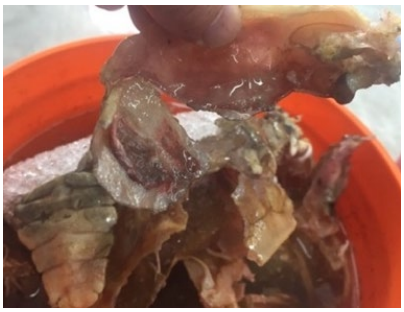
A

D

B

E

C



F

Figure 3. 6 Visual observations of 2018 crab seawater treated samples after frozen storage: A - May feedstock & seawater; B - June feedstock; C - June seawater; D - blocked grinder barrel; E - July feedstock with minor surface discolouration; F - July feedstock with no discolouration

and July seawater, respectively) had the lowest scores. Based on the 100 mm line scores, the samples ranked in order of highest score (lowest quality) to lowest score (highest quality) as follows: 1c > 7 c> 5c > 3sw > 8sw > 6sw. This ranking indicates that in terms of visual appearance and odor, the control samples displayed lower quality characteristics

than the seawater treated samples. The May control sample (1c) received the highest score (i.e., lowest quality ranking) due to its slight fishy off-odour and black discoloration, followed by the July control sample (7c) which also had a slight fishy off-odour, but less severe discolouration. The June control sample (5c) was rated as the best quality of the control samples with no off-odours, and only slight surface discolouration. Sample 3sw (May seawater) had a slightly higher score (slightly lower quality) than samples 6sw and 8sw (June and July seawater, respectively). All three seawater treated samples had a fresh seaweed/briny odour characteristic of fresh seafood, however, the May sample (3sw) had signs of discolouration which were not present in the June (6sw) and July (8sw) seawater treated samples. This discolouration was attributed to the handling of the May sample.

Table 3. 5 Sensory assessment of thawed ground crab biomass feedstock samples - May, June, and July 2018

Sample #	Sample Description	100 mm Line Score*	Comments
1c	May 2, 2018 - Control	Odour = 18 Colour = 100	Neutral to slight fishy odour; feedstock dark grey to black
3sw	May 2, 2018 - Seawater	Odour = 5 Colour = 39	Feedstock dark grey in colour; fresh seaweed odour; a lot of moisture in sample; water dark and discoloured
5c	June 30, 2018 - Control	Odour = 8 Colour = 49	Fresh seaweed odour; surface feedstock dark grey; middle feedstock was pink in colour
6sw	June 30, 2018 - Seawater	Odour = 3 Colour = 5	Feedstock bright pink colour; neutral to briny odour
7c	July 25, 2018 - Control	Odour = 13 Colour = 49	Slight fishy odour; greyish colour feedstock with pink mixed throughout
8sw	July 25, 2018 - Seawater	Odour = 3 Colour = 10	No discolouration; bright pink feedstock; fresh salty/briny odour

** The lower the score the better the quality. Score < 50 = pass, score > 50 = fail.*

The sensory assessments indicate that the treatment, collection, and storage methods affect the sensory characteristics of crab processing by-products, which are

extremely susceptible to biochemical discolouration reactions, such as bluing and melanosis. These reaction mechanisms are discussed in section 3.4.1.4.

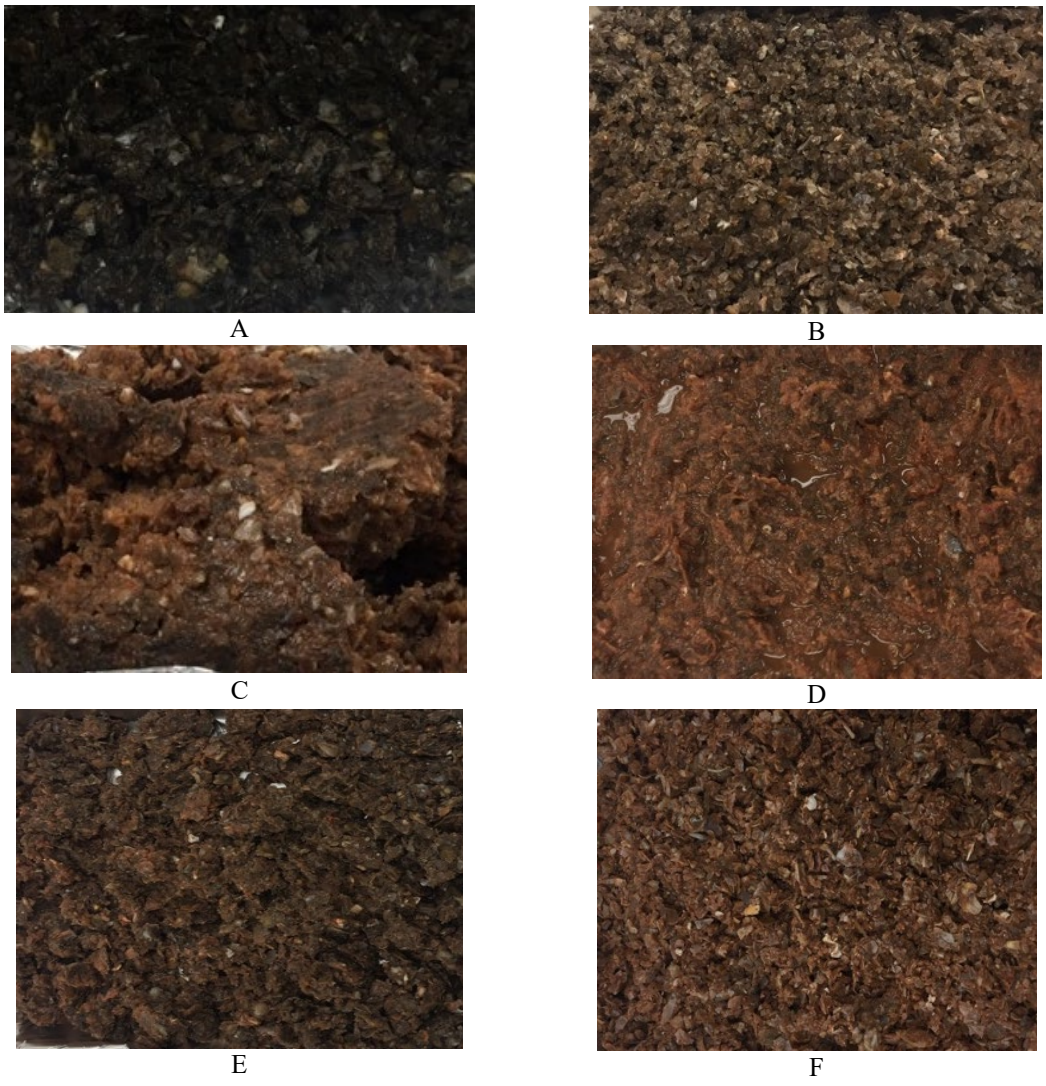


Figure 3. 7 Visual appearance of 2018 thawed ground crab biomass feedstock samples: A - May control; B - May seawater; C - June control; D - June seawater; E - July control; F - July seawater

The May 2018 control sample (1c) received the lowest quality ranking largely due to the black discolouration which developed in the crab feedstock during storage prior to freezing. This sample had a longer holding time prior to freezing due to overnight storage, and further exposure to atmospheric oxygen during transfer to 1kg trays. The July 2018 control sample (7c) was the second lowest quality feedstock sample, primarily due to the fishy off-odour. Warmer temperatures during sample collection and the larger collection pails used likely resulted in longer cooling and freezing times thus contributing to the development of off-odours. The seawater treated samples were better visually and had a more pleasing odour compared with the control samples.

3.4.1.4 Discolouration Reactions in Crab

Crab meat and shell discolouration caused by oxidative enzymatic reactions is not a food safety issue, but it is a major quality concern for the NL snow crab processing industry as buyers will not purchase these products because they associate the discolouration with spoilage [47]. Boon [18] identified five types of crab meat discolouration: (1) blue - due to a crab blood reaction; (2) brown – due to the Maillard reaction, a non-enzymatic browning reaction that occurs between protein and reducing sugars when heated; (3) black – due to the formation of iron sulfide; (4) discolouration due to oxidation in frozen crab; and (5) discolouration from the diffusion of pigments in canned crab meat. The most important crab discolouration reactions of concern to NL processors include crab-bluing and melanosis. Although this phenomenon is a major concern for the industry, and tremendous effort has been exerted to prevent bluing in crab (e.g., new cookers, careful control over process temperatures, etc.), other than the study conducted by

Boon [18] and a few others prior to that, there have been very few studies conducted to fully understand the reaction mechanisms that cause crab discolouration and how to prevent it. A comprehensive review of melanosis in crustaceans was conducted in 2016 by Gonçalves and de Oliveira [47]. This review shows that most studies on melanosis have focused on warm water shrimp species with only a few focusing on crab (*Charybdis japonica* and *Chionoecetes japonicas*) [48, 49, 50, 51], but nothing specifically related to *Chionoecetes opilio* was reported.

'Bluing' in crabmeat is linked to the haemolymph content of the meat. According to Boon [18], the degree of bluing varied between crab species, and the species that underwent bluing had similar haemolymph characteristics. These included high copper content in the haemolymph and the presence of polyphenol oxidase enzymes such as tyrosinase [18]. Although this type of discolouration is called bluing, the actual colour may range from light blue to blue gray to black. While there is uncertainty surrounding the specific blood constituents and reaction mechanisms involved in the formation of the blue pigment there are 5 proposed categories [18]: iron compounds; copper compounds; melanin; copper proteins or biuret complexes; hemocyanin compounds.

Melanosis or blackspot occurs postharvest in crustaceans. It is a recognized post-mortem phenomenon caused by the polymerization of phenol into a black pigment, melanin. Phenol polymerization is initiated by polyphenol oxidase, an enzymatic complex [19]. According to Ogawa *et al*. [20], the amount of stress suffered by crustaceans, such as physical damage to the cuticle, moult stage and gender all effect the amount of blackspot.

In crustaceans, both bluing and melanosis (i.e., black spot) are attributed to the presence and activation of the polyphenol oxidase (PPO) enzyme complex which involves

two copper units and causes blue, gray, brown and black discolouration [19]. The reaction mechanism (Figure 3.8) includes: (a) hydroxylation of monophenols like tyrosine (mono phenol oxidase reaction) to *o*-diphenol (Dopa); and (b) oxidation of *o*-diphenols to di-quinones (diphenol oxidase reaction) [19]. A series of non-enzymatic reactions are involved with the conversion of di-quinones to black melanin. Tyrosine is the principal substrate for crustacean PPOs (e.g., tyrosinase) [19]. Many researchers agree that copper and iron are related to blue discolouration, and are either directly involved in the reaction, or act as catalysts [18]. The interaction of tyrosine with the copper protein hemocyanin (Hc) in crab blood (haemolymph) has also been implicated in blue discoloration of crabmeat [18]. Hemocyanin which accounts for 90-95% of total plasma protein in crustaceans [47], has recently been shown to display PPO activity (HcPO) when exposed to various activators (e.g., perchlorate, antibacterial peptides, serein proteinases) due to induced structural changes that occur in Hc [47, 52, 53]. The effect of PPO and HcPO help explain the rapid onset of melanosis in crustaceans.

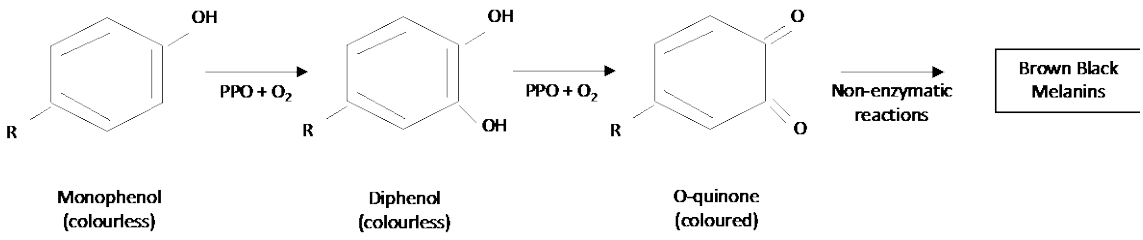


Figure 3. 8 Melanosis reaction pathway [19, 47].

The discoloration observed in the crab feedstock samples in this study showed a range of colours from blue gray to brown, to black. Elemental analysis (Table 3.6) confirmed that the June and July samples contained high levels of copper and iron (May samples were not assessed), and amino acid analysis (Table 3.17 and Table 3.18) confirmed

the presence of tyrosine (8.61-14.15 mg/g) in all crab feedstock samples. Previous studies conducted by the CASD confirmed the presence of PPO and iron in raw crab meat and copper in the blood [21]. In addition, studies conducted by Reiber and McGaw [22] showed that snow crab have a partially closed hybrid circulatory system which was confirmed through corrosion casting techniques used to map the circulatory system of various crab species. This hybrid circulatory system has a vast and complex structure of arterioles, capillaries and sinuses which allows for the vast distribution of the hemolymph to the meat. This suggests that snow crabs are naturally susceptible to bluing and melanosis discolouration reactions.

The pattern of discolouration observed in the raw crab feedstock samples during this study may be attributed to bluing and melanosis discolouration reactions occurring during sample collection, storage, and thawing. Although Sample 6sw (June seawater) had the highest levels of iron, copper, and tyrosine, it ranked highest based on sensory characteristics and had minimal discolouration compared with the other crab feedstock samples. This suggests that storage in seawater may be effective in preventing discolouration in crab processing by-products by preventing contact of the crab feedstock with atmospheric oxygen, and/or causing partial inactivation of the PPO enzyme complex due to salt content and freezing [19]. Sample 6sw had the highest salt content (24%), which was 4-6 times higher than that found in all other samples, including control and seawater treated samples (Tables 3.10 and 3.11). NaCl is commonly used in the food industry at concentrations of 2-4% to prevent browning, and studies have shown that PPO activity decreases with increasing NaCl concentrations [19]. Sample 6sw also had minimum

exposure to oxygen in comparison to the May and July samples likely affecting the onset of bluing and melanosis.

For the seawater treated samples, it is also possible that some constituents that cause discolouration reactions (e.g., proteins, amino acids, PPO, HcPO, and copper) leached into the seawater due to cell disruption caused by freezing and thawing resulting in the reaction occurring in the storage water, rather than in the solid constituents of the crab feedstock. Samples of the storage water were not analyzed during this study as this was not the focus of this chapter.

3.4.2 Elemental Composition of Raw Crab Feedstock

The elemental compositions of the 2018 crab feedstock samples are presented in Table 3.6. Due to the small sample size available for the May 2018 samples, only the June 2018 and July 2018 samples were selected for elemental compositional analysis. The purpose of this analysis was to determine which heavy metal contaminants are present in the samples and if their concentrations are within the acceptable limits for seafood established by Health Canada (Table 3.7). Comparison of heavy metal levels in the crab samples against the industry standard for the maximum levels of metal contaminants permitted in chitosan intended for biomedical use (Table 3.8) was also conducted. Copper, calcium and phosphorous were also assessed due to their role in crab discolouration reactions (Cu), shell hardness (Ca, P) and chitin/chitosan yield and purity (Ca, P).

Table 3. 6 Elemental composition of June-July 2018 crab biomass feedstock samples in ppm (mg/kg original sample) on a dry weight basis[a]

Analytes	June 2018		July 2018	
	5c - Control	*6sw - Seawater*	*7c - Control*	*8sw - Seawater*
Aluminum	102.7 ± 0.02	154.5 ± 2.8	77.0 ± 2.4	60.9
Antimony	nd[b]	nd	nd	nd
Arsenic	3.64 ± 0.07	2.77 ± 0.01	3.83 ± 0.15	1.65
Barium	27.7 ± 0.2	10.4 ± 0.2	27.5 ± 0.1	27.1
Beryllium	nd	nd	nd	nd
Bismuth	0.014 ± 0.001	0.058 ± 0.0004	0.014 ± 0.0004	0.010
Boron	nd	nd	nd	nd
Cadmium	2.35 ± 0.05	3.47 ± 0.04	1.55 ± 0.00	0.67
Calcium (%)	10.8 ± 0.1	4.8 ± 0.11	14.2 ± 0.2	14.1
Caesium	0.015 ± 0.001	0.022 ± 0.0004	0.012 ± 0.0004	0.006
Cerium	0.25 ± 0.003	0.39 ± 0.006	0.24 ± 0.001	0.19
Chromium	0.87 ± 0.032	2.67 ± 0.04	0.77 ± 0.017	0.59
Cobalt	0.50 ± 0.033	0.62 ± 0.028	0.47 ± 0.020	0.15
Copper	40.6 ± 1.8	57.2 ± 2.4	35.5 ± 1.8	14.9
Iron	159 ± 18	376 ± 29	112 ± 19	38.5
Lanthanum	0.26 ± 0.001	0.29 ± 0.002	0.27 ± 0.004	0.23
Lead	0.34 ± 0.008	0.78 ± 0.017	0.28 ± 0.000	0.25
Lithium	1.34 ± 0.03	2.13 ± 0.07	1.75 ± 0.09	0.96
Magnesium	nd	nd	nd	nd
Manganese	6.26 ± 0.27	7.06 ± 0.40	6.60 ± 0.16	4.81
Mercury	< DL[c]	< DL	< DL	< DL
Molybdenum	0.39 ± 0.01	0.57 ± 0.01	0.28 ± 0.004	0.23
Nickel	2.70 ± 0.50	2.70 ± 0.24	2.28 ± 0.18	1.02
Phosphorous	40606 ± 1892	14420 ± 461	36530 ± 1820	37021
Potassium	nd	nd	nd	nd
Rubidium	2.76 ± 0.01	2.98 ± 0.01	2.31 ± 0.08	1.01
Selenium	nd	nd	nd	nd
Silver	2.25 ± 0.00	4.01 ± 0.07	1.83 ± 0.00	0.97
Sodium	nd	nd	nd	nd
Strontium	2300 ± 34	847 ± 5	2531 ± 104	2747
Tellurium	nd	nd	nd	nd
Thallium	< DL	< DL	< DL	< DL
Tin	17.4 ± 0.6	342 ± 7	15.7 ± 0.1	9.9
Titanium	3.30 ± 0.15	8.05 ± 0.14	4.08 ± 0.09	2.50
Uranium	0.19 ± 0.01	0.13 ± 0.001	0.17 ± 0.01	0.18
Vanadium	0.67 ± 0.01	0.89 ± 0.01	0.58 ± 0.02	0.41
Zinc	52.1 ± 1.2	93.5 ± 4.2	37.3 ± 10.5	30.9

[a] Results are the mean ± standard deviation of two replicates (n=2), except for the July Seawater treated sample where the results represent the determination of one composite sample (n=1). [b] nd = not determined. [c] < DL = below detection limit

Table 3. 7 Main heavy metals of concern for seafood and Health Canada maximum allowable levels [43]

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

The main heavy metals of concern for edible seafood and for which Health Canada has established maximum allowable levels (Table 3.7) include arsenic (3.5 ppm), lead (0.5 ppm) and mercury (0.5-1.0 mg/kg).[61] For chitosan intended for use in biomedical/pharmaceutical applications, the heavy metals of concern for which industry has established maximum levels (Table 3.8) 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 biomedical/pharmaceutical chitosan applications also recommends a total heavy metal content of <40 ppm [23, 24].

Copper is an important factor impacting crab quality due to its role as a co-factor for PPO enzymatic reactions causing discolouration of crab known as bluing and melanosis. These biochemical reactions result in blue, blue-gray, brown, and black discolouration in crab meat and shell as previously discussed in section 3.4.1.4.

Calcium and phosphorous were also evaluated as these elements are an indicator of shell hardness which has an impact on chitin extractability, yield, and purity. The exoskeleton of crustaceans, such as lobster and crab, typically consists of an organic matrix of alpha-chitin with an inorganic mineral such as calcium carbonate [25]. Bobelmann *et al.* [25] conducted X-ray spectroscopy studies on American lobster (*Homarus americanus*) shells and *Cancer pagurus* crab shells and found that calcium, magnesium and phosphorous are main components of the cuticles. They also found that the mineral content of the shell increased from the carapace to the claw to the finger, all of which have different requirements for hardness, and that the exoskeleton of the Cancer crab was harder (i.e., had

[61] https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/chemical-contaminants/maximum-levels-chemical-contaminants-foods.html

a higher mineral content) than that of lobster, as crab require a hard, highly mineralized shell. Lage-Yusty *et al.* [7] reported that snow crab shells contained high amounts of calcium > phosphorous > magnesium. Due to limitations of the ICP-MS equipment, magnesium content of the crab samples was not determined.

Table 3. 8 Industry standard for heavy metals in chitosan intended for use in biomedical/pharmaceutical applications [44, 45]

Heavy Metal	Maximum 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

3.4.2.1 Arsenic

Arsenic levels in the samples followed the order 7c > 5c > 6sw > 8sw. Arsenic levels in samples 6sw (June) and 8sw (July) were below the Health Canada maximum level of 3.5 ppm, however samples 5c (June) and 7c (July) were slightly above the Health Canada maximum allowable level for seafood. Samples 6sw and 8sw were treated with seawater which may have had a dilution effect on the levels of arsenic in the samples. All samples had levels of arsenic well above the industry requirement of <0.5 ppm for biomedical chitosan applications.

3.4.2.2 Lead

Levels of lead in the samples followed the order 6sw > 5c> 7c > 8sw. All samples, except sample 6sw, had levels of lead below the Health Canada maximum level (0.5 ppm), and below the industry standard for biomedical chitosan applications (<0.5 ppm).

3.4.2.3 Chromium

Levels of chromium in the samples from highest to lowest were as follows: 6sw > 5c > 7c > 8sw. All samples, except sample 6sw (2.67 ± 0.04 ppm), exhibited levels of chromium below 1.0 ppm, meeting the industry standard for biomedical chitosan applications.

3.4.2.4 Nickel

All samples exceeded the industry standard of <1.0 ppm nickel for biomedical chitosan applications and followed the order 5c > 6sw > 7c > 8sw. Levels of nickel were comparable in samples 5c and 6sw (June control and seawater, respectively). However, sample 7c (July control) had a much higher level of nickel than sample 8sw (July seawater).

3.4.2.5 Cadmium

Cadmium levels in the samples followed the order 6sw >5c >7c >8sw. Levels in all samples exceeded the industry standard of <0.2 ppm for biomedical chitosan applications.

3.4.2.6 Iron

Levels of iron were highest in sample 6sw followed by sample 5c, sample 7c, and sample 8sw respectively. Levels of iron were 3.85 (8sw) to 37.55 (6sw) times higher than the maximum level (<10 ppm) established for biomedical chitosan applications.

3.4.2.7 Copper

Copper concentrations were higher in the June samples (5c and 6sw) compared with the July samples (7c and 8sw) and followed the order 6sw > 5c > 7c > 8sw.

3.4.2.8 Calcium and Phosphorous

The calcium content was lowest in sample 6sw (June seawater) and highest in sample 7c (July control). The July samples, 7c and 8sw, had similar calcium concentrations of 14.25% and 14.10%, respectively. However, the June samples had lower calcium contents with sample 5c (June control) having 10.8% and sample 6sw having only 4.77% calcium. The concentration of phosphorous was lowest in sample 6sw (14,420 ± 416 ppm) and highest in sample 5c (40,605 ± 1892 ppm). However, phosphorous was comparable in the July samples with Sample 7c having 36,530 ppm and sample 8sw containing 37,021 ppm phosphorous. This suggests that sample 6sw contained a higher percentage of meat and organs, and a lower shell content than the other samples. It is also possible that sample 6sw contained soft shell (or new hard shell) crab by-products. Soft shell crabs have been reported to contain lower mineral contents in comparison to hard shell crabs [60]. Soft shell crabs may occur in the spring due to molting and become more prevalent in the summer as the molted crabs become more mobile [26]. While there are fisheries management measures in place to minimize the capture of soft-shell crabs, such as closure of fishing areas during the fishing season when the percentage of soft-shell crabs is high, it can take up to a year for shells to fully harden [26]. Thus, it is possible that some soft shell and/or new hard shell crabs were captured during the 2018 fishing season. Further studies into the variability of

calcium and phosphorus levels within these samples from the 2018 fishing season were beyond the scope of this thesis.

3.4.2.9 Summary of Elemental Analysis

The total concentration of the heavy metals described above ranged from 88.5 ppm (8sw - July seawater) to 538.5 ppm (6sw - June seawater) in the crab samples tested. Metals were highest in the June samples with sample 6sw having the highest total concentration of metals overall. This is consistent with the higher protein and lipid contents found in the June seawater treated samples (Table 3.10 and Table 3.11) as discussed in section 3.4.3. Heavy metals tend to accumulate in the organs and tissues of crustaceans such as crabs and prawns [27, 28]. The higher protein and lipid contents indicate that samples 6sw and 5c may contain higher amounts of hepatopancreas and gills in comparison to the July samples (7c and 8sw). Kim and Yoon [29] demonstrated that copper, arsenic, cadmium, and chromium tend to bioaccumulate in the hepatopancreas and gills of Korean Yeongdeok crab and Russian snow crab. Zinc is most highly concentrated in the leg and body meat of both species, whereas nickel is highest in the shell of the Korean Yeongdeok crab. Rouleau *et al*. [31] measured the accumulation of cadmium in snow crabs (*Chionoecetes opilio*) caught in the Estuary and Gulf of St. Lawrence and reported that cadmium concentrations were highest in the hepatopancreatic tissues. While the concentrations of heavy metals in crabs will vary depending on the marine environment from where they are harvested, these previous studies provide insight regarding the crab components (i.e., tissues and organs) in which these metals are most likely to accumulate.

Sample 6sw had elevated levels of iron, zinc, and copper in comparison to samples 5c, 7c and 8sw. Although sample 6sw had the highest level of copper and iron, both of which may be catalysts for bluing and melanosis discolouration reactions in crab, it ranked highest based on sensory characteristics and had minimum discolouration in comparison to the other crab feedstock samples. Sample 6sw also had the highest salt content of all the samples which may have inactivated the PPO enzyme involved in the bluing and melanosis discoloration reactions. This suggests that storage in seawater which has a salt content of 3.5% may be effective in preventing the blue-black discolouration reactions in crab as previously discussed in section 3.4.1.4.

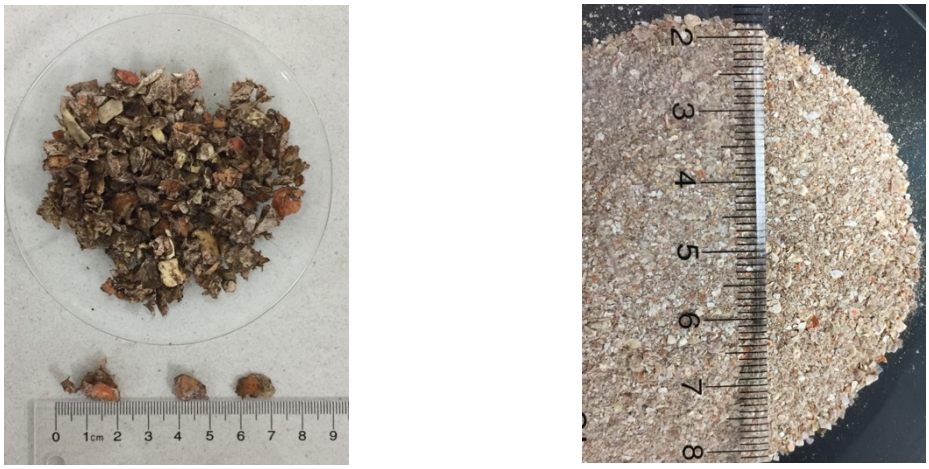
Sample 6sw had the lowest concentrations of calcium and phosphorous in comparison to samples 5c, 7c and 8sw suggesting the presence of softer-shell (i.e., soft shell or new hard shell) crab by-products, or a higher proportion of meat and organs and lower shell content, in the sample. This result may also explain the lower chitin content obtained for sample 6sw (Table 3.13) which is discussed in section 3.4.5.

All samples exceeded the total maximum allowable limit for heavy metals of 40 ppm for biomedical chitosan applications. Samples 5c and 7c slightly exceeded the Health Canada maximum allowable level for arsenic, while sample 6sw exceeded the Health Canada maximum allowable level for lead. However, depending on the end use application and final product form, purification of the crab feedstock materials will likely be required to reduce the concentrations of metals in the final product. However, if the end use application is for a feed application the high levels of iron, zinc and calcium may be beneficial from a nutritive perspective and therefore, it would be important to develop a process to maintain these elements while removing other metals such as arsenic and

cadmium. It is also worth noting that these measurements were performed on the bulk by-product sample and not on the chitin obtained from it. The process of chitin isolation from the bulk sample involves a stepwise series of purification steps (demineralization, deproteination, depigmentation). This process may lead to a product that has a reduced arsenic and/or lead level when compared with these values. The transfer of heavy metals from crab by-products to extracted crab bioproducts is further explored in Chapters 4 and 5 of this thesis. Conclusions regarding the origin of these metals in crab feedstock is beyond the scope of this thesis but is of interest to promote scientific knowledge.

3.4.3 Proximate Composition of Air-Dried and Freeze-Dried Crab Feedstock Samples

Figure 3.9 and Tables 3.9 - 3.11 describe the composition of the crab feedstock samples based on collection pre-treatment method, drying method, and seasonality. Results for proximate composition of the air-dried and freeze-dried samples are reported on a dry weight basis (i.e., % composition after removing all the moisture from the samples).



A B

Figure 3. 9 A - Air dried crab biomass feedstock before milling (13 mm particle size). B - Air dried crab biomass feedstock after milling (1-2 mm particle size)

3.4.3.1 Moisture Content Analysis of Raw Crab Feedstock Samples

The moisture contents of the raw ground crab biomass feedstock samples are presented Table 3.9. The % moisture on a wet weight basis was in the order of sample 6sw > 3sw > 5c > 7c > 8sw > 1c and ranged from 65.60% (1c) to 87.30% (6sw). The seawater treated samples had significantly ($p < 0.05$) higher moisture contents compared to the control samples. The seawater samples ranged from 71.65% (8sw) to 87.30% (6sw) moisture, whereas the control samples ranged from 65.60% (1c) to 75.88% (5c) moisture. The moisture contents of the control samples were similar to that reported by Lage-Yusty *et al*. (72%) [7] and Beaulieu *et al*. (78%) [2] for snow crab shells and snow crab by-products, respectively.

Table 3. 9 Moisture content of raw crab biomass feedstock samples - May, June, and July 2018 [1]

Sample #	Sample Description	% Moisture[2]
1c	May 2, 2018 - Control	65.60 ± 0.41^{a}
3sw	May 2, 2018 - Seawater	84.75 ± 0.73^{b}
5c	June 30, 2018 - Control	75.88 ± 0.55^{c}
6sw	June 30, 2018 - Seawater	87.30 ± 0.52^{b}
7c	July 25, 2018 - Control	75.15 ± 0.36^{c}
8sw	July 25, 2018 - Seawater	71.65 ± 0.12^{d}

[1] *Results represent the mean of 3 determinations (n=3) ± standard deviation.*
[2] *Values with different letters are significantly different ($p < 0.05$) according to Tukey's test.*

The effect of seasonality and pre-treatment method on moisture content was determined by two-factor ANOVA and Tukey's Test. For both the control samples and the seawater treated samples, the June samples had the highest moisture contents which were 75.88% (control) and 87.30% (seawater). ANOVA results indicate that the effects of both seasonality and treatment method on moisture content are significant ($p < 0.05$). For the control samples, moisture content of the May sample was different from June and July (p

< 0.05), whereas the moisture contents of June and July samples were the same ($p > 0.05$). However, for the seawater treated samples, the moisture contents of the May and June samples were the same ($p > 0.05$), whereas the mean moisture content for July was different ($p < 0.05$).

The moisture content of the crab biomass feedstock material is an important consideration for stabilization and further processing of this material into intermediate products such as the dried powders produced for this study. The higher the moisture content of the crab feedstock material, the more complex the stabilization, extraction and purification processes become since the excess moisture must be removed. This requires additional processing steps, additional energy input, and longer processing times, leading to increased processing costs. The seawater pre-treatment method used in this study provides an example of the additional processing steps needed for the higher moisture samples. The seawater treated samples required longer thawing time, a draining step, and a longer drying time compared with the control samples. The seawater treated samples were also more difficult to grind as the shells were softer causing the material to become stuck in the grinder barrel. The control samples thawed within 1-4 hours at 4ºC, whereas the seawater treated samples required 16-36 hours to thaw at 4ºC. The seawater treated samples had to be drained after thawing which is an additional step not required for the control samples. The drained seawater treated samples contained 6.05-11.42% more moisture than the control samples, and therefore required additional drying time for stabilization. For example, even after 48 hours of drying time, the freeze-dried seawater treated samples had moisture contents above 20%, whereas the control samples had moisture contents around 10%. While it may be possible to reduce process times through

modification of the thawing/draining/drying process, optimization of this methodology was beyond the scope of this thesis.

3.4.3.2 Proximate Composition of Air-Dried and Freeze-Dried Crab Feedstock Samples

The proximate compositions of the air-dried and freeze-dried crab feedstock samples are presented in Table 3.10 and Table 3.11 respectively. Proximate composition data for the air-dried and freeze-dried samples were compared using a paired T-Test to identify: (1) if there were significant differences between the two drying methods; and (2) if there were significant compositional differences between the control and the seawater treated samples. One-way ANOVA and Tukey's test were conducted to determine whether seasonality contributed to variances in proximate composition of the samples.

It should be noted that for the May samples, only the control sample was freeze-dried due to limited availability of the May seawater treated sample. Therefore, for the freeze-dried samples only results for June and July were compared for compositional differences between the control and the seawater treated samples.

3.4.3.2.1 Ash Content

For the air-dried samples (Table 3.10) the ash content ranged from 34.40% to 39.71% and was in the order 8sw > 3sw > 6sw > 5c > 7c > 1c. The seawater treated air-dried samples had higher ash contents ranging from 37.18% to 39.71%, compared with a range of 34.40% to 35.53% for the control samples. The difference in ash contents between the control and seawater treated air-dried samples was significant ($p < 0.05$). The ash content of the June samples was also significantly different ($p < 0.05$) than the May and

July samples. These results indicate that both seasonality and pre-treatment method affect the ash content of the air-dried crab by-product samples ($p < 0.05$).

The ash contents of the freeze-dried samples (Table 3.11) ranged from 35.52% to 38.50% and was in the order of Sample 6sw > 8sw > 7c > 1c> 5c. The seawater treated freeze-dried samples had higher ash contents ranging from 38.28% to 38.50%, compared with a range of 35.52% to 36.41% for the control samples. This difference in ash contents between the control and seawater treated freeze-dried samples was significant ($p < 0.05$). There were no differences in ash content between the June and July ($p > 0.05$) freeze-dried samples. The higher ash contents found in the seawater treated samples are mainly attributed to the salt content (3.5% NaCl) of seawater. The seawater treated samples had significantly higher ($p < 0.05$) salt contents in comparison to the control samples as discussed in section 3.4.3.2.4.

3.4.3.2.2 Total Nitrogen Content and Protein Estimation

Air-Dried Samples: Total nitrogen content of the air-dried samples (Table 3.10) followed the order 6sw > 5c = 7c > 1c > 3sw > 8sw and ranged from 4.75% to 6.43% (db). The % total nitrogen was highest in sample 6sw (June seawater) and lowest in samples 3sw (May seawater) and 8sw (July seawater) at 4.75% and 4.88%, respectively. The mean total nitrogen contents were similar for all the control samples with a narrow range between 5.93% and 6.05%. The difference in the total nitrogen contents of the control and seawater air-dried samples were significant ($p < 0.05$) overall. However, the difference in total nitrogen content between the control samples and the June seawater (6sw = 6.43%) samples

was not significant ($p > 0.05$). There were no differences in total nitrogen content due to seasonality ($p > 0.05$).

Freeze-Dried Samples: Total nitrogen content of the freeze-dried samples (Table 3.11) were higher in the control samples compared with the seawater treated samples in the following order 1c > 5c > 7c > 6sw > 8sw and ranged from 5.37% to 6.57%. The % total nitrogen was highest in sample 1c (May control) and lowest in 8sw (July seawater). The mean nitrogen contents were similar for all the control samples with a narrow range of 6.36-6.57%. The difference in total nitrogen content of the control and the seawater treated freeze-dried samples was significant ($p < 0.05$). However, there were no differences in total nitrogen content due to seasonality at $p > 0.05$.

Summary: The results indicate that treatment with seawater resulted in lower total nitrogen content for all samples (except air-dried sample 6sw which had the highest nitrogen content of all the samples). This suggests that some of the protein was either suspended and/or solubilized in the seawater, possibly due to the salting in effect, however, the remaining storage seawater was not analyzed to confirm this hypothesis. The higher nitrogen content found in air-dried sample 6sw is likely the result of a non-homogenous sample containing more meat, organs and tissues, and less shell (perhaps due to the presence of soft shell or new hard shell crab as previously discussed in section 3.4.2.8), in comparison to the other samples. This likely contributed to a higher protein nitrogen content in this sample. A wide range of factors affect the aqueous solubility of proteins including pH, ionic strength, addition of salts and the type of protein, which can have either a salting in (increased solubility) or salting out (decreased solubility) effect on the protein [42]. Dahal & Schmit [42] developed a simplified protein aggregation model to predict

Table 3. 10 Proximate composition of air-dried crab biomass feedstock samples - May, June, July 2018 [1]

Sample #	Sample Description	% Ash (db)	% Total Nitrogen (db)	% Protein (db)	% Lipid (db)	% NaCl (db)
1c	May 2, 2018 - Control	34.40 ± 0.45[a]	5.93 ± 0.08[a]	29.30 ± 0.40[a]	3.22 ± 0.63[a]	4.95 ± 0.12[a]
5c	June 30, 2018 - Control	36.94 ± 2.45[b]	6.05 ± 1.16[a]	29.88 ± 5.73[a]	8.99 ± 0.42[b]	7.47 ± 0.27[b]
7c	July 25, 2018 - Control	34.52 ± 0.36[a]	6.05 ± 0.19[a]	29.88 ± 0.95[a]	3.16 ± 0.82[a]	6.57 ± 0.18[c]

Sample #	Sample Description	% Ash (db)	% Total Nitrogen (db)	% Protein (db)	% Lipid (db)	% NaCl (db)
3sw	May 2, 2018 - Seawater	38.64 ± 0.15[c]	4.75 ± 0.05[b]	23.48 ± 0.23[b]	7.09 ± 0.01[c]	12.95 ± 0.08[d]
6sw	June 30, 2018 - Seawater	37.18 ± 0.08[b]	6.43 ± 0.04[a]	31.78 ± 0.19[a]	9.69 ± 0.57[b]	24.46 ± 0.04[e]
8sw	July 25, 2018 - Seawater	39.71 ± 0.03[c]	4.88 ± 0.27[b]	24.13 ± 1.34[b]	1.51 ± 0.74[d]	7.16 ± 0.26[f]

[1] Results represent the mean of 3 determinations (n=3) ± standard deviation and are reported on a dry weight basis (db). [2] Values in the same column with different letters are significantly different ($p < 0.05$) according to Tukey's test.

Table 3. 11 Proximate composition of freeze-dried crab biomass feedstock samples - May, June, July 2018 [1]

Sample #	Sample Description	% Ash (db)	% Total Nitrogen (db)	% Protein (db)	% Lipid (db)	% NaCl (db)
1c	May 2, 2018 - Control	35.91 ± 0.41[a]	6.57 ± 0.19[a]	32.47 ± 0.94[a]	3.62 ± 0.29[a]	4.14 ± 0.16[a]
5c	June 30, 2018 - Control	35.52 ± 1.74[a]	6.37 ± 0.04[a]	31.46 ± 0.22[a]	4.52 ± 0.77[b]	6.64 ± 0.02[b]
7c	July 25, 2018 - Control	36.41 ± 0.33[a]	6.36 ± 0.18[a]	31.40 ± 0.88[a]	0.84 ± 0.27[c]	6.55 ± 0.11[b]

Sample #	Sample Description	% Ash (db)	% Total Nitrogen (db)	% Protein (db)	% Lipid (db)	% NaCl (db)
3sw	May 2, 2018 - Seawater	Not Determined				
6sw	June 30, 2018 - Seawater	38.50 ± 0.36[b]	5.78 ± 0.10[b]	28.55 ± 0.49[b]	7.53 ± 0.35[d]	12.21 ± 0.12[c]
8sw	July 25, 2018 - Seawater	38.28 ± 0.36[b]	5.37 ± 0.15[c]	26.51 ± 0.72[c]	0.28 ± 0.27[c]	6.26 ± 0.08[d]

[1] Results represent the mean of 3 determinations (n=3) ± standard deviation and are reported on a dry weight basis (db). [2] Values in the same column with different letters are significantly different ($p < 0.05$) according to Tukey's test.

salting-in and salting-out trends and concluded that Coulomb energy, salt entropy, and protein-ion binding are the key factors affecting protein solubility. While beyond the scope of this chapter, understanding how such factors affect protein solubility may be an important consideration for the future development of stabilization methods for crab by-products.

The protein content of the crab feedstock samples was calculated from the total Kjeldahl nitrogen content according to equation (3.3) using a nitrogen-to-net protein conversion factor 4.94 [46]. The traditional nitrogen-to-net protein conversion factor of 6.25 is typically used to calculate the protein value of a food, however, this value has been shown to vary significantly, and has been reported to be 21% lower for fish and fish products which contain a significant amount of non-protein nitrogen [46].

$$\% \text{ Net Protein} = \% \text{ Total Kjeldahl Nitrogen} \times 4.94 \qquad \text{(Eqn. 3.3)}$$

For crustaceans, a significant amount of non-protein nitrogen comes from chitin. Therefore, to avoid over estimation of the true protein content of the crab feedstock samples, the protein content was calculated using the corrected nitrogen-to-net protein conversion factor of 4.94. Ideally, the true protein content, or net protein, can be calculated by the sum of the 20 amino acid residues. Although the amino acid composition of each sample was evaluated (refer to section 3.4.7) only 15 amino acid residues were detected and therefore this method could not be used.

The protein content of the crab feedstock samples evaluated in this study were comparable to that reported by other authors [2, 7, 9, 31], but higher than that reported by

Shahidi & Synowiecki [4] who reported a total protein content of 15.68-23.95% (db) for snow crab shell waste collected from the same crab processor who supplied the raw material used for this study. The main difference between the protein content reported in this study and that reported by Shahidi & Synowiecki [4] is likely because this study used crab by-products that were coarsely ground as a composite sample without separation of the shells and protein. Whereas Shahidi & Synowiecki [4] separated the crab shell by-products into various parts (e.g., backs, legs, claws) and analyzed the protein content in each individual component. Other sources of variation between the two studies may include differences in the sample collection and preparation methods, differences in the processing equipment used at the plant (e.g., the plant was destroyed by fire in 2016[62] and was completely rebuilt and equipped with state-of-the-art crab processing equipment including new butchering machines, crab leg cutters, and offal collection and shredding systems), biological variations within the crab stock from 1991 and 2018, and the inherent heterogeneous nature of snow crab processing by-products.

3.4.3.2.3 Lipid Content

Air-Dried Samples: Lipid contents of the air-dried samples (Table 3.10) varied from 1.51% in sample 8sw (July Seawater) to 9.69% in sample 6sw (June seawater) and followed the order 6sw > 5c > 3sw > 1c > 7c > 8sw. The variation between lipid contents of the control and seawater treated samples was not significant overall ($p > 0.05$). However, seasonal variations in lipid contents were suggested by one-way ANOVA and Tukey's Test ($p < 0.05$). The June samples (control and seawater) had higher lipid contents than the May

[62] https://www.cbc.ca/news/canada/newfoundland-labrador/bay-de-verde-fire-homes-damaged-by-smoke-1.3530490

and July samples. Lipid content followed the seasonal order of June > May = July for the control samples and June > May > July for the seawater treated samples. These results were significant at the 95% confidence level.

Freeze-Dried Samples: For the freeze-dried samples (Table 3.11), lipid contents varied from 0.28% in sample 8sw (July Seawater) to 7.53% in sample 6sw (June seawater) and followed the order 6sw > 5c > 1c > 7c > 8sw. Both the June samples (control and seawater) had higher lipid contents than all other samples whether control or seawater treated. The variation between lipid contents of the control and seawater treated samples were not significant overall ($p > 0.05$), whereas the difference in lipid contents between the June control and June seawater treated samples was significant ($p < 0.05$). ANOVA and Tukey's Test indicate that seasonal variations in lipid contents are significant ($p < 0.05$). For the control samples, lipid contents were significantly different ($p < 0.05$) for May, June and July and followed the order June > May > July with the lipid content being significantly lower for most, but not all, of the July samples.

Summary: The mean and range of total lipid contents for the crab biomass samples evaluated in this study (0.28-9.69%) are higher than that reported by Shahidi & Synowiecki (0.1-1.4%) [4] for snow crab shell waste collected from the same crab processor, but were lower than the total lipid contents for snow crab shell by-products reported by Beaulieu *et al*. [2], and Lage-Yusty *et al*. [7], which were 14.8% and 17.1%, respectively. Variations in the reported lipid contents are likely due to differences in the sample collection and preparation methods, and the analysis methods (i.e., different extraction reagents) used for each study. Crab by-products in this study were collected as composite samples whereas in the Shahidi [4] study crab shell by-products were separated into their component parts prior

to analysis. Other sources of variation include seasonal variability, which was not studied by the other authors, biological variations in crab stocks, and the heterogeneous nature of snow crab processing by-products.

3.4.3.2.4 Salt Content

Air-Dried Samples: The salt contents of the air-dried samples (Table 3.10) were higher overall in the seawater treated samples (7.16-24.46%) compared with the control samples (4.95-7.47%). The % salt followed the order 6sw > 5c > 3sw > 8sw > 7c > 1c. Variations in salt contents were significant ($p < 0.05$) between the control and seawater treated samples and is attributed to the salt content of the seawater (3.5%). Salt contents also varied significantly ($p < 0.05$) based on seasonality. For the control samples salt content followed the order June > July > May, however, for the seawater treated samples the order was June > May > July.

Freeze-Dried Samples: The % salt in the freeze-dried samples (Table 3.11) was similar to the air-dried samples and followed the order 6sw > 5c > 7c > 8sw > 1c. Variations in salt contents between the control and seawater treated samples were significant ($p < 0.05$). Salt contents also varied significantly based on seasonality ($p < 0.05$). For the control samples differences in salt contents: (1) were significant for May and June, and May and July; (2) were similar for June and July; and (3) followed the order June = July > May. However, for the seawater treated samples the difference between the June and July samples was significant ($p < 0.05$). This could be the result of the higher protein content in the June seawater treated sample allowing more salt to be absorbed.

Summary: The seawater treatment increased the % salt content of the samples by 2-4 times over the control treatment. However, there was also some variability in salt contents between the air-dried and freeze-dried seawater samples. This could have been influenced by the ratio of crab by-product and seawater added to each collection pail, and the distribution of shell and protein/meat in each sample. These variables were not well controlled due to the nature of sample collection at the processing plant. Furthermore, no studies were performed to try and reduce % salt content in the seawater treated samples. This might be an option in future studies if pre-treatment with seawater is deemed desirable.

3.4.3.2.5 Summary of Proximate Composition of Crab Feedstock Samples

There were no significant differences ($p > 0.05$) identified in the % ash, % protein and % salt between the air dried and freeze-dried crab feedstock samples. A significant difference was identified between the lipid contents of the air-dried and freeze-dried samples at $p < 0.05$. These results indicate that the drying method does not affect the ash, protein, or salt content of the samples, but may have a moderate effect on the lipid content of the samples. The variations in lipid content between the air dried and freeze-dried samples may also be affected by the heterogeneous nature of the crab by-product material due to the difficulty in obtaining a homogenous sample from the processing plant's by-product collection container, as well as the difficulty in grinding and milling some of the samples at the MI pilot plant.

The differences in the ash, protein and salt contents between the control samples and the seawater treated samples were significant ($p < 0.05$). However, the difference in lipid contents between the control and seawater treated samples was not significant ($p >$

0.05). These results suggest that treating the crab biomass samples with seawater: (1) has a major impact on the % ash, % protein and % salt content; but (2) does not influence the lipid content of the samples. The increases in % ash, and % salt, were expected upon seawater treatment due to sodium. However, the significant decrease in % protein was unforeseen and may be due to the salting in effect during storage in seawater resulting in solubilization of some proteins.

Results of ANOVA and Tukey's test confirmed that seasonal variations affect the % lipid content and % salt content of the samples but have little effect on % protein and % ash content. The June samples had a higher lipid content and salt content compared with the May and July samples

3.4.4 Astaxanthin Content of Air-Dried and Freeze-Dried Crab Biomass Feedstock Samples

Astaxanthin is a carotenoid pigment that gives crab its orange-red colour. It is used as a valuable feed ingredient in salmonid and poultry diets, and as an antioxidant natural health supplement. This section provides comparative data of the effect of collection treatment method, drying method, and seasonality on the total astaxanthin content of the crab feedstock samples (Table 3.12). Results represent the total astaxanthin content of the air-dried and freeze-dried samples and are reported on a dry weight basis (i.e., µg of astaxanthin per gram of sample after removing all the moisture from the samples).

Results for the air-dried and freeze-dried samples were compared using a paired T-Test to identify if there were significant differences between the two drying methods. Additionally, the results were compared using the paired T-Test to determine whether there

were significant compositional differences between the control and the seawater treated samples. One-way ANOVA and Tukey's test were used to assess seasonal variations.

Table 3. 12 Total astaxanthin content (µg/g) of air-dried and freeze-dried crab feedstock samples - May, June, July 2018 [1]

Sample #	Sample Description	Air Dried µg/g (db)	Freeze Dried µg/g (db)
1c	May 2, 2018 - Control	19.34 ± 0.82[a]	58.45 ± 0.38[a]
5c	June 30, 2018 - Control	13.75 ± 0.28[b]	59.40 ± 0.14[a]
7c	July 25, 2018 - Control	8.76 ± 1.43[c]	30.02 ± 3.62[b]
3sw	May 2, 2018 - Seawater	19.62 ± 0.62[a]	n.d.[2]
6sw	June 30, 2018 - Seawater	19.37 ± 2.92[a]	46.46 ± 0.74[c]
8sw	July 25, 2018 - Seawater	3.26 ± 0.29[d]	34.99 ± 3.73[d]

[1] Results represent the mean of 2-4 determinations (n=2-4) ± standard deviation and are reported on a dry weight basis (db). [2] n.d. = not determined. [3] Values in the same column with different letters are significantly different ($p < 0.05$) according to Tukey's test.

The results show that the freeze-dried samples had much higher astaxanthin contents compared with the air-dried samples ($p < 0.05$). The astaxanthin content for the air-dried samples ranged from 3.26 µg/g to 19.62 µg/g and followed the order 3sw = 6sw = 1c > 5c > 7c > 8sw. The astaxanthin content of the freeze-dried samples ranged from 30.03 µg/g to 59.40 µg/g and followed the order 5 c = 1c > 6sw > 8sw > 7c. For the air-dried samples, the results indicate that the difference in astaxanthin content between the control and seawater treated samples may be significant ($p = 0.05$). However, the difference in astaxanthin content between the May control (1c) and the May seawater treated (3sw) samples was not significant ($p > 0.05$). For the freeze-dried samples, the paired T-Test results indicate that the difference in astaxanthin contents between the control and sea water treated samples is not significant ($p > 0.05$). These results confirm that the astaxanthin

content is affected by the drying method with astaxanthin content being higher in the freeze-dried samples. The effect of the seawater treatment on astaxanthin content is inconclusive.

With respect to seasonal variations, the May and June samples had similar astaxanthin contents, whereas the July samples had much lower astaxanthin contents. This trend was the same for both the air-dried and freeze-dried samples suggesting that seasonality also influences astaxanthin content of the samples. For the air-dried samples the mean astaxanthin contents for all three months were different for the control samples ($p < 0.05$). For the seawater treated samples, the astaxanthin content was the same for May and June with these samples having significantly higher ($p < 0.05$) astaxanthin contents than the July sample. For the freeze-dried samples, the mean astaxanthin contents for the control samples were the same for May and June, with these samples having significantly higher ($p < 0.05$) astaxanthin contents than the July sample. For the freeze-dried seawater treated samples the astaxanthin content for June was significantly higher ($P < 0.05$) than the July sample. These results suggest that there are seasonal variations in astaxanthin content with astaxanthin contents being similar and higher in May and June, but much lower in July.

Shahidi & Synowiecki [4] reported that the crab back shell (carapace) contained the highest amounts of carotenoids in snow crab by-products and ranged from 119 – 139 μg/g (db) in hard- and soft-shell crab backs respectively, however, other segments of crab shell contained only 16-34 μg/g (db) carotenoids. Shahidi & Synowiecki [4] also reported that the astaxanthin diester was the major carotenoid present making up 56.47-56.57% of the total carotenoids. The total astaxanthin content in hard shell crab backs on a dry matter basis was 82.84% (98.58 μg/g) and 89.45% (124.34 μg/g) in soft shell crab backs [4].

Manu-Tawiah & Haard [32] reported that shell waste from snow crab contain about 330 μg/g total astaxanthin. According to Higuera-Ciapara *et al*. [33], the carotenoid content in shrimp and crab by-products ranges from 119-148 μg/g. Lage-Yusty *et al*. [7] reported an astaxanthin content of 94.9 μg/g (db) in snow crab shells.

The astaxanthin contents of snow crab by-products reported in the literature are much higher than the values obtained in this study and are likely due to differences in sample collection and preparation methods, and the evaluation methods used. The samples in this study were prepared as a composite sample and therefore contained other crab processing by-products, not just the shell backs, which would partially account for the lower astaxanthin contents present. The air-drying method used in this study is not suitable for recovery/preservation of carotenoids/astaxanthin due to their high susceptibility to oxidative degradation under thermal processing conditions [33, 34]. Therefore, the low astaxanthin contents in the air-dried samples can be attributed to the high temperature drying treatment. The higher astaxanthin contents in the freeze-dried samples also support this observation. The levels of astaxanthin present in the freeze-dried samples were comparable to that reported by Shahidi & Synowiecki [4] for the "other segments of crab shell". These data suggest that if astaxanthin recovery is desirable, the different components of crab by-product would likely have to be separated. This may be challenging to implement at a crab processing plant and may require reconfiguration of the processing line and by-product collection systems.

3.4.5 Chitin Content of Air-Dried and Freeze-Dried Crab Biomass Feedstock Samples

Table 3.13 compares the theoretical crude chitin content (calculated from the proximate compositions presented in Tables 3.10 and 3.11 using equation 3.4), to the actual crude chitin content obtained through demineralization and deproteination of the air-dried and freeze-dried crab feedstock samples. Chitin content is reported on a dry weight basis as [mass chitin/mass sample] x 100. Recovered yield is calculated as [actual chitin content/theoretical chitin content] x 100. Quality parameters, including residual protein (determined by Modified Lowry method), ash content, and chitin nitrogen (determined by calculation) of the extracted chitin are presented in Table 3.14. Each parameter is reported as [mass/mass sample] x 100 on a dry weight basis.

$$\% \text{ Theoretical Crude Chitin} = 100\% - \% \text{ ash} - \% \text{ crude protein} - \% \text{ fat} \qquad \text{(Eqn. 3.4)}$$

Since carbohydrate in crustacean shell by-products is mainly present in the form of chitin [58], and assuming the nitrogen-to-net protein conversion factor of 4.94 is an accurate estimate of net protein content [46], Eqn. 3.4 should deliver a reasonable estimate of the theoretical crude chitin content in the samples, provided the composition of the other components (water, mineral, and lipids) are accurately known. Using total nitrogen content, Diaz-Rojas *et. al*. [58] similarly proposed that a more complex mathematical model could be used to estimate the chitin and protein content during the isolation of chitin from shrimp shell waste, provided the proximate composition of the sample is accurately known. For this study and the calculation of % theoretical crude chitin it was assumed that the

proximate composition (moisture, ash, protein, and lipid) of the samples was accurate based on the proximate compositions presented in Tables 3.10 and 3.11, and that all carbohydrate present in the samples was in the form of chitin.

Table 3. 13 Theoretical and actual chitin yield of air-dried and freeze-dried crab feedstock samples - May, June, July 2018

Sample #[b]	Air Dried[a]			Freeze Dried[a]		
	% Crude Chitin			% Crude Chitin		
	Theoretical[c]	Actual[d]	Recovered Yield[e]	Theoretical[c]	Actual[d]	Recovered Yield[e]
1c - May	33.08	19.26	58.22	28.00	16.59	59.25
5c - June	24.19	20.15	83.30	28.5	12.16	42.67
7c - July	32.44	16.59	51.14	31.35	17.03	54.32
6sw - June	21.35	8.34	39.06	25.42	9.70	38.16
8sw - July	34.65	25.12	72.50	34.93	19.11	54.71

[a] Due to the small sample size available n=1. [b] c = control, sw = seawater. [c] Calculated using equation (3.4). [d] % Actual chitin yield is reported as [mass chitin ÷ mass sample] x 100 and is reported on a dry weight basis (db). [e] % Recovered Yield = [Actual chitin content ÷ Theoretical chitin content] x 100.

Chitin is an important product that has commercial applications and can be extracted from snow crab shells. A preliminary assessment of the chitin content, recovery and quality was conducted to determine how these parameters were affected by the various pre-treatment and drying methods, and seasonality. However, the chitin extraction process was not optimized and was based on chemical extraction methods using HCl and NaOH which have been shown to cause modifications to the native chitin molecule, such as depolymerization and deacetylation [35, 40]. A more detailed assessment of chitin content, recovery and quality is undertaken in Chapter 6.

The crude chitin content and percent chitin recovery (Table 3.13) for the air-dried and freeze-dried crab biomass samples were compared using a paired T-test. The paired T-test was also used to compare the chitin content of the control and seawater treated samples.

Due to the small sample size available only one determination for actual chitin content was possible. Based on the results, the theoretical chitin yields for both the air-dried and freeze-dried samples were higher than the actual chitin yields obtained.

For the air-dried samples: (1) Theoretical chitin content ranged from 21.35% to 34.65% and followed the order 8sw > 1c > 7c > 5c > 6sw; (2) Actual chitin yield ranged from 8.34% to 25.12% and followed the order 8sw > 5c > 1c > 7c > 6sw; and (3) % recovered chitin ranged from 39.06% to 83.30% and followed the order 5c > 8sw > 1c > 7c > 6sw. The paired T-test indicates that there is a significant difference between the theoretical and actual chitin yields for the air-dried samples ($p < 0.05$). There was no difference between the chitin contents of the air-dried control and the air-dried seawater treated samples ($p > 0.05$). These results indicate that the chitin content of the air-dried samples is not affected by the pre-treatment method (i.e., control vs seawater).

For the freeze-dried samples: (1) Theoretical chitin content ranged from 28.0% to 34.93% and followed the order 8sw > 7c > 5c > 1c > 6sw; (2) Actual chitin content ranged from 9.70% to 19.11% and followed the order 8sw > 7c > 1c > 5c > 6sw; and (3) % chitin recovery ranged from 54.37 – 85.56% and followed the order 1c > 7c > 8sw > 5c > 6sw. The paired T-test results indicate that there is a significant difference between the theoretical and actual chitin yields of the freeze-dried samples ($p < 0.05$). There was no difference between the chitin contents of the freeze-dried control and the freeze-dried seawater treated samples ($p > 0.05$) indicating that the chitin content of the freeze-dried samples is not affected by the pre-treatment method (i.e., control vs seawater).

A comparison of the chitin contents (actual and theoretical) and the % chitin recovery of the air-dried and freeze-dried samples revealed no significant differences ($p >$

0.05), confirming that the drying method had no effect on chitin content. However, there was a significant difference ($p < 0.05$) between the actual chitin content and % chitin content recovered for the June control and June seawater treated samples with the % recovery being higher for the control samples. The June seawater treated samples also had the highest salt contents suggesting that the seawater treatment (or salt) may interfere with chitin recovery. The % yield recovery was higher for the air-dried samples (avg = 60.8%) in comparison to the freeze-dried samples (avg = 49.8%), and while this variance was not statistically significant ($p > 0.05$), in a commercial application this 11% yield difference could have major implications for the economic viability of the process.

The effect of seasonality on chitin content was also evaluated. This assessment resulted in the observation that actual chitin yield followed the order: July (avg = 19.5%) > May (avg = 17.9%) > June (avg = 12.6%). These results, while not significant ($p > 0.05$), indicate that chitin contents are similar and higher in the May and July samples, and lower in the June samples. This result is likely due to a higher proportion of meat and organs as suggested by the higher lipid and protein contents in the June samples (Tables 3.10 and 3.11).

The chitin contents of the May and July crab biomass samples are comparable with chitin contents reported for snow crab and other crustacean by-products, whereas the June samples had lower chitin contents as previously discussed. Shahidi & Synoweicki [4] reported chitin contents of 18.7-32.2% in snow crab by-products; Younes & Rinaudo [35] reported that shrimps and crabs contain 15-40% chitin; and Beaulieu *et al*. [2] reported 16.25% chitin in snow crab by-products. The % chitin recovered from the crab biomass samples in this study ranged from 38.2-83.3%, with an average recovery of 55%, whereas

Shahidi & Synowiecki [4] reported 86% chitin recovery from snow crab shells. Factors affecting the % chitin recovery in the current study may include shell particle size; effectiveness of the demineralization and deproteination steps; loss of fine shell particles during washing; shell:meat ratio of crab processing by-products; seasonality; presence of soft shell/new hard-shell crab; and salt content.

Chitin quality (Table 3.14) was evaluated for each of the chitin samples obtained. The results obtained for residual protein, chitin nitrogen and ash content are consistent with values reported in the literature for shellfish chitin [4, 5, 36]. The residual protein content was < 1% for all samples which is an indication that the deproteination step of the chitin extraction process was effective. The % chitin nitrogen was also consistent with that reported in the literature and ranged from 5.20-6.47% but is lower than the %N for pure fully acetylated chitin (6.9%). The June seawater treated sample (6sw) had the lowest % chitin nitrogen. This suggests there are impurities in the recovered chitin samples such as salt and protein which can be seen in Table 3.14. The ash content was <1% for all samples except for the May freeze-dried control sample (1c) and the June seawater treated sample (6sw). This indicates that the demineralization step for these sample was incomplete. The June seawater treated sample may also have been contaminated with salt. The demineralization step was effective for all other samples as indicated by the low ash contents (0.09-0.64%). Statistically there were no significant differences ($p > 0.05$) in the residual protein, chitin, or ash contents between the air-dried and freeze-dried samples. However, for commercial applications even small differences of 1-2% can have major impacts on scalability of the process.

Table 3. 14 Quality of chitin recovered from air-dried and freeze-dried crab feedstock samples - May, June, July 2018

Sample ID	Air Dried			Freeze Dried		
	% Residual Protein [a]	% Chitin Nitrogen [b]	% Ash [c]	% Residual Protein [a]	% Chitin Nitrogen [b]	% Ash [c]
1c - May	0.59 ± 0.31	6.22	0.22	0.08 ± 0.03	6.24	1.13
5c - June	0.41 ± 0.45	6.32	0.28	0.05 ± 0.002	6.34	0.64
6sw - June	0.002	5.20	1.08	0.04 ± 0.04	6.18	0.19
7c - July	0.13 ± 0.06	6.39	0.09	0.103	6.46	0.43
8sw - July	0.65 ± 0.61	6.07	0.14	0.041	6.20	0.13

[a] % residual protein = [mass protein ÷ mass sample] x 100 and represents mean ± standard deviation where n=2. n = 1 where standard deviation is not reported. Results are reported on a dry weight basis (db).
[b] % chitin nitrogen = % total nitrogen - % protein nitrogen. Results are reported on a dry weight basis. (db)[c] % ash represents value of n=1 due to the small sample size available. Results are reported on a dry weight basis (db).

3.4.6 Lipid Profiles and Fatty Acid Composition of Air-Dried and Freeze-Dried Crab Feedstock Samples

The lipid profiles and fatty acid compositions of the air-dried and freeze-dried crab feedstock samples are presented in Table 3.15 and Table 3.16, respectively. Results are expressed as percentage (%) of the total lipid in the extract by weight. The standard errors of the variability across the samples are shown in and Figure 3.10 and Figure 3.11 for the lipid profiles and fatty acid compositions, respectively.

The total lipid content for the air-dried samples was previously reported and ranged from 1.51 - 9.69% (Table 3.10) with the highest fat content found in the June samples. For the freeze-dried samples the lipid content, as previously reported in Table 3.11, ranged from 0.28 - 7.53%, with the June samples also containing the highest fat content. The total lipid content and range were different in this study than that reported in other studies [2, 4, 7]. Differences in the sample collection and preparation methods used, and the heterogeneous nature of snow crab processing by-products resulting in different shell:meat ratios in some samples were thought to be the main sources of variation between the different studies.

The lipid class profiles (Table 3.15 and Figure 3.10) indicate that triacylglycerols (avg = 29.65-30.31%), phospholipids (avg = 25.44-29.89%), free fatty acids (avg = 15.04-22.78%), sterols (avg = 13.13-13.69%) and acetone mobile polar lipids (avg = 7.00-9.84%) make-up the major lipid classes in the snow crab processing by-products. Similar results were reported by Beaulieu *et al*. [2]. Seasonal variations in the lipid class content were not significant ($p > 0.05$). There were no differences in the lipid class content between the air-dried and freeze-dried samples for triacylglycerols, phospholipids, sterols, or acetone mobile polar lipids ($p > 0.05$). However, a significant difference was identified between the free fatty acid (FFA) content of the air-dried and freeze-dried samples ($p < 0.05$) with the free fatty acid content being higher in the freeze-dried samples except for sample 7c (July control). While this result suggests that freeze-drying may increase the free fatty acid content of the samples, a more likely reason for the higher FFAs in the May and June freeze-dried samples could be due to lipid hydrolysis during frozen storage prior to drying. Milled samples were removed from frozen storage within 5 months for air drying and within 6-8 months for freeze drying. The freeze-dried samples were held in frozen storage for an additional 1-3 months prior to drying which may have resulted in lipid hydrolysis and a subsequent higher FFA content in the May and June freeze-dried samples in comparison to the air-dried samples.

There were no differences in the lipid class content between the control and seawater treated samples for triacylglycerols, or sterols ($p > 0.05$). However, differences in phospholipids, free fatty acids and acetone-mobile polar lipids between the control and seawater treated samples were significant ($p < 0.05$). The seawater treated samples were higher in free fatty acids and acetone mobile polar lipids, and lower in phospholipids

compared to the control samples. These results suggest that seawater may have contributed to hydrolysis of phospholipids possibly due to the additional water content and the presence of NaCl. Studies have been conducted on the effect of NaCl content on lipid hydrolysis with varying results. Some studies indicate that the increase of NaCl content can promote lipid hydrolysis, yet others found that the effect of NaCl content was not significant [54, 55]. This ambiguous result was evaluated by Tunieva *et al*. [59] in salted meat products with the conclusion that addition of salt up to 2.0% had an inhibitory effect on fat hydrolysis and oxidation, but at levels above 3.5% NaCl acted as a catalyst initiating oxidation of fatty acids.

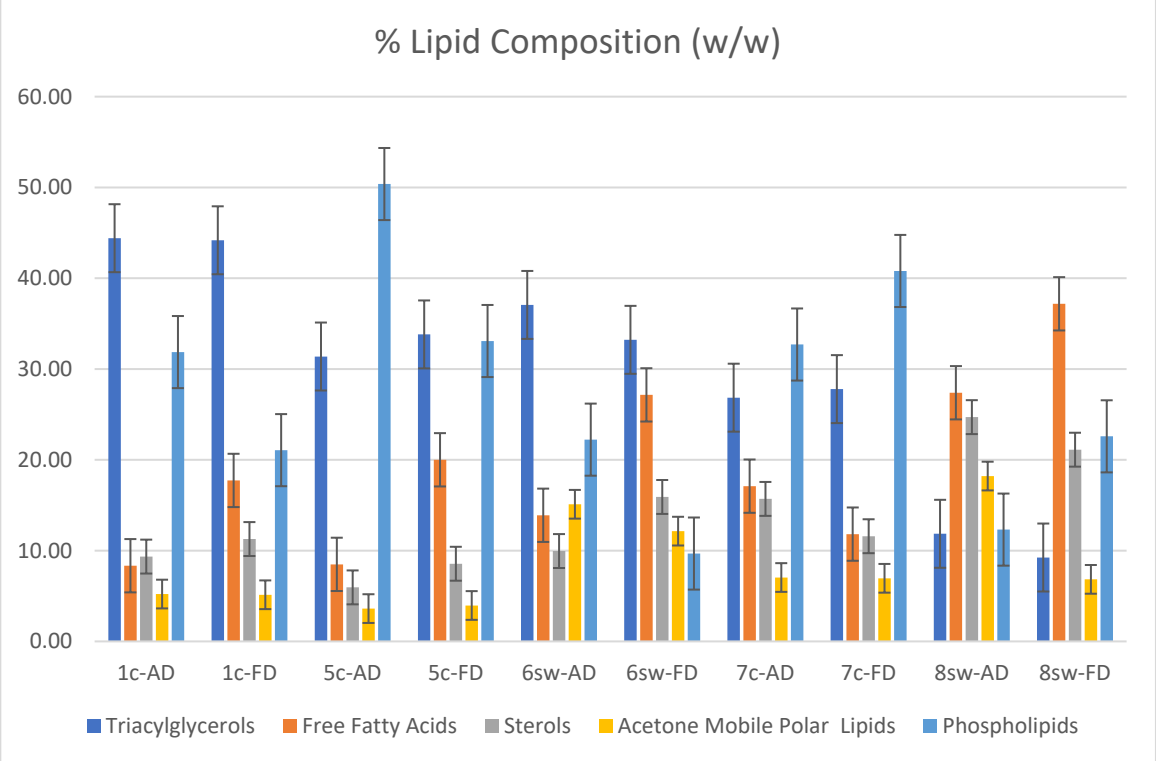


Figure 3. 10 Lipid profiles of extracted lipids obtained from air-dried and freeze-dried crab biomass feedstock samples for May, June, July 2018 with standard error bars, and where c = control, sw = seawater treated, AD = air-dried and FD = freeze-dried.

Table 3. 15 Lipid profiles of extracted lipids obtained from air-dried and freeze-dried crab biomass feedstock samples - May, June, July 2018 [1]

% Lipid Composition	May[6]		June[6]				July[6]			
	1c-AD	1c-FD	5c-AD	5c-FD	6sw-AD	6sw-FD	7c-AD	7c-FD	8sw-AD	8sw-FD
Triacylglycerols[2]	44.41	44.18	31.38	33.81	37.06	33.21	26.84	27.79	11.85	9.24
Free Fatty Acids[3]	8.34	17.73	8.49	20.00	13.89	27.15	17.10	11.82	27.39	37.18
Sterols[2]	9.34	11.27	5.95	8.55	9.95	15.91	15.69	11.58	24.70	21.12
Polar Lipids[4]	5.22	5.14	3.61	3.96	15.10	12.14	7.04	6.95	18.21	6.83
Phospholipids[5]	31.87	21.06	50.37	33.09	22.22	9.68	32.70	40.80	12.31	22.59
Other	0.83	0.62	0.20	0.59	1.77	1.90	0.63	1.06	5.53	3.04

[1] Results are the determination of individual samples (n=1) expressed as % of the total lipid in the extract by weight, where c = control, sw = seawater treated, AD = air-dried and FD = freeze-dried.
[2] Differences between air-dried and freeze-dried samples (n=5), and control and seawater treated samples (n=4), are not significant ($p > 0.05$).
[3] Differences between air-dried and freeze-dried samples (n=5), and control and seawater treated samples (n=4), are significant ($p < 0.05$).
[4] Differences between air-dried and freeze-dried samples (n=5) are not significant ($p > 0.05$), while differences between control and seawater treated samples (n=4) are significant ($p < 0.05$).
[5] Differences between air-dried and freeze-dried samples (n=5) are not significant ($p > 0.05$), while differences between control and seawater treated samples (n=4) are significant ($p < 0.05$).
[6] Seasonal variations between May, June and July samples (n=2-4) were not significant ($p > 0.05$).

The major fatty acids and summary of total fatty acids given in Table 3.16 and Figure 3.11 indicate that the crab biomass samples are mainly composed of monounsaturated fatty acids (MUFA), followed by polyunsaturated fatty acids (PUFA), omega-3 (a subset of PUFA), and saturated fatty acids, respectively. These results show a similar trend to that reported by Shahidi & Synowiecki [4] and Beaulieu *et al*. [2] in that both studies reported MUFA > PUFA > Omega-3 > Saturated. However, the average values obtained in this study for saturated fatty acids and omega-3 values were higher with a range of 13.67-17.56% and 22.78-29.87%, respectively, compared with 9.36% (saturates) and 18.31% (omega-3) reported by the Shahidi [4] study. However, Beaulieu *et al*. [2] reported similar values for saturated fatty acids (15.0%) and omega-3 (21.1%) as found in this study. This study also shows that snow crab by-products have exceptionally high DHA + EPA values. All three studies demonstrated that MUFAs are the major components of lipids in snow crab (*C. opilio*) shell by-products and contain high levels of eicosenoic (20:1) and octadecenoic (18:1) acids. Levels of DHA (22:6ω3) were similar in all three studies ranging from 5.59 – 8.9%, however, levels of EPA (20:5ω3) varied from 9.9-18.71%. The DHA/EPA ratio in this study was comparable to that reported by Shahidi & Synowiecki [4]. Differences in fatty acid composition may be due to seasonal variations in the lipid content; the collection methods used; storage conditions of the by-product material; the composition of the snow crab processing discards; and the by-product preparation method used in each study. In the Shahidi study [4] the shell components were separated into different segments (e.g., backs, legs, claws), whereas in this study and the Beaulieu study

[2], the processing discards were combined by crude grinding of the snow crab processing discards.

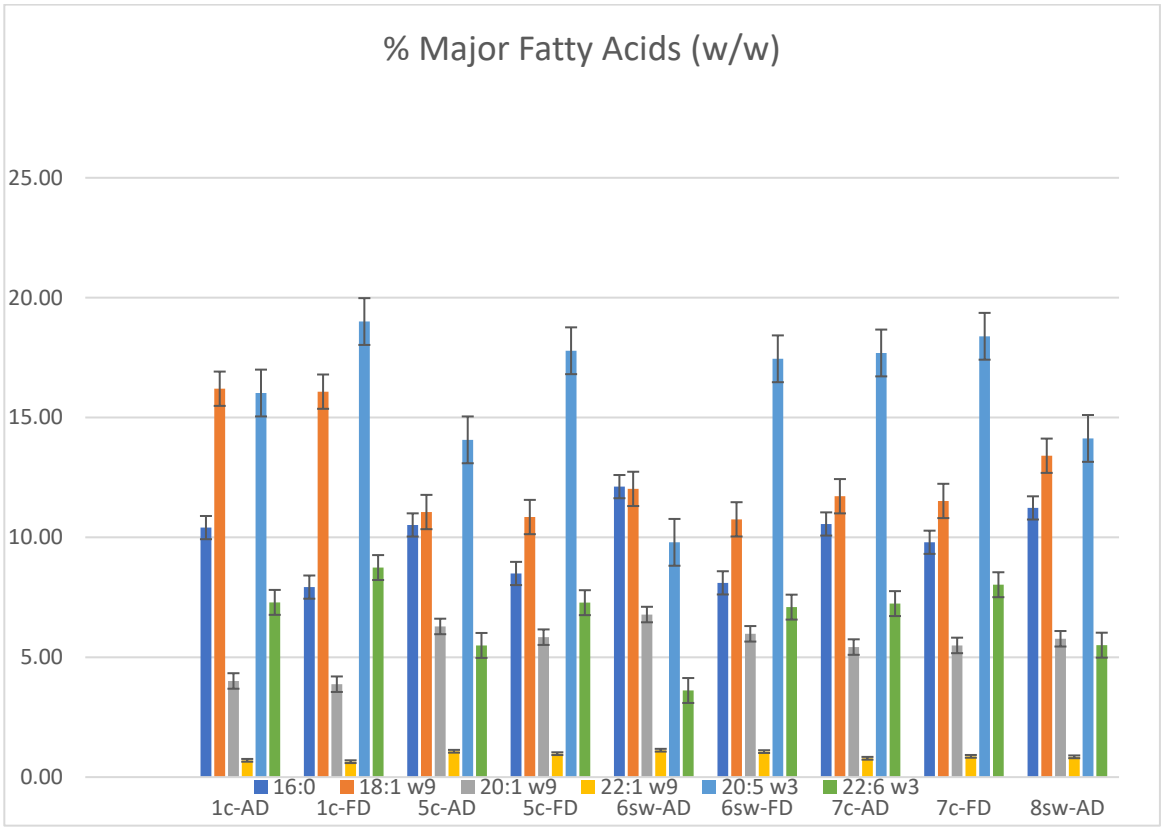


Figure 3. 11 Major fatty acids found in lipids extracted from air-dried and freeze-dried crab biomass feedstock samples from May (1), June (5, 6), July (7, 8) 2018 with standard error bars, where c = control, sw = seawater, AD = air dried and FD = freeze dried.

Table 3. 16 Major fatty acids found in lipids extracted from air-dried and freeze-dried crab biomass feedstock samples - May, June, July 2018 [1]

% Major Fatty Acids		May[3]		June[3]				July[3]			
		1c-AD	1c-FD	5c-AD	5c-FD	6sw-AD	6sw-FD	7c-AD	7c-FD	8sw-AD	8sw-FD
16:0		10.41	7.93	10.52	8.49	12.12	8.10	10.56	9.80	11.23	8.36
18:1 ω9		16.20	16.08	11.06	10.85	12.02	10.75	11.72	11.52	13.41	12.48
20:1 ω9		4.01	3.88	6.28	5.84	6.78	5.98	5.42	5.49	5.77	4.96
22:1 ω9		0.70	0.64	1.08	0.98	1.12	1.06	0.79	0.87	0.85	0.49
20:5 ω3		16.02	19.00	14.07	17.79	9.79	17.45	17.69	18.39	14.13	20.95
22:6 ω3		7.29	8.74	5.49	7.28	3.61	7.09	7.24	8.03	5.50	8.55
Sum	Sat[2]	16.79	12.87	16.78	13.49	19.22	13.07	16.89	15.58	18.12	13.35
Sum	MUFA[2]	46.85	45.82	51.89	48.91	57.14	49.92	46.49	46.01	51.60	44.73
Sum	PUFA[2]	35.04	40.19	29.89	36.45	22.13	35.81	35.42	37.27	29.04	40.85
PUFA/Sat	ratio[2]	2.09	3.12	1.78	2.70	1.15	2.74	2.10	2.39	1.60	3.06
Sum	ω6[2]	6.77	7.32	5.63	6.21	4.87	6.21	6.67	6.60	6.13	7.38
Sum	ω3[2]	26.33	31.01	22.53	28.66	15.65	27.88	27.57	29.25	21.81	32.54
ω3/ω6	ratio[2]	3.89	4.24	4.00	4.61	3.21	4.49	4.13	4.43	3.56	4.41
DHA/EPA	ratio[2]	0.45	0.46	0.39	0.41	0.37	0.41	0.41	0.44	0.39	0.41

[1] Results are the determination of individual samples (n=1) expressed as % of the total lipid in the extract by weight, where c = control, sw = seawater treated, AD = air-dried and FD = freeze-dried

[2] Differences between air-dried and freeze-dried samples (n=5) are significant ($p < 0.05$), while differences between control and seawater treated samples (n=4) are not significant ($p > 0.05$) according to the two-tailed t-test.

[3] Seasonal variations between May, June and July samples (n=2-4) were not significant ($p > 0.05$) according to ANOVA.

In the current study, the fatty acid compositions differed significantly ($p < 0.05$) for the air-dried and the freeze-dried samples but were not affected by the seawater treatment ($p > 0.05$). The freeze-dried samples had a higher average omega-3 content (29.87%) and PUFA content (38.11%) compared with 22.78% and 30.30% for the air-dried samples, respectively. The air-dried samples contained higher MUFA (17.56%) and saturated fatty acid (50.80%) contents compared with 13.67% and 47.08% for the freeze-dried samples, respectively. These results confirm that the drying method affects the fatty acid composition with higher PUFA and omega-3 contents, and better DHA/EPA and PUFA/Sat ratios obtained through freeze-drying. Due to their high content of PUFAs, marine oils are highly susceptible to thermal oxidative degradation which increases with temperature and cooking time [56, 57], which explains the lower content of PUFAs and omega-3 found in the air-dried crab feedstock samples.

From a nutritional perspective, oils with high PUFA and Omega-3, and low saturated fatty acids are considered optimal for fish health and human nutrition. For aquatic species, lipid levels between 16-35% in the diet protect protein and support growth [7]. In the case of *Chionoecetes opilio* processing by-products, the fatty acid composition is high in PUFA and omega-3. EPA and DHA are considered essential fatty acids for marine species and must therefore be available in the diet [7]. The ratio of omega-3 to omega-6 (ω3/ω6) is used as an index of nutritional value with a higher ratio being an indicator of high nutritional value. This index should be around 10 for snow crab oil by-products [2, 7, 37]. In this study the average ω3/ω6 ratio was 3.9 for the air-dried samples and 4.4 for the freeze-dried samples. The lower ω3/ω6 ratio for the air-dried samples may be attributed to

the higher heat treatment resulting in thermal oxidative degradation of the omega-3 and omega-6 fatty acids. The ω3/ω6 ratios in this study were lower than that reported by other authors [2, 7, 37] and may be a function of the challenges encountered during by-product handling which resulted in prolonged exposure to oxygen and increased surface area during the grinding/milling process of the raw crab by-product.

3.4.7 Amino Acid Composition of Air-Dried and Freeze-Dried Crab Biomass Feedstock Samples

The crude protein content for the air-dried samples was previously reported and ranged from 23.48% to 31.78% (db) (Table 3.10) with the highest protein content found in the June seawater sample (6sw). For the freeze-dried samples the protein content was previously reported in Table 3.11 as 26.51-32.47% (db), with the May control sample (1c) exhibiting the highest protein content. The crude protein contents of the crab feedstock samples evaluated in this study were comparable to that reported by other authors [2, 7, 9, 31], but higher than that reported by Shahidi & Synowiecki (17.21-23.95% db) [4]. As mentioned previously, in the Shahidi study [4] the shell components were separated into different segments (e.g., backs, legs, claws), whereas in this study and the Beaulieu study [2], the processing discards were combined by crude grinding of the snow crab processing discards without separation of meat and shell and is thought to be the main reason for differences in crude protein content between the studies.

Results of the amino acid analysis for the air-dried and freeze-dried crab feedstock samples are presented in Table 3.17 and Table 3.18 and are reported on a dry weight basis as mg amino acid/g sample. In comparison to studies by Beaulieu *et al*. [2] and Vilasoa-

Martinez *et al*. [9] which reported the presence of 17 and 16 amino acids, respectively, in snow crab by-products, our analysis shows that 15 amino acids are present. The two amino acids missing in our data set include histidine and tryptophan. These amino acids were likely destroyed by acid hydrolysis [38] and were therefore not detected. However, some samples (June and July) did contain hydroxyproline which was not reported in the literature. Also, while arginine was not detected in our samples, ornithine was present in all samples. According to Corso *et al*. [39] arginine is converted to ornithine during the derivatization procedure for analysis of amino acids by GC-MS as used in this study.

The differences in the essential amino acid composition between the air-dried and freeze-dried crab samples, the control samples and seawater treated samples, and the May-June-July samples, were not significant ($p > 0.05$) indicating that the pre-treatment, drying methods, and seasonality have a minimal effect on the amino acid composition of the crab biomass material. Sample 6sw (air-dried) however, did have an unusually high total amino acid content, likely a result of its higher protein content (Table 3.10).

The essential amino acid composition of the crab biomass samples in this study were comparable with the literature values obtained from other snow crab (*C. opilio*) studies [2, 4, 7, 9] with the following exceptions: (1) histidine and tryptophan were not detected in this study; (2) the samples had lower lysine (1.17-6.57 mg/g) contents; (3) the samples also had lower threonine (2.87-7.24 mg/g) contents except sample 6sw (air-dried) which had a higher threonine content of 19.37 mg/g.

Table 3. 17 Amino acid composition of air-dried crab feedstock samples - Many, June, July 2018 [a,b]

		May	May	June	June	July	July
Amino Acids	**Literature [6, 8]**	**1c-AD**	**3sw-AD**	**5c-AD**	**6sw-AD**	**7c-AD**	**8sw-AD**
	mg/g	**mg/g**	**mg/g**	**mg/g**	**mg/g**	**mg/g**	**mg/g**
Essential [c,d]							
L-Histidine	13.85	nd	nd	nd	nd	nd	nd
Isoleucine	11.45	9.75	11.93	11.07 ± 2.48	14.87 ± 1.72	6.41	8.14 ± 4.64
L-Leucine	19.90	14.00	9.59	14.99 ± 2.82	23.92 ± 2.93	13.06	8.03 ± 0.35
L-Lysine	19.80	1.23	1.06	1.99 ± 0.85	6.57 ± 8.53	1.17	1.41 ± 0.39
L-Methionine	6.65	4.24	4.64	5.36 ± 0.22	8.25 ± 1.20	4.31	4.52 ± 0.53
L-Phenylalanine	11.90	7.95	7.53	9.62 ± 1.34	13.43 ± 0.32	8.11	6.75 ± 0.91
L-Threonine	14.40	2.88	4.97	7.24 ± 0.09	19.37 ± 0.09	4.86	2.87 ± 1.27
Tryptophan	9.80	nd	nd	nd	nd	nd	nd
L-Valine	13.95	18.38	11.72	16.27 ± 3.45	18.92 ± 2.44	13.53	13.33 ± 4.24
Non-Essential							
L-Alanine	13.95	11.30	8.11	13.24 ± 0.54	18.34 ± 0.84	10.18	6.39 ± 0.54
L-Aspartic acid	14.20	1.88	2.40	4.83 ± 1.75	18.29 ± 0.30	4.43	1.87 ± 0.28
L-Glutamic acid	19.70	6.49	6.88	11.11 ± 3.29	30.82 ± 2.56	9.80	7.02 ± 0.95
Glycine	13.00	12.21	9.07	15.10 ± 0.87	22.13 ± 0.58	12.08	6.43 ± 0.01
L-Proline	8.80	11.42	13.05	16.19 ± 2.94	17.90 ± 8.32	14.53	8.74 ± 1.10
L-Ornithine	22.90	4.05	5.29	6.94 ± 0.10	19.37 ± 4.60	5.63	4.58 ± 0.28
L-Serine	15.75	2.43	3.91	5.20 ± 0.03	11.80 ± 0.004	4.29	1.82 ± 0.39
L-Tyrosine	10.90	8.61	10.06	10.66 ± 0.06	14.15 ± 1.92	8.93	9.24 ± 0.90

[a] Results are the mean of two determinations (n=2) ± standard deviation where indicated. n=1 where there is no standard deviation reported. Results are reported on a dry weight basis as mg aa/g sample. [b] nd = not determined. [c] Differences between air-dried and freeze-dried samples, and control and seawater treated samples, are not significant ($p > 0.05$) according to the two-tailed T-Test. [d] Differences between May, June and July samples are not significant ($p > 0.05$) according to ANOVA.

Table 3. 18 Amino acid composition of freeze-dried crab feedstock samples - May, June, July 2018 [a,b]

		May	June	June	July	July
Amino Acids	**Literature [6, 8]**	**1c-FD**	**5c-FD**	**6sw-FD**	**7c-FD**	**8sw-FD**
	mg/g	**mg/g**	**mg/g**	**mg/g**	**mg/g**	**mg/g**
Essential [c,d]						
L-Histidine	13.85	n.d	n.d	n.d	n.d	n.d
Isoleucine	11.45	9.20 ± 3.45	11.13	6.07 ± 1.78	9.90 ± 4.64	3.54 ± 0.34
L-Leucine	19.90	16.65 ± 6.60	13.57	11.10 ± 3.01	11.33 ± 2.81	7.42 ± 0.88
L-Lysine	19.80	3.84 ± 4.35	2.08	1.41 ± 0.28	1.53	1.52 ± 0.59
L-Methionine	6.65	4.56 ± 1.21	6.91	4.97 ± 0.37	6.21 ± 1.21	4.07 ± 0.16
L-Phenylalanine	11.90	7.00 ± 1.42	10.74	8.71 ± 1.92	9.42 ± 1.28	7.24 ± 0.22
L-Threonine	14.40	8.37 ± 5.44	11.82	7.92 ± 0.66	7.55 ± 1.87	4.83 ± 2.42
Tryptophan	9.80	n.d	n.d	n.d	n.d	n.d
L-Valine	13.95	12.38 ± 4.39	14.94	11.15 ± 2.88	13.44 ± 4.59	13.62 ± 6.53
Non-Essential						
L-Alanine	13.95	12.02 ± 5.75	12.00	7.73 ± 2.56	10.28 ± 0.47	6.74 ± 0.43
L-Aspartic acid	14.20	3.38 ± 0.66	9.66	5.66 ± 0.93	6.72 ± 1.78	3.89 ± 1.26
L-Glutamic acid	19.70	8.57 ± 0.59	18.80	11.92 ± 2.23	13.39 ± 1.87	8.79 ± 0.86
Glycine	13.00	5.46 ± 4.94	15.02	5.14 ± 6.29	11.70 ± 0.30	7.71 ± 1.05
L-Proline	8.80	9.62 ± 3.83	11.76	8.01 ± 2.90	13.37 ± 2.87	10.69 ± 3.04
L-Ornithine	22.90	7.22 ± 4.40	5.60	4.44 ± 1.54	6.70 ± 1.91	4.67 ± 1.82
L-Serine	15.75	3.41 ± 1.05	7.55	6.33 ± 1.13	6.79 ± 0.15	4.62 ± 1.87
L-Tyrosine	10.90	10.55 ± 2.32	12.65	10.15 ± 0.50	10.55 ± 0.01	10.04 ± 0.73

[a] Results are the mean of two determinations (n=2) ± standard deviation where indicated. n=1 where there is no standard deviation reported. Results are reported on a dry weight basis as mg aa/g sample. [b] nd = not determined. [c] Differences between air-dried and freeze-dried samples, and control and seawater treated samples, are not significant (p > 0.05) according to the two-tailed T-Test. [d] Differences between May, Jun and July samples are not significant (p > 0.05) according to ANOVA.

Other non-essential amino acids were also comparable to the literature values [7, 9] except aspartic acid, ornithine and serine which were lower in all samples, except sample 6sw (air-dried - June seawater treated) which was higher in aspartic acid and ornithine. Sample 6sw (air-dried) also had a higher total amino acid content compared with the other samples, which is consistent with its higher crude protein content (Table 3.10).

In the literature [2, 4, 9], essential amino acid contents for snow crab (*C. opilio*) shell biomass samples followed the order Leucine > Lysine > Threonine > Valine > Histidine > Phenylalanine > Isoleucine > Tryptophan > Methionine. In this study the amino acid contents, on average, followed the order Valine > Leucine > Isoleucine > Phenylalanine > Threonine > Methionine > Lysine. The total amino acid content of the air-dried samples followed the order 6sw > 5c > 7c > 1c > 3sw > 8sw. The total amino acid contents of the freeze-dried samples were similar to the air-dried samples except for sample 6sw, and followed the order 5c > 7c > 1c > 6sw > 8sw. Variations in the amino acid compositions observed between the samples evaluated in this study and in other studies [2, 4, 7, 9] may be due to differences in the sample collection and preparation methods used, the method of analysis (GC-MS vs HPLC), and the components of the crab used for the analysis (i.e., combined vs individual components).

Methionine and lysine which are essential amino acids for aquatic species [7] are limiting in the snow crab processing by-products evaluated in this study. A similar result for lysine was previously reported by Shahidi & Synowiecki [4]. This reduces the quality of the protein in comparison to proteins obtained from other crustacean sources such as northern shrimp and red crab [4]. The samples in this study are also lower in quality than snow crab by-products reported elsewhere [2, 4, 9], apart from sample 6sw which had

higher levels of essential amino acids, other than lysine. Due to limitations in the essential amino acid content of crab by-product proteins, they may not be as beneficial as a nutritional ingredient in aqua feeds in comparison to shrimp shell (*Pandalus borealis*) proteins, which, as reported by Shahidi & Synowiecki [4], are well-balanced in their essential amino acid composition. This may limit the use of snow crab by-product proteins as a nutritional supplement for aqua feeds, animal feeds, and as a protein hydrolysate for nutraceutical applications.

Overall, the crab biomass samples evaluated in this study have high protein levels (23.48-32.47%) with amino acid compositions comparable to that reported for other snow crab processing by-products. This may make them suitable for higher value products such as protein hydrolysates or aqua feed ingredients, however additional amino acid supplementation with methionine and lysine may be required.

3.5 Conclusions

Based on the characterization studies conducted, crab processing by-products from the Newfoundland and Labrador seafood processing industry have potential applications as feedstock for intermediate bioproducts including proteins, lipids, chitin, pigments and minerals, which in turn may have further applications as higher value bioproducts such as amino acids (protein hydrolysates), fatty acids (omega-3), calcium, astaxanthin and chitosan as illustrated in the proposed snow crab bioproducts value chain (Figure 3.12).

The suitability of crab processing by-products as feedstock for intermediate and high value crab bioproducts, however, will be affected by seasonality, collection pre-treatment methods, and stabilization methods. The effects of these variables on the quality

of the raw and dried crab feedstock samples are summarized in Table 3.19. Seasonality and pre-treatment methods had the greatest impact on crab feedstock quality followed by drying method. Understanding the effects of these variables and how to control them will be critical to implementing effective methods for the collection and stabilization of crab processing by-products intended for intermediate and higher value bio-product applications (Figure 3.12).

Snow crab (*Chionoecetes opilio*) processing by-products also have unique intrinsic characteristics that make their utilization more challenging in comparison to northern shrimp (*Pandalus borealis*) processing by-products. Snow crab by-products are highly susceptible to bluing and melanosis reactions which can cause rapid blue-black discolouration within 24-48 hours. Therefore, these raw materials require rapid stabilization. They are low in two essential amino acids, lysine, and methionine, and may require supplementation for use in aqua feeds and protein supplements. Their shell hardness creates challenges with chitin extraction resulting in lower chitin yields due to more aggressive extraction methods (e.g., additional grinding and particle size reduction). They are high in some heavy metals which may require additional purification steps to render the by-products suitable for use in animal feeds, or natural health products. In addition, the snow crab fishing season is quite short spanning only a 3-month period (May - July) resulting in a large volume of processing by-products that processors must handle in short order. Processing plants in NL are currently not set-up to properly collect and stabilize this material for further use and consequently it is destined for landfills or dumping at sea by permit.

Despite these challenges, crab processing by-products represent a potentially valuable feedstock for high value bioproducts due to their high protein and calcium contents, high astaxanthin content, high chitin content and favorable lipid class profile. Further research to address the above challenges is therefore warranted.

3.6 Future Opportunities

Before snow crab processing by-products can be utilized for higher value bioproducts further research and investment to develop suitable collection and stabilization technologies will be needed. Processors will have to decide which bioproducts they wish to produce and develop appropriate technologies to take care of the by-products throughout the crab bioproducts value chain. Ideally, the goal should be to recover multiple co-products such as pigments, proteins, chitin, lipids, etc., so that quality and yields are maximized while keeping production costs low. Crab processing by-product collection and stabilization systems must be simple and inexpensive; easily integrated into the process flow; and must be adaptable to allow for biological and seasonal variability in the raw material.

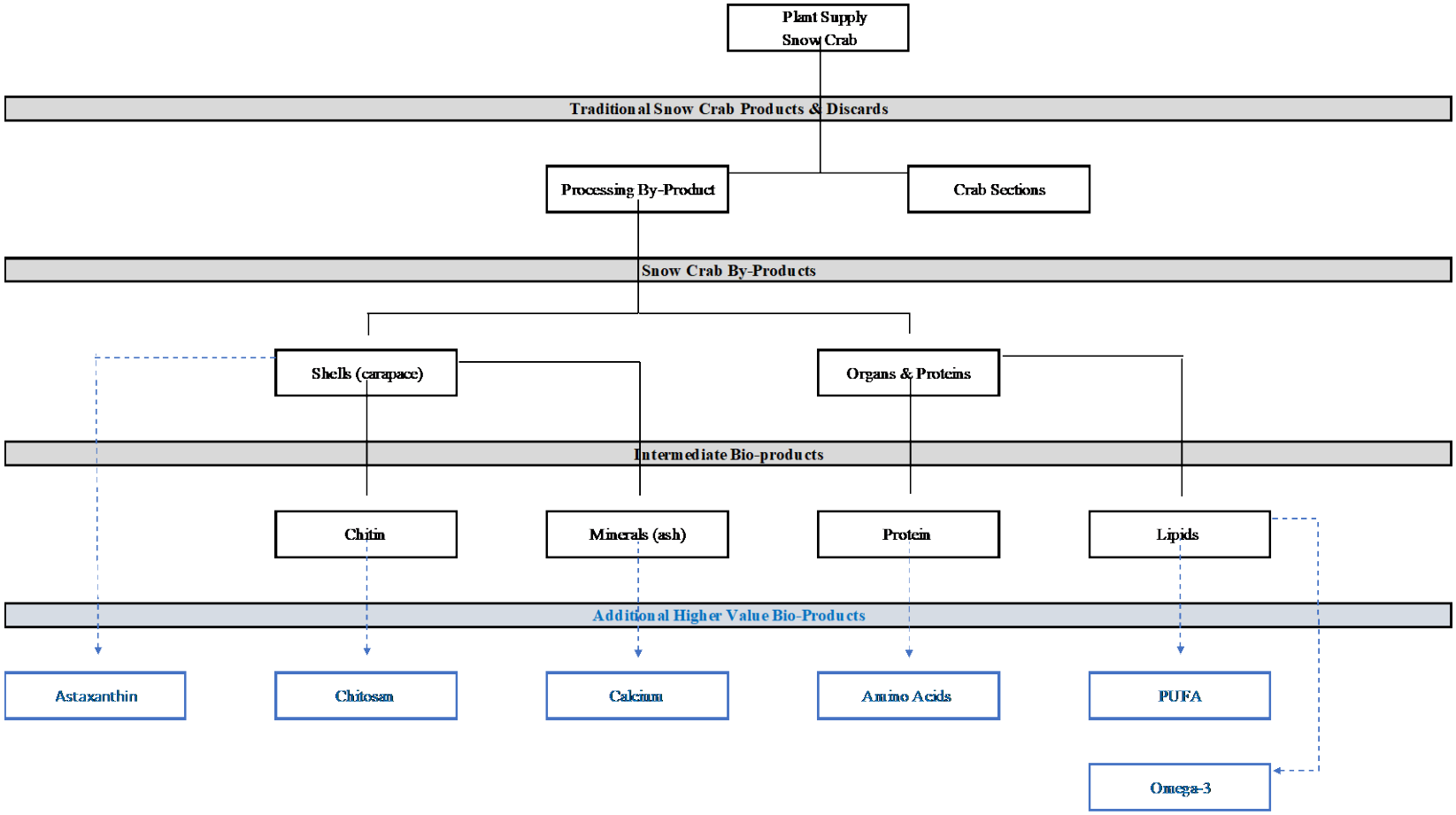


Figure 3. 12 Proposed snow crab bioproducts value chain for the NL seafood processing industry

Table 3. 19 The effect of pre-treatment method, seasonality, and drying method on quality of crab feedstock samples

Quality Parameter	Variables Affecting Quality Parameters		
	Pre-Treatment Method	**Seasonality**	**Drying Method**
Raw Crab By-product			
Sensory	x [a]	+ [b]	n/a [c]
Moisture Content	x	x	n/a
Heavy Metals	inconclusive	+	n/a
Calcium Content	- [d]	x	n/a
Dried Crab By-product			
Ash Content	x	-	-
Protein Content	x	-	-
Amino Acid Composition	-	-	-
Lipid Content	-	x	-
Lipid Class Profile	x	-	inconclusive
Fatty Acid Composition	-	-	x
Astaxanthin Content	inconclusive	x	x
Chitin Content	-	+	-
Chitin Recovery	inconclusive	+	-
Salt Content	x	x	-

[a] x = significant effect. [b] + = moderate effect. [c] n/a = not applicable. [d] - = no effect.

Factors that should be considered when developing collection and stabilization technologies for snow crab processing by-products include:

1. Stabilization such as freezing of the by-products immediately following production will be critical to maintaining their suitability for future applications.
2. Minimizing exposure to oxygen during collection and storage will be critical to prevent/minimize enzymatic and oxidative spoilage reactions such as bluing/melanosis, as well as lipid oxidation and oxidation of carotenoid pigments if recovery of these components is desired.

3. Whether purification of the feedstock and/or the extracted bioproducts is required to eliminate or reduce heavy metals to acceptable levels.
4. The effect of seawater treatment on additional handling requirements, quality, chitin extraction and recovery, and lipid hydrolysis, should be further evaluated to determine its techno-economic feasibility as a stabilization pre-treatment method.
5. The selected drying method must be appropriate for the bio-product being produced. For example, if lipids, PUFA, omega-3 and astaxanthin are important bioproducts to be recovered, high temperature air drying is not appropriate. Low temperature drying methods such as freeze drying, spray drying, or vacuum drying should be considered. While equipment for these drying methods is potentially more expensive to purchase (e.g., cost of a 50 Kg capacity freeze dryer is $150,000-200,000 USD), their lower operating costs and ability to produce higher quality higher value bioproducts, may make these options economically attractive.
6. If calcium is a primary product as a feed ingredient or natural health product supplement, it will be important to avoid soft-shell crab by-products which typically occur in spring to early summer due to their lower calcium content.

Note to Reader:

- The Appendix for Chapter 3 begins on page 227
- Chapter 4 begins on page 244

3.7 References

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3.8 Appendix

Appendix 1 - Sensory Analysis Using the 100 mm Line Scaling Method

*Sensory analysis performed by H. Burke - CASD Marine Bioprocessing Lab, MI

Procedure:

Step 1: Examine each sample and decide whether it is tainted and/or decomposed.

Step 2: Record on the ballot sheet whether the sample passes or fails the quality standard by checking the appropriate box.

Step 3: Indicate opinion of the quality of the sample by recording the "intensity" or "degree" of the pass/fail decision by placing a vertical mark on the 10-centimetre line scale provided on the worksheet.

Positions from the extreme-left end of the line to the midpoint indicate that the sample is of acceptable quality and has been passed. Positions to the right of the midpoint indicate that the sample has been failed.

As one moves from the left to the right of the line, the quality of the sample becomes worse. The midpoint of the line must NOT be used in this exercise.

Step 4: Samples are failed if they contain indicators of taint and/or decomposition that are distinct and persistent. If a sample is failed, the reason for failing the sample must be recorded in the "comments" column (e.g., T = Taint or D = Decomposition).

Step 5: Comments, such as descriptions of odour and/or flavour, are also to be recorded in the comments column on the worksheet.

Step 6: To decode the results, use a 6-inch ruler to measure the vertical line "score" for each sample. The vertical line "score" is recorded in millimetres.

(Adapted from CFIA Standard, Chapter 13)

Sensory Workshop - COD - FINAL EXAM
Tuesday April 5, 2005 - St. John's, NL

Participant ID #_____

NAME:_____ SESSION: **Final Exam**

	SAMPLE CODE	DECISION P	DECISION F	COMMENTS
1	**119**	P	F	
2	**293**	P	F	
3	**926**	P	F	
4	**455**	P	F	
5	**834**	P	F	
6	**662**	P	F	
7	**341**	P	F	
8	**787**	P	F	
9	**578**	P	F	
#	**814**	P	F	
#	**498**	P	F	
#	**675**	P	F	
#	**581**	P	F	
#	**137**	P	F	
#	**226**	P	F	
#		P	F	
#		P	F	
#		P	F	
#		P	F	
#		P	F	

Appendix 2 - Sample Preparation Method for ICP-MS Analysis

*Metal analysis conducted by John Allen - ICP-MS lab, Earth Science MUN

Procedure

1. Weigh out approximately 10g of sample into Aluminum pan. Weigh empty pan and sample + pan.
2. Heat for 24 hours at 105°C and allow to cool in desiccator. Weigh sample + pan and calculate Moisture Content or (LOD).
3. Ash samples in furnace starting at 100°C then ramping at 55°C/min to 550°C and hold for 6 hours and then return to 100°C. Allow to cool in Desiccator and weigh sample + pan. Calculate Loss on Ignition (LOI).
4. Weigh approximately 0.1000g of ashed sample into Teflon (Savillex) Beaker.
5. Add 3mL of conc. HNO3 to samples and reflux at 100°C overnight and sonicate for 1 hour in the morning. Evaluate if sample completely digested. If not continue refluxing.
6. After several days, if sample is still not completely digested, might be necessary to add HF.
7. After samples appear digested, (clear or slightly milky, no visible material), dry completely down.
8. After dry, add 3mL of 6M HCl and put on hotplate overnight at 100°C and sonicate for 1 hour in the morning.
9. Dry samples completely down and add 0.5mL of conc. HNO3 to convert to nitrates. Heat for 3 hours at 100°C and sonicate for 1 hour.
10. Dry samples completely down and dilute to approximately 10mL with 0.2M HNO3 in preparation for ICP-MS analysis.

Appendix 3 - Proximate Analysis

*All proximate analysis procedures performed by H. Burke - CASD Marine Bioprocessing Lab, MI

Moisture Content

Method AOAC 950.46B/AOAC 930.15
Scope Measurement of moisture in wet or dry (sea)food and shellfish samples
Apparatus

- Aluminum weighing dish
- Drying oven, set at 105°C ±2°C
- Balance, capable of weighing 0.0001g

Procedure

1. Place moisture pans in drying oven at 105ºC for one hour. Cool in desiccator for 20 minutes.
2. Weigh each to nearest 0.0001g.
3. Comminute sample well and add 5-10g to pan and spread evenly over bottom. Weigh pan and contents.
4. Dry in drying oven overnight, cool in desiccator for at least 20 minutes, and weigh again.
5. Samples may be checked for constancy of weight by returning to oven for 1 hour and re-weighing.
6. Calculate moisture content (expressed as %) as follows:

$$\%\ Moisture = \left(\frac{(W_1 - W_{2)}}{W_1 - W_0}\right) \times 100$$

where: W_0 = weight of empty dish
W_1 = weight of dish and sample before drying
W_2 = weight of dish and sample after drying

Ash Content

Method: AOAC 938.08 Ash of Seafood

Scope: Measurement of ash in wet or dry (sea)food and shellfish samples

Procedure

- Heat crucibles in a muffle furnace at 550°C for a minimum of 1 hour, or overnight. Cool in desiccator and weigh to the nearest 0.0001g.
- Add ~2g sample into each crucible and weigh to nearest 0.0001g.
- If samples have a high moisture content place samples in drying oven set at 105°C for one hour to remove excess moisture.
- For high fat samples carefully char material on an electric plate or with a Bunsen burner (low flame) taking care not to allow sample to burst into flame. If flaming occurs quickly cover crucible with cover. (Note: Pre-charring may be omitted if low fat samples are placed in a cold muffle furnace and temperature is raised gradually.)
- Place crucibles with samples in a cold muffle furnace. Set furnace to 550°C and leave overnight.
- With tongs carefully remove crucibles from furnace and allow to cool in desiccator. Do not close desiccator completely; allow heated air to escape otherwise glass may crack under vacuum.
- If ash appears creamy white or grey and is free of black particles weigh the crucible with the ash.
- If black particles are observed add 1-2 mL distilled water, evaporate, and return to furnace at 550°C until ash becomes white, probably overnight. Cool and recheck color.
- Weigh crucible (and ash) to nearest 0.0001g.
- Calculate Ash content as follows:

$$\%\, Ash = \left(\frac{(W_3 - W_{1)}}{W_2 - W_1}\right) \times 100$$

Where: W1 = weight of empty crucible
W2 = Weight of crucible and wet sample
W3 = Weight of crucible and ash

Crude Protein Content - Kjeldahl Nitrogen Method

Method AOAC 954.01/988.05 (Kjeldahl)

Scope Determination of total protein by calculation from total Nitrogen content using Kjeldahl method. Can be used for analysis of wet or dry (sea)food and shellfish samples.

Sample preparation

- Comminute and completely dry raw sample. Grind sample prior to extraction.

Equipment

- Digestion unit w/DigiPrep touch screen controller
- Distillation unit
- Digestion tubes
- Eppendorf Varispenser (for sulfuric acid dispensing)
- Erlenmeyer flasks, 250 ml
- Automatic titrator/burette

Chemicals

- Acetanilide (Nitrogen Standard)
- Glycine (Nitrogen Standard)
- Nicotinic acid (Nitrogen Standard)
- Kjeltabs (3.5 mg copper)
- Sulfuric acid (conc)
- Boric acid (4%) with methyl red/methylene blue indicator
- Sodium Hydroxide (40%)
- Hydrochloric Acid

Part I: Digestion

- Preparation:
    - Wash tubes and rinse with deionized water
    - It is not necessary to fill all positions.
    - ***Use same shaped tubes***
    - Organize and make a record of sample positions (test each sample in triplicate, run two-three blanks).
    - Run two standards – preferably two of each if possible. *E.g., Glycine (*18.66 % N)*, acetanilide* (10.36%N) or *Nicotinic acid* (11.4% N).

Nicotinic acid tests that the temp is not too low and *acetanilide* tests that the temp is not too high.
    - Recovery of N standards should be within ± 1% of the reference % N (e.g., within ± 1% of 18.66% for glycine).
    - Turn on DigiPrep Touch screen- next to fume hood. Open the required program from the menu (e.g., **Protein 1**) or write/edit a new program.
- Sample preparation:
    - Comminute samples well before analysis
    - Weigh approximately 0.5g of sample on weighing paper. Record the exact weight to the closest 0.0001g.
    - Transfer the sample to a digestion tube.
    - Check inside of digester for liquid, chemicals or broken glass and remove if necessary.
    - Add 2 Kjeldahl tablets to each tube (3.5mg Copper each).
    - Transfer the rack into the fume hood.
    - Add 20ml concentrated Sulfuric acid to each tube.
- Put rack with filled tubes onto lower rack of digestor.
- Put manifold/reflux head on tubes.
- Carefully put the tube rack with manifold in the digester
- On the touch screen press 'Start' for the selected program to begin heating the digester
- When the temperature of the digester reaches approximately 380°C (~80 minutes), fumes should begin to develop in the tubes. Once fumes are present, turn on the water (to the right of fumehood-FH2) on 'high'. (~15-25 minutes)
- As fume formation decreases, reduce the water to 'medium-low'
- The digestion cycle should run at least 75 minutes starting when the temperature reaches 380°C.*
- At the end of the digestion cycle (≥75 minutes), remove unit from digester and lift the tube rack – with the MANIFOLD EXHAUST STILL IN PLACE and put it back on the racking system (or lay on a heat resistant surface).
    - Turn the water back up to 'high' for an additional 15 minutes.
    - Hit 'stop' on the touch screen and turn it off. This will turn off the heat to the digester.
- After cooling, remove exhaust manifold to the upper rack and immediately cover with the drip tray to prevent acid from dropping on the digester.
- Turn off the water.
- Allow sample tubes to cool for at least 30 minutes.

- *The digestion is not complete if the digest is cloudy or hazy, or if black carbonized particles are floating in the acid or clinging to the side walls of the digestion tube. The additional 60 minutes may be sufficient, but difficult to digest samples could take longer.
- A complete digestion must be clear although it might contain a light color. This color can range from a pale blue-green to a pale yellow-orange depending on the catalyst used.

Part II: Distillation

- Prepare equipment
  - Turn on new FOSS distillation unit (switch on left hand side)
  - Turn on tap (fully open) behind distillation unit
  - Select Program II (70 ml H_2O, 100ml NaOH, 4min distillation time)
  - Perform one distillation run with an empty distillation tube (Program II) to heat up steam generator. Collect distillate in empty Erlenmeyer flask and discard.
  - Prepare receiving flasks: Add 25 ml of 4% (w/w) boric acid with methyl red/methylene blue indicator to each 250 ml Erlenmeyer flask.
- Insert a receiving flask into the distillation unit. Make sure the end of the glass tube is below the surface of the boric acid solution. If not, add a bit of water.
- Insert a digestion tube into the unit and close the protective shield.
- Start the program and wait until completed.
- Remove flask and set aside for titration.
- Carefully remove the digestion tube with tongs (it's hot).
- Put in next digestion tube and receiving flask. While the distillation is running, titrate the previous sample.

Part III: Titration

- Clean burette and flush with 0.1 N HCl before use. Then fill up with 0.1 N HCl.
- Titrate the condensate with 0.1 N HCl to a red endpoint. Record the volume of HCl needed to reach the endpoint in ml.
- Calculate the % Nitrogen:

$$\%N = \frac{(Vol_{HCl\ sample} - Vol_{HCl\ blank}) \times Normality\ of\ acid \times 14.0067}{weight\ of\ sample\ [mg]} * 100$$

- Report the % crude protein as: $\%\ crude\ protein = \%N * 4.94$

Lipid Content - Soxhlet Method

Method AOAC 920.39 (Soxhlet)

Scope Determination of total lipids using Soxhlet extraction

Sample preparation: Comminute and completely dry raw sample. Grind sample prior to extraction.

Equipment

- Extraction thimbles
- Balance
- Glass wool
- Boiling flask
- Soxhlet flask
- Condenser
- Heating mantle
- Drying oven
- Boiling chips

Chemicals

- Hexane (or petroleum ether)

Procedure

- Pre-dry extraction thimbles (105°C for 4 hours or overnight).
- Pre-dry boiling chips (200°C overnight) in drying oven. Boiling chips are *extremely hygroscopic*. Store in desiccator until use.
- Weight to the nearest mg, about 2 g pre-dried sample into a pre-dried extraction thimble, with porosity permitting rapid flow of hexane. Cover sample in thimble with glass wool.
- Weigh pre-dried boiling flask with 6-10 boiling chips.
- Put ~125 ml hexane in each boiling flask.
- Assemble boiling flask, Soxhlet flask and condenser.
- Extract in Soxhlet extractor at heating mantle setting #7 (5-6 drops condensation per second) for a minimum of 4 hours.
- Remove condenser and let the system cool down, approximately 20 minutes, until the boiling flask can be handled.
- Remove condenser and either allow solvent to evaporate in fume hood (heating mantle setting 2.5-3.0) -OR- recover the solvent using rotary evaporator. All solvent must be removed from the flask before the next step.

- Dry boiling flask containing lipid reside in drying oven at 105°C for 30 min. Cool in desiccator for at least 20 min and weigh.
- Calculate the lipid content as follows:

$$\%\ fat\ on\ dry\ weight\ basis = \frac{(g\ fat\ in\ smp)}{(g\ dried\ smp)} * 100$$

Salt Content

Method: DFO Technical Report No.1448 – Recommended Laboratory Methods for Assessment of Fish Quality

Scope: Determination of salt content in wet or dry (sea)food and shellfish samples

Apparatus

- Disposable weigh boat
- Balance, capable of weighing 0.0001 g
- Conductivity meter (e.g., Hach Multimeter and conductivity probe)
- Blender (e.g., Ninja)
- Glass beakers
- Magnetic stir plate
- Magnetic stir bar
- Graduated cylinder, 200 ml
- Weighing spatulas
- Waste beaker

Preparation of Calibration Standard

Before starting the "Calibration Procedure", review the manufacturer's instructions for the device that will be used.

1. Chose the appropriate standard salt for calibration. Conductivity standard solution, 1000 µS/cm ±10 µS/cm at 25°C, 491 mg/L ±2.5 mg/L is good for most seafood samples. This can be purchased (Hach Canada) or prepared.
2. To prepare fresh conductivity standard dry ACS grade NaCl overnight at 110°C. After drying allow NaCl to come to room temperature in desiccator.
3. Accurately weigh 491 (± 2) mg of dried NaCl in weigh boat.
4. Add NaCl to a 1000 ml volumetric flask. Make up solution to 1000 ml mark.

Calibration

1. Pour a small amount of room temperature NaCl standard into a clean beaker, enough to fully submerge the probe. Add magnetic stirrer and place beaker on stirring plate.
2. Start-up the conductivity meter and place the system into "Calibration" mode.
3. Connect conductivity probe to meter or check that it is already attached.
4. Clean conductivity electrode: Rinse the electrode with distilled water. Once rinsed, blot dry with Kimwipes or similar delicate wipe cloths. Do not rub the electrode. If electrode is very dirty, refer to manual for additional cleaning procedures.
5. Put the probe in the standard solution and stir gently, making sure the temperature sensor is completely submerged.
6. Push Read. When the reading has stabilized press DONE to view the calibration summary.
7. Record if calibration passed or failed on the calibration log.
8. Start testing your samples.

Procedure- Wet Samples

1. Determine moisture content of samples.
2. Accurately weigh comminuted, wet samples 10, 20 or 40 g according to anticipated salt content for 18, 8 and 4% NaCl (wet weight) respectively
3. Add 200 ml of D- H2O
4. Mix with hand mixer for 1 minute on high speed.
5. If possible, add stir bar and place sample on stir plate.
6. Rinse the conductivity electrode with distilled water and blot dry with a delicate task wiper (e.g., Kimwipe).
7. Put the probe in the sample solution, making sure the temperature sensor is completely submerged.
8. If necessary, stir gently.
9. Push Read.
10. When the reading has stabilized record reading.

Procedure- Dry Samples

1. Determine moisture content of samples.
2. Accurately weigh dry samples 5g. This amount may require adjustment based on expected salt content of sample. For very high expected salt start with 2 g, for low salt start with 7g.

3. Add 200 ml of D-H2O or Milli-q water. Cover with watch glass.
4. Mix with stir bar on magnetic stirrer for at least 5 minutes.
5. Rinse the conductivity electrode with distilled water and blot dry with a delicate task wiper (e.g., Kimwipe).
6. Put the probe in the sample solution, making sure the temperature sensor is completely submerged.
7. Push Read.
8. When the reading has stabilized record reading.

The salt content is calculated as:

$$C = \frac{R}{W}(\mathrm{V} + \frac{(M \times W)}{100})$$

where:
C = concentration of NaCl in sample expressed as percent on a wet weight basis
M = Moisture as percent by weight
R = % NaCl (converted from ‰ NaCl reading on meter)
V = volume (ml) distilled water added
W = weight (g) of sample used

Appendix 4 - Astaxanthin Extraction and Quantification

*Analysis performed by H. Burke - CASD Marine Bioprocessing Lab, MI

Method: Lopez-Cervantes *et al.* [14] & Davies [15]

Scope: Simplified methanol method for extraction of (total) astaxanthin from dried powder

Methanol - Simplified Extraction Procedure

- 0.240 g of dried powder were transferred to 10 ml HPLC-grade Methanol and immediately vortexed for 20 seconds using a Thermodyne Maxi-Mix 16700 mixer.
- For completion of the extraction, the samples were then sonicated for 5 min at 25°C in a Branson 2800 sonicator.
- Solid particles were removed by centrifugation for 15 min at 1500 rpm (465 x g) in a Thermo Scientific Sorvall Lynx 4000 centrifuge.
- Remaining suspended particles were removed by filtration through a 0.2 µm syringe filter.
- The samples were stored under dark conditions at 2-8°C until analysis could be performed.

Astaxanthin Quantification by Spectrophotometry

- Immediately following extraction, the samples were placed in a cuvette and the absorption was measured at λ_{max} (476 nm) in a HACH DR600 Spectrophotometer.
- The pigment concentration was calculated using the following formula:

$$Astaxanthin\ \left[\frac{\mu g}{g}\right] = \frac{A * D * 10^6}{100 * G * d * E}$$

Where:

A: Absorption at λ_{max},
D: Volume of extract [ml],
G: Sample weight [g],
d: Cuvette distance (10 mm),
E: Extinction coefficient (2100 for Astaxanthin) (Davies,1976).

Appendix 5 - Modified Lowry Method for Total Protein

*Analysis performed by H. Burke - CASD Marine Bioprocessing Lab, MI

Method: Modified Lowry based on Lowry *et al.* (1951), Petterson (1977), and Waterborg (2002)

Scope: Determination of total protein by the Lowry method.

Note: Lowry method is based on the conversion of Cu^{2+} to Cu^{+} under alkaline conditions. The reactions result in a strong blue color, which depends partly on the tyrosine and tryptophan content. Sensitivity of the method is down to about 0.01 mg of protein/ml and is best used on solutions with concentrations in the range 0.01-1.0 mg/ml of protein.

Sample preparation

- Dry samples to completion at 105°C. Grind to a fine powder.

Equipment

- Microcentrifuge
- HACH COD digester
- HACH glass tubes (10ml) that fit into the COD digester including caps
- Photometer
- Vortex mixer
- Analytical balance
- 1.5 ml disposable cuvettes
- Serological pipettes
- Micropipettes

Chemicals

- 2M KOH (prepare 500 ml)
- 2N NaOH (prepare 100 ml)
- Folin reagent: Dilute the stock (2N) to 1N immediately before use (1ml + 1ml is sufficient for up to 18 test tubes)
- Complex forming reagent: Prepare fresh the day of testing
    - Solution A: 2% (w/v) Na_2CO_3 in distilled water
    - Solution B: 1% (w/v) $CuSO_4*5H_2O$ in distilled water
    - Solution C: 2% sodium tartrate in distilled water

- Standards: use a stock solution (e.g., BSA) containing 2 mg/ml protein in distilled water. Make a dilution series. Keep in mind that at higher concentrations, the calibration curve is not going to be linear.

Part I: Extraction of protein residue (Samples only)

- Preheat COD digester to 90°C (Program: CHIT)
- Procedure: Add 5 ml of KOH to 0.1 ml sample
    - Tare a glass tube standing in a small beaker.
    - Add approximately 0.1 g of sample to the glass tube
    - Return the tube to the scale
    - Record the precise weight
    - Tare again
    - Pipet 5 ml of 2M KOH into the tube
    - Record precise weight
- Transfer tubes to the pre-heated COD digester and incubate at 90°C for 60 minutes.
- Mix briefly on vortex mixer
- Fill a 2 ml centrifuge tube by pouring.
- Centrifuge for 10 minutes at high speed
- Prepare fresh 2 ml centrifuge tubes containing 950 µl distilled water
- Add 50 µl of supernatant from the centrifuged tubes to the water. Vortex.
- Place in zip lock bag and store in fridge.

Part II: Lowry Analysis

- Label HACH 10 ml test tubes
- Pipet 0.1 ml of sample or standard into the test tube, then add 0.1 ml of 2 N NaOH
- Mix, then hydrolyze at 100°C for 10 min in the COD heating block
- Cool the hydrolysate to room temperature, 5-10 minutes
- Add 1 mL of freshly mixed complex-forming reagent
- Protect the test tubes from light and let stand at room temperature for 10 min
- After 8 min, prepare 1 M Folin reagent by mixing 1 ml Folin reagent (2 M) with 1 ml distilled water. This amount is sufficient for 18 test tubes.
- Add 0.1 ml of Folin reagent, cap the tubes and immediately vortex.
- Let the solution stand in the dark at room temperature for 30–60 min (do not exceed 60 min).
- Read the absorbance at 750 nm if the protein concentration was below 500 micrograms/ml or at 550 nm if the protein concentration was between 100 and 2000 micrograms/ml.
- Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentration.

Appendix 6 - Lipid Class Analysis and Fatty Acid Composition

*Analysis performed by J. Wells - Lipid Lab, Dept. Ocean Sciences MUN

Method: Lipid Extraction Parrish (1999)
Lipid Class Composition Parrish (1987)

Scope: Lipid class analysis via Iatroscan and fatty acid composition via GC FID

Procedure

Samples were weighed into 15ml vials tubes that had been ashed at 450C for 8 hours. The caps were rinsed three times with methanol followed by three rinses with chloroform. After the wet weights were recorded the samples were covered with 2 mL of chloroform, the headspace in the tube was filled with nitrogen, the caps sealed with Teflon tape and the samples were stored at -20ºC.

Lipid samples were extracted according to Parrish (1999). Samples were homogenized in a 2:1 mixture of ice-cold chloroform: methanol. Samples were homogenized with a Tissue Master 125 Homogenizer with a 7mm Probe (Omni International, Inc., Kennesaw, Georgia, USA). Chloroform extracted water was added to bring the ratio of cholorform:methanol:water to 8:4:3. The sample was sonicated for 4 minutes in an ice bath and centrifuged for two minutes. The bottom organic layer was removed using a double pipetting technique, placing a long lipid cleaned pasture pipette inside a short one, to remove the organic layer without disturbing the top aqueous layer. Chloroform was then added back to the extraction test tube and the entire procedure was repeated 3 more times. All the organic layers were pooled into a lipid-cleaned vial. The samples were concentrated under a gentle stream of nitrogen.

Lipid class composition was determined using an Iatroscan Mark VI TLC-FID, silica coated Chromarods and a three-step development method (Parrish, 1987). The lipid extracts were applied to the Chromarods and focused to a narrow band using 100% acetone. The first development system was hexane:diethyl ether:formic acid (99.95:1:00.05). The rods were developed for 25 minutes, removed from the system for 5 minutes and replaced for 20 minutes. The second development was for 40 minutes in hexane:diethyl ether:formic acid (79:20:1). The final development system had two steps, the first was 100% acetone for two 15 minute time periods, followed by two 10 minute periods in chloroform: methanol:chloroform-extracted water (5:4:1). Before each solvent system, the rods were dried in a constant humidity chamber. After each development system, the rods were scanned in the Iatroscan and the data collected using Peak Simple software (ver 3.67, SRI

Inc.) The Chromarods were calibrated using standards from Sigma Chemicals (Sigma Chemicals, St. Louis, Mo., USA).

For all samples lipid extracts were transesterified using sulfuric acid and methanol for 1 hour at 100ºC. The FAME were analysed on a HP 6890 GC FID equipped with a 7683 autosampler. The GC column was a ZB-WAXplus (Phenomenex, U.S.A.). The column length was 30m with an internal diameter of 0.32mm. The column temperature began at 65ºC and held this temperature for 0.5 minutes. The temperature ramped to 195 ºC at a rate of 40 ºC/min, held for 15 minutes then ramped to a final temperature of 220 ºC at a rate of 2 ºC/min. This final temperature was held for 0.75 minutes. The carrier gas was hydrogen and flowed at a rate of 2 ml/minute. The injector temperature started at 150 ºC and ramped to a final temperature of 250 ºC at a rate of 120 ºC/minute. The detector temperature stayed constant at 260 ºC. Peaks were identified using retention times from standards purchased from Supelco, 37 component FAME mix (Product number 47885-U), Bacterial acid methyl ester mix (product number 47080-U), PUFA 1 (product number 47033) and PUFA 3 (product number 47085-U). Chromatograms were integrated using the Agilent OpenLAB Data Analysis - Build 2.203.0.573. A quantitative standard purchased from Nu-Chek Prep, Inc. (product number GLC490) was used to check the GC column about every 300 samples (or once a month) to ensure that the areas returned are as expected.

CHAPTER 4. Heavy Metals in Snow Crab (*Chionoecetes opilio*) Bioproducts - Part 1

This Chapter was published in the Spring 2022 edition of the Journal of Ocean Technology (JOT), Volume 17 (1). While writing this manuscript for JOT, additional studies were conducted on heavy metals in snow crab bioproducts.

Part 2 of the heavy metal study is presented in CHAPTER 5. Heavy Metals in Snow Crab (*Chionoecetes opilio*) Bioproducts - Part 2, and includes the additional data, results, and conclusions.

4.1 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 (NL), Canada. In 2019, snow crab landings were 26,894 t of which 16,658 t were exported to the United States (77%), China (8%), Indonesia (6%) and Vietnam (4%), at a value of $415 million [1]. Crab processing plants in Newfoundland and Labrador have historically discarded on average about 30% of their total raw material supply in the form of waste and by-products. In 2019 this amounted to an estimated 8,100 t. Over the last 5 years the average annual plant supply of snow crab in NL has been approximately 30,000 t [2].

In NL, snow crab is primarily processed as Individually Quick Frozen (IQF) cooked sections which generates waste comprised of carapace (cephalothorax shells), viscera and hepatopancreas, haemolymph [3], residual meat and gills. This material is currently not being utilized commercially (personal communications with industry stakeholders) but

could potentially be recovered from processing plant butchering stations as a by-product and converted into intermediate bioproducts (chitin, crab meal, proteins, lipids) or transformed into higher value bioproducts (chitosan, peptides, omega-3, astaxanthin). Potential crab processing by-products and bioproducts that could be produced in NL based on an average annual plant supply of 30,000 t are depicted in the crab bio-product value chain in Figure 4.1.

Many of the identified snow crab bioproducts (Figure 4.1) have potential applications as feed ingredients (for terrestrial and aquatic animals), natural health products (e.g., nutraceuticals, dietary supplements), bio-medical & 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 bioproducts 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 [5, 6, 7, 8, 9] we evaluated the levels of trace metal contaminants in crab processing by-products (i.e., crab meal) and their transfer to selected crab bioproducts: crab protein hydrolysate; and crab chitin.

According to Health Canada, heavy metals including arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) are considered toxic contaminants in seafood and natural health products (NHPs) if present in certain levels. The main heavy metals of concern (Table 4.1) for edible seafood and for which Health Canada have 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 4. 2.

For chitosan intended for use in biomedical applications the heavy metals of concern for which industry [13, 14] has established maximum levels (Table 4. 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 also recommends that the total heavy metal content should not exceed < 40 ppm [13, 14].

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 [5, 6]. Metals are naturally present in the environment including soil, water, and air, and therefore end up in food [6, 7, 8, 9]. Heavy metals tend to accumulate in the organs and tissues of crustaceans such as crabs and prawns [15, 16, 17]. Organs and tissues account for 80% of the crab by-products available from NL crab processing plants (Figure 4.1). Therefore, understanding the levels of heavy metals in snow crab by-products and how they are transferred throughout the crab bio-product value chain will be key to developing safe marketable crab bioproducts for natural health product and biomedical/pharmaceutical applications.

Table 4. 1 Heavy metals of concern for seafood and Health Canada maximum allowable levels [11]

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

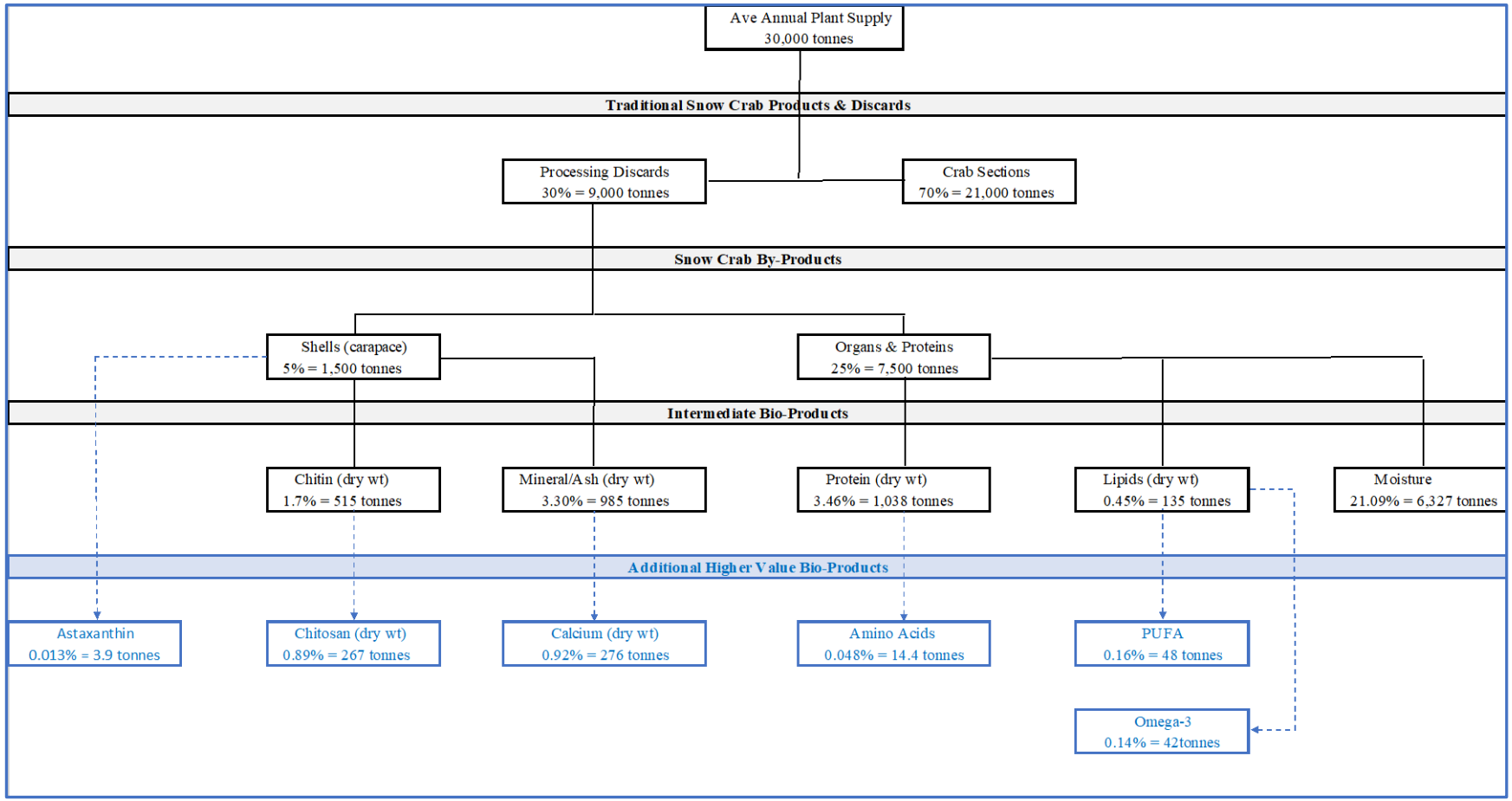


Figure 4. 1 Value chain of NL snow crab processing by-products and bioproducts based on an average annual plant supply of 30,000 t of crab [4].

Table 4. 2 Acceptable limits for elemental impurities in natural health products [12]

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

Table 4. 3 Industry standard for heavy metal levels in chitosan intended for use in biomedical applications [13, 14]

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

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 [18, 19, 20, 21]. Therefore, despite the many published studies on chitosan drug delivery products, they are still not approved by the FDA as they require studies demonstrating they are safe for human use [18, 19, 20,]. 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.

4.3 Purpose and Scope

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

4.4 Selection of Crab Bioproducts

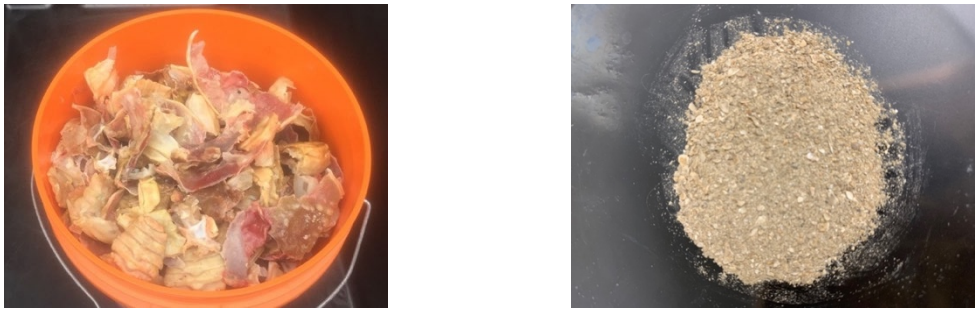
Figure 4.1 identified various bulk intermediate bioproducts that could be extracted from snow crab processing by-products including protein, lipids, chitin, minerals (ash), and astaxanthin. Due to the estimated low yields of lipids and astaxanthin likely to be extracted from the available crab by-products these bioproducts 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.

4.5 Methods

4.5.1 Collection and Preparation of Crab By-product

Snow crab processing by-products (Figure 4.2 A) collected from a processing plant located in Newfoundland and Labrador (NL), Canada in June 2018 were milled and dried to produce crab meal (Figure 4.2 B). 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.



A B

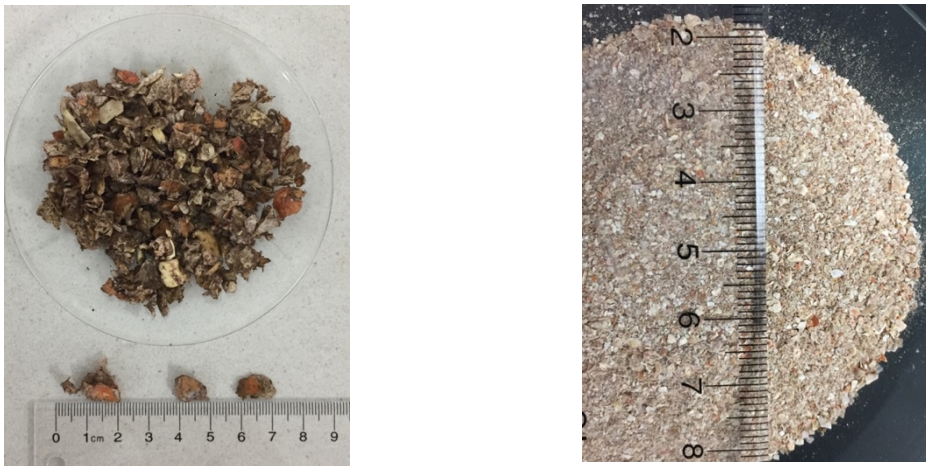
Figure 4. 2 A - Snow crab processing by-products; B- Snow crab meal

4.5.2 Extraction of Snow Crab Bioproducts

Raw, fresh unseparated snow crab processing by-products were collected in 10L plastic pails (Figure 4.2 A), packed in flake ice, and transported to the Marine Institute's Marine Bioprocessing pilot plant in St. John's, NL where the by-product was immediately frozen at –20°C until it could be further processed. The frozen crab by-product was later thawed at 4°C and crushed in a Hobart grinder (Figure 4.3) in a 2-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 by-product was then placed on aluminum 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 4.4). This dried crab meal product was later used for the extraction of additional crab bioproducts, protein hydrolysate and chitin.

A B

Figure 4. 3 A - Hobart grinder; B - snow crab by-product milled through the 17 mm cutting plate



A B

Figure 4. 4 Air dried crab by-product; A - before milling (13 mm); B - after milling (1-2 mm)

4.5.3 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 [52] 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 [53]; (2) Gildberg and Stenberg [58] 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 [53] and shrimp [58]. The hydrolysis was carried out at pH 8-8.55 and 55°C for 120 minutes using a crab by-product-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 [54]. The protein hydrolysate liquid was centrifuged at 7000 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 4.5) to collect the protein hydrolysate (Figure 4.6). The spray drier operating parameters were set at: Inlet temperature 180°C; Outlet temperature 40°C; Aspirator 100%; Pump 20%; Q-Flow 30.

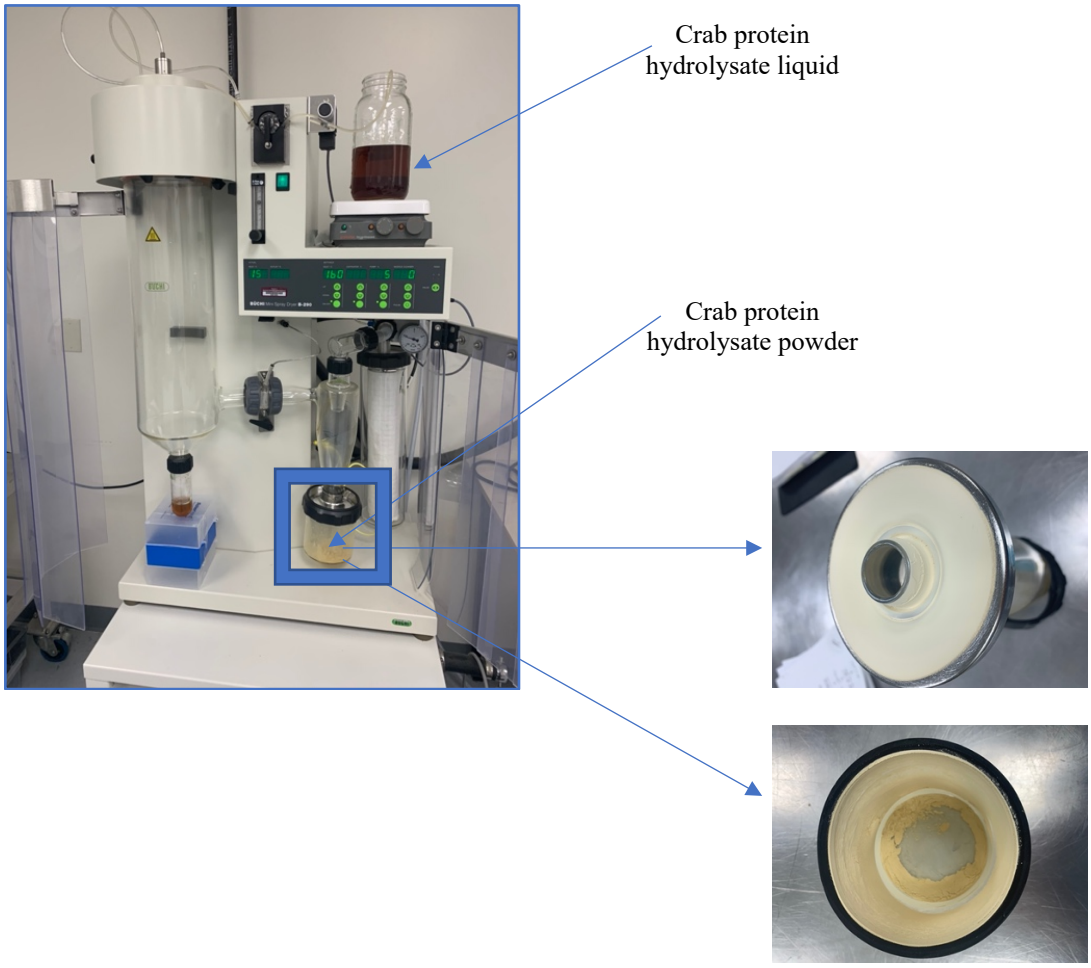


Figure 4. 5 Spray drying Snow crab protein hydrolysate using the Buchi mini spray dryer

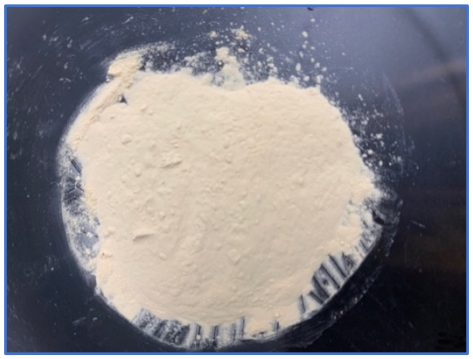


Figure 4. 6 Spray dried snow crab protein hydrolysate powder

4.5.4 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 2 hours); (2) demineralization - removal of minerals, mainly calcium carbonate, by treatment with strong acid (e.g. 5-7% w/v HCl for 2 hours at room temperature); and (3) depigmentation/decolouration - removal of pigment using an organic solvent (e.g., ethanol, acetone), or bleaching using an oxidizing agent (e.g. hydrogen peroxide, sodium hypochlorite) to obtain a colourless product [61, 62, 63]. This process may be carried out on fresh or dried shells, and the demineralization and deproteination steps may be carried out in reverse order [62, 63].

A schematic illustration summarizing the extraction, recovery and purification processes used to prepare crab bioproducts for this study is presented in Figure 4.8. 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 3 hours at 25°C; and (2) Deproteination with 10% NaOH (1:10 shells:NaOH) for 2 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 [62, 63].

The resulting chitin (Figure 4.7) 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.

Figure 4. 7 Snow crab chitin (not depigmented)

4.5.5 Proximate Composition

Proximate composition was determined for the dried crab by-product samples and included determination of: Moisture Content - Air Oven Method - AOAC Method 930.14; Kjeldahl Nitrogen - AOAC Method 954.01/988.05; and Ash Content - AOAC Method 938.08 Ash of Seafood. Detailed methods were previously described in Chapter 3, Appendix 3.

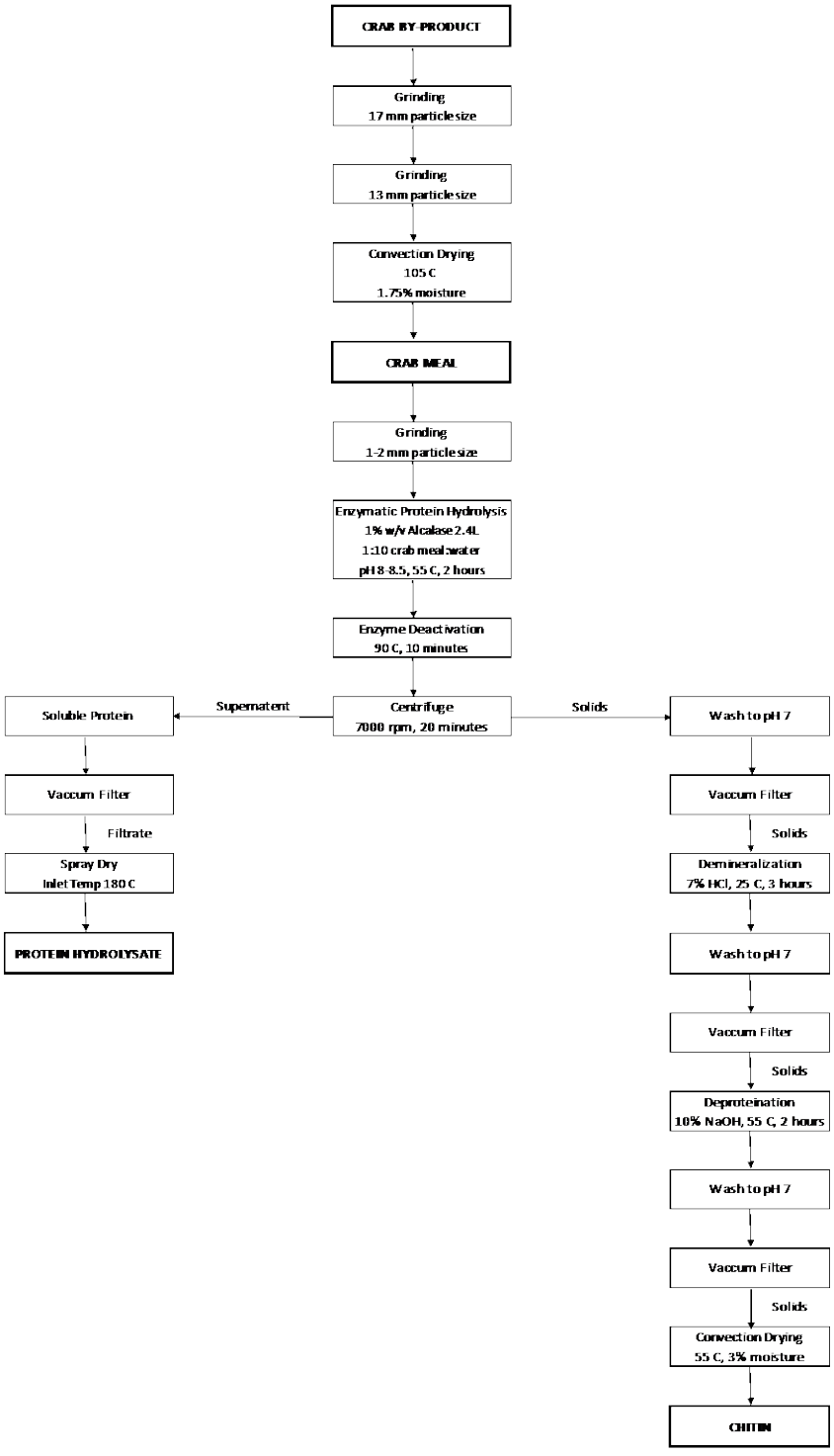


Figure 4. 8 Schematic illustration of the extraction, recovery and purification processes used to prepare crab bioproducts.

4.5.6 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 3 hours at 25°C, followed by deproteination with 10% NaOH (1:8 of crab:NaOH) for 2 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 Equation 4.1:

% Chitin Yield = [weight of chitin (g)/weight of crab meal (g)] x 100 (Eqn. 4.1)

Chitin content was calculated for crab chitin using Equation 4.2:

% Chitin Content = % Nitrogen x 14.5 (Eqn. 4.2)

Where: 14.5 is the nitrogen-to-chitin conversion factor based on an average nitrogen content of 6.9% for fully acetylated chitin.

4.5.7 Elemental Analysis (ICP-MS) Raw Crab By-Products

Samples of raw (unprocessed) crab by-products were analyzed by Memorial University's Department of Earth Sciences for elemental analysis. Samples were prepared by ashing for 6 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. The step-by-step sample preparation method was previously described in Chapter 3, Appendix 2.

4.5.8 Elemental Analysis (ICP-MS) Dried Crab Bioproducts

Due to a maintenance shutdown of the Memorial University lab that conducted the elemental analysis on the raw (unprocessed) crab by-products, the subsequently isolated crab bioproducts (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.

4.6 Results and Discussion

4.6.1 Proximate Composition

The proximate compositions of the extracted crab bioproducts are presented in Table 4.4. 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 4.94 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 by-product. The protein hydrolysate contained 50.93% protein and 25.2% ash. The chitin fraction had an acceptable low ash content below 1%, and a high chitin content (88%).

Table 4. 4 Proximate composition of extracted crab processing bioproducts [a]

Sample	% Moisture	% Chitin	% Total Nitrogen	% Protein	% Ash	% Lipid
Crab Meal [b]	1.75	16.74 ± 1.26	6.12 ± 0.07	30.23	36.01 ± 0.85	8.99 ± 0.42
Protein Hydrolysate [c]	2.33	-	10.31	50.93	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 by-product. [b] Results are the mean of 3 determinations ± standard deviation, except % Moisture for which there was only a single determination (n=1). [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.

4.6.2 Elemental Composition of Crab By-Products and Crab Bioproducts

The purpose of this analysis was to understand the transfer of heavy metals from snow crab processing by-products during the extraction of bulk intermediate bioproducts - crab meal, protein hydrolysate and chitin. Elemental compositions of the raw (unprocessed) crab by-products and the extracted crab bioproducts are presented in Table 4.5. 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.

Table 4. 5 Elemental composition of raw snow crab by-products and extracted bioproducts on a dry weight basis in parts per million (ppm)

Analytes	Raw Crab By-product [a]	Whole Crab Meal [b]	Crab Protein Hydrolysate [b]	Crab Chitin [b]
Aluminum	102.7 ± 0.02	185	5	151
Antimony	nd	0.05	0.03	< 0.02
Arsenic	3.64 ± 0.07	21.2	54.6	< 0.2
Barium	27.7 ± 0.2	23.9	0.4	< 0.2
Beryllium	nd	< 0.02	< 0.02	< 0.02
Bismuth	0.0141 ± 0.001	< 0.2	< 0.2	< 0.2
Boron	nd	34	76.2	0.8
Cadmium	2.35 ± 0.05	1.8	0.833	0.004
Calcium	108000 ± 1414	117000	16800	< 10
Chromium	0.871 ± 0.032	1.1	0.3	0.3
Cobalt	0.503 ± 0.033	0.56	1.29	< 0.02
Copper	40.6 ± 1.8	36.1	17.3	7.5
Iron	159 ± 18	179	16	< 4
Lead	0.343 ± 0.008	0.31	0.06	0.03
Lithium	1.34 ± 0.03	1.47	2.96	< 0.02
Magnesium	nd	12900	8260	3
Manganese	6.26 ± 0.27	5.4	0.6	< 0.2
Mercury	<DL	0.16	0.03	< 0.01
Molybdenum	0.39 ± 0.01	0.36	0.65	< 0.02
Nickel	2.70 ± 0.50	2.1	4.6	< 0.2
Potassium	nd	7040	19000	13
Rubidium	2.76 ± 0.01	2.87	7.83	< 0.02
Selenium	nd	2.2	3.2	< 0.2
Silver	2.25 ± 0.00	1.79	0.07	0.07
Sodium	nd	23500	69700	530
Strontium	2300 ± 34	2210	329	< 0.2
Tellurium	nd	< 0.02	< 0.02	< 0.02
Thallium	<DL	< 0.02	< 0.02	< 0.02
Tin	17.4 ± 0.6	46.2	1.94	0.05
Uranium	0.192 ± 0.005	0.16	< 0.02	< 0.02
Vanadium	0.66 5 ± 0.007	0.6	0.4	< 0.2
Zinc	52.1 ± 1.2	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 RPC.*

The level of heavy metals in the crab bioproducts evaluated in this study followed the order: crab meal > crab by-product > protein hydrolysate > chitin. Heavy metals tend to accumulate in the organs and tissues of crustaceans such as crabs and prawns [15, 16]. Kim and Yoon [17] 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 bio-product (Table 4.4) indicate 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 by-product 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 bioproducts 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 by-product (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 by-products, 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 by-product 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 by-product (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 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 [66]. This, coupled with reports of increasing concentrations of aluminum in the environment, food, and drink [8, 33, 48], 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 [65], limits for aluminum in food and natural health products have not been established.

4.6.3 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 have established maximum allowable levels (Table 4.1) 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 4.6. Mercury and lead levels were below

the Health Canada maximum level of 0.5-1 ppm [11] 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 [11], and 8 ppm in livestock feed [10]. Arsenic was more concentrated in the protein hydrolysate sample in comparison to the crab meal sample. The high levels of sodium and potassium (Table 4.5) 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.

Table 4. 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) [11]	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

4.6.3.1 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 [5, 6]. Sources of arsenic in the environment come from industrial sources, natural mine deposits, use of pesticides containing arsenic, and inappropriate disposal of arsenic chemicals [6].

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 [24]. 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 [5, 23, 24, 25]. Organo-arsenicals such as arsenobetaine, have low toxicity due to their low biological reactivity and their rapid excretion in the urine [26].

Dietary exposure to arsenic is largely influenced by the amount of seafood in the diet [26]. 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 [5, 25, 26, 27]. 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 [25, 29]. Lynch *et al.* [28] reported that crustaceans may contain high levels of iAs.

Total arsenic concentrations in some crustaceans have been reported to be > 100 mg/kg [26, 31, 64]. Anacleto *et al*. [30] 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.* [31] reported total arsenic levels of 1.69-137.32 ppm in crustaceans, and Fabris *et al.* [32] reported a total arsenic level of 50.7 ppm in Australian lobster. The levels of arsenic found in our snow crab by-product, 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*. [5] and Lorenzana *et al.* [29]. Cubadda *et al*. [5] 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 organoarsenic species (other than arsenobetaine), which may or may not be toxic. Lorenzana *et al*. [29] 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 [67]. At this dosage, based on our theoretical estimate of iAs, our crab protein hydrolysate exceeds Health Canada's daily acceptable limits for NHPs (Table 4.2) resulting in 164-218 µg/day of total arsenic, and 8.19-54.6 µg/day of iAs.

4.6.4 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 [34]. The control over molecular weight (MW), viscosity and degree of deacetylation (DD) 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 [34, 37, 38, 39]. Safety is determined by the levels of residual protein, bacterial endotoxins, and heavy metals present [34, 41].

Currently, chitosan is approved in Canada as a natural health product (NHP) for oral administration as a supplement for weight management and maintaining healthy cholesterol levels [42]. In the United States (US), chitosan has been approved by the FDA (Food and Drug Administration) for wound healing applications [43], and as a GRAS (Generally Recognized as Safe) food additive [36], while its complete approval by the FDA for all biomedical applications is still pending [43]. It is also approved as a food ingredient in Japan and Korea [36].

Morin-Crini *et al*. [36] 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 2 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 [39], 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 [35] covered general characteristics, chemical and microbiological purity levels, physiological properties, and biological activity [39]. More recently, ASTM [13] and USP-NF [14] 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, GMP (Good Manufacturing Practices) or GLP (Good Laboratory Practices) and must meet the requirements of the importing countries health regulations [41].

The chitin sample produced meets the USP-NF standard for biomedical/pharmaceutical chitin-chitosan applications for arsenic, cadmium, chromium, iron, lead, mercury, and nickel, but exceeds the total maximum allowable level of heavy metals when aluminum is considered (Table 4.7). The chitin sample also meets the Health Canada requirements for levels of arsenic, lead and mercury in seafood (Table 4.7). However, Health Canada has not established limits for levels of total aluminum in food or natural health products.

Table 4. 7 Comparison of heavy metals in chitin with industry standard for biomedical chitosan use and Health Canada levels for seafood [11, 13, 14, 40]

Heavy Metal	Maximum Allowable Level (ppm) for Biomedical Use (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.

4.6.4.1 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 [33]. 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 [6, 7, 8, 44, 46]. Mining and processing of aluminum increases its level in the environment [6, 45, 46] as does acidification of the soils [8, 46]. 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 [8]. Other sources of aluminum include food additives, aluminum utensils and tea consumption [49]. However, aluminum has no known biological role. It is a non-essential toxic metal to microorganisms, animals, fish, aquatic life, and potentially humans [8, 47]. 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 [8, 33, 48, 65, 66], however, studies to date have been inconclusive.

Maximum dietary limit intake levels for aluminum have been established by various organizations. The EFSA (European Food Safety Authority) has established a Tolerable Weekly Intake (TWI) of 1 mg Al per kg of bodyweight [22]. The FAO/WHO Expert Committee on Food Additives has set a Provisional Tolerable Weekly Intake (PTWI) of 2 mg/kg of bodyweight/week [8, 23], stating that a daily aluminum intake of up to 7 mg/Kg body weight is tolerable [59]. Dietary limit intake levels have not been established by

Health Canada, 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*. [6] 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 [50, 51]. Pereira *et al*. [33] reported that in marine samples, aluminum levels vary and can range from 0.1 to 19.2 μg/g in a variety of fish to as high as 71.9 μg/g in mussels (*Mytilus edulis*). The aluminum levels determined for snow crab products in this study are within the range reported by Jaishankar *et al*. [6].

Ingestion, inhalation, and dermal contact have all been identified as routes of aluminum exposure [6]. Drugs.com reported that in clinical trials the dosage of chitosan administered for glucose control is 1.5g/day yet could be as high as 15g/day for weight loss applications [68]. 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 15g/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 [55, 66]. Another proposed use of chitin and chitosan is as a drug delivery agent in inhalation products, and in the manufacture of biodegradable sutures [20, 43], 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 [65, 66]. Since aluminum has no biological function [8, 47], and it is not overtly toxic, it could become covertly toxic because it accumulates in the brain as we age [65, 66]. Until further scientific data is available regarding safe vs unsafe exposure levels, a precautionary approach to reduce human exposure to aluminum is advisable [65].

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

4.7 Conclusions and Future Opportunities

Understanding the levels of heavy metals in snow crab by-products and how they are transferred throughout the crab bio-product value chain will be key to developing safe marketable crab bioproducts for natural health product and biomedical/pharmaceutical applications. Two metals of concern were identified in the crab bioproducts 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 [5]. 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 by-product, and to ensure the extraction process does not increase the concentration of these metals and inadvertently facilitate their transfer to the final bioproducts. The processing steps should be further evaluated with the aim of reducing the arsenic and aluminum content in the bioproducts 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 by-products from NL, and their final concentrations in extracted crab bioproducts.

Note to Reader:

- Chapter 5 begins on page 277

4.8 References

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CHAPTER 5. Heavy Metals in Snow Crab (*Chionoecetes opilio*) Bioproducts - Part 2

5.1 Introduction

This chapter is an addendum to Chapter 4 in which we evaluated the levels of trace metal contaminants in crab processing by-products collected during the 2018 fishing season and their transfer to selected crab bioproducts: crab protein hydrolysate; and crab chitin. This Chapter provides additional data related to arsenic and aluminum in snow crab bioproducts extracted from crab processing by-products collected during the 2021 fishing season.

5.2 Purpose and Scope

The purpose of this second heavy metal study was to address the following recommendations from the 2018 study: (1) to evaluate the effect of separating the crab backs (shell) from the whole crab by-product prior to extraction of chitin on the trace metal content in the final chitin product; and (2) to evaluate the effect of the grinding and additional purification steps on trace metal levels in crab meal, protein hydrolysate and chitin extracted from whole (unseparated) crab by-product.

Snow crab by-product samples for this study were collected in May 2021 from the Quinlan Brothers Ltd. processing plant in Bay de Verde, Newfoundland.

5.3 Methods

The methods used for the 2021 study were the same as reported in Chapter 4 section 4.5 for the 2018 study but with the following modifications.

5.3.1 Collection and Preparation of Crab By-product

Snow crab processing by-products were collected from the Quinan Brothers Ltd. plant located in Bay de Verde, Newfoundland and Labrador (NL), Canada in May 2021. Two samples were collected; coarsely milled crab by-product (similar to the samples collected in June 2018) was collected from the processing plant offal grinder (Figure 5.1 A), and whole crab backs were collected prior to entering the processing plant offal grinder (Figure 5.1 B).

A B

Figure 5. 1 A - Coarsely milled crab by-product collected from the processing plant offal grinder; and B - Whole crab backs collected before entering the processing plant offal grinder

Crab by-products were collected in 44L Styro fish boxes. A layer of flake ice was placed in the bottom of each fish box and the crab by-product samples were collected in plastic bags which were tied, placed on top of the flake ice in the fish box, and then covered with additional flake ice (Figure 5.2). Lids were placed securely on top of each fish box for transport back to the Marine Institute in St. John's, NL.

Upon arrival at the Marine Institute, crab by-products were separated into smaller lots (~ 1 kg), vacuumed packaged, and placed in frozen storage at -20°C until required for

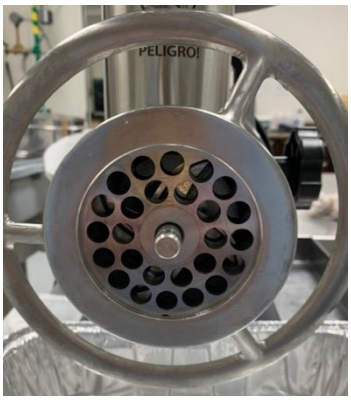
analysis. When required, samples of the crab by-products were removed from frozen storage and allowed to partially thaw at 4°C overnight.

Figure 5. 2 Crab by-product packed in Styro fish box with flake ice

The coarsely ground crab by-product was further processed through a Cabela's Carnivore electric meat grinder using a 10 mm plate (Figure 5.3). A 100 g sample of the raw (wet) ground crab by-product was removed and used to produce a dried crab meal sample by placing it on a watch glass and drying at 105ºC overnight to constant weight. A second 100 g sample of the raw (wet) ground crab by-product was removed and used to prepare protein hydrolysate and chitin.

The whole crab backs were first cleaned to remove belly flaps, mandibles, gills, and any remaining protein. The cleaned crab backs were placed in a convection drying oven (Figure 5.4) and dried at 55°C overnight. The dried crab shell backs were then milled/ground to about 1-2 mm in a Ninja high speed blender using a two-step process; coarsely ground to 5-10 mm followed by finer grinding to 1-2 mm. A 25 g sample of the dried milled crab shell was removed and used to prepare chitin.

A



B

Figure 5. 3 Cabela's Carnivore electric meat grinder (A) with 10 mm stainless steel cutting plate (B)

Figure 5. 4 Cleaned crab shell backs in drying oven

5.3.2 Extraction of Crab Bioproducts

A schematic illustration summarizing the extraction, recovery and purification processes used to prepare crab bioproducts from the raw (unseparated) ground crab by-product and the dried crab shell backs for this study are presented in Figure 5.6 and Figure 5.7, respectively. While the extraction processes were like those used in the 2018 study (Chapter 4), there were some key differences as shown in the illustrations and further described in sections 5.3.2.1 Protein Hydrolysis and 5.3.2.2 Chitin Extraction.

5.3.2.1 Protein Hydrolysis

Protein hydrolysis was carried out on the raw (wet) ground whole crab by-product sample according to the procedure described in Chapter 4 section 4.5.3 Protein Hydrolysis. The protein hydrolysate was dried in a Labcono freeze dryer as the material was too sticky to spray dry. Freeze drying was conducted as described in Chapter 3 section 3.3.3.4 Evaluation of Drying Methods. Approximately 600 ml of soluble protein (24 cupcakes x 25 ml each) were placed into silicon cupcake trays, frozen at -80°C overnight (Figure 5.5) and then placed in the freeze dryer. The dried samples were milled to a particle size of ~1 mm using an IKA high speed grinder.

Figure 5. 5 Frozen crab protein hydrolysate in silicon trays in preparation for freeze drying.

5.3.2.2 Chitin Extraction

Chitin extraction was carried out on the raw (wet) ground deproteinated whole crab by-product sample, and on the dried milled crab shell backs using the same procedure that was used for the 2018 study (refer to section 4.5.4 Chitin Extraction). In addition, the chitin samples were further processed by washing three times with acetone for removal of carotenoid pigments [1] and other contaminants.

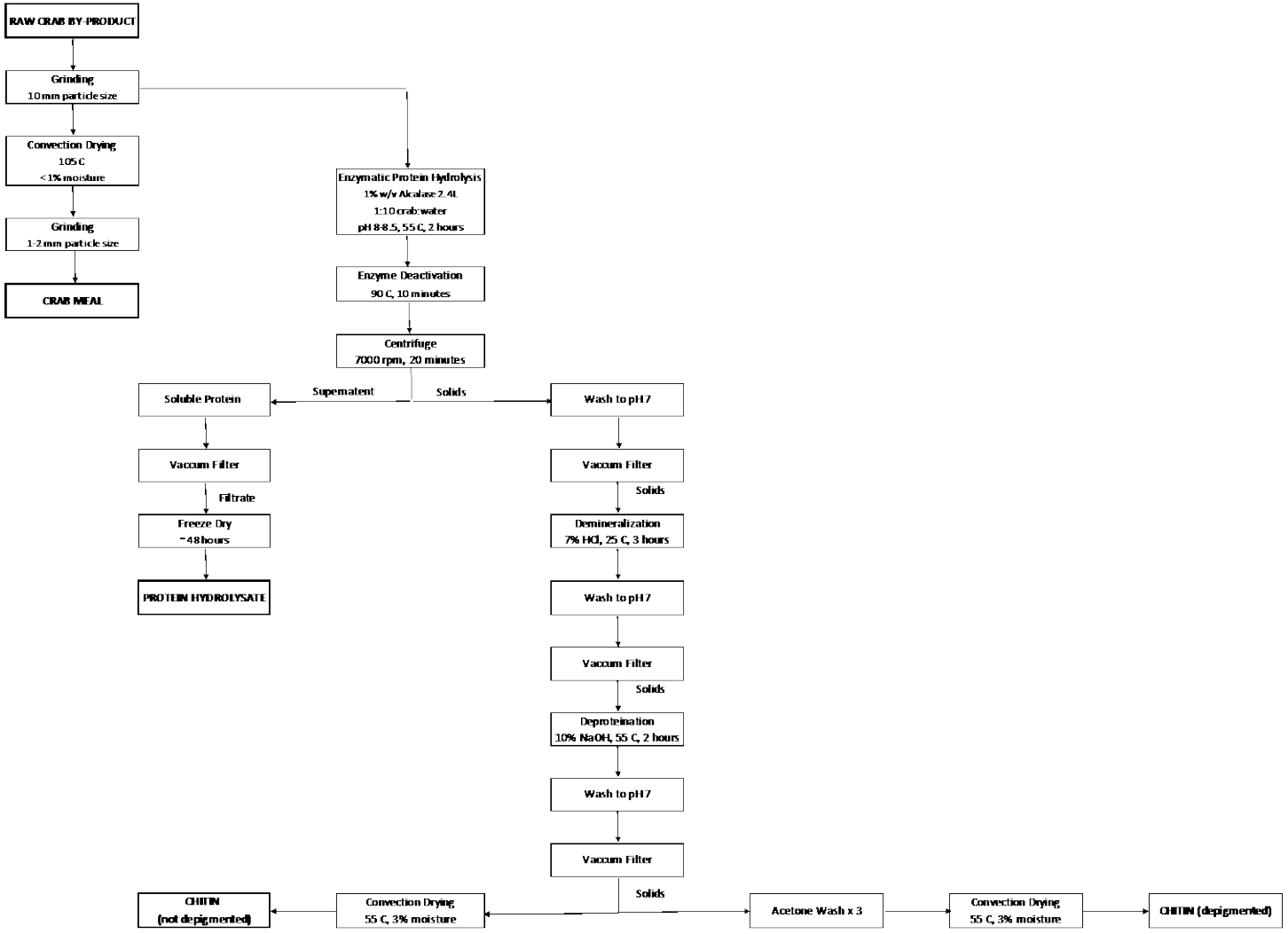


Figure 5. 6 Schematic illustration of the extraction, recovery and purification processes used to prepare crab bioproducts from raw ground crab by-product

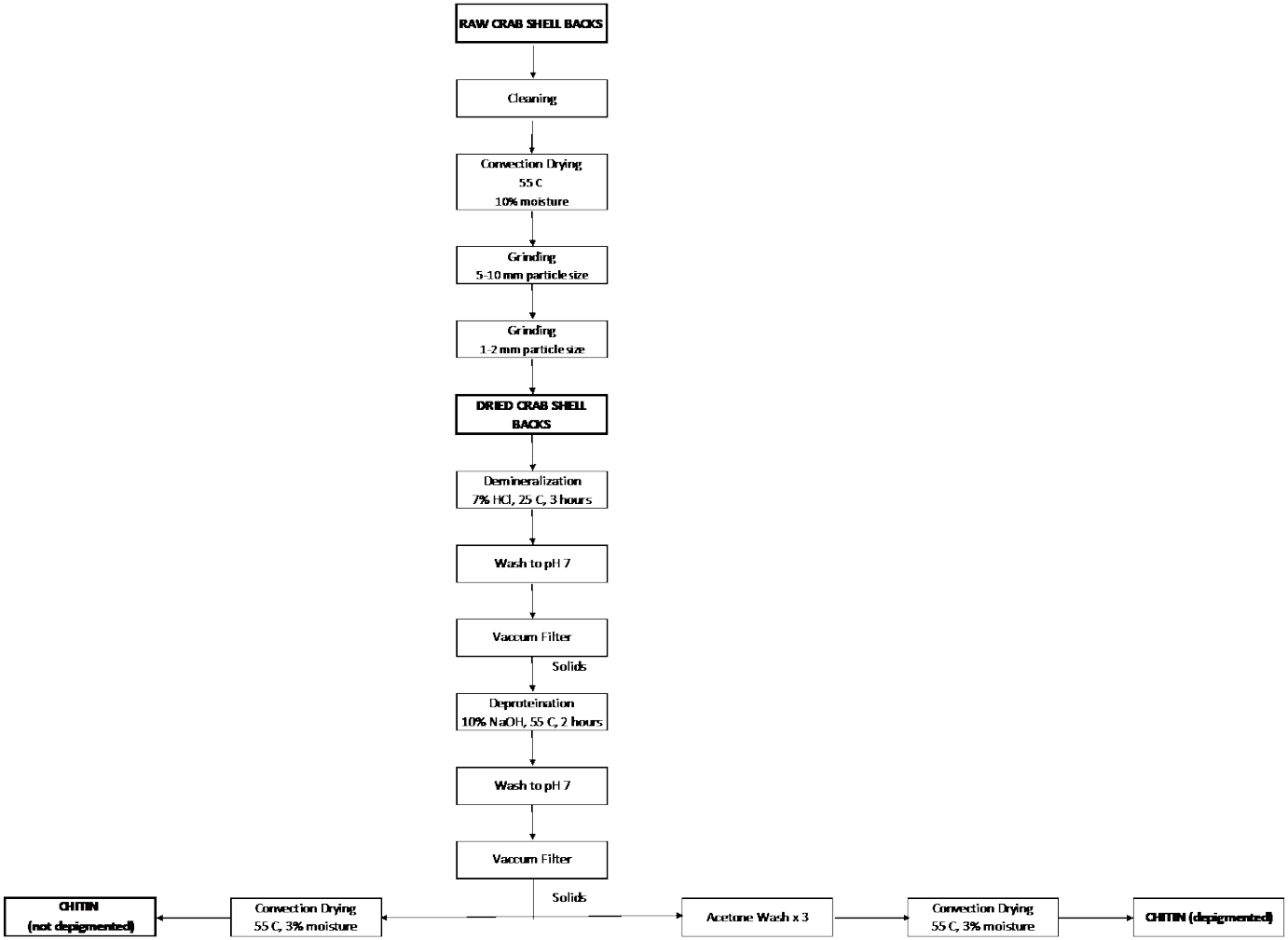


Figure 5. 7 Schematic illustration of the extraction, recovery and purification processes used to prepare crab bioproducts from dried crab shell back

5.3.3 Proximate Analysis

Proximate composition was determined for the dried crab bio-product samples and included determination of: Moisture Content - Air Oven Method - AOAC Method 930.14; Kjeldahl Nitrogen - AOAC Method 954.01/988.05; and Ash Content - AOAC Method 938.08 Ash of Seafood. Detailed methods were previously described in section 3.8 Appendix.

5.3.4 Elemental Analysis (ICP-MS) Dried Crab Bioproducts

The dried crab bioproducts (crab shell backs, crab meal, protein hydrolysate and chitin samples) were submitted to the Research and Productivity Council (RPC) in New Brunswick, Canada for analysis of trace metals and mercury as previously described in Chapter 4 section 4.5.8 Elemental Analysis (ICP-MS) Dried Crab Bioproducts.

5.4 Results and Discussion

5.4.1 Proximate Composition

The proximate composition of each crab bio-product is presented in Table 5.1. These results demonstrate that the extraction methods were effective in separating the protein and the chitin fractions from the crab meal by-product, and in removing the mineral (ash) from the crab backs and crab meal samples. The protein hydrolysate contained 9.55% protein nitrogen and 23% ash which is comparable to the results obtained in June 2018 (Table 4.4). All chitin fractions had acceptable nitrogen contents between 6-7% [2, 3] and low ash contents below 0.60%. Depigmentation had no effect on the nitrogen content ($p > 0.05$) or the ash content of the chitin samples. The ash content was much lower in the crab shell chitin in comparison to the crab meal chitin, resulting in an ~85-90% reduction in ash

content in chitin extracted from crab shell backs and therefore a higher purity. The crab meal chitin had a 3-5% higher nitrogen content than the crab shell (backs) chitin suggesting there may be some residual protein remaining in the crab meal chitin, or it may have a higher chitin content than the crab shell (backs) chitin. Shahidi and Synowiecki [1] reported that crab shell backs contained less chitin at ~ 18% (db) in comparison to other parts of snow crab which had chitin contents of 23.7-32.25% (db). Since the crab meal contains shell from the carapace, mandibles, belly flap and any small shell particles from the butchering process, it is likely that the crab meal chitin has a higher chitin nitrogen content than the crab shell (backs) chitin. The chitin nitrogen of the crab meal chitin (6.47-6.52) was comparable to that reported by Shahidi and Synowiecki [1] for crab shell chitin (6.42%), but our crab shell (backs) chitin had a slightly lower nitrogen content (6.17-6.23%).

Table 5. 1 Proximate composition of extracted snow crab bioproducts May 2021[1]

Sample Description	% Total Nitrogen [2]	% Ash [3]
Dried Crab Shell Backs	-	41.67 ± 0.027
Crab Shell Chitin (not depigmented)	6.17 ± 0.003[a]	0.05
Crab Shell Chitin (depigmented)	6.23 ± 0.004[a]	0.08
Dried Crab Meal (whole crab by-product)	5.07 ± 0.001[b]	41.81
Crab Meal Chitin (not depigmented)	6.52 ± 0.002[c]	0.59
Crab Meal Chitin (depigmented)	6.47 ± 0.005[c]	0.55
Dried protein Hydrolysate (whole crab by-product)	9.55 ± 0.002[d]	23.06

[1] Results are reported on a dry weight basis.
[2] Results are the mean of 3 determinations ± standard deviation.
[3] Results represent one determination due to the small sample size available except for the dried crab shell backs which is the mean of 3 determinations ± standard deviation.
[4] Values in the same column with different letters are significantly different ($p < 0.05$) according to Tukey's test.

5.4.2 Elemental Composition of Crab By-Products and Crab Bioproducts May 2021

Elemental compositions of the 2021 crab by-products and their extracted crab bioproducts are presented in Table 5.2. The total metal content followed the order crab shell backs > whole crab meal > protein hydrolysate > crab meal chitin > crab shell (backs) chitin.

Comparison of the metal content of each of the starting raw materials used in this study reveals that concentrations of aluminum, arsenic, cadmium, chromium, copper, iron, lead, mercury, potassium, sodium, tin, and zinc are higher in the crab meal by-product than in the crab shell backs which is expected since the crab meal by-product also contains meat, gills, hepatopancreas and other organs were such metals tend to accumulate. These metals were also higher in the 2018 crab meal sample in comparison to the 2021 crab meal sample, except for cadmium which was slightly higher in the 2021 sample.

Levels of calcium and magnesium are higher in the crab shell backs and contribute to > 92% of the total metal content. These elements are main components of the crab exoskeleton and contribute to crab shell hardness. Lage-Yusty *et al.* [4] also reported that snow crab shells contained high amounts of calcium, phosphorous and magnesium. Similarly, BoBelmann *et. al.* [5] found that calcium, magnesium and phosphorous are main components of the cuticles in crab and lobster. However, calcium was higher in the crab meal chitin samples (1640-1780 ppm) than the crab shell (backs) chitin (40-41 ppm) which is consistent with the higher ash content in the crab meal chitin samples and indicates that a higher purity is obtained by separating the crab shell backs from the whole crab by-product.

Table 5. 2 Elemental composition of the May 2021 crab by-products and their extracted bioproducts [a]

Analytes	May 2021 Samples						
	Whole Crab Meal	Crab Meal Chitin (not depigmented)	Crab Meal Chitin (depigmented)	Crab Protein Hydrolysate	Crab Shell Backs	Crab Shell Chitin (not depigmented)	Crab Shell Chitin (depigmented)
Aluminum	111	9.3	3	38.11	34.84	4.4	3.5
Antimony	0.05	< 0.02	< 0.02	0.09	< 0.02	< 0.02	< 0.02
Arsenic	16.9	< 0.2	< 0.2	59.70	4.64	< 0.2	< 0.2
Barium	13.8	1.2	1.2	1.0	14.48	< 0.2	< 0.2
Beryllium	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Bismuth	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
Boron	41	0.6	0.3	94.6	43.06	< 0.2	< 0.2
Cadmium	2.97	0.011	0.008	2.94	0.492	< 0.002	< 0.003
Calcium	145000	1780	1640	30400	169991	41	40
Chromium	1.8	0.3	0.3	5.84	< 0.2	0.2	< 0.2
Cobalt	0.51	0.19	0.48	3.04	0.19	0.49	< 0.02
Copper	14.6	22.4	17.2	78.8	2.6	0.2	< 0.2
Iron	143	7	8	99.1	32	< 4	< 4
Lead	0.19	0.84	1.26	2.94	0.084	0.062	0.02
Lithium	1.11	< 0.02	< 0.02	2.07	0.71	< 0.02	< 0.02
Magnesium	12800	62	51	11675	13924	3.08	3
Manganese	5.5	0.4	0.3	1.65	5.9	< 0.2	< 0.2
Mercury	0.04	< 0.01	0.01	0.055	< 0.01	< 0.01	< 0.01
Molybdenum	0.18	0.02	0.05	0.86	< 0.02	0.03	< 0.02
Nickel	1.1	0.6	0.8	7.5	0.3	0.7	0.7
Potassium	4790	6	< 4	16742	3562	< 4	< 4
Rubidium	2.04	< 0.02	< 0.02	6.36	0.96	< 0.02	< 0.02
Selenium	2.4	< 0.2	< 0.2	4.96	0.89	< 0.2	< 0.2
Silver	1.46	0.02	< 0.02	2.64	0.15	0.48	0.46
Sodium	13500	250	120	46041	8421	< 10	< 10
Strontium	2470	16	13.9	555	2762	< 0.2	< 0.2
Tellurium	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Thallium	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Tin	4.66	0.21	0.35	2.19	< 0.02	0.02	< 0.02
Uranium	0.07	< 0.02	< 0.02	0.05	0.05	< 0.02	< 0.02
Vanadium	0.5	< 0.1	< 0.1	0.55	< 0.02	< 0.1	< 0.1
Zinc	26	0.3	< 0.2	24.9	4.4	< 0.2	< 0.2

[a] *Results represent the determination of one composite sample due to limited sample size available and cost of analysis. Analysis was conducted by RPC.*

Generally, all metals were reduced to acceptable levels in the chitin products obtained from crab meal and crab shell backs, while most metals became more concentrated in the protein hydrolysate. Arsenic levels in the 2021 crab meal bioproducts followed a similar trend to that observed in the 2018 study (Table 4.5) with arsenic increasing from 16.9 ppm in the crab meal to 59.7 ppm (Table 5.2) in the protein hydrolysate. Arsenic in the crab shell backs was 4.64 ppm and was reduced to < 0.2 ppm in the depigmented crab shell chitin. These results indicate that the source of arsenic is bioaccumulation from the marine environment, that it is mainly present in the crab organs, and that during extraction it becomes concentrated in the protein hydrolysate.

Aluminum levels however followed a different trend and were reduced by 92% from 111 ppm in the 2021 crab meal to 9.3 ppm in the crab meal chitin (not depigmented) in comparison to only an 18% reduction in the 2018 study. Aluminum was reduced by 65.8% to 38.11 ppm in the 2021 protein hydrolysate in comparison to a 97% reduction in the 2018 study. Aluminum in the 2021 crab meal product was lower at 111 ppm than that reported for 2018 (185 ppm). In the 2018 study the aluminum content remained high in the chitin sample (151 ppm) and was lower in the protein hydrolysate sample (5 ppm). This suggests that in the 2018 study, the aluminum may have adsorbed to chitin which may be the result of differences in the extraction process used in 2018 (extraction from dried crab meal) vs. 2021 (extraction from wet crab by-product).

Our results suggest that in addition to bioaccumulation from the marine environment, contamination from the grinding equipment contributed to the high aluminum content in the 2018 crab meal and chitin samples. In 2018, a Hobart meat grinder was used to prepare the initial crab by-product sample. This grinder has a heat-treated steel knife and

cutting plate with a tinned auger made from alloyed steel [8]. It has also an aluminum auger and barrel. The Carnivore meat grinder used for the 2021 study has a stainless-steel grinder head and ring-nut assembly [9]. Visual examination of the two cutting plates showed that the Hobart grinding plate made from steel alloy has evidence of mild corrosion/rust whereas there is no corrosion/rust evident on the Carnivore cutting plate and grinder assembly made from stainless-steel (Figure 5.8). This may partially account for the overall higher metal content in the 2018 crab meal sample in comparison to the 2021 crab meal sample.

Depigmentation had a minimal effect on removal of metals from the non-depigmented chitin samples, however there was some effect on the reduction in aluminum. Depigmentation reduced aluminum by 67.7% in crab meal chitin and 20% in crab shell chitin.

A B

Figure 5. 8 Hobart cutting plate (A); Carnivore grinder head ring-nut assembly (B)

5.5 Conclusions and Future Opportunities

Based on the 2018 and 2021 results it is concluded that: (1) the source of arsenic in snow crab by-products is bioaccumulation from the marine environment; (2) that arsenic is mainly present in the non-shell (i.e., meat, organs, protein) fraction; and (3) that during extraction arsenic becomes concentrated in the protein hydrolysate. Because arsenic and other metals accumulate in the protein hydrolysate, this product is not suitable as a natural health product in its current form and may not be suitable as a feed ingredient. It currently exceeds Health Canada's acceptable levels of arsenic for seafood, natural health products, and animal feed [6, 7].

Other conclusions drawn include: (1) the grinding process is a source of aluminum and other metal contamination in our crab by-products; (2) extraction of chitin from wet (never dried) crab by-product seems to facilitate removal of aluminum from the extracted chitin but increases the aluminum content in the protein hydrolysate; (3) separation of the crab shell from the whole crab by-product facilitates removal of aluminum, and other metals from the chitin; (4) Depigmentation had a minimal effect on removal of metals from the non-depigmented chitin, but had some effect on the reduction of aluminum.

Due to the limited sample size available, additional studies should be conducted to confirm these results. Based on the information obtained from the 2018 and 2021 study, the following recommendations are provided to improve the purity of snow crab bioproducts extracted from crab processing discards; (1) the processing by-products should be separated into shell and meat/organs/protein prior to extraction of intermediate crab bioproducts (i.e., protein hydrolysate and chitin); (2) grinding equipment that comes into

direct contact with the crab by-product, such as the cutting plates, augers, and knives, should be made from stainless steel, not alloyed steel, to minimize metal contamination; (3) including a purification step such as depigmentation with acetone may increase the removal of aluminum and other contaminants from chitin; (4) additional purification studies for the protein hydrolysate, such as ultrafiltration, to facilitate removal of arsenic and salt are also recommended.

Note to Reader:

- Chapter 6 begins on page 293

5.6 References

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CHAPTER 6. Green Extraction Technologies for the Recovery of Valuable Intermediate Snow Crab (*Chionoecetes opilio*) Bioproducts

6.1 Introduction

As discussed in the preceding chapters, Snow crab has replaced cod as "King" in the NL seafood industry. It is the province's most valuable export and was worth $648 million in 2020 [1]. Snow crab is primarily processed as Individually Quick Frozen (IQF) cooked sections, which generates 25-30% (~ 10,000 t/year) waste, comprised mainly of shells, protein, and organs. This material is currently not being utilized commercially but could potentially be recovered from processing plant butchering stations as a by-product and converted into intermediate bioproducts (crab meal, proteins, lipids, ash, chitin, pigments) or transformed into higher value bioproducts (chitosan, peptides, omega-3, astaxanthin, marine calcium). These bioproducts have a wide range of applications in several fields such as agriculture, aquaculture, biopharma, biomedicine, cosmetics, environment, food science & technology, health & nutrition [2]. Despite this, most crab processing by-products are being landfilled, or sea-dumped under permit. However, environmental restrictions are becoming more stringent making it more difficult and costly for processors to continue this type of waste disposal. In early 2022 for example, the crab plant in Bay de Verde received notification that it could no longer dispose of plant processing waste in the local landfill, leaving at sea dumping as the only disposal option. This option is neither logistically, economically nor environmentally favorable (2.8.5 Semi-structure Interviews - personal communications with industry stakeholders).

A key goal of this thesis is to identify ways to better utilize crab processing by-products generated by NL processing plants thereby keeping this material out of the environment and providing new economic opportunities for coastal communities. An additional goal is to implement inexpensive green technologies as an alternative to harsh chemical treatments and costly equipment that are traditionally employed for the recovery of crustacean bioproducts. Replacing harmful chemicals with green alternatives is of particular importance to many NL coastal communities. A key issue being the transportation and storage of dangerous chemicals in areas where there are limited health and safety emergency response resources in the event of a chemical spill or chemical fire. In addition to the environmental concerns of using harsh chemicals, there is an added health and safety concern for the intended end use of the bioproducts due to possible contaminants from the chemical treatments used. Chemical treatments such as deproteination with NaOH and demineralization with HCl can also have a negative impact on the quality of the recovered bioproducts by damaging the protein (e.g., hydrolysis and denaturation) and chitin molecules (e.g., decrease in MW and DP) [7, 38].

6.2 Purpose and Scope

This Chapter focuses on evaluating simple green technologies for the extraction of bulk intermediate bioproducts from snow crab processing by-products which were used as the feedstock material. Key objectives were to avoid the use of hazardous chemical solvents, conduct as close to a 100% green extraction as possible, and to develop simple processes that could facilitate implementation into existing snow crab processing plants, or a centralized crustacean by-product processing facility (refer to Chapter 2, Section 2.10

Recommendations), without the need for complicated and expensive equipment. Specific attention was placed on using green chemistry and simplified processes to extract carotenoid pigments in the form of pigmented vegetable oil, pigmented protein powder, and chitin from crab processing by-products collected during the 2021 crab harvesting season from the Quinlan Brothers Ltd. processing plant located in Bay de Verde, NL. To understand the effect of the selected green chemistry treatments on the quality of the extracted bioproducts, and their effectiveness for extracting the targeted bioproducts, characterization studies were conducted on the raw material and each crab bio-product.

6.3 Methods

Crab processing by-products were collected in May 2021 from Quinlan Brothers Ltd. processing plant, Bay de Verde, Newfoundland. Following preparation and stabilization of the by-product material (refer to section 6.3.1 Raw Materials), raw materials were characterized as described in section 6.3.2 Characterization of Raw Materials. A combination of mechanical, enzymatic, and green chemical treatments was used to isolate carotenoids, proteins, and chitin as described in section 6.3.3 Isolation of Crab Bioproducts.

6.3.1 Raw Materials

Coarsely shredded crab by-product samples were collected and prepared as described in Chapter 5, section 5.3.1 Collection and Preparation of Crab By-product. Whole crab backs were not evaluated in this study. All analyses were conducted within 6 months.

6.3.2 Characterization of Raw Materials

6.3.2.1 Proximate Composition

Proximate composition was determined for the raw crab by-product samples and included determination of: Moisture Content - Air Oven Method - AOAC Method 930.14; Kjeldahl Nitrogen - AOAC Method 954.01/988.05; Lipid Content - AOAC 920.39 (Soxhlet); and Ash Content - AOAC Method 938.08 Ash of Seafood. Detailed methods were previously described in Chapter 3 Appendix 3 - Proximate Analysis.

6.3.2.2 Chitin Content

Chitin content was determined as previously described in Chapter 3 section 3.3.3.7 Chitin Content.

6.3.2.3 Total Astaxanthin Content

Astaxanthin was extracted from the raw crab by-product samples using hexane:isoproponal (3:2 v/v) as described by Sindhu and Sherief [3]. A known weight of homogenized samples (approximately 1 g) was mixed with 10 ml of hexane:isopropanol (3:2 v/v) and immediately vortexed for 20 seconds. For completion of the extraction, the samples were then sonicated for 5 min at 25°C in a Branson 2800 sonicator. Solid particles were removed by filtration through Whatman #42 filter paper. The recovered solids were repeatedly extracted with fresh solvent until the filtrate was clear. The pooled filtrate was collected in a separatory funnel. An equal volume of 1% NaCl solution was added. After thorough mixing, the epiphase was collected and dehydrated with anhydrous sodium sulphate, and then evaporated to dryness in a 60°C water bath. The residue was re-dissolved in 5 ml of hexane. The samples were stored in the dark at 2-8°C until needed for analysis.

Immediately following extraction, the samples were placed in a cuvette and the absorption was measured at λ_{max} (476 nm) in a HACH DR600 Spectrophotometer. The pigment concentration was calculated using Equation (6.1) and reported as total astaxanthin.

$$Astaxanthin\ \left[\frac{\mu g}{g}\right] = \frac{A*D*10^6}{100*G*d*E} \quad \text{(Eqn 6.1)}$$

With A: Absorption at λ_{max}, D: Volume of extract [ml], G: Sample weight [g], d: Cuvette distance (10 mm), E: Extinction coefficient (2100 for Astaxanthin) [3].

6.2.3.4 Crude PPO Activity

In Chapter 3 the results indicated that snow crab by-products may be high in polyphenol oxidase (PPO) rendering this material prone to discolouration reactions such as bluing and melanosis, which will impact handling and storage requirements of crab by-products. To confirm this hypothesis, PPO activity was evaluated in the 2021 samples.

Enzyme Extract

Polyphenol oxidase (PPO) enzyme was extracted from crab by-product samples by homogenizing with distilled water using a ratio of 2:1 (crab by-product: water), stirring for 10 minutes, and then centrifuging at 10,400 x *g* for 20 minutes at 4ºC [5, 6]. The resulting supernatants (Figure 6.1) were removed and frozen at –20ºC until analysis could be performed.

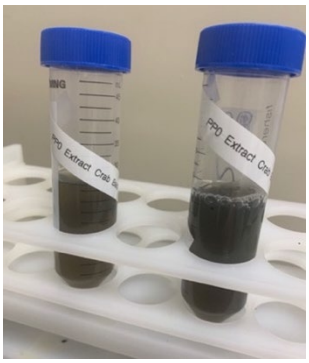


Figure 6. 1 Crab PPO extract

PPO Analysis

1 mL of the crude enzyme solution was placed into a test tube containing 3.5 mL of 0.066 M phosphate buffer solution at pH 7.0, and 0.5 mL of catechol solution containing 1 mg substrate. The final volume was adjusted to 6.0 mL by adding distilled water and it was incubated at 25°C for 15-30 minutes. Before reading, 1 mL of 1 mol NaOH was added to the solution and mixed. The absorbance was read at 480 nm in a HACH DR600 spectrophotometer. Enzymatic activity was expressed in U, where 1 U = increase in absorbance of 0.001 per min per mg protein in the extract [6, 15]. Protein content of the extract was determined by the modified Lowry method (Chapter 3 Appendix 5). Enzyme activity was measured on two samples of crab by-product. For each sample 3 replicate measurements were taken of the supernatant enzyme activity.

6.3.3 Isolation of Crab Bioproducts

Isolation of crab bioproducts involved simple processes using a combination of mechanical, enzymatic, and green chemical treatments to extract carotenoids (astaxanthin), proteins, and chitin as illustrated in Figure 6.2.

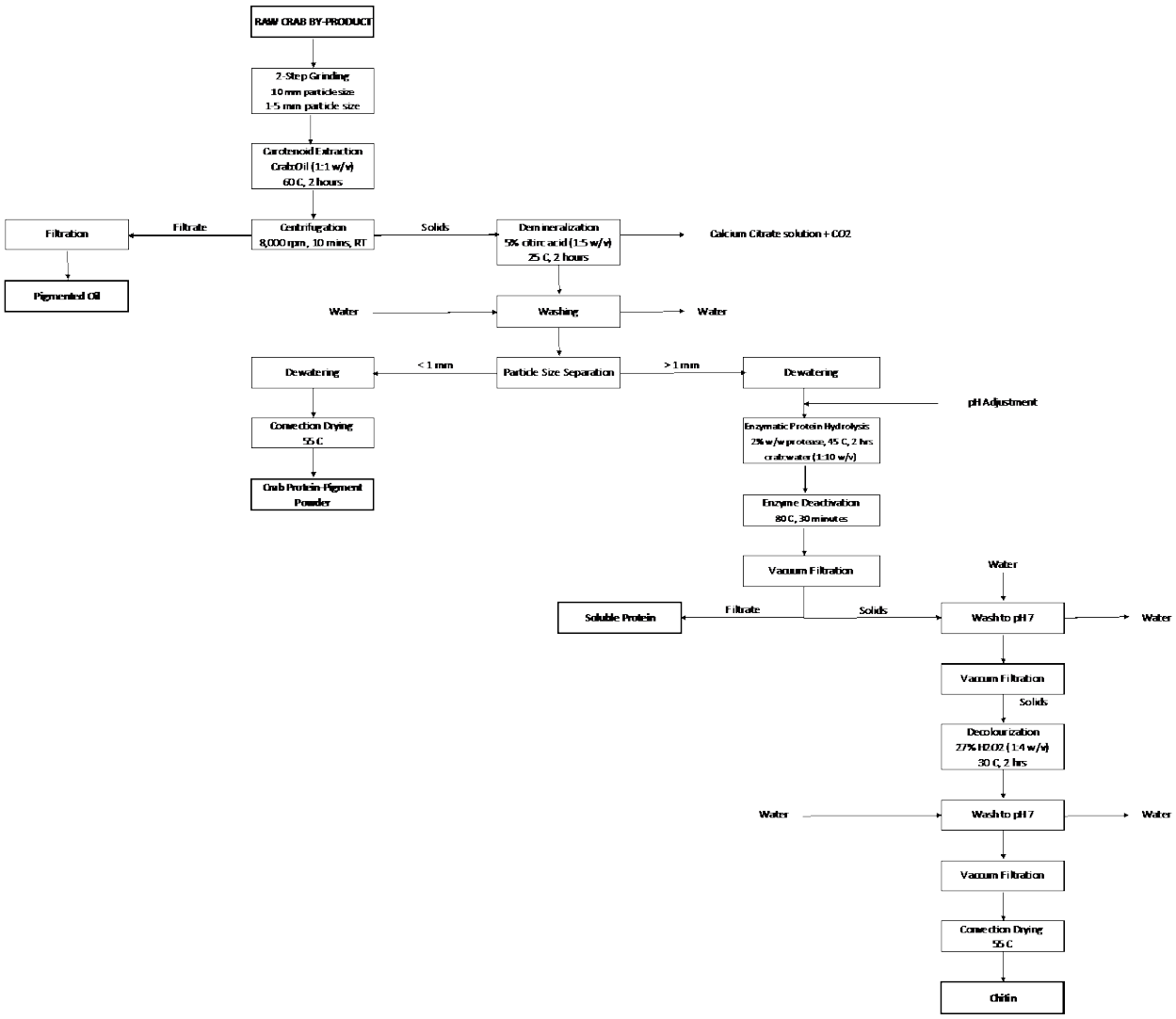


Figure 6. 2 Isolation of Snow crab bioproducts using a green chemistry approach

6.3.3.1 Astaxanthin Extraction in Different Vegetable Oils

Astaxanthin, the principal carotenoid in crustaceans, is a lipid soluble orange-red pigment found in both the free and esterified forms in crustacean shells [7, 61]. Its solubility in fats and oils is due to its long unsaturated aliphatic chains (Figure 6.3). Because of this lipophilic property, different vegetable oils were selected as green solvent alternatives to

replace the use of flammable solvents such as ethanol and acetone which are often used for the extraction of natural health products and for depigmentation of chitin.

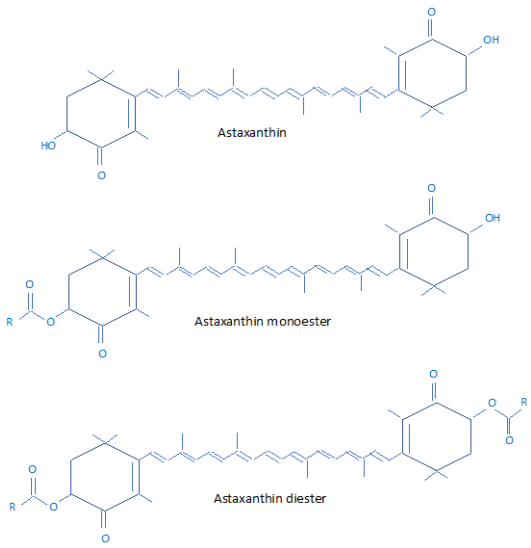


Figure 6. 3 Chemical structure of astaxanthin and its esters.

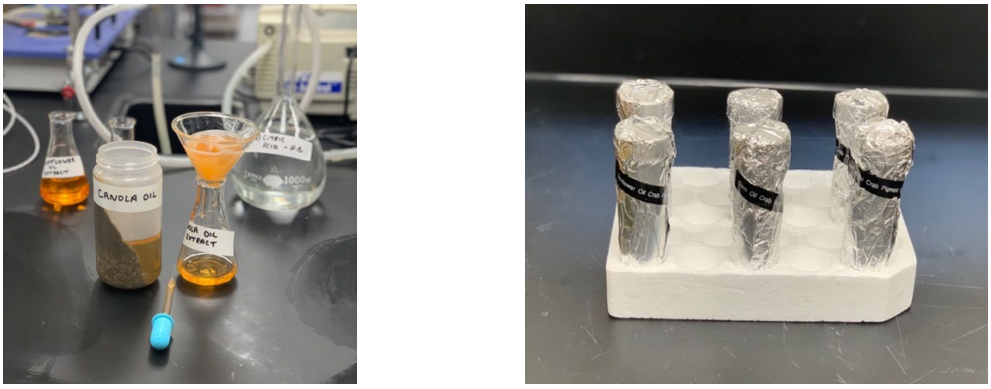
Three common vegetable oils were selected based on availability from Canadian producers, and wholesale price (in Canadian $) including canola oil ($1.01/kg), corn oil ($0.88/kg), and sunflower oil ($0.71/kg)[63]. Fish oil was also considered but was not included due to its higher cost ($2.92/kg USD)[64] and limited availability. The physical and chemical properties of oils are dependent on their fatty acid and triacylglycerol compositions, while thermo-oxidative stability depends on the amount of PUFAs as well as the content and type of tocopherols present [62, 63]. Oils with lower PUFAs and high oleic acid and saturated fatty acids are more stable than oils with high PUFAs, while oils

[63] Prices were obtained from https://www.selinawamucii.com/
[64] Price was obtained from tridge.com

with higher gamma and delta tocopherols are more stable than those with more alpha and beta tocopherols [64]. Various characteristics of the 3 selected vegetable oils were compared (Appendix 2) revealing that: (1) Canola oil is highest in MUFAs, mainly oleic acid (60%); (2) Sunflower oil is highest in PUFAs, mainly linoleic acid (68%); (3) Corn oil is highest in saturated fatty acids (13%) as well as gamma and delta tocopherols (451 ppm); and (4) Sunflower oil is highest in alpha and beta tocopherols (630 ppm). This comparison indicates that of the oils selected, corn oil is the most stable, and sunflower oil is least stable. Viscosity is also thought to impact the effectiveness of vegetable oil solvents compared with lower viscosity organic solvents for pigment extraction. At 25°C and 60°C viscosities of the selected oils were highest for sunflower oil (51-59 mm^2/sec and 16.9-20 mm^2/sec), followed by corn oil (49.7 mm^2/sec and 16.9 mm^2/sec), and canola oil (48.7 mm^2/sec and 16 mm^2/sec), when measured using a capillary tube viscometer [65].

An amount of ~100 g of crab by-product was blended with 50 mL distilled water in a Ninja blender to a particle size of 1-5 mm. The blended crab samples were mixed with each oil in a 1:1 (w/v) ratio of crab-by-product:oil in 500 mL glass mason jars and incubated for 2 hours in a Thermo Scientific Enviro Shaker at 60°C with continuous agitation at 165 rpm [7, 17, 18]. After incubation each sample was transferred to a 500 ml centrifuge bottle and centrifuged in a Thermo Scientific Sorvall Lynx 4000 Centrifuge for 10 minutes at 8,000 rpm and 20°C. The pigmented oil layer was carefully decanted, and gravity filtered through a Whatman #40 ashless filter paper and collected in a 125 mL Erlenmeyer flask (Figure 6.4 A). The volume of pigmented oil recovered was noted. The aqueous layer was discarded. The pigmented oils were collected in 50 mL centrifuge tubes, wrapped in foil, and stored at -80°C until required for testing (Figure 6.4 B).

The total astaxanthin content in each pigmented oil was measured spectrophotometrically at 470 nm against the particular oil as a blank [18]. The total astaxanthin yield was calculated using Equation (6.1) and the extinction coefficient for the particular oil.



A B

Figure 6. 4 Recovery of pigmented oil extracted from snow crab by-product (A) and pigmented oils packaged for frozen storage (B)

6.3.3.2 Demineralization Using Organic Acid

Following carotenoid extraction, the remaining solids were collected on a 45 mesh (355 µm) screen (Figure 6.5) and washed several times with distilled water to remove residual oil. The collected solids contained protein, shell, and some residual oil. This material was demineralized using citric acid ($H_3C_6H_5O_7$ - an organic acid found in citrus fruit) instead of hydrochloric acid (HCl - a strong inorganic acid) traditionally used for shell demineralization [7, 8].

Acetic acid ($HC_2H_3O_2$ - vinegar) [8] was also considered as an alternate organic acid. A preliminary study was conducted comparing acetic acid (7.5% v/v) applied at 4.8× the stoichiometric amount needed for neutralizing calcium carbonate, and citric acid (7.5%

w/v) applied at 4.5× the stoichiometric amount needed for neutralizing calcium carbonate [7]. Crab by-product was mixed with each acid solution in a 1:10 ratio of crab:liquid over a 3-hour period at room temperature with constant agitation at 150-200 rpm. Citric acid, a triprotic acid, and acetic acid, a monoprotic acid, react differently with calcium carbonate ($CaCO_3$). Citric acid can transfer 3 H+ ions per molecule whereas acetic acid can transfer only 1 H+ ion per molecule during an acid-base reaction. Citric acid therefore has 3 pKa values (pKa_1 =3.08, pKa_2 = 4.74, pKa_3 = 5.40) [67] with the first proton being released much more quickly than the second and third. Acetic acid has only one pKa value (4.75) [67] making it a weaker acid. Stoichiometrically, 2 moles of acetic acid are needed to neutralize 1 mole of $CaCO_3$ (Eqn. 6.2) whereas 2 moles of citric acid will neutralize 3 moles of $CaCO_3$ (Eqn. 6.3).

$$CaCO_{3\,(s)} + 2HC_2H_3O_{2\,(aq)} \rightleftharpoons Ca(CH_3O_2)_{2\,(aq)} + H_2O_{\,(l)} + CO_{2\,(g)} \quad \text{(Eqn. 6.2)}$$

$$3CaCO_{3\,(s)} + 2H_3C_6H_5O_{7\,(aq)} \rightleftharpoons Ca_3(C_6H_5O_7)_{2\,(aq)} + 3H_2O_{\,(l)} + 3CO_{2\,(g)} \quad \text{(Eqn. 6.3)}$$

Effectiveness of the acid treatment was determined based on the residual ash content of the demineralized crab by-product. It was concluded that citric acid was more effective for demineralization of the crab by-product resulting in a 36% reduction in ash residue (14.16% db) over acetic acid (22.12% db). There was an initial rapid drop in pH within the first 20 minutes of demineralization (Figure 6.6), with this drop being greater for citric acid (pH 8.55 to pH 2.53) than acetic acid (pH 8.55 to pH 3.53) due to the difference

in pKa values of the two acids. The pH increased slightly as the acid was used up in the reaction with no increase in pH after 120 minutes (citric acid pH 2.80, acetic acid pH 3.72). The acetic acid treated shells contained a heavy white residue (Figure 6.7) perhaps due to precipitation of unreacted calcium carbonate once the acid was used up in the reaction accounting for the higher ash content.

Figure 6. 5 Collection of crab by-product solids following pigment extraction

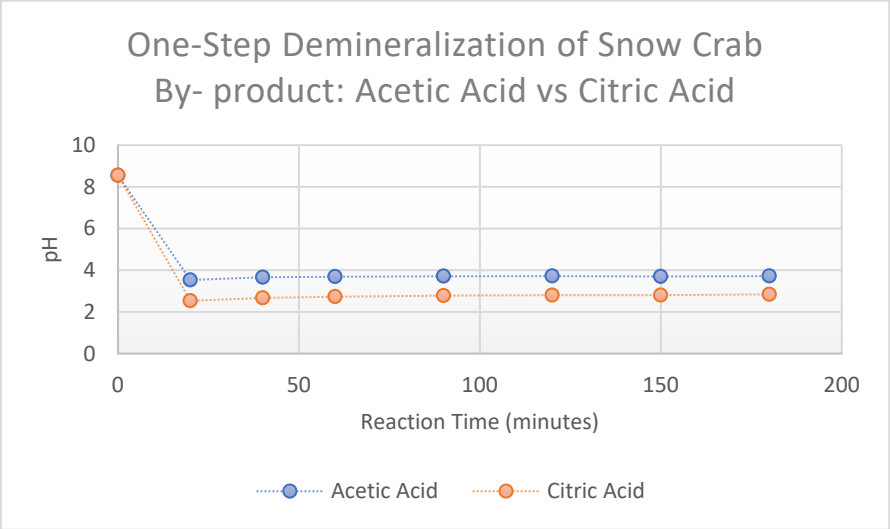


Figure 6. 6 Effect of acetic acid and citric acid on pH of crab by-product during one-step demineralization

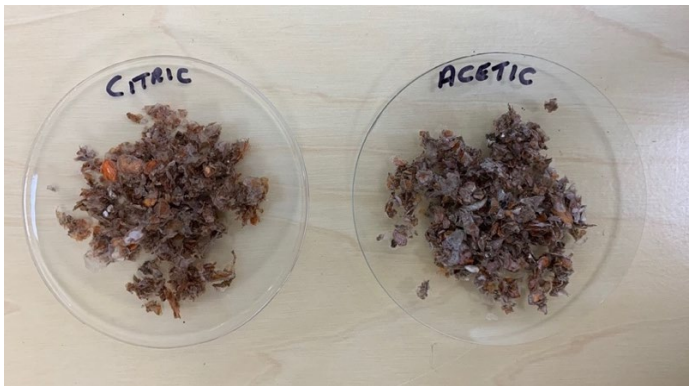


Figure 6. 7 Citric acid demineralized crab shells (left) vs acetic acid demineralized crab shells (right)

A preliminary study was also conducted to evaluate the merit of a two-step demineralization process using citric acid (7.5% w/v). The previous one-step process did not achieve full demineralization of the shell and a white precipitate, likely calcium citrate, was noticeable on the demineralized shells (Figure 6.7). The one-step citric acid demineralization process also resulted in excessive foaming (Figure 6.8). Similar results were reported by Pohling *et al.* [11] who also used a stepwise citric acid demineralization process for *Pandalus borealis* shrimp shells.

Figure 6. 8 Excessive foaming and precipitation of calcium citrate during one-step citric acid demineralization

To improve demineralization of the crab shells and minimize contamination of the shells with calcium citrate precipitate, a two-step process was evaluated. Crab shells were mixed with citric acid (7.5% w/v) in a 1:10 ratio of crab:liquid at room temperature for 30 minutes. Intense foaming, from the rapid neutralization of the carbonate to produce CO_2, was observed for the first 10 minutes but had dissipated completely after 30 minutes. The pH dropped initially to 3.32 upon addition of the citric acid and increased to 3.80 after 30 minutes as the acid was used up. Calcium citrate is a sparingly soluble tri-calcium salt whose solubility increases at pH < 3 [66]. Therefore, at pH 3.32-3.80, it is likely that calcium citrate precipitate may form during the first demineralization step and be deposited on the shells. After 30 minutes, the shells were drained, rinsed, and mixed with fresh citric acid (7.5% w/v) and allowed to react for an additional 90 minutes at room temperature. Upon the second addition of citric acid, minimal foaming was observed, the pH dropped to 2.39 and increased to 2.82 after 90 minutes. This process resulted in nearly complete removal (98.1%) of minerals as determined by the low ash content (0.82% db) of the demineralized shell perhaps a result of the lower pH of the reaction in step two causing any calcium citrate precipitate formed in step one to be redissolved. Therefore, a stepwise demineralization process using citric acid was selected for further evaluation as described below.

Partially depigmented crab by-product solids were treated with 5% (w/v) citric acid solution in a 3-step process using a ratio of 1:5 (solids:liquid) as described below. The lower concentration and ratio of citric acid was attempted to reduce the amount of citric acid needed for demineralization.

Step 1 of the demineralization process was a pretreatment step whereby each crab by-product sample was mixed with 5% (w/v) citric acid solution (1:5 ratio crab:citric acid solution) in a 1 L beaker and stored at 4°C overnight. The citric acid was added in 2 equal portions to avoid excess foaming. This pretreatment step was added to prevent melanosis during overnight storage and to partially demineralize the samples. The next day, the pretreated crab by-product samples were drained, washed, and dewatered.

In step 2, the pretreated samples were mixed with fresh, 5% (w/v) citric acid solution (1:5 ratio crab:citric acid solution) in a 1 L beaker with stepwise addition of citric acid as described in step 1. The mixture was stirred at room temperature using a VWR magnetic stirrer for ~30 minutes and foaming had stopped. The pH of the mixture was monitored using a HQ40D portable multi-meter (HACH, London, ON, Canada). The samples were then drained, washed, and dewatered.

Step 3 followed the same procedure as described in step 2; however, the reaction was allowed to proceed until there was no change in pH. The pH of the mixture was monitored using a HQ40D portable multi-meter (HACH, London, ON, Canada). The samples were then drained, washed, and strained through a 1 mm and 0.5 mm sieve to collect the demineralized shell (> 1 mm) and the residual protein fraction (< 1 mm). Each fraction was washed several times with distilled water to pH 7 and pressed to remove excess moisture. The protein fraction was dried in a convection oven at 55°C overnight to constant weight.

6.3.3.3 Enzymatic Deproteination

Following demineralization, the recovered shell material was enzymatically deproteinated using three different proteases, replacing the traditional method of deproteinating with KOH or NaOH [7]. The enzymes selected for this study were Fungal Acid Protease (FAP) *Aspergillus oryzae* purchased from Sigma-Aldrich, Sea-B-Zyme L200 purchased from Specialty Enzymes (Chino, CA, USA), and Acid Protease from Weifang Yuexiang Chemical Co. Ltd. provided by Ensymm UG & Co. KG.

FAP and Sea-B-Zyme L200 were selected based on a preliminary study which evaluated both enzymes as well as Alcalase 2.4L *Bacillus licehniformis* for their effectiveness in deproteinating the crab shell by-products collected in May 2021. Enzyme concentrations of 0.5-2.0% (based on weight of crab shell by-product), and treatment times of 1-4 hours were evaluated to identify which enzyme(s), concentrations, and reaction times were most effective. Determinations were performed in duplicate. pH and temperature of the crab:enzyme mixture were based on the supplier recommended optimum conditions for each enzyme. Enzymes for the current study were selected based on the residual protein content of deproteinated crab by-products obtained from the preliminary study. Alcalase 2.4L was eliminated from further evaluation as it gave the highest residual protein contents (2.91-11.59% db) regardless of concentration used in comparison to FAP (1.65-3.98% db) and Sea B Zyme (2.28 - 5.87% db). Weifang Acid Protease was included in the current study for comparison as a cheaper commercially available alternative.

The demineralized shells were divided into 3 equal portions for treatment with each of the 3 selected protease enzymes. The enzyme characteristics and reaction parameters for each enzyme treatment are presented in Table 6.1.

Table 6. 1 Enzyme characteristics and reaction parameters for enzymatic deproteination vs chemical deproteination of demineralized snow crab (Chionoecetes opilio) shells

Parameter	Fungal Acid Protease (*Aspergillus oryzae*)	Sea-B-Zyme L200	Weifang Acid Protease	NaOH
Type of Protease	Endo and Exo	Acid Protease	Acid Protease	
Enzyme Activity	> 500 U/g	Not specified	> 100,000 U/g	
% Enzyme[1]	2%	2%	2%	
pH	7.0	5.0	3.0	
Reaction Time	2 hours	2 hours	2 hours	2 hours
Optimum Temp Range	30-55°C	40-55°C	45°C	
Reaction Temp	45°C	45°C	45°C	55-90°C
Shell:Water	1:10	1:10	1:10	1:8 or 1:10
Bulk Cost ($/kg in USD)[2]	$80.00	$32.50	$12.00	$14.00

[1] *Enzyme concentration = % (w/w) of demineralized shell.* [2] *2021 prices provided by suppliers.*

The pH of the demineralized shells was monitored using a HQ40D portable multi-meter (HACH, London, ON, Canada). The demineralized shells were added to a 500 mL glass mason jar and either mixed with distilled water, or phosphate buffer depending on pH, in a ratio of 1:10 shells:liquid. The pH was monitored and adjusted as needed according to the parameters outlined in Table 6.1. The shell:liquid mixture was heated to 45°C, at which time the enzyme was added. The jars were capped and incubated for 2 hours in a Thermo Scientific Enviro Shaker at 45°C with continuous agitation at 165 rpm. The pH was monitored at 30-minute intervals and adjusted using 1 M HCl or 1 M NaOH as required. Enzyme deactivation was achieved by heating the mixture to 80°C and holding for 20-30 minutes. The samples were removed from the incubator and cooled to room temperature.

After enzyme deactivation and cooling, the shells (crude chitin) and filtrate (protein hydrolysate) were separated by vacuum filtration on a Whatman #40 ashless filter paper. The filtrate (protein hydrolysate) was collected in 50 mL centrifuge tubes, capped, wrapped in foil, and stored at –80°C. This represents a protein hydrolysate product which can be spray dried or freeze dried into a powder containing about 50% protein and around 25% mineral as demonstrated in Chapters 4 and 5. Further analysis of the protein hydrolysates was not possible due Covid-19 related delays. The chitin was washed several times to pH 7 with distilled water using a ratio of 1:10 chitin:water and vacuum filtered.

6.3.3.4 Decolourization with Hydrogen Peroxide

The wet chitin fraction was decolourized using laboratory grade 27% H_2O_2. Hydrogen peroxide was selected as an alternative to flammable solvents (e.g., acetone, ethanol, hexane) and hazardous (i.e., corrosive, oxidizers, toxic to aquatic life) bleaching agents (e.g., NaOCl) that are traditionally used for chitin depigmentation and decolourization [7]. Hydrogen peroxide is a strong bleaching agent which breaks down into water and oxygen leaving no harmful by-products and it is biodegradable, therefore meeting the selection criteria for green reagents and conditions. Chitin samples were placed in 500 mL mason jars, mixed with 27% H_2O_2 in a ratio of 1:4 chitin:H_2O_2 and heated to 30°C using a VWR hot plate stirrer. The jars were capped and incubated for 2 hours in a Thermo Scientific Enviro Shaker at 30°C with continuous agitation at 165 rpm. The samples were removed from the incubator and cooled to room temperature. The samples were vacuum filtered using a Whatman #40 ashless filter paper, washed with distilled water

to a final pH of 7, transferred to a watch glass and dried at 55°C overnight to a constant weight (Figure 6.9).

The above parameters were selected based on previous industrial studies (unpublished) conducted using H_2O_2 to decolourize shrimp shell (*Pandalus borealis*) and BSF (black soldier fly pupae) chitins. When used at higher temperatures and longer duration H_2O_2 can damage the chitin molecule, causing changes in molecular weight and degree of polymerization [30, 31]. Also, at higher temperatures H_2O_2 is unstable and highly reactive. The treatment time and temperature were selected based on minimums used in previous studies (unpublished) that were effective in decolourizing the chitin fraction and caused only a slight decrease in MW.

Figure 6. 9 Crab shell chitin in drying oven following attempted depigmentation with hydrogen peroxide

6.3.4 Characterization of Snow Crab Bioproducts

Selected crab bioproducts were characterized using the methods outlined in Table 6.2. Some of these methods were previously described in section 6.3.2 Characterization of Raw Materials.

6.3.4.1 Tristimulus Colour Parameters

The tristimulus colour parameters, L (lightness/darkness), a* (red /green), and b* (yellow/blue) of the ground chitin samples were measured using a portable handheld ColorTech-PCM Colorimeter (ColorTec, Clinton, NJ, USA), with a measurement angle of 10°, Illuminator D65, and aperture of 8 mm. The chromatic properties were defined by the L* a* b* color method of the CIE (Commission Internationale de l'Eclairage) and were expressed as L* (lightness; 100 = white, 0 = black), a* (red +; green -), and b* (yellow +; blue -) coordinates [7, 9]. Each sample was milled to a particle size of ~ 0.5 mm using an IKA WERKE MF 10 basic Microfine grinder equipped with a MF 1.2 impact grinding head and MF 0.5 stainless steel sieve. Approximately 2 g of each milled sample was placed on a watch glass. The dishes were placed on a white surface and measurements were carried out in at least triplicate [9, 10].

6.3.4.2 Powder X-Ray Diffraction

Powder X-ray diffraction was used to compare the effect of chemical and green extraction methods on the crystalline structure of the extracted chitin. X-ray powder diffractograms for chitin were recorded with a Rigaku Ultima IV automated X-ray diffractometer with a copper X-ray source (40 kV / 44 mA current) and a scintillation counter detector. The diffraction profile was recorded at room temperature at a scan speed of 1.0 deg/min, scan axis of 2theta, and scan range of 3-100°. The crystallinity Index (CI) was calculated using Equation (6.4) where I_{110} is the maximum intensity of the (110) peak at around 2theta = 19°, and I_{am} is the amorphous diffraction at 2theta = 12.6° [40, 41, 42].

$$CI\ (\%) = [(I_{110} - I_{am})/I_{110}] \times 100 \qquad \text{(Eqn 6.4)}$$

Table 6. 2 Methods used for characterization of snow crab bioproducts

Crab Bio-Product	Parameters Analyzed	Method
Pigmented Oils	Total Astaxanthin	hexane:isopropanol [2] (section 7.2.3.2)
Protein-Pigment Powder	Moisture	AOAC Method 930.14
	Total Nitrogen	AOAC Method 954.01/988.05
	Ash Content	AOAC Method 938.08 Ash of Seafood
	Total Astaxanthin	hexane:isopropanol [2]
Chitin	Moisture	AOAC Method 930.14
	Total Nitrogen	AOAC Method 954.01/988.05
	Protein Nitrogen	Modified Lowry
	Ash Content	AOAC Method 938.08 Ash of Seafood
	Total Astaxanthin	hexane:isopropanol [2] (section 7.2.3.2)
	Chitin Yield	refer to section 4.5.3.7
	Chitin Nitrogen	by calculation (refer to section 4.5.3.7)
	Tristimulus Colour Parameters	ColorTec PCM Colorimeter
	Powder X-ray Diffraction	Rigaku Ultima IV x-ray diffractometer

6.3.5 Statistical Analysis

Results were compared using analysis of variance (ANOVA) and the differences between the means by Tukey's test. Regression analysis was also performed to determine correlations between the Hunter L*a*b* colour parameters. All analyses were performed using the Data Analysis ToolPak in Microsoft Excel for Mac, Version 16.44. Alpha level 0.05 was selected as the threshold of significance to test the null hypothesis that all sample means are the same.

6.4 Results and Discussion

6.4.1 Pre-treatment of Raw Crab By-products

The raw material collection and pre-treatment methods used for this study were determined based on the results and observations identified in Chapter 3, section 3.4.1 Sample Preparation and Sensory Assessment of Raw Crab Biomass Feedstock. In Chapter 3 it was established that to maintain quality of crab processing by-products:

1. Stabilization such as freezing or seawater treatment of the by-products immediately following production will be critical.
2. Minimizing exposure to oxygen during collection and storage will be necessary to prevent/minimize enzymatic and oxidative spoilage reactions (e.g., bluing and melanosis, lipid oxidation, oxidation of carotenoid pigments).
3. The effect of pre-treatment steps, such as seawater treatment, on quality improvement *vs* processing challenges and associated production costs must be considered.

Crab by-products collected in May 2021 were handled as described in section 6.3.1 Raw Materials, vacuum packaged (Figure 6.10) and kept frozen at –20°C until required for analysis. The crab by-products handled in this way maintained their pink-red colour and did not exhibit any signs of the discolouration that was evident in the 2018 study (Figure 3.7).

A B

Figure 6. 10 Vacuum packaged frozen crab processing products (A) and pre-treated crab processing by-products (B)

Vacuum packaging and freezing were selected as the preferred stabilization methods for this study based on industry structure and the results of the 2018 study. Nearly all crab processing plants in Newfoundland and Labrador have commercial vacuum packaging machines, freezing capability, and cold storage facilities, therefore, additional infrastructure investment would not be necessary. Pre-treatment of crab with saltwater in the 2018 study caused significant processing issues and contributed to minor quality issues, therefore, this pre-treatment method was omitted to simplify the crab bioproducts stabilization and extraction processes. Drying was not used as a stabilization method for the 2021 study for the following reasons: (1) Only a few plants have drying capability; (2) Drying technology commercially in use in NL is based on hot air drying which is not optimal for higher-value bio-product applications; and (3) there are no large commercial scale freeze drying systems in use in NL at this time.

6.4.2 Characterization of Raw Crab By-products

Results for proximate composition and chitin content of the crab processing by-products collected in May 2021 are presented in Table 6.3 and are reported on a dry weight basis (i.e., % composition after removing all the moisture from the samples). Results for

total astaxanthin content are presented in Table 6.4 and are reported on a wet weight and a dry weight basis. The results of the polyphenol oxidase (PPO) activity are presented in Figure 6. 11 and are reported in units (U) as defined in section 6.2.3.4 Crude PPO Activity.

6.4.2.1 Proximate Composition

The proximate compositions of the May 2021 crab by-product samples (Table 6.3) are comparable to the proximate compositions of the May 2018 crab by-product control samples (Table 3.10 and Table 3.11). There were no significant differences ($p > 0.05$) between the moisture contents of the May 2018 and May 2021 samples. Both samples contained approximately 65% moisture. Lipid contents ranged between 2.1% (May 2021) and 3.2% (May 2018) and differences were significant ($p < 0.05$) according to ANOVA and Tukey's test. Significant differences ($p < 0.05$) however were also noted in the ash contents and nitrogen contents of the May 2018 and May 2021 samples. The ash content of the May 2021 samples (37-38% db) was slightly higher than the ash content of the May 2018 control samples (34-36% db). The nitrogen contents of the May 2021 samples were slightly lower at 5.3-5.4% N compared to 5.8-6.0% N in the May 2018 control samples. Variations in the lipid, ash and nitrogen contents between the May 2018 and May 2021 samples are likely due to biological variations within the samples and differences in the homogeneity of the samples. Obtaining a homogenous sample during collection from the offal bin was difficult as samples had to be manually collected from the top of the bin using pails. This may have resulted in differences in the shell:meat ratio of the samples.

Table 6. 3 Proximate composition of crab processing by-products collected in May 2021

Parameter	Sample # 1	Sample # 2	May 2018
% Moisture in Original Sample [1]	65.23 ± 0.85[a]	65.12 ± 0.34[a]	65.84 ± 0.41[a]
% Ash [2]	37.06 ± 1.10[a]	38.06 ± 1.60[a]	34.40 ± 0.44[b]
% N [2]	5.30 ± 0.16[a]	5.40 ± 0.23[a]	5.93 ± 0.08[b]
% Lipid [2]	2.04 ± 0.002[a]	2.19 ± 0.23[a]	3.22 ± 0.63[b]
% Chitin Yield (Theoretical)[3]	34.75 ± 1.45[a]	33.07 ± 2.69[a]	30.54 ± 3.59[a]
% Chitin Yield (Actual)[4]	24.57 ± 1.90[a]	24.94 ± 2.64[a]	17.93 ± 1.89[b]
% Chitin Recovery [5]	71.79 ± 0.02[a]	73.41 ± 0.01[a]	58.48 ± 0.36[b]

[1] Results represent the mean of 3 determinations (n=3) ± standard deviation.[2] Results represent the mean of 2-3 determinations (n=2 or n=3) ± standard deviation and are reported on a dry weight basis (db).[3] Chitin yield was estimated based on calculation by difference on a dry weight basis as [mass of chitin ÷ mass of dry sample] x 100 and represents a theoretical yield. [4] Results represent the mean of 2 determinations (n=2) ± standard deviation. Yield is reported as [mass chitin ÷ mass sample] x 100 and is reported on a dry weight basis (db). [5] Chitin recovery was calculated as [Actual Chitin Yield ÷ Theoretical Chitin Yield] x 100 and is reported on a dry weight basis (db).[6] Values in the same row with different letters are significantly different ($p < 0.05$) according to Tukey's test.

Theoretical chitin contents, although slightly higher in 2021, ranging from 28-33% (db) for the May 2018 control samples and 31-36% (db) for the May 2021 samples, were not statistically significant ($p > 0.05$). The actual chitin yield (~25% db) and % recovered chitin (~72%) of the May 2021 samples (Table 6.3) however were significantly higher ($p < 0.05$) than that of the May 2018 control samples (actual chitin yield ~ 18% db and 59% recovery) (Table 3.13). The higher chitin yield and % recovery obtained for the May 2021 samples, may be the result of: (1) the lower protein and lower lipid content in the May 2021 samples; (2) the chitin extraction being performed using wet crab by-product as the starting

material versus dried crab by-product which was used in May 2018. Differences in chitin yield have significant implications in a production setting where economically it is important to maximize yields without increasing production costs. An added benefit of using fresh crab by-products is that it negates the need for additional and often expensive drying equipment (due to the initial high capital cost and subsequent high operating costs), thus adding to the green chemistry principal of energy efficiency. One drawback, however, is that frozen crab by-products have a much shorter shelf-life. Based on observations and results from the 2018 and 2021 studies, fresh-frozen crab processing by-products should be used within 6-8 months, whereas dried crab by-products could have up to a 2-year shelf-life.

6.4.2.2 Crude PPO Activity

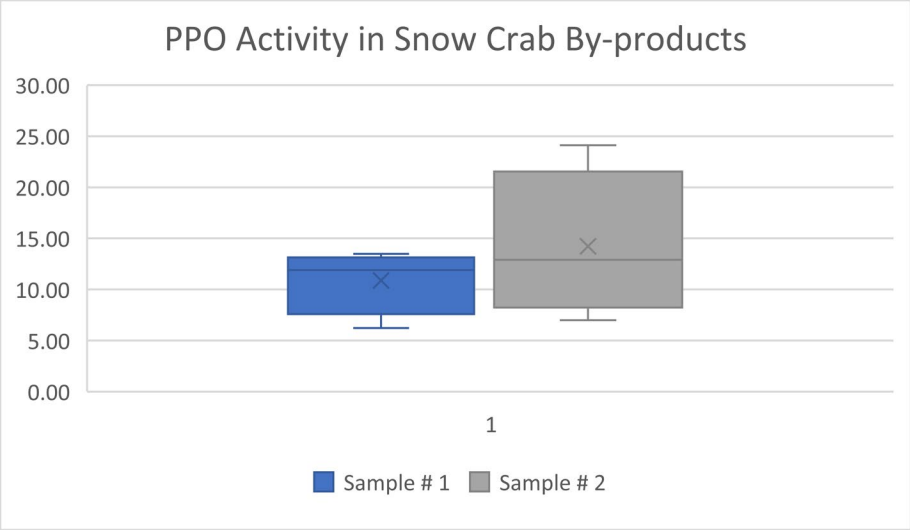


Figure 6. 11 Polyphenol oxidase activity in snow crab by-products

As discussed in Chapter 3, section 3.4.1.1 Discolouration Reactions in Crab, snow crab by-products are highly susceptible to bluing/melanosis discolouration reactions caused

by the presence and activation of the polyphenol oxidase (PPO) enzyme complex [16]. Polyphenol oxidase (PPO) activity varied between the two crab by-product samples ranging from 6.23 U to 13.49 U (mean = 10.88 U) in sample # 1 and 7.0 U to 24.11 U (mean = 14.23 U) in sample # 2 (Figure 6.11). The variance between the two samples was not significant ($p > 0.05$). Overall, the PPO activity in the snow crab by-product samples was higher, and had a wider spread, than that reported by Bartolo and Birk for Norway lobster cuticle which peaked around 6.5 U [6].

The PPO results reported here confirm that snow crab by-products contain high levels of PPO activity. The high PPO activity combined with the presence of other co-factors/catalysts (i.e., iron, copper, tyrosine) as previously identified in Chapter 3, make snow crab by-products prone to discolouration reactions. Seasonality and handling methods were also cited in Chapter 3 as factors affecting discolouration in snow crab by-products. Bartolo and Birk [6] reported that cuticular PPO activity in Norway lobster varied throughout the season showing peaks in May and September that coincided with moulting. Bartolo and Birk [6] also reported that rough handling of lobsters led to higher PPO activity but found no direct relationship between initial PPO activity and black spot development suggesting that the development of PPO activity during storage is more important. Following the development of PPO activity and discolouration during storage was beyond the scope of this thesis. PPO activity is presented here only to confirm its presence and support my previous hypothesis in Chapter 3 that snow crab by-products contain intrinsic properties rendering them susceptible to bluing/melanosis discoloration reactions, and therefore must be considered in the design of a snow crab by-product utilization strategy.

Although these discoloration reactions are not a health and safety concern, they do represent a major quality concern from a market perspective where colour of the extracted bioproducts such as chitin/chitosan affect the market value. In my previous experience working with crab shell chitin, when the chitin was extracted from discoloured shells, the chitin sustained an unacceptable grey colour even following depigmentation with organic solvents.

6.4.2.3 Astaxanthin Content

The average total astaxanthin content of the May 2021 samples was 60.05 µg/g (db) (Table 6.4). Total astaxanthin was extracted from the raw fresh/frozen crab by-product material. In comparison, the total astaxanthin content of the May 2018 control samples ranged from 19.34 µg/g (db) in the air-dried sample to 58.45 µg/g (db) in the freeze-dried sample (Table 3.12). ANOVA analysis and Tukey's test revealed that there is a significant difference ($p < 0.05$) in astaxanthin content between the air-dried and freeze-

Table 6. 4 Total astaxanthin content (µg/g) of snow crab feedstock samples collected May 2021

Sample #	Astaxanthin (µg/g)	
	wet basis	dry basis
1 [a]	20.96 ± 1.15	59.32 ± 3.25
2 [b]	21.20 ± 0.82	60.78 ± 2.36

[a] *Results represent the mean of 3 determinations (n=3) ± standard deviation.*
[b] *Results represent the mean of 4 determinations (n=4) ± standard deviation.*

dried samples, and the air-dried and fresh/frozen samples, however the difference is not significant ($p > 0.05$) between the freeze dried and fresh/frozen samples. This result indicates that freeze-drying is effective for stabilization of carotenoid pigments in crab by-products as previously discussed in Chapter 3, section 3.4.4 Astaxanthin Content of Air-

Dried and Freeze-Dried Crab Feedstock Samples. This result also confirms the 2018 results that the astaxanthin content in the combined crab by-product is lower than that reported elsewhere for specific crab shell components such as crab backs [7, 12, 13, 14]. Therefore, separation of crab by-products at the processing plant may be required if pigment recovery is a priority.

6.4.3 Characterization of Extracted Crab Bioproducts

6.4.3.1 Extraction of Carotenoids with Vegetable Oils

In this study, sequential extraction of carotenoid pigments, pigmented protein powder and chitin from crab processing by-products was attempted. Direct pigment extraction from crab shell waste was carried out using various refined vegetable oils (Figure 6.12) prior to protein and chitin extraction to avoid degradation of the carotenoid pigments. A similar approach was used by Shahidi and Synoweicki [7] using cod liver oil to extract carotenoids from shrimp (*Pandalus borealis*) shell waste.

The recovery of astaxanthin from crab processing by-products using different vegetable oils is presented in Table 6.5. Recovery is reported as the percent of total astaxanthin in the original crab by-product sample (21.094 μg/g wet basis). The highest astaxanthin recovery was obtained using corn oil (37.93%) followed by canola oil (31.23%) and sunflower oil (24.85%), respectively.

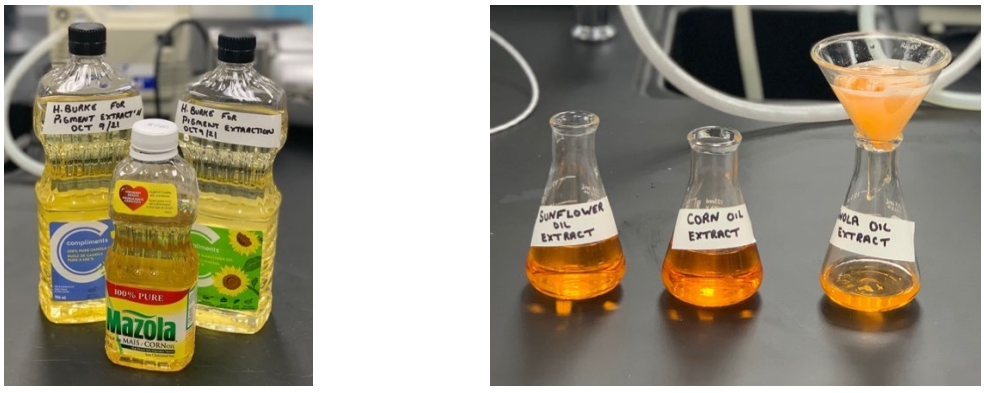
Shahidi and Synoweicki [7] reported that the best recovery of carotenoids (74.23%) from shrimp shell waste was obtained using a ratio of 1:2 (w/v) offal:oil at 60°C. Chen and Myers [17] reported ~40-52% carotenoid recovery from crawfish shell wastes using a single-stage extraction process with soybean oil in a 1:1 (v/w) ratio of oil:shell waste and

heated at 80-90°C for 30 minutes [17]. Sachindra and Mahendrakar [18] evaluated several vegetable oils for the extraction of carotenoids from shrimp shell wastes and obtained higher carotenoid yields using refined sunflower oil in an oil to waste ratio of 2:1 and heating the mixture at 70°C for 150 minutes. Hooshmand *et al*. [19] also reported higher yields of carotenoids from crab wastes using a multi-stage extraction process with sunflower oil in a ratio of 5:1 (v/w) oil to waste at 78°C for 95 minutes in comparison to other vegetable oils, but extraction using organic solvents such as acetone was more efficient than vegetable oil extraction. In addition, Hooshmand *et. al*. [19] reported carotenoid yields were higher in shrimp wastes than crab wastes.

Table 6. 5 Recovery, as percent of total astaxanthin from snow crab processing by-products by extraction with different vegetable oils [1]

% Astaxanthin Recovery		
Sunflower	Corn	Canola
24.85 ± 0.01^{a}	37.93 ± 0.04^{b}	31.23 ± 0.03^{c}

[1] *Results are the mean of three replicates ± standard deviation. Total astaxanthin in crab processing by-products was 21.09 µg/g. Values with different letters are significantly different ($p < 0.05$) according to Tukey's test.*



A B

Figure 6. 12 Vegetable oils used for pigment extraction (A) and pigmented oils recovered from crab processing by-products May 2021 (B)

The only factor evaluated in the current study was type of vegetable oil used. Other factors which may affect optimization of pigment extraction for each oil such as time, temperature, ratio of oil to waste, influence of moisture content and particle size, were not studied. This was a preliminary study to determine if commonly available vegetable oils are effective for pigment recovery from crab processing by-products. The crab by-product material had a particle size range of 1-5 mm and a moisture content of 65%. The results show that corn oil was most effective for recovering astaxanthin from crab processing by-products using a single-stage extraction in a 1:1 (v/w) ratio of oil to waste, at 60°C for 2 hours. Sunflower oil did not perform as well as reported elsewhere for shrimp [19, 20] and was the least effective in recovering astaxanthin from the crab by-product material under the study extraction parameters. An interesting observation was the odour of the pigmented oils. All pigmented oils had strong crab meat and fishy aromas. According to Chen and Zhang (2010) the two major odorants in Chinese mitten crab with high odour intensity were dimethyl sulfide (crab meat aroma) and TMA (fish and amines odour) [28]. These highly odiferous pigmented oils may therefore be useful as an attractant in aquaculture feeds, or as a flavourant in soup bases or formulated crab-based foods [2]. If the intent, however, is for use in a nutraceutical product, or as a colourant in beverages, the oil would require a deodorization step.

Currently, the lack of environmentally friendly, efficient technologies for the extraction of valuable bioproducts such as astaxanthin has limited the use of crab processing by-products. Traditional technologies using organic solvents, Soxhlet, and ultrasound as reported elsewhere [21, 22, 23, 24, 25] are expensive, inflexible and may

cause structural changes of valuable compounds resulting in loss of functionality or a decrease in nutritional value [20]. A promising alternative is the use of edible oils since astaxanthin is oil soluble [18, 20], and meets the Six Principles of Green Extraction as proposed by Chemat *et al*. [26]. The use of edible oils offers other advantages such as protecting the pigment against oxidation and acts as a pigment carrier and an energy source in aquaculture feed [20, 27]. However, previous studies using vegetable oil alternatives reported lower yields of carotenoids from crustacean processing wastes than that obtained using organic solvents [7, 17, 18, 19, 20]. Parjikolaei *et al*. [20] have suggested that the lower yields are due to the high viscosity of vegetable oils resulting in less diffusivity and point to a lack of comprehensive studies on effective extraction methods and optimized processing conditions using vegetable oil solvents.

Further evaluations using corn and canola oils as solvents for pigment extraction and recovery are recommended and should focus on (1) optimizing the crab:oil ratio; (2) comparing single *vs* multi-stage extraction processes; (3) determining the effects of moisture, particle size, time, and temperature on pigment recovery; and (4) determining the effect of using co-solvent mixtures such as vegetable oil and ethanol to reduce viscosity. Additionally, comparison of the lipid profiles and free fatty acid compositions should be evaluated and compared against nutritional profiles of commercial aqua feeds to determine their suitability for this purpose.

Based on the astaxanthin recovery obtained in this study, corn oil and canola oil performed better than sunflower oil. Although the wholesale price of sunflower oil is lower than corn and canola oil, it is also much less abundant. However, sunflower oil is non-GMO, unlike canola and corn, which may offer an advantage in some markets. In 2020-21,

the major oil seeds grown in Canada included Canola > Soybeans > Flaxseed > Sunflower, while corn is mainly grown for grain, not oil production [33, 34]. Canadian vegetable oil production is dominated by Canola oil (83%), followed by soybean oil (16%) [35]. Flaxseed, mustard seed and sunflower oils, make-up the difference (1%) [35]. In 2021, Canada produced 4.22 MT of Canola oil with an export value of CAD $5.2 billion [36], 308,000 t of soybean oil with an export value of CAD $3.0 billion [36], and 101,300 t of sunflower oil [34]. Soybean oil was not available for this study but may be worth further evaluation due to the earlier success noted by Chen and Meyers [17] for extraction of astaxanthin from crawfish shell waste.

6.4.3.2 Pigmented Protein Powder

The composition of the pigmented protein powders (Figure 6.13) recovered following carotenoid extraction with vegetable oils and demineralization with citric acid is provided in Table 6.6. The protein contents of all three samples were ~ 51% (db) and were not statistically different ($p > 0.05$). The low ash contents (<1%) indicate that demineralization was effective for removal of minerals. The astaxanthin content varied between the samples and was determined to be significantly different ($p < 0.05$) for all three samples. About 56-66% (db) of the total astaxanthin was retained in the protein fraction. The astaxanthin content of the pigmented protein powders was in the order of Sunflower > Canola > Corn, confirming that corn oil was most effective for the recovery of astaxanthin from crab processing by-products followed by canola oil and sunflower oil, respectively.

Table 6. 6 Composition of protein-pigment powders following carotenoid extraction with vegetable oils and demineralization with citric acid

Composition	Vegetable Oil Treatment [4]		
	Sunflower	Corn	Canola
% Protein [1] (db)	51.69 ± 0.53[a]	51.53 ± 1.23[a]	51.05 ± 0.52[a]
% Lipid [2] (db)	24.58	19.72	16.08
% Ash [2] (db)	0.86	0.72	0.76
Total Astaxanthin [1] (µg/g)	39.56 ± 1.04[a]	33.77 ± 0.35[b]	37.67 ± 0.64[c]
% Astaxanthin Retention [3]	65.87 ± 1.72[a]	56.23 ± 0.59[b]	62.73 ± 1.07[c]

[1] Results are the mean of 2-3 replicates ± standard deviation and are reported on a dry weight basis.[2] Results represent one determination due to limited sample size available. Reported on a dry weight basis.[3] Results are the mean of 3 replicates (n=3) ± standard deviation, reported on a dry weight basis as % total astaxanthin in the original crab by-product sample. [4] Values in the same row with different letters are significantly different (p < 0.05) according to Tukey's test.

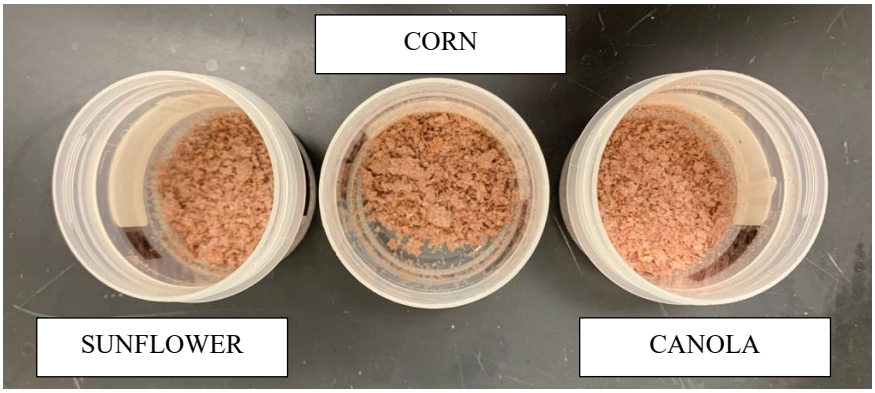


Figure 6. 13 Pigmented protein powders from crab processing by-products following pigment extraction with vegetable oils and demineralization with citric acid

These results show that the vegetable oil extraction method was not efficient for full recovery of astaxanthin which may be the result of its association with protein in the form of a water-soluble pigment-protein complex, or carotenoprotein, in which the pigment and protein are associated via ionic bonding [57, 58]. This carotenoprotein complex is thought to stabilize carotenoids [54, 55, 56] making them less susceptible to photo-oxidation [57, 60]. Organic solvents such as ethanol and acetone can split the carotenoprotein into carotenoid and apoprotein facilitating extraction of the lipophilic astaxanthin molecule [57,

59]. Shahidi and Synowiecki [6] reported that astaxanthin is found in both the free (21.16%) and esterified forms (61.68%) in crab by-products, with astaxanthin di-ester (56.57%) being the major carotenoid present. However, since astaxanthin mainly exists as a carotenoprotein in crustacean shells [61], a longer heat treatment and/or higher temperature may be required to disrupt the ionic bond and optimize astaxanthin extraction using vegetable oil solvents.

The high protein (~51%), lipid (~16-25%), and astaxanthin contents (33.8-39.6 μg/g) plus the low ash content (< 1%) of the protein-pigment powders may make them suitable for use in aquaculture feeds and poultry feeds. By calculation, the pigmented protein powders contain ~22-32% (db) carbohydrate, likely chitin, which could affect the nutritional properties and applications as an ingredient. For example, nutritional studies in the US showed that incorporating small amounts of chitin in diets of chicks caused increased weight gain and enabled the chicks to digest milk lactose and utilize whey more efficiently [51]. Characterization of the amino acid composition, fatty acid composition, lipid profile, and composition of the carotenoid pigments will be critical in making this determination. In Chapter 3 it was determined that the crab meal powders were low in two essential amino acids, methionine and lysine, therefore, the crab protein powder will also likely be low in these amino acids. In Chapters 4 and 5 it was also established that the crab meal and enzymatically obtained protein hydrolysates were high in arsenic, exceeding regulatory limits for use in feeds, foods and natural health products, therefore heavy metal testing will be a key quality parameter requiring further evaluation. It is not known if the high lipid contents of the protein powders are the result of the vegetable oil treatment, or the result of crab oil naturally present in the raw material. The lipid contents in the powders

represent 26.68-42.94% (w/w) of the original lipid content in the crab by-products (Table 6.7), with the corn oil treated sample having the lowest percentage. Further characterization of the pigmented protein powders will be a key factor in further identifying opportunities for commercial applications. Unfortunately, further characterization studies were not possible due to Covid-19 related delays and limited access to lab facilities in 2021.

Protein Pigment Powder Sample	Lipid in Original[1] Crab By-product (g)	Lipid in Protein[2] Pigment Powder (g)	% Lipid in protein[3] pigment powder based on lipid in original sample
Sunflower	0.7433	0.3192	42.94%
Corn	0.7293	0.1946	26.68%
Canola	0.6041	0.2468	40.85%

Table 6. 7 Comparison of lipid content in original crab by-product sample and extracted protein-pigment powder

[1] Obtained by calculation based on % lipid in original crab by-product samples. [2] Results represent one determination due to limited sample size available and are reported on a dry weight basis. [3] % Lipid = [Lipid in Protein Pigment ÷ Lipid in Origin Crab By-product] × 100.

6.4.3.1 Chitin

Characteristics of chitins prepared from crab by-products using green extraction methods are presented in Table 6.8. Chitin yields are comparable to that reported for the May 2018 chitin samples (Table 3.13). In the current study, the oil treatment did not affect the chitin yield ($p > 0.05$). However, the enzyme treatment did have a significant effect ($p < 0.05$) on chitin yields, which were 1.5-2% higher for samples treated with Weifang enzyme in comparison to FAP and Sea-B-Zyme.

The demineralization reaction between calcium carbonate and citric acid produces calcium citrate, carbon dioxide and water (Eqn. 6.3). The amount of acid must be stoichiometrically equal to or greater than all the minerals present in the sample to ensure complete reaction, which depends on the acid concentration, and the ratio of shell to acid [7, 11]. For stoichiometric calculations, it was assumed that all mineral deposits were due

to calcium carbonate. In this study, 1.5× the stoichiometric amount of citric acid needed for demineralization was attempted and applied in a two-step process.

A residual ash content below 2.5% is required for food-grade chitin [11, 29], and a lower target of < 1% residual ash is recommended for higher quality applications such as chitosan [11, 20]. The ash contents of the chitin samples ranged between 0.75% up to 2.16%, meeting food-grade quality, however only one sample (Canola + FAP) met the residual ash content recommended for chitosan applications. The two-step citric acid process used in this study effectively removed 94-98% of the mineral content from the crab shells, however, full demineralization was not achieved. This could be due to the presence of insoluble calcium citrate formed during step one at the reaction (pH of 3.06-3.50).

A slightly higher concentration of acid, or lower pH (pH < 3), and a higher ratio of acid to shells will be required to reduce the ash content of snow crab shells to < 1%. This target was initially achieved in a preliminary two-step demineralization study (6.3.3.2 Demineralization Using Organic Acid) using 7.5% (w/v) citric acid and 1:10 ratio of shells to acid (4.5x stoichiometric amount of acid). However, double demineralization using 5% (w/v) citric acid added in a 1:5 ratio of shells to acid (1.5x stoichiometric amount of acid) was less effective leaving some residual mineral content in the resulting chitin.

These preliminary results are a strong indication that citric acid can effectively replace HCl as a green solvent for crab shell demineralization. A similar result was reported by Pohling *et al*. [11] using a two-step citric acid demineralization process for shrimp shells (*Pandalus borealis*). Further research to optimize a citric acid two-step demineralization process for snow crab by-products is therefore recommended.

Table 6. 8 Characteristics of chitins prepared from crab by-products using a green chemistry approach

Parameter	Sunflower Oil			Corn Oil			Canola Oil		
	FAP	Sea B Zyme	Weifang	FAP	Sea B Zyme	Weifang	FAP	Sea B Zyme	Weifang
% Chitin Yield[a] (db)	17.06	17.09	18.57	18.02	18.20	20.08	17.53	17.46	19.15
% Moisture[b]	2.24 ± 0.68	1.65 ± 0.75	1.46 ± 0.50	1.10 ± 0.65	1.14 ± 0.37	1.26 ± 0.42	1.05 ± 0.55	1.26 ± 0.55	1.42 ± 0.42
% Ash[c] (db)	2.16	1.8	1.05	1.17	1.24	1.44	0.75	1.06	1.92
% Total Nitrogen[d] (db)	6.31 ± 0.10	6.34 ± 0.01	6.35 ± 0.03	6.38 ± 0.15	6.19 ± 0.01	6.61 ± 0.12	6.43 ± 0.07	6.24 ± 0.001	6.55 ± 0.21
% Residual protein[e] (db)	0.26 ± 0.17	0.39 ± 0.14	0.63 ± 0.21	0.28 ± 0.22	0.77 ± 0.37	0.95 ± 0.15	0.45 ± 0.28	0.76 ± 0.46	0.92 ± 0.15
% Chitin Nitrogen[f] (db)	6.26 ± 0.10	6.26 ± 0.01	6.22 ± 0.03	6.32 ± 0.15	6.03 ± 0.01	6.42 ± 0.12	6.26 ±0.07	6.09 ± 0.00	6.36 ± 0.21
% Lipids[g] (db)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Astaxanthin[h] (µg/g) (db)	17.61 ± 0.09	18.59 ± 0.57	9.91 ± 0.14	14.76 ± 0.77	20.70 ± 0.28	13.12 ± 0.10	15.64 ± 0.12	20.44 ± 0.21	12.36 ± 0.16
Hunter Colour Parameters[i]									
L	65.76 ± 0.54	70.25 ± 0.51	67.08 ± 0.15	69.87 ± 0.26	68.89 ± 0.34	70.82 ± 0.47	66.35 ± 1.45	63.45 ± 1.06	66.76 ± 0.58
a	23.64 ± 0.11	23.58 ± 0.13	23.46 ± 0.04	23.48 ± 0.12	23.85 ± 0.10	22.45 ± 0.14	24.28 ± 0.37	24.35 ± 0.26	23.12 ± 0.23
b	3.44 ± 0.23	0.36 ± 0.79	1.28 ± 0.27	0.84 ± 0.31	0.26 ± 0.15	1.09 ± 0.29	-0.71 ± 0.39	-0.66 ± 0.64	1.25 ± 0.71

[a] Results represent one determination based on extraction from ~ 100 g of raw crab by-product material. Reported on a dry weight basis. Chitin yields are significantly different and significantly affected by the enzyme treatment ($p < 0.05$).[b] Results represent the mean of two determinations (n=2) ± standard deviation. [c] Results represent one determination due to limited sample size available. Reported on a dry weight basis. Ash contents are not significantly different ($p > 0.05$). [d] Results represent the mean of two determinations (n=2) ± standard deviation. Effect of enzyme treatment was significant ($p < 0.05$).
[e] Results represent the mean of four determinations (n=4) ± standard deviation. Residual protein contents were not significantly different ($p > 0.05$).
[f] Result calculated as the difference between total Kjeldahl nitrogen and Lowry protein nitrogen. Results represent the mean of two determinations (n=2) ± standard deviation. The enzyme treatment had a significant effect ($p < 0.05$). [g] Lipid content was assumed to be zero. [h] Results represent the mean of three determinations (n=3) ± standard deviation. All means are significantly different ($p < 0.05$). The oil treatment and the enzyme treatment had a significant effect ($p < 0.05$) on astaxanthin content. The interaction between the oil and enzyme treatments was significant ($p < 0.05$) [i] Results represent the mean of six determinations (n=6) ± standard deviation. The oil and enzyme treatments had a significant effect ($p < 0.05$) on the colour characteristics. The interaction between the oil and enzyme treatment was significant ($p < 0.05$).

The total nitrogen content of the green chitin samples ranged from 6.19 to 6.61% (db) which is comparable to that reported by Shahidi and Synowiecki at 6.42-6.48% (db) [7]. The effect of the oil treatment was not significant ($p > 0.05$), and there was no significant interaction ($p > 0.05$) between the oil treatment and enzyme treatment on total nitrogen content. However, the enzyme treatment did have a significant effect ($p < 0.05$) on total nitrogen content of the samples with the Weifang treated samples having higher total nitrogen contents overall.

Residual protein contents ranged from 0.26 to 0.95% (db) however these differences were not significant ($p > 0.05$). Residual protein content was highest in Weifang (0.63-0.95% db) treated samples, followed by Sea B Zyme (0.39-0.77% db) and FAP (0.26-0.45% db) treated samples, respectively. Shahidi and Synowiecki [7] reported 0.45-0.49% (db) protein residue in crab shell chitin obtained using a traditional chemical process. The results of the current study indicate that treatment with various protease enzymes is effective for reducing the residual protein in crab chitin samples to < 1% and can be used as a replacement for chemical deproteination with NaOH. The selection of enzyme will therefore depend on cost of the enzyme treatment and the targeted chitin application. If a lower residual protein content is required, it may be possible to optimize the enzyme treatment by adjusting the enzyme concentration and time-temperature application. Another approach used in other studies, is to treat the chitin with dilute NaOH solutions to remove any residual protein following enzymatic deproteination [37]. However, the goal here is to use 100% green chemistry, thus avoiding the use of corrosive chemicals, for the extraction of crab bioproducts. Based on these results, Weifang acid protease is

recommended for industrial grade chitin. Sea B Zyme and FAP can be considered for higher grade chitin applications.

The nitrogen content of pure chitin is 6.9%. The chitin nitrogen content of the chitin samples in this study were lower ranging from 6.09 to 6.42% (db). This indicates that chitin samples contain impurities and that the green extraction process requires additional optimization. The enzyme treatment had a significant effect on chitin nitrogen ($p < 0.05$) with FAP and Weifang treated chitins having similar and higher chitin nitrogen contents in comparison to Sea B Zyme treated chitins. The Corn Oil-Sea B Zyme treated chitin had the lowest chitin nitrogen content, while the highest was obtained for the Corn Oil-Weifang treated sample. The oil treatment had no effect on chitin nitrogen ($p > 0.05$).

The colour of the final chitin samples had an array of off-white, light pink, and pink (Figure 6.14) indicating that decolourization with hydrogen peroxide was not effective. The desired white to off-white colour was not achieved using the green extraction methodology employed for this study.

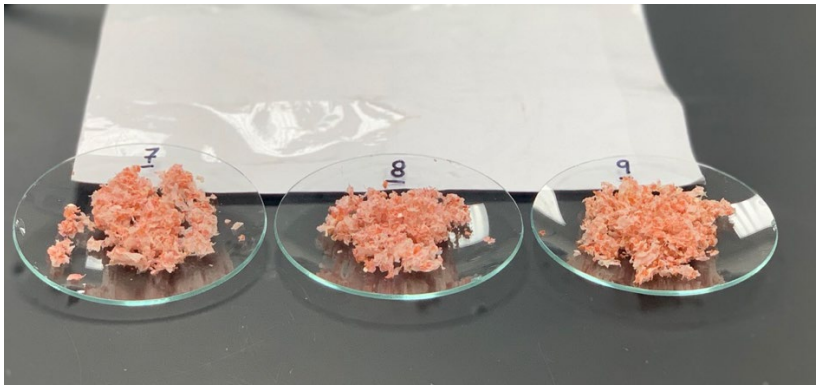


Figure 6. 14 Chitin samples depigmented with 37% hydrogen peroxide

The distribution of astaxanthin amongst the various crab bioproducts is summarized in Table 6.9. Results are reported on a dry weight basis (db) as µg astaxanthin/g sample. Evaluation of the total astaxanthin content confirmed that the chitin samples contained 9.91-20.70 µg/g (db) total astaxanthin representing 16.5-34.5% (db) retention of the total astaxanthin from the original crab by-product. Weifang treated chitin samples had the lowest astaxanthin contents (9.91-13.12 µg/g) overall.

Table 6. 9 Distribution of astaxanthin in extracted crab bioproducts [1,2,3]

Oil Treatment	Pigmented Oil	Protein Pigment Powder	FAP Chitin	Sea B Zyme Chitin	Weifang Chitin
Sunflower Oil	5.24 ± 0.19^{a}	39.56 ± 1.04^{a}	17.61 ± 0.09^{a}	18.59 ± 0.57^{d}	9.90 ± 0.14^{f}
Corn Oil	8.00 ± 0.87^{b}	33.77 ± 0.35^{b}	14.77 ± 0.77^{b}	20.70 ± 0.28^{e}	13.12 ± 0.10^{g}
Canola Oil	6.59 ± 0.60^{c}	37.67 ± 0.64^{c}	15.64 ± 0.12^{c}	20.44 ± 0.21^{e}	12.36 ± 0.16^{h}

[1] Mean of 3 determinations ± standard deviation and are reported on dry weight basis as µg astaxanthin/g sample. [2] Values in the same column with different letters are significantly different ($p < 0.05$) according to Tukey's test. [3] For the chitin samples, values in the same column or row with different letters are significantly different ($p < 0.05$) according to Tukey's test.

Two factor ANOVA indicated that both the oil treatment and the enzyme treatment had a significant effect ($p < 0.05$) on the astaxanthin content of the extracted chitin, and that there were significant crossed effects between the oil and enzyme treatments ($p < 0.05$). The Sunflower Oil-Weifang treated chitin sample had the lowest astaxanthin content (9.91 µg/g) of all the samples. The effect of protease enzymes on pigment removal is likely due to hydrolysis of carotenoproteins.

The Hunter colour characteristics of the prepared chitin samples were also evaluated. Two factor ANOVA indicated that both the oil and enzyme treatments had a significant effect ($p < 0.05$) on the colour characteristics of the chitin, and the interaction between the oil and enzyme treatment was also significant ($p < 0.05$). Further ANOVA

analysis identified that: (1) The oil treatment had a significant effect ($p < 0.05$) on the L-values with the corn oil treated samples having higher L-values overall indicating these samples are lighter (whiter) in colour; (2) The Weifang treated samples had different a-values ($p < 0.05$) than FAP and Sea B Zyme treated samples with a-values being slightly lower overall indicating the Weifang samples are less red; and (3) b-values were significantly different ($p < 0.05$) for the sunflower oil treated samples which were higher overall all indicating these samples are more yellow.

A regression analysis was conducted to determine if there is a correlation between the astaxanthin content and the L*a*b values (Table 6.10) of the chitin samples. The results indicate that astaxanthin and L-values (whiteness) are not correlated ($r = -0.19$), and astaxanthin and b-values (yellow-blue) are not correlated ($r = -0.29$). However, astaxanthin and a-values (redness) are moderately positively correlated ($r = 0.598$), L-values and a-values are moderately negatively correlated ($r = -0.64$), while a-values and b-values are slightly positively correlated ($r = 0.48$). Therefore, a-values (redness) increase with increasing astaxanthin content and decrease with increasing L-values (whiteness). Astaxanthin content, however, and L-values (whiteness) are independent of each other. This suggests that whiteness of the chitin samples is affected by the hydrogen peroxide treatment, whereas redness is a result of the astaxanthin in the sample which is affected by the oil and enzyme treatments.

All three treatments (oil, enzyme, and hydrogen peroxide) influence the colour characteristics of the chitin samples. The oil treatment has a significant effect on the L-values and the b-values, whereas the enzyme treatment had a significant effect on the a-values. This suggests that the combined effects of the oil treatment and the enzyme

*Table 6. 10 Correlation coefficients (r) for astaxanthin and L*a*b values of chitin[1, 2]*

	AXT	L	a	b
AXT	1			
L	-0.19	1		
a	0.60	-0.64	1	
b	-0.29	0.06	-0.48	1

[1] AXT = astaxanthin, L = L-value (whiteness), a = a-value (redness), b = b-value (blueness).
[2] Correlations were not significant (p > 0.05).

treatment could be further optimized to recover more astaxanthin in the pigmented oils and facilitate further pigment removal during deproteination. The combined effect of corn oil treatment with Weifang enzyme had the most significant effect on the colour characteristics of the chitin in terms of whiteness and redness justifying further investigation. It may be possible to eliminate a final depigmentation step if the oil and enzyme treatments can be optimized to maximize pigment recovery (oil treatment) and subsequent removal of residual colour (enzyme treatment). The results also suggest that H_2O_2 may be more effective as a whitening agent when more astaxanthin is removed from the samples prior to H_2O_2 treatment. The H_2O_2 treatment may have some effect on colour removal based on the correlation between L-values and a-values; however peroxide treatment alone was not effective for full decolourization of the chitin samples. It may be possible to optimize treatment with hydrogen peroxide considering the effects of other process variables such as particle size, shell to liquid ratio, time, and temperature, in addition to oil and enzyme treatments.

X-ray diffraction (XRD) has been used to characterize the crystalline structure of chitin which varies depending on the source, with α-chitin being the most abundant form in nature, and the predominant form found in shrimp and crab shells [42, 43, 44].

Characterized by strong intermolecular hydrogen bonding and a structure of antiparallel chains in the crystalline regions, α-chitin is highly crystalline and unable to swell in water limiting its bioactivity [42, 45, 48]. Jang *et al.* [46] studied the XRD pattern of α-chitin and identified 4 sharp crystalline reflections at 9.6, 19.6, 21.1 and 23.7°. Abdou *et al.* [47] reported strong reflections around 9-10° and 20-21°, and minor reflections at higher 2θ values of $\geq$ 26.4°.

For the current study, XRD patterns (Appendix 6.8) and crystallinity index (CI) (Table 6.11) for selected crab shell chitins from the 2021 study and the 2018 study were compared. The XRD patterns were characteristic of α-chitin with reflections at 9-10°, 19-21° and $\geq$ 26° (Appendix 6.8, Figure 6.15). Crystallinity index (% CI) of the extracted chitins (Table 6.11) was 80.18-84.81% CI for the May 2021 chitin samples, and 81.94-93.18% for the chemically extracted air-dried chitin samples from the 2018 study but were not statistically different ($P > 0.05$). The crystallinity index of all the samples was > 80% which is typical for α-chitin [50]. The crystallinity was in the order of June 2018 (chemical) > crab backs 2021 (chemical) > July 2018 (chemical) > May 2021 (chemical) > May 2018 (chemical) > May 2021 (green). The green extracted chitin sample had the lowest % CI (80.18%) and the highest amorphous diffraction intensity at 2θ = 12.6° (611.11) suggesting partial degradation of the chitin molecule presumably due to hydrogen peroxide treatment. H_2O_2 has been shown to decrease the crystallinity of chitosan due to structural rearrangements, decrease MW, decrease the degree of polymerization, and increase solubility [48, 49]. Qin *et al*. [49] suggested that H_2O_2 first attacks the amorphous regions of chitosan degrading it to water soluble molecules, as the degradation progresses deeper

into the molecule, crystallinity is destroyed, while the rate of degradation increased with increasing temperature and H_2O_2 concentration.

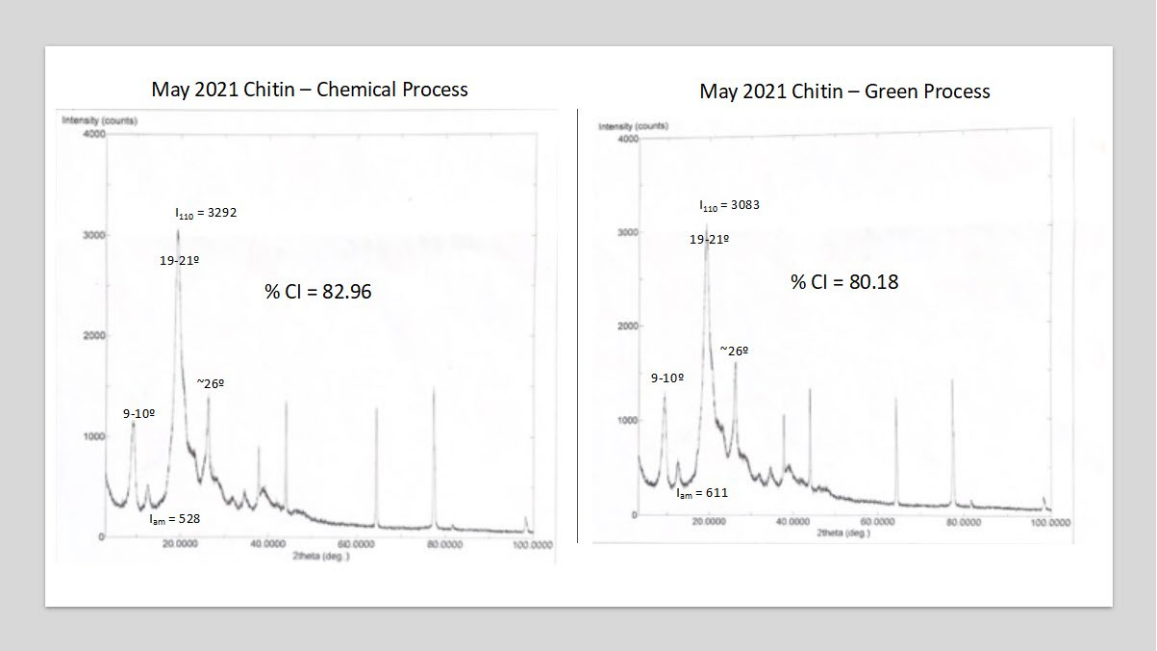


Figure 6. 15 Powder X-ray diffractograms of chemically extracted and green extracted chitins showing characteristic peaks of α-chitin

The XRD data show strong correlation between I_{110} - maximum intensity of the (110) peak at around 2θ = 19°, the I_{am} - amorphous diffraction at 2θ = 12.6°, and crystallinity index (CI). I_{am} and I_{110} have a strong negative correlation (-0.90). I_{am} and CI % also have a high negative correlation (-0.97), whereas I_{110} and CI % have a strong positive correlation (0.97). Consequently, as amorphous intensity increases, both the I_{110} intensity and CI % decrease, whereas CI% increases as I_{110} intensity increases.

Table 6. 11 Crystallinity index (CI %) of Snow crab chitin samples - 2018 and 2021 [1]

Sample Description	I_{am}	I_{110}	% CI
Crab Shell Backs 2021 (Chemical Process)	500	3291.7	84.81
May 2021 (Chemical Process)	527.78	3097.2	82.96
May 2021 (Green Process - Corn oil, FAP, H_2O_2)	611.11	3083.33	80.18
May 2018 (Chemical Process)	541.67	3000	81.94
June 2018 (Chemical Process)	333.33	4888.89	93.18
July 2018 (Chemical Process)	555.56	3555.56	84.37

[1] *Results represent one XRD scan (n = 1) per chitin sample.*

6.5 Conclusions

Sequential extraction of carotenoid pigments, pigmented protein powder and chitin from crab processing by-products was attempted using simple extraction methods and green solvents to conceptualize an environmentally friendly cost-effective crab by-product utilization process that would be easy to implement in a commercial crab processing plant, or a centralized crustacean by-product processing facility. A key factor to full utilization of crab by-products is stabilization of the raw material immediately following processing. Stabilization was achieved by vacuum packaging and freezing the by-products at –20°C. On a commercial scale, bag-in-box industrial vacuum sealers designed for heavy bags (> 25lbs) used as a box liner are available and relatively inexpensive.

To simplify the collection process, composite samples of unseparated crab by-products were collected directly from the processing plant offal bin. This included shell backs, belly flaps, mandibles, hepatopancreas, gills, other organs, and residual meat

protein. This material was then subjected to a two-step grinding process to obtain as homogenous a sample as possible, without separating the shells from the meat and organs. This approach allowed for an acceptable yield and quality of pigmented protein powder and chitin. However, separation of crab by-products may be required for effective pigment recovery. Freeze drying was shown to be effective in stabilizing astaxanthin in dried crab by-products but was omitted from further consideration at this time as there are no commercial freeze-drying facilities located in NL, and this technology is expensive.

Corn oil was most effective for extraction of astaxanthin followed by canola oil and sunflower oil to produce pigmented oils resulting in 37.9%, 31.2% and 24.9% recovery of astaxanthin from the original crab by-product sample. The extraction process was not optimized, however optimization to improve recovery of up to 60-80% astaxanthin from crustacean shell waste using vegetable oils has been reported in the literature [19] and may be a possible green solvent alternative to organic solvents with the added benefits of: protecting astaxanthin from oxidation and degradation; acting as a source of energy, a pigment carrier, and attractant in aqua feeds. Sunflower oil, while not as effective for astaxanthin extraction under the current study parameters, offers a non-GMO option and has been shown to be effective in recovering up to 60% astaxanthin from crustaceans in other studies [19]. Deodorization may be required if the oils are intended to be used as a natural health product (e.g., antioxidant supplement).

The pigmented protein powder obtained was high in protein (51%), fat (16-25%) and astaxanthin (33.8-39.6 μg/g), and low in ash (<1%). Therefore, it may be suitable for use in aqua feeds as a source of protein, energy and astaxanthin, but may require additional supplementation with lysine and methionine as these amino acids were limiting in the 2018

crab meal samples (Chapter 3). Arsenic levels were not measured but may be a concern due to high levels obtained in earlier studies for crab meal and protein hydrolysates.

A two-step demineralization process using citric acid effectively removed 94-98% of the mineral content in crab by-products offering a green alternative to HCl. Enzymatic deproteination reduced the residual protein content to < 1% in all samples regardless of the enzyme used. The H_2O_2 treatment was not effective for decolourization of the chitin samples although it did have a slight bleaching effect. Treatment with H_2O_2 may be more effective for whitening if more of the astaxanthin is removed in earlier process steps. The powder X-ray diffractograms and crystallinity index also indicate that treatment with H_2O_2 may have degraded the chitin molecule causing a slight decrease in crystallinity.

Enzymatic deproteination using commercial enzymes was effective in reducing residual protein to < 1%. Therefore, the choice of enzyme for deproteination of chitin will depend on the cost of the enzyme treatment and the quality of the chitin required. Weifang acid protease treated chitins had the highest residual protein contents, but this enzyme was also the cheapest and is therefore recommended for industrial grade chitin. FAP and Sea B Zyme produced chitins with lower residual protein contents, but were more expensive, $80/kg and $32.50/kg respectively, and therefore are recommended for higher grade chitins.

It may be possible to eliminate a final decolourization step if the oil and enzyme treatments can be optimized to maximize pigment recovery. For example, in a study by De Holanda and Netto [53] treatment of shrimp waste with Alcalase was performed prior to oil extraction resulting in good recovery of pigments which was thought to be due to weakening of the carotenoprotein bond by enzymatic hydrolysis. Eliminating the hydrogen

peroxide treatment has added benefits of minimizing structural changes to the extracted chitin, further reduction in the use of chemicals and the number of processing steps, less production time, and lower production costs. While decreased crystallinity may have desirable effects in terms of the physicochemical characteristics of chitin, the reaction may be difficult to control, resulting in inconsistent quality such as variations in MW, DP, and solubility. Producing a consistent chitin product will be advantageous since it will allow chitosan manufacturers to customize the deacetylation and depolymerization process resulting in chitosans with specified MW, DDA and pattern of acetylation for customized applications [42].

6.6 Future Opportunities

Based on the results of this proof-of-concept study, further optimizations studies using simple, low cost, green technologies for the extraction of snow crab bioproducts are recommended.

Further studies using corn, canola and soybean oils as solvents for pigment extraction and recovery are recommended and should focus on (1) optimizing the crab:oil ratio; (2) comparing single *vs* multi-stage extraction processes; (3) determining the effects of moisture, particle size, co-solvents, time, and temperature on pigment recovery; (4) comparing the lipid class and free fatty acid profiles, and astaxanthin compositions against nutritional profiles of commercial aqua feeds to determine their suitability for this purpose; (5) evaluating simple low cost technologies for deodorization of the oils.

Full characterization of pigmented protein powders will be a key factor in further identifying opportunities for commercial applications. Specifically, the following

parameters should be fully assessed: amino acid, fatty acid and astaxanthin compositions, as well as chitin content and heavy metal content. If pigmented oils are not important as a commercial product, a pigmented protein powder with higher astaxanthin concentrations can be targeted.

Further optimization of the two-step citric acid demineralization process should focus on minimizing the amount of citric acid and duration of the acid treatment required to achieve full demineralization of chitin. Based on this study, the optimum citric acid concentration will be somewhere between 5% (w/v) and 7% (w/v) applied in a stoichiometric concentration $>1.5\times$ and $\leq 4.5\times$.

The vegetable oil, protease, and hydrogen peroxide treatments were found to influence the colour characteristics of the chitin samples. Optimizing these three treatments to maximize astaxanthin recovery in the pigmented oils and pigmented protein powders with subsequent removal of residual protein and colour from chitin, requires a dedicated research effort. Elimination of a final depigmentation step by optimization of vegetable oil and enzyme treatments makes both environmental and economic sense by reducing the amount of chemical needed, processing time, and costs. Optimization studies should focus on the effects of other process variables such as particle size, shell to liquid ratio, time, and temperature applications for each treatment, as well as the order of processing steps (e.g., effect of enzyme treatment before oil extraction); and if needed alternative green oxidation chemicals (e.g., tetra-amido macrocyclic ligands (TAML) - activated H_2O_2) [68].

Note to Reader:

- The Appendix for Chapter 6 begins on page 348
- Chapter 7 begins on page 351

6.7 References

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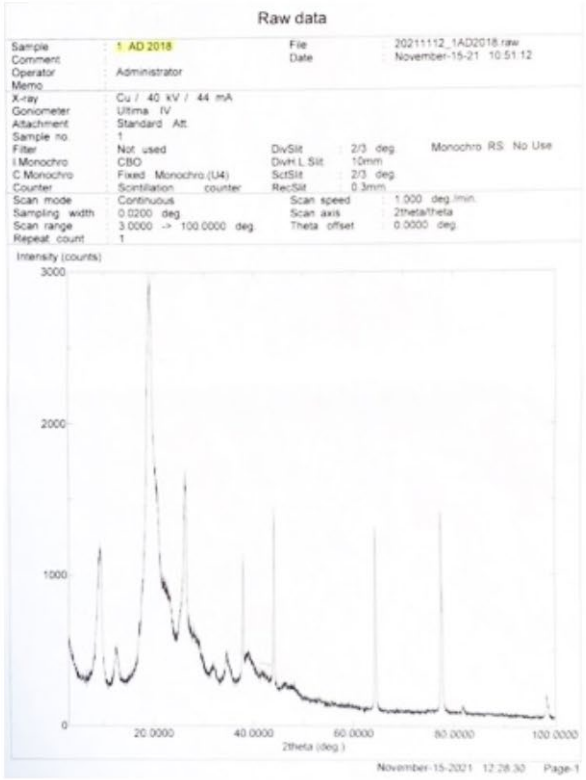
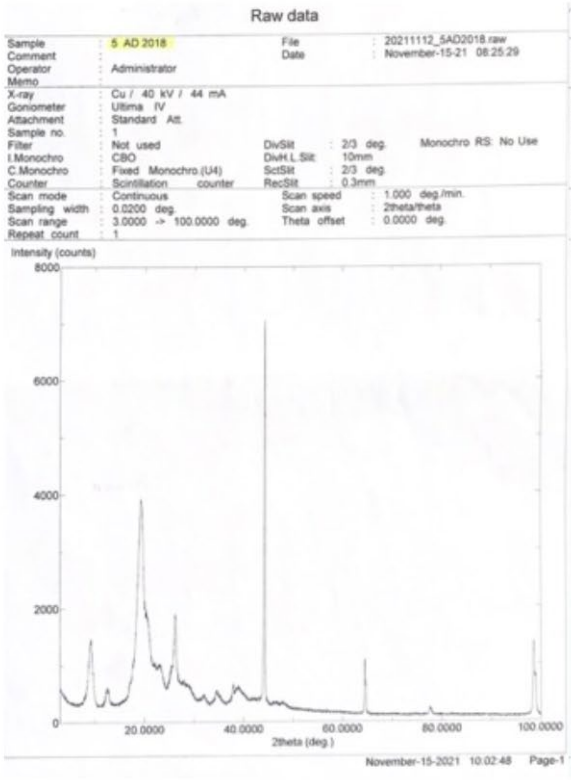
6.8 Appendix

Appendix 1 - Powder X-ray Diffractograms of Selected Chitin Samples

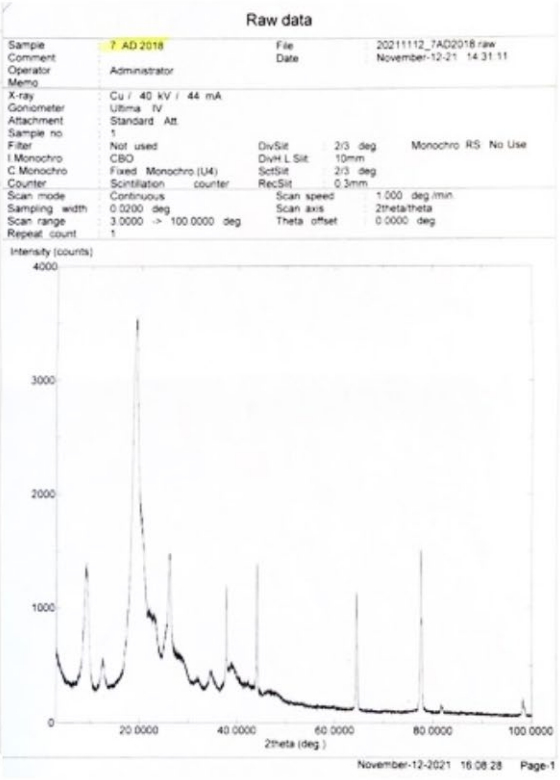
May 2018 Chitin – Chemical Process



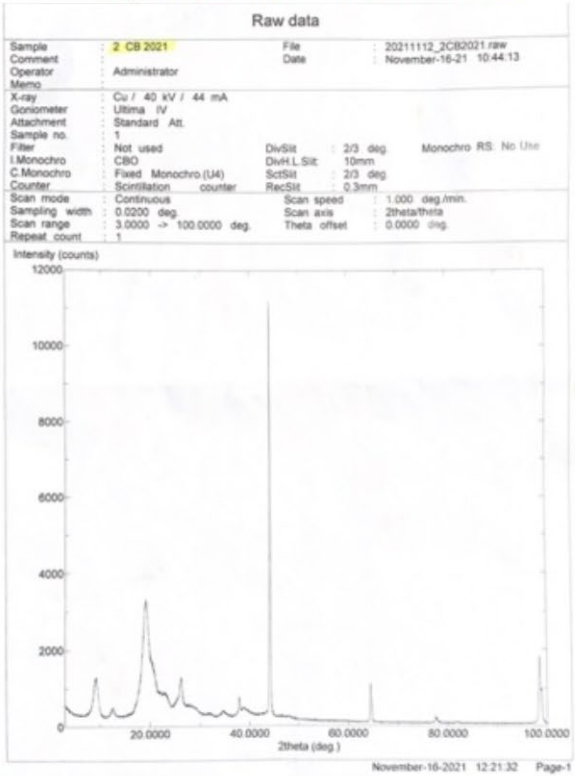
June 2018 Chitin – Chemical Process



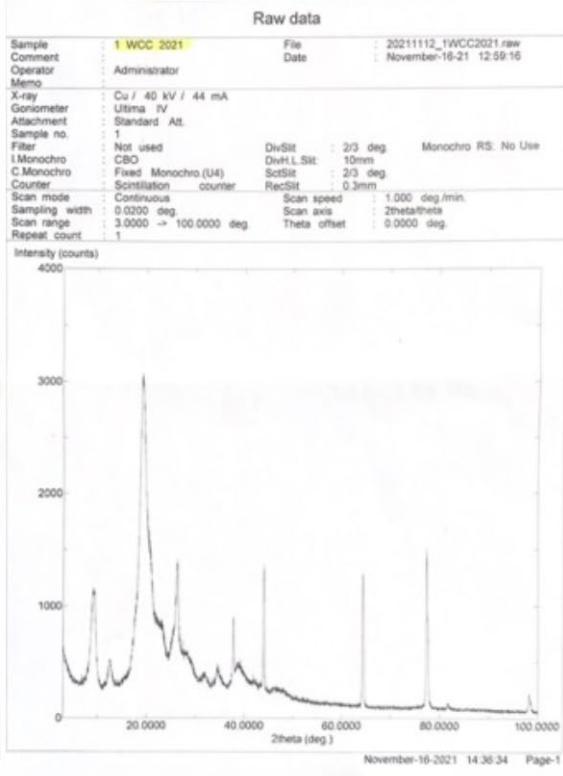
July 2018 Chitin – Chemical Process



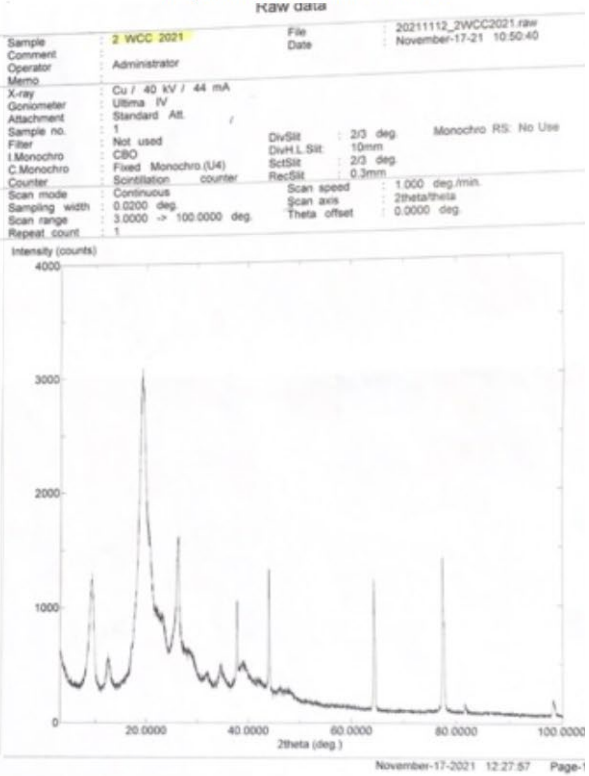
May 2021 Crab Backs Chitin – Chemical Process



May 2021 Chitin – Chemical Process



May 2021 Chitin – Green Process



Appendix 2 - Properties of Canola Oil, Corn Oil and Sunflower Oil

Component/ Characteristic	Vegetable Oil (Standard Refined Oil)		
	Canola[1]	Corn[2]	Sunflower[3]
Triglycerides (%)	94.4-99.1	98.80	
Free Fatty Acids (%)	0.4-1.2	0.05	
Major Fatty Acids[1]			
Total Saturated	6.0	12.9-13.3	10.9
C16:0 (palmitic)	3.5	11.4	6.2 - 7.0
C18:0 (steric)	1.5	1.9	4.7-6.0
Total MUFA	61.9	24.4-25.4	20.6
C18:1 (oleic)	60.1	25.3	20.4-29
Total PUFA	29.7	60.7-61.1	68.8
C18:2n-6 (linoleic)	20.1	60.7	58-68.6
C18:3n3 (linolenic)	9.6	-	-
Tocopherols (ppm)[1]	700-1200	800-1200	600-737[4]
alpha	272.1	134.0	613.0
beta	0.1	18.0	17.0
gamma	423.2	412.0	18.9
delta	-	39.0	-
Chlorophylls (ppm)			
Carotenoids (ppm)	9.5		
Density (g/cm3) 20-30°C[5]	0.914-0.917	0.915-0.920	0.910-0.915
Refractive Index (40°C)[5]	1.465-1.467	1.470-1.474	1.4640-1.4800
Viscosity (mm2/sec)[5]			
25°C	48.666	49.676	51.428-59.254
60°C	15.987	16.935	16.935-20.124
Iodine Value[6]	163	103-135	100-140

[1] *Przybylski, R. [nd]. Canola Oil: Physical and chemical properties. Canola Council of Canada. Retrieved May 28, 2022 from https://www.academia.edu/15838770/PHYSICAL_PROPERTIES_CANOLA*

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CHAPTER 7. Summary and Recommendations: Green Chemistry and an Ocean Based Biorefinery Approach for the Valorization of NL Snow Crab Processing Discards

7.1 Overview of Thesis

About 25-30% (~10,000 -15,000 t) of Newfoundland and Labrador's Atlantic snow crab (*Chionoecetes opilio*) landings are discarded annually in landfills or at sea, as processing waste. The NL snow crab industry faces several challenges which have historically limited its use of crab processing by-products. The key challenges include high volumes landed during a short harvesting season (April-July), the rapid spoilage rate of the discards, the unique intrinsic quality characteristics of this raw material, and the lack of crustacean by-product processing facilities in the province, all of which make it difficult to utilize this material and divert it away from traditional disposal options. Another complicating factor is that traditional crustacean bioproducts extraction methods are chemically intensive requiring the use of harmful organic solvents and inorganic reagents contributing to air and water pollution. This presents a significant challenge in NL coastal communities due to limited health & safety emergency response resources which are needed to deal with transportation and storage of dangerous chemicals, and to respond to a chemical spill or chemical fire. Additionally, environmental restrictions are increasing making it more difficult and costly for crab processors to avail of traditional waste disposal options.

In this thesis I have investigated methods of redirecting snow crab processing discards toward extracting valuable bioproducts using simple green chemistry alternatives and an ocean based biorefinery approach to; (1) provide an alternative to current at-sea

dumping and landfilling practices, (2) reduce environmental pollution, (3) mitigate the need for additional health & safety emergency response resources, and (4) increase crab by-product utilization opportunities, for NL rural coastal communities.

7.2 Major Findings and Significance

The research studies conducted in this thesis have made significant research contributions towards the application of green chemistry and an ocean based biorefinery approach for the valorization of NL snow crab processing discards. Since this is a relatively new approach for the valorization of crustacean waste, this thesis will provide direction for future research in this area. The biorefinery concept did not gain significance in the scientific literature until 2007 [1], and most research has focused on land-based feedstocks from agriculture and forestry (e.g., corn, soybean, wood) [1-4]. The ocean based biorefinery concept using a green chemistry approach was first proposed by Kerton *et al.* [3] in 2013. Dedicated research efforts for the valorization of NL's snow crab processing discards, however, have not been published since 1991 [5] and these prior studies did not focus on green chemistry solutions. More recent studies on the valorization of Atlantic snow crab processing discards (2009-2011) have been conducted on crabs harvested from the North Atlantic between Greenland and Canada [6], and Gaspé, Quebec [7]. The most recent study was conducted by Nofima in Norway on snow crabs harvested from the Barents Sea, 2021 [8]. In that study a combination of extractions using edible oils and proteases were used to obtain astaxanthin and protein hydrolysates, however traditional chemical solvents were used to extract chitin. My approach differed in that I focused on green chemistry alternatives for all bioproducts of interest, including chitin, and included consideration of

seasonal effects on crab by-product quality, as well as an evaluation of trace metals in the extracted bioproducts. These considerations were not included in the studies mentioned above, but they all have a significant effect on the valorization of crab processing by-products. Studies on the valorization of Atlantic snow crab processing discards remain limited, yet in NL it is our most valued fisheries resource. It is anticipated that this thesis will lay the foundation for additional research enabling NL to develop a viable snow crab bioproducts value chain and increase the province's contribution to Canada's Blue Economy[65].

7.2.1 Summary of Major Findings

1. In Chapter 2, it was proposed that regional crustacean by-product facilities should be strategically located in regions where several shrimp and crab processing plants are operating. These regional by-product processing facilities were based on the plants in operation in 2015 which included 10 shrimp plants and 27 crab plants spread across the Northern Peninsula; Northeast Coast; and Avalon Peninsula. In 2019 there were only 7 licensed shrimp plants and 22 licensed crab plants in operation. The highest concentration of crab plants was located on the Northeast Coast (7 plants) and the Avalon Peninsula (7 plants) accounting for 86% of the total provincial crab discards. A more recent assessment conducted by Norsworthy and Burke in 2021 [9] recommended that crustacean by-product processing facilities

[65] Canada's Blue Economy refers to how ocean industries can contribute to the environmental sustainability of our oceans' resources and ensure long-term economic benefits to our coastal communities [https://eco.ca/blog/what-is-the-blue-economy/].

could be regionally located in Central NL, the Avalon Peninsula, and the Northern Peninsula.

2. Based on observations and results from the 2018 and 2021 studies (Chapters 3 & 6), fresh-frozen crab processing by-products should be used within 6-8 months, whereas dried crab by-products could have up to a 2-year shelf-life. However, yield and quality of extracted crab bioproducts are better when fresh-frozen crab by-products are used as the raw material vs dried crab by-products.
3. Snow crab by-products have intrinsic properties (e.g., PPO, iron, copper, tyrosine) rendering them susceptible to bluing/melanosis discoloration reactions, which must be considered in the design of a snow crab by-product utilization strategy therefore requiring either immediate processing or freezing (Chapters 3 & 6).
4. The difference in astaxanthin content between the freeze dried and fresh/frozen samples is not significant ($p > 0.05$). This result indicates that freeze-drying is effective for stabilization of carotenoid pigments in snow crab by-products, however, this is an expensive technology and is not currently available on a commercial scale in NL.
5. The astaxanthin content in the composite crab by-product is lower than that reported elsewhere for specific crab shell components such as crab backs. Therefore, separation of crab by-products at the processing plant, or at a regional processing facility, may be required if pigment recovery is a priority.
6. Based on the astaxanthin recovery method used in Chapter 6, corn oil and canola oil performed better than sunflower oil, but overall, vegetable oil extraction was not efficient for full pigment recovery and requires optimization of time-temperature

applications, ratio of crab:oil, solvent viscosity, and single vs multi-stage extractions.

7. The high protein (~51%), lipid (~16-25%), and astaxanthin contents (33.8-39.6 μg/g) plus the low ash content (< 1%) of the protein-pigment powders may make them suitable for use in aquaculture feeds and poultry feeds. However, these powders may be low in methionine and lysine and high in arsenic, and therefore require additional characterization studies.
8. A two-step demineralization process using citric acid was effective for removal of 94-98% of the mineral content in crab shells and can therefore be used as a green reagent to replace HCl in the chitin extraction/purification process.
9. Enzymatic deproteination using different commercial proteases was effective in reducing the residual protein content to < 1% in the final chitin samples and can therefore be used as a green alternative to NaOH. The selection of protease enzyme will depend on the cost and the grade of chitin required (e.g., industrial use vs medical use).
10. H_2O_2 was not effective for bleaching of the residual pigment in chitin. As suggested in Chapter 6, it may be possible to eliminate the decolourization step by optimizing the oil and enzyme treatments which would make the green chemistry-biorefinery option presented in this thesis even more cost-effective, environmentally friendly, and a safer process for plant workers.

7.2.2 Discussion of Major Findings and Significance

Using the combined green chemistry-ocean based biorefinery approach as described in Chapter 6, higher value, more environmentally safe, and lower cost crab by-product options have been identified as an alternative to the chemically intensive processes typically used for the extraction of crustacean bioproducts. Based on the approach as applied in this thesis, a summary of snow crab bioproducts, their potential applications, advantages, and disadvantages, is presented in Table 7.1. While the proposed green chemistry-biorefinery model offers several advantages over traditional chemical extraction processes for astaxanthin, protein, and chitin, there are also some challenges with the current model which have been discussed throughout this thesis. Some additional environmental and cost considerations of the model are discussed in this section.

7.2.2.1 Environmental Considerations

Edible oils, food grade citric acid, and protease have minimal environmental health and safety requirements for their use compared to ethanol, HCl, and NaOH. Ethanol is a highly flammable liquid, an inhalation hazard, carcinogenic, and toxic to aquatic life. HCl and NaOH are highly corrosive, represent an inhalation danger, are considered environmental hazards and are harmful to aquatic life. Specialized transportation, storage, ventilation, and spill prevention systems are needed to use ethanol, HCl and NaOH on an industrial scale [10]. Since these chemicals are usually purchased in concentrated forms, they require specialized equipment for dilution and mixing. HCl and NaOH can cause corrosion of high-grade stainless steel over time and eventually lead to corrosion of plant processing equipment limiting the equipment life span [9, 10]. Workers also need

specialized health and safety training, appropriate PPE, and local storage and handling infrastructure for hazardous chemicals is required [10].

In contrast, citric acid and enzymes are common food ingredients and have few handling requirements, however, both can cause eye, lung, and skin irritations [15, 16], therefore use of gloves, eye and face protection, and proper ventilation is recommended. Citric acid is mildly corrosive and therefore has less impact on equipment lifespan than HCl. Commercially, both citric acid and proteases are typically used/purchased as a powder for food and industrial applications, therefore dust control is needed to prevent formation of combustible dust concentrations in air during processing [15, 16]. However, neither ingredient is considered an environmental contaminant and as such do not require specialized transportation, storage, or handling [10, 15, 16].

Edible oils, while not considered hazardous substances, can cause adverse environmental impacts in the event of an oil spill such as coating animals and plants with oil causing suffocation; suffocating aquatic life due to oxygen depletion from oil spills in water; fouling of shorelines; produce rancid odours; and can be a fire hazard if ignition sources are present [11]. Therefore, for commercial volumes, appropriate spill response measures will be needed such as booms, vacuum trucks, and firefighting equipment [11]. Some of this equipment (e.g., booms, trucks, seiners) may already be in place in NL rural coastal areas because of the 2019 south coast Atlantic salmon mortality event which required extensive clean-up of salmon mortalities and salmon oil [12].

Table 7. 1 Summary of snow crab bioproducts obtained using a combined green chemistry-biorefinery approach

Crab Bio-product	Application	Advantages	Disadvantages
Crab Meal	Feed ingredient for aquaculture, poultry, livestock	Exisiting technologies available May be a better option than disposal	High capital costs, low value High ash, low in methionine & lysine, low astaxanthin Source of air pollution, offensive odours
Pigmented Oils	Feed ingredient for aquaculture & poultry Food ingredient as natural flavourant or colourant Natural health product - antioxidant	High value if high astaxanthin content obtained Oil less flammable and lower cost than traditional solvents Solvent is non-toxic, food grade edible oil Oil provides protection of astaxanthin pigment Source of energy and fatty acids when used as a feed ingredient No processing waste generated	May require deodorization for use as NHP or poultry feed Lower astaxanthin yield in comparison to solvent extraction Can cause environmental harm in case of an oil spill Will require spill response measures
Pigmented Protien Powder	Feed ingredient for aquaculture & poultry Protein supplement - Natural Health Product	High in protein and astaxanthin, low in ash, may be high in chitin Low temperatrue drying, low cost of input materaials	May be high in arsenic May be high in chitin May be low in methionine and lysine May cause offensive odours during drying
Protein Hydrolysate	Feed ingredient for terrestrial animals Nutritional Supplement - Natural Health Product	Low cost enzymatic treatment High in protien and calcium and other minerals Minimal processing waste	Recovery may require expensive drying treatments - spray drying or freeze drying May be high in Arsenic May require additional purification steps
Chitin	Industrial applications - waste water treatment, agriculture Chitosan natural health products, biopharma, medical	Lower cost option to traditional chemical process Residual protein and ash contents <1%, acceptable chitin N ~6% More environmentally friendly process HCl replaced with citric acid for demineralization step NaOH replaced with protease for deproteination step	Chitin was pink in colour May require chemical solvent to remove residual pigment if unable to optimize process Ash and residual protein slightly higher than that required for medical grade Hydrogen peroxide caused some damage to chitin structure Hydrogen peroxide requires additional health & safety measures Large volume of effluents generated may require treatment After demineralization to neturalize pH After deproteination to decrease BOD

Although hydrogen peroxide is considered eco-friendly since it biodegrades to water and oxygen, and is therefore not an environmental pollutant, when used in concentrations approaching 35% it can pose a health risk to workers causing eye, lung and skin irritations and even burns [13]. Therefore, appropriate transportation, storage, handling, and spill response measures must be in place, and workers must be properly trained and provided with appropriate PPE when high concentrations are used. H_2O_2 is also an explosion risk and must be properly stored to prevent decomposition and release of oxygen which can lead to pressure build-up and explosion. This requires the use of explosion-proof electrical equipment and fittings in areas where it is used, handled, and stored [13, 14].

7.2.2.2 Cost Considerations

The input costs for the green chemistry-biorefinery extraction process for pigmented oil, pigmented protein powder and chitin products were compared with the input costs for the chemical extraction of chitin and crab meal. The comparison is based on the estimated costs of the input materials for a 5 MT/day crustacean by-product processing facility - the smallest processing capacity that might be economically feasible for NL [9]. For this comparison, only the Corn Oil-Weifang extraction process was considered as this process provided the highest yields of astaxanthin and chitin and was the third lowest cost option of the combined green chemistry-biorefinery process. The Sunflower-Weifang process was the lowest cost option but had the lowest yield of astaxanthin, and lowest yield of chitin for the Weifang treated samples, making it less economical than the Corn Oil-

Weifang option. This assessment did not include capital cost estimates or other production or operational costs.

Table 7. 2 Comparison of input costs for a 5 MT/day traditional chemical and a green chemistry-biorefinery approach for valorization of NL Snow crab processing discards[1]

Traditional Chemical Process for Chitin			Green Chemistry Biorefinery Process		
Starting Weight of Crab By-product (kg)	5,000	% of Total Cost	Starting Weight of Crab By-product (kg)	5,000	% of Total Cost
Demineralization 7% HCL (1:10)		26.0%	**Pigment Recovery Corn Oil (1:1)**		3.1%
QTY (L) 20 baum HCL	10,938		QTY (kg)	4,605	
Unit Cost	$ 4.17		Unit Cost	$ 0.88	
Total Cost	$ 45,611.46		Total Cost	$ 4,052.40	
Deproteination 10% NaOH (1:8)		20.5%	**Two-step Demineralization 7.5% citric acid (1:10)**		65.7%
QTY (kg)	2,000		QTY (kg)	7500	
Unit Cost	$ 18.00		Unit Cost	$ 11.60	
Total Cost	$ 36,000.00		Total Cost	$ 87,000.00	
Depigmentation Ethanol (1:10)		53.5%	**Deproteination Weifang Protease 2% w/w**		0.6%
QTY (L)	25,000		QTY (kg)	47.58	
Unit Cost	$ 3.75		Unit Cost	$ 15.32	
Total Cost	$ 93,750.00		Total Cost	$ 728.93	
			Depigmentation H2O2 (1:4)		30.7%
			QTY (L) 25% H2O2	9,516	
			Unit Cost	$ 4.28	
			Total Cost	$ 40,728.48	
Total Input Cost	$175,361.46	100.0%	**Total Input Cost**	$ 132,509.81	100.0%
Chitin Yield & Cost			**Chitin Yield & Cost**		
Dry Chitin from Raw Crab By-product (%)		6.63%	Dry Chitin from Raw Crab By-product (%)		7.03%
Dry Chitin from Raw Crab By-product (kg)		331.5	Dry Chitin from Raw Crab By-product (kg)		351.5
Input Cost per kg of Chitin		$ 528.99	Input Cost per kg of Chitin		$ 376.98

[1] *All costs are in $CDN. Unit costs for HCl, NaOH, ethanol and citric acid were obtained from https://www.ingredientdepot.com/ (retrieved Feb 19, 2022). Unit cost for H_2O_2 was obtained from https://www.laballey.com/products/buy-25-food-grade-hydrogen-peroxide-for-sale (retrieved Feb 19, 2022) Unit cost for Weifang protease was obtained from the supplier based on pricing for 2021. Unit cost for corn oil was obtained from https://www.selinawamucii.com/insights/prices/canada/maize-corn-oil/ (retrieved Feb 19, 2022 - prices reported for 2019).*

The results of the input cost comparison of the combined green chemistry-biorefinery process and the traditional chemical process for crab by-product valorization are presented in Table 7.2. It should be noted that the chemical costs are likely

overestimated since pricing was obtained from internet sources which may not be reflective of supplier pricing for commercial scale processing, but it does allow us to compare the relative cost between both process methods.

The estimated input costs for the chemical process based on a starting weight of 5,000 kg of crab by-product were $45,611 for demineralization with HCl, $36,000 for deproteination with NaOH, and $93,750 for depigmentation with ethanol for a total input cost of $175,361. Based on the same starting weight of crab by-product the input costs for the Corn Oil-Weifang process were estimated to be $4,052 for pigment recovery with corn oil, $87,000 for demineralization with citric acid, $730 for deproteination with protease, and $40,728 for depigmentation with H_2O_2 for a total input cost of $132,510. Based on this analysis the green chemistry-biorefinery process is a lower cost option with input costs about 25% less than a traditional chemical extraction method, and results in additional high value bioproducts (i.e., pigmented oil, pigmented protein powder).

In the chemical process, the depigmentation solvent represents 53.5% of the total input costs, however, in the green chemistry-biorefinery process the two-step demineralization with citric acid was the costliest representing more than 65% of the total input cost. As suggested in Chapter 6, it may be possible to eliminate the depigmentation step by optimizing the oil and enzyme treatments which would make the green chemistry-biorefinery option even more cost-effective and environmentally friendly. In addition, the two-step demineralization process may be more cost effective once this process is optimized. Citric acid is also a more environmentally friendly and safer alternative than HCl and requires no specialized transportation, storage, or handling equipment. Because it

is less corrosive than HCl, citric acid will extend equipment lifespan thereby decreasing overall process costs (refer to section 7.2.2.1).

When product yields are factored in, the green chemistry-biorefinery model is more economical as the input costs per kg of chitin produced are nearly 30% lower than that obtained with the chemical method ($376.98 vs $528.99 respectively) and produces 2 additional products at no additional cost.

7.3 Green Chemistry-Biorefinery Model for Valorization of NL Snow Crab Discards

Based on my research findings as summarized in section 7.2, a combined green chemistry-biorefinery model for the valorization of NL snow crab processing discards which includes the extraction of multiple bioproducts is presented in Figure 7.1. This model offers a lower cost, eco-friendly, and safer alternative to traditional chemically intensive approaches evaluated in other studies for the valorization of NL Snow crab processing discards. The potential market value of each bio-product is indicated in the diagram however, these prices will vary depending on the quantity and quality of the bioactive components contained within each. Market prices were estimated based on prices reported by Norsworthy & Burke [9] for protein, chitin, and chitosan. Market prices for pigmented oils and astaxanthin were estimated based on prices reported by www.pondtech.com/astaxanthin/ (retrieved Feb 19, 2022). Market prices for all products represent the mid-range price.

It should also be noted that for some bioproducts, additional processing steps will be required to increase their value which will also increase the production costs for those bioproducts. However, the model may only be economically viable if these higher value

bioproducts can be incorporated. For example, due to the low market value of chitin, Norsworthy & Burke [9] recommended that crab by-product utilization may only be economically viable if chitin is further processed to chitosan due to its higher market value. A stand-alone crab meal plant did not appear viable [9]. Based on the currently available bioproduct market prices, pigmented oils and chitosan offer the best value if the extraction methods can be optimized to improve yield and quality while keeping production costs low. While carotenoprotein and protein hydrolysate market values are lower than that of pigmented oils and chitosan, these bioproducts may still add value and offer a means of reducing waste and maximizing utilization of crab by-products, which are key objectives of the model. Missing from the current model is the recovery of calcium citrate which represents another potentially valuable bioproduct (e.g., as a calcium supplement) that could be incorporated.

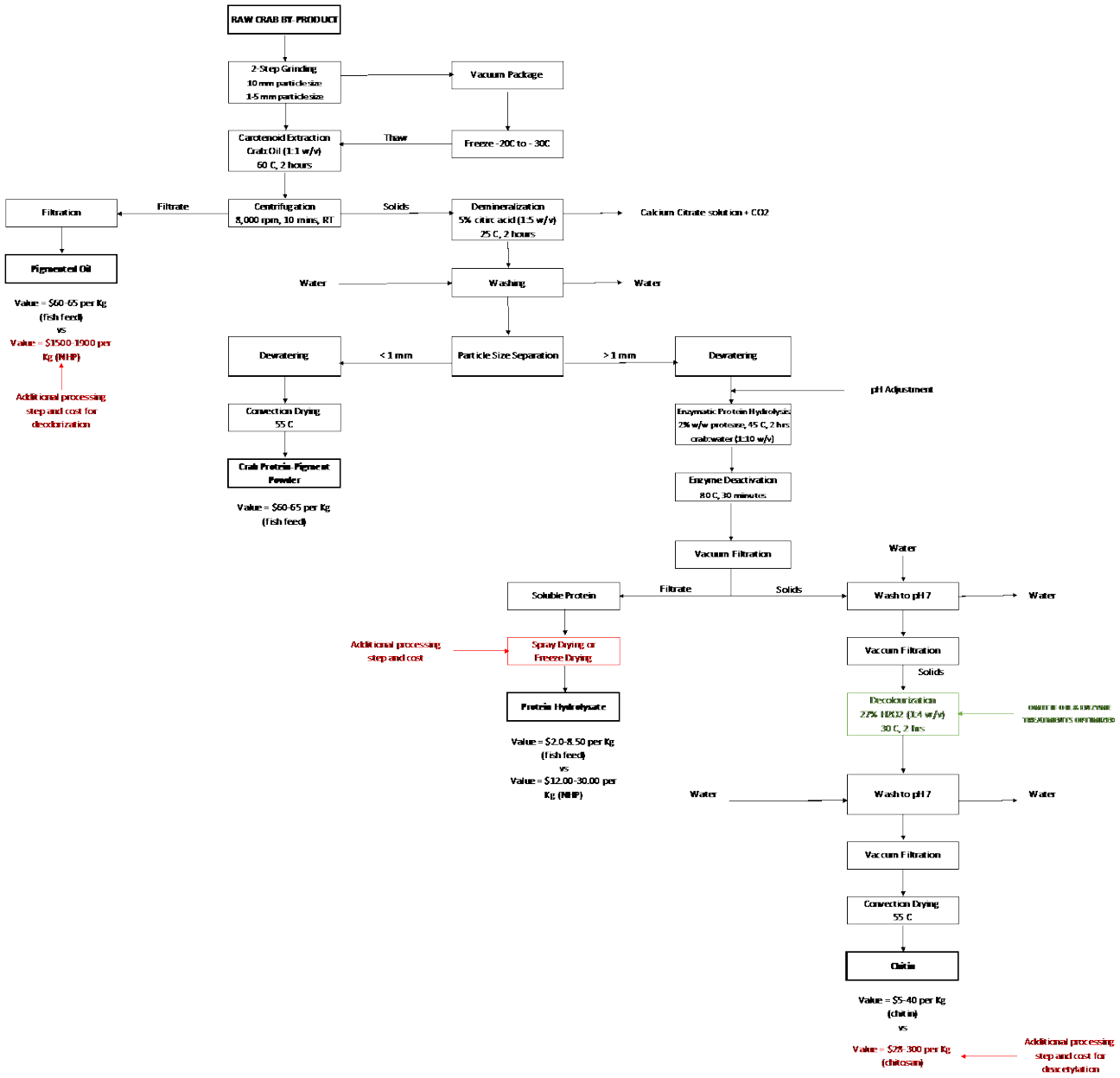

Figure 7. 1 Green chemistry-biorefinery model for the valorization of NL snow crab processing discards

7.4 Limitations

As mentioned in section 7.2, there are some limitations to the proposed green chemistry-biorefinery approach for valorizing snow crab processing discards. These limitations are further described below.

1. Obtaining homogenous samples during collection from the offal bin was difficult as samples had to be manually collected from the top of the bin using pails. This may have resulted in differences in the shell:meat ratio of the samples.
2. Due to the hardness of the crab shells, obtaining a uniform particle size was challenging which may have impacted the effectiveness of the extraction methods used for pigment recovery, demineralization and deproteination.
3. Studies comparing the extraction of bioproducts from combined crab by-products with separated crab by-products (i.e., protein and shell) were not evaluated, but my results suggest that yield and quality of some bioproducts may be improved if crab by-products are separated prior to extraction.
4. Due to Covid-19 related challenges in obtaining samples in 2020 and 2021, a limited volume of sample was available for the trace metal study and therefore should be repeated to confirm the sources of aluminum and arsenic. Due to time and cost limitations, arsenic speciation was not conducted. Due to limited sample size and time constraints, further purification of the protein hydrolysates was not investigated. This study was conducted on chemically extracted crab bioproducts, (with the exception of the protein hydrolysate which used an enzymatic method),

and should be repeated on crab bioproducts extracted using the green chemistry-biorefinery method presented in this thesis.

5. Pigment recovery using vegetable oils was not optimized. Other than the type of oil, additional factors which may impact the effectiveness of pigment extraction such as time, temperature, ratio of oil to waste, solvent viscosity, influence of moisture content and particle size, single vs multi-stage extraction, were not studied due to time and cost limitations.
6. Further characterization of the pigmented protein powders (i.e., amin acids, fatty acids, trace metals) will be a key factor in identifying opportunities for commercial applications. Unfortunately, further characterization studies were not possible due to Covid-19 related delays and limited access to lab facilities in 2020-2021.
7. Recovery of calcium citrate was not included in the current study but represents another potentially valuable bioproduct (i.e., calcium supplement) that could be incorporated into the green chemistry-biorefinery model for crab by-products.
8. Complete optimization of my green chemistry-biorefinery approach was not possible due to Covid-19 related delays and limited access to lab facilities in 2020-2021.
9. A full economic analysis of the proposed model will be required to determine the feasibility of such an approach for rural NL. This was beyond the scope of my thesis but will be an important part of a provincial crustacean waste valorization strategy and would require the skills of an economist rather than a scientist to be meaningful.
10. A life cycle assessment of the proposed green chemistry-biorefinery model was beyond the scope of this thesis but should be considered to determine the full

environmental impact of the new approach. This will also be an important component for the development of a provincial crustacean waste valorization strategy.

7.5 Recommendations for Further Research

Despite the limitations discussed in section 7.4, the research conducted in this thesis has offered some useful results and conclusions that could be applied to the valorization of NL's snow crab processing discards. Based on the results of this thesis, further studies are required to optimize the green chemistry-ocean based biorefinery model presented for snow crab discards. Optimization studies should focus on using simple, low cost, green technologies: (1) to improve yield and quality of extracted snow crab bioproducts; (2) that can be easily incorporated on a commercial scale; and (3) that minimize environmental health and safety concerns for remote coastal areas. The suggested areas for further research are described below.

1. A feasibility study should be undertaken jointly by the Government of NL, seafood processors, industry associations and academia to assess the technical, environmental, and financial feasibility of establishing regional crustacean by-product processing facilities in the province.
2. Industry and the province will have to decide which crab/crustacean bioproducts to produce, and design dedicated processing facilities around these products.
3. Further research is needed to optimize collection and stabilization technologies for fresh-frozen crab processing by-products on a commercial scale. Technologies must minimize exposure to oxygen, avoid high temperature processes, be easy to

implement and inexpensive, and must be suitable for the intended bioproducts (e.g., astaxanthin recovery vs chitin extraction).

4. Seasonality and pre-treatment methods were shown to have the greatest impact on crab by-product quality, and therefore warrant additional studies. While seawater treatment proved useful in preventing oxidative deterioration reactions, it also created additional handling and quality issues (e.g., lipid hydrolysis, chitin extraction and recovery).
5. Studies comparing the extraction of bioproducts from combined crab by-products with separated crab by-products (i.e., protein/organs vs shell) should be explored to determine effects on yield and quality of crab bioproducts.
6. Understanding the levels of arsenic and aluminum in snow crab by-products and how they are transferred throughout the crab bio-product value chain will be key to developing safe marketable natural health and biomedical products. This study should be repeated with a larger sample size and should include: (1) speciation of arsenic; (2) evaluation of process steps to determine the effect of separation of the shell and protein/organs on the levels of arsenic and aluminum in the extracted bioproducts; (3) evaluation of additional purification steps (e.g., ultrafiltration) as a means of reducing/removing environmental contaminants; and (4) comparison with green extracted crab bioproducts to determine if/how the extraction solvents affect the removal of trace metal contaminants.
7. Further studies using corn, canola and soybean oils as solvents for pigment extraction and recovery should focus on (1) optimizing the crab:oil ratio; (2) comparing single vs multi-stage extraction processes; (3) determining the effects of

moisture, particle size, time, temperature, and solvent viscosity on pigment recovery; (4) comparing the lipid profiles, free fatty acid, and astaxanthin compositions against nutritional profiles of commercial aquafeeds to determine their suitability for this purpose; (5) evaluating simple low cost technologies for deodorization of the oils.

8. Full characterization of pigmented protein powders will be required for commercial applications, and should include analysis of amino acid, fatty acid and astaxanthin compositions, chitin content and heavy metal content. If pigmented oils are not important as a commercial product, a pigmented protein powder with higher astaxanthin concentrations can be targeted.
9. Further optimization of the two-step citric acid demineralization process should focus on minimizing the amount of citric acid and duration of the acid treatment required to achieve full demineralization of chitin, while incorporating steps to recover marine calcium. Calcium citrate would be one form of calcium produced during demineralization and has potential as a food supplement if food grade citric acid is used in the process.
10. Elimination of a final H_2O_2 decolourization step by optimization of vegetable oil and enzyme treatments will reduce the amount of chemical needed, processing time and costs. Optimization studies should focus on the effects of other process variables such as particle size, shell to liquid ratio, time, and temperature applications for each treatment, as well as the order of processing steps (e.g., effect of enzyme treatment before oil extraction), and if needed alternative green oxidation chemicals (e.g., TAML - activated H_2O_2) [68].

11. A life cycle assessment of the optimized green chemistry-biorefinery model will be required to determine the environmental impacts, positive and negative, of the new approach to snow crab by-product valorization for NL coastal communities.

7.6 Conclusion

This thesis represents the first dedicated research study using a combined green chemistry-biorefinery approach for the valorization of NL's snow crab processing discards. In comparison to the chemically intensive processes typically used for the extraction of crustacean bioproducts, this new approach for the valorization of snow crab waste offers higher value, eco-friendly, and lower cost options. The results are informative and may help industry and government develop an eco-friendly strategy for utilization of the province's snow crab processing discards. These results can also be applied in other jurisdictions such as Atlantic Canada, Quebec, Alaska, and Norway, where there are significant snow crab fisheries.

In summary, the findings from this thesis demonstrate that a green chemistry-biorefinery model offers advantages over traditional disposal and utilization methods for valorizing our snow crab processing discards. Once optimized, this model has potential to reduce environmental pollution, increase utilization and maximize the value of our snow crab resource. The model could also be adapted for the valorization of other crustaceans, or adapted to combine processing wastes from shrimp, crab, and lobster, thereby making significant contributions to Canada's Blue Economy.

7.7 References

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