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COMPARAISON DE DIFFÉRENTES MÉTHODES DE CONSERVATION ET D'EXTRACTION DES COMPOSÉS PHÉNOLIQUES DANS L'ALGUE MARINE *ASCOPHYLLUM NODOSUM* APPLICABLES DANS LES RÉGIONS CÔTIÈRES ET ÉLOIGNÉES

COMPARING THE EFFECTS OF DIFFERENT PRESERVATION AND EXTRACTION METHODS ON PHENOLIC COMPOUNDS IN THE SEAWEED *ASCOPHYLLUM NODOSUM* APPLICABLE TO REMOTE COASTAL COMMUNITIES

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FORWARD

The research and work of this dissertation was carried out from May 2016 to August 2019, as part of a Master's degree in Environmental Science. It was conducted under the supervision of Professor Simon Barnabé PhD, in the department of Chemistry, Biochemistry, and Physics, and co-directed by Louis-Charles Rainville PhD, from the marine research institute Merinov. The thesis presents the article called "Bioextracting phenolic compounds from the brown seaweed *Ascophyllum nodosum* from Quebec's North Shore coastline", which was published in the journal "Industrial Biotechnology" in June 2019.

This Master's degree corresponds to one of the initiatives managed by the Coaster's Association, to develop the bio-economy on the Lower North Shore region of Quebec. Located in a region furthest east in the province and along the Gulf of the Saint-Lawrence, the Lower North Shore spans over 500 kilometers of coastline, most of which is completely isolated. The region contains a vast abundance and diversity of interesting natural resources, evolved to adapt to its cold and harsh climate. Throughout history and still to this day, the communities highly depend on fishing as the only economic source. However, fishery cuts in cod and crab harvesting have left some municipalities extremely devitalized and caused population sizes to decrease. Diversifying industries in these communities have proven difficult since most of the skilled human resources have moved away for employment. The Coaster's Association aims to offer support to local entrepreneurs wanting to develop an innovative industry using local resources sustainably. The organization had to first educate and train scientists, preferably from the region, and then determine the valuable resources and analyze the resource's characteristics and production opportunity.

Seaweed growing in abundance along the coastline was identified as a valuable resource containing polyphenols and high antioxidant activity for ingredients in the nutraceutical and cosmeceutical industry. However, it required the scientific expertise

from UQTR and Merinov to help develop an extraction procedure to produce a phenolic compound rich extract. The results and procedures developed from this research project could aid a new and different business start-up in the remote region and could severely improve the socio-economic conditions of the Lower North Shore.

In this project, Louis-Charles Rainville and Simon Barnabé developed the project breakdown and details, as well as offer suggestions and recommendations to the Coaster's Association and the enterprise, the Lower North Shore Bioproducts Cooperative, on valorization of a bioproduct, processing marine algae, and extraction technologies. Simon and his team of post-doc biochemistry specialists offered themselves on the extraction and analytic methods of the seaweed material. Thus, this research study was carried out with a goal in mind to provide information to develop a bioactive ingredient using environmentally friendly technologies that could help the economy of remote coastal communities. The results presented in this dissertation gives recommendations on the procedures and yields of seaweed extracts that could help progress the industry in the future.

RÉSUMÉ

Le développement et la relance économique des communautés éloignées et côtières passent souvent par l'obtention de divers produits à partir de ressources naturelles. Des ressources telles que les algues marines sont présentes dans ces régions et contiennent des molécules d'intérêt pour divers marchés, dont celui des cosmétiques et de la biotechnologie.

Cette étude porte sur les composés polyphénoliques riches en antioxydants que l'on trouve dans l'espèce d'algue marine *Ascophyllum nodosum* qui est fortement présente sur le littoral de la Basse-Côte-Nord du Québec. Différentes méthodes de conservation et d'extraction ont été comparées pour optimiser le rendement en polyphénols, y compris des méthodes applicables et accessibles aux régions éloignées. Les analyses des extraits ont été effectuées à l'aide de différents tests colorimétriques pour mesurer les polyphénols totaux et les phlorotannins, ainsi que pour estimer l'activité antioxydante.

Les résultats de l'étude ont démontré que les échantillons immédiatement congelés présentaient une concentration plus élevée en polyphénols et la meilleure activité antioxydante. L'analyse a également démontré que la méthode d'extraction assistée par micro-ondes a amélioré l'efficacité du rendement en polyphénols pour les extractions à l'eau. Cependant, la méthode conventionnelle d'extraction par solvant avec du 1,3-propanediol à 75 % (v/v) a permis d'obtenir le contenu phénolique le plus élevé, totalisant 9,8 % (p/p) du poids sec de l'échantillon et la meilleure activité antioxydante.

Mots-clés : algues marines, extraction assistée aux micro-ondes, polyphénols, antioxidants, phlorotannins

ABSTRACT

Developing innovative industries in rural communities require researching valuable finished products using local natural resources and feasible equipment and technology. Resources like seaweed, are popular in today's global cosmetic ingredient and biotechnology market and are commonly found in remote communities, making it an ideal opportunity for rural economic development. The research in this study focuses on the antioxidant rich phenolic compounds found in the seaweed species *Ascophyllum nodosum*, local to Québec's North Shore coastline. Different marine processing technologies were compared to optimize phenolic content yields. It analyzed the efficiency of extracts following four different preservation methods of the seaweed raw material; freezing, shock freezing, dehydrating, and air-drying in a greenhouse. Analysis also compared bioextraction techniques, such as water and organic solvent liquid extractions and microwave-assisted extractions, that are applicable and accessible to remote regions. Evaluations of extracts were performed using different colorimetric assays to quantify phenolic compounds and phlorotannins, as well as to analyze antioxidant efficiency. Results from the study found that the samples immediately frozen displayed higher polyphenol concentration and expressed the highest antioxidant efficiency. Analysis also showed that a microwave-assisted extraction method improved phenolic compound concentration yield efficiency by 36% when extracted at 50°C in water. However, the conventional solvent extraction method using 75% aqueous 1,3-propandiol solvent resulted in the highest phenolic content, totalling 9.8% of its dry weight, and the optimal antioxidant activity.

Keywords: seaweed, microwave assisted extraction, polyphenol, antioxidant, phlorotannin.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of variance
DFO	Department of Fisheries and Oceans Canada
DMBA	2,4-Dimethoxybenzaldehyde
DPPH	1,1-Diphenyl-2picrylhydrazyl
IC_{50}	Concentration at 50% inhibition of free radicals
MAE	Microwave-assisted extraction
N	North
PGE	Phloroglucinol equivalent
QC	Québec
US$	American dollars
USA	United States of America
UQTR	Université du Québec à Trois-Rivières
UVB	Ultraviolet B radiation
W	West

LIST OF SYMBOLS

cm	Centimeter
°C	Degree Celsius
%dw	Percentage of dry weight
g	Gram
h	Hour
L	Liter
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Micromolar
nm	Nanometer
p	Probability
R^2	Determination coefficient
W	Watts
% v/v aq.	Percent of volume in aqueous mixture
µL	Microlitre

CHAPTER I

INTRODUCTION

1.1 Coastal industry opportunities

1.1.1 Building sustainable rural communities

Developing the bio-economy in remote regions poses challenges other urban areas do not have to face. Rural communities tend to maintain traditional industries, harvesting common renewable resources usually for the common fishing and farming industries. Local businesses first off must overcome challenges caused by isolation, such as minimal and expensive transportation options, difficulty to import goods required for processing, and so on. Locals are also continuing to sell raw materials externally, doing little transformation internally and receiving little profit. Remote regions continue this primary transformation industry since they tend to lack the scientific expertise and they are not given the opportunity for research and development.

In order to begin the process towards building sustainable rural communities through bio-economic development, the region must identify its bio-resources and research the production possibilities the resource could eventually be used for. It is then necessary to follow up with stock assessments and abundance estimates of the resources to know whether it is sufficient enough to meet the demand of the industry. It is also recommended to consider the harvesting methods and how sustainable the practices are. The resource selected should be renewable and growing back frequently for future harvests. And finally, it is very important that the community involved is passionate about the resource and has a desire to sell the product externally. Developing a new industry in a remote region will require innovative thinking and persistence in order to be successful.

The process explained above had been done for a remote coastal region of the Lower North Shore in the province of Québec, to assist the development of the local bio-economy. An interesting identified resource that was revealed in the process was the local macroalgae species found in abundance along the coastline of this region. Seaweed harvesting and production businesses has been recommended as an opportunity to improve the wealth and socio-economic conditions of coastal communities in developing countries, as long as sustainable practices are followed (Rebours *et al.* 2014). Therefore, it was essential to investigate the production opportunities of the species found locally on the Lower North Shore and find sustainable harvesting practices to share with local harvesters to protect the marine environment.

1.1.2 A macroalgae industry opportunity

Seaweed-based products are growing in popularity in our modern world, growing the global macroalgae market each year. The concept of harvesting seaweed in coastal communities began centuries ago, where the resource provided locals with food, fuel, feed, and fertilizer (Mac Monagail *et al.* 2017). The industry has since expanded drastically, and in 2015 the global production of seaweed reached 30.4 million tonnes, with wild harvests only contributing 1.1 million tonnes of the total (FAO 2018). The remainder 96% of seaweed production is cultivated in aquaculture settings, mostly in Asian countries. The value of the worldwide macroalgae industry is valued at approximately US$10 billion annually, and production continues to increase about 5.7% each year (Rebours *et al.* 2014).

Majority of seaweeds in the industry are used to produce human foods, and the remainder is used to make a variety of extracted materials, such as food additives, animal feed, fertilizers, biofuel, cosmetics, and medicines (Valderrama *et al.* 2013). In Asian markets, seaweed is a popular food source where it is eaten raw, dried, or cooked in soups and stews (FAO 2018). Besides producing for human foods, US$1 billion of the industry is profited making macroalgae extract products, like hydrocolloids and fertilizers (Lee 2008). Approximately 61% of macroalgae extracts are used to produce

hydrocolloids, like alginate, carrageenan, and agar (Figure 1.1) (Nayar & Bott 2014). Hydrocolloid substances are usually used as a gelling food ingredient for thickening or emulsifying. Alginate, carrageenan, and agar are used in products like jellies, creams, and puddings (Saha & Bhattacharya 2010). Alginates are also in variety of other non-food ingredient purposes, such as stabilizing for color agents, waterproof textiles, coating paper, and treating wastewater (Mac Monagail *et al.* 2017). The minority of the profit margin is derived from producing agricultural materials food (McHugh 2003). Processed macroalgae used in soil additives, fertilizers, and animal feed, also called seaweed meal, contribute to 38% of the macroalgae extracts produced worldwide (Figure 1.1). The high fiber content in them help condition and hold moisture in the soil, and the mineral content and trace elements in seaweeds make for good fertilizing. The algae fertilizer industry is now popular, especially with increasing organic farming interest (McHugh 2003). And finally, a small 1% of the seaweed extract derives from producing ingredients for the pharmaceutical, cosmeceutical, nutraceutical, and bioactives market (Figure 1.1). Extracts are sometimes added to lotions to help the skin with moisture retention. As well, fresh seaweed can be crushed and applied to a person's body, in thalassotherapy, which aids in rheumatism and osteoporosis relief (McHugh 2003). However, the potential of seaweed extracts in cosmetic or nutraceutical ingredients are currently being researched more frequently to replace synthetic ingredients with natural materials, which could easily be sourced from algae.

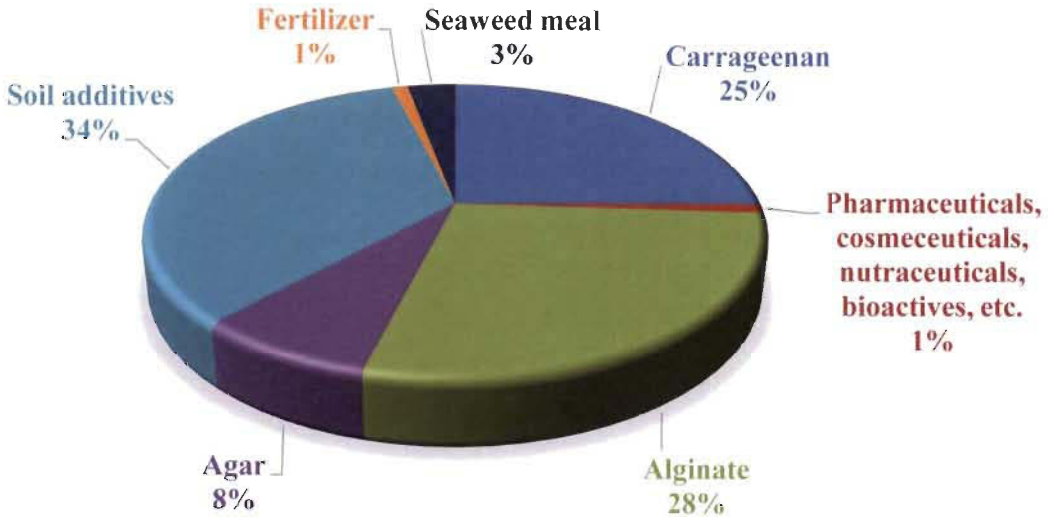

Figure 1.1 Percentage of raw macroalgae biomass used for industrial applications.

While majority of the raw biomass of harvesting macroalgae produced into extracts is used for hydrocolloids and agriculture ingredients, it does not correspond with the greatest profit margin a business can achieve using the raw material. Seaweed used to make nutritional and food products, as well as cosmetics, nutraceuticals, and pharmaceutical ingredients not only produce the highest value, the final product requires less raw biomass harvested (Figure 1.2) (Côté-Laurin *et al.* 2016). Value-addition ensures investment return, and this will come from innovative products marketed for the benefit of human health (Hafting *et al.* 2015). The high-value combined with minimal biomass harvesting required should be an incentive for business developers to focus on researching and innovating extracts for the nutritional and cosmeceutical markets.

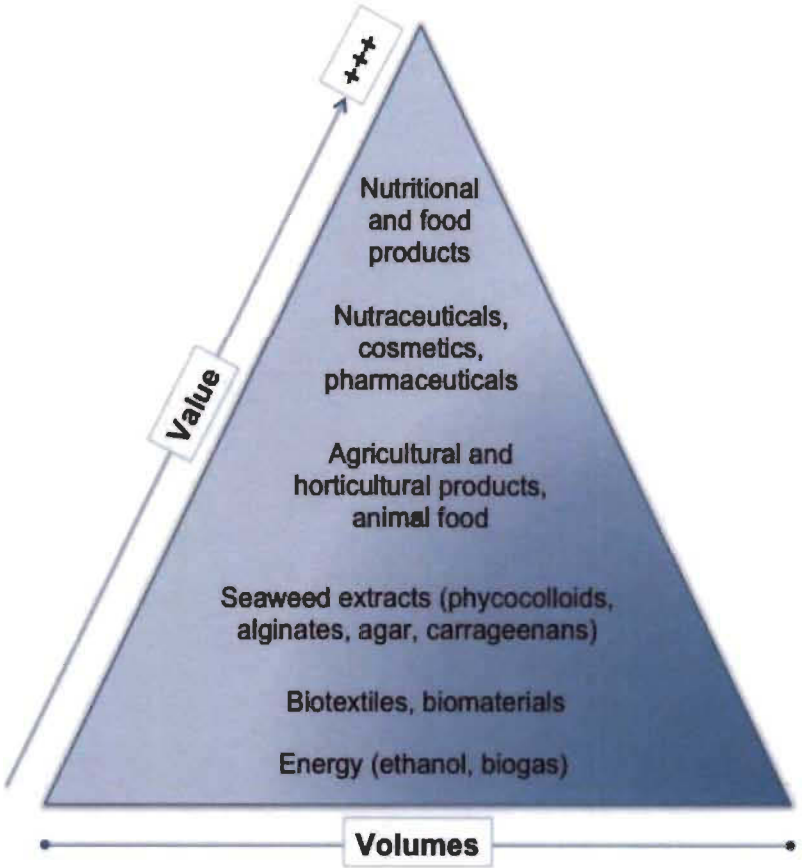

Figure 1.2 Diagram of the different potential markets for seaweed, and its corresponding value and volume of harvest required.

1.1.3 Québec's seaweeds

Macroalgae are marine algae that are multicellular and macroscopic (Mac Monagail *et al.* 2017). These are plant-like organisms that grow under sea water and are attached to a bottom substrate. Different species can be found in the intertidal zones or growing in deeper waters. Most seaweeds have blades that keep afloat under water and use sunlight for photosynthesis. The whole organism is then held down to the ocean floor by securing onto heavy substrates using its holdfast (Figure 1.3).

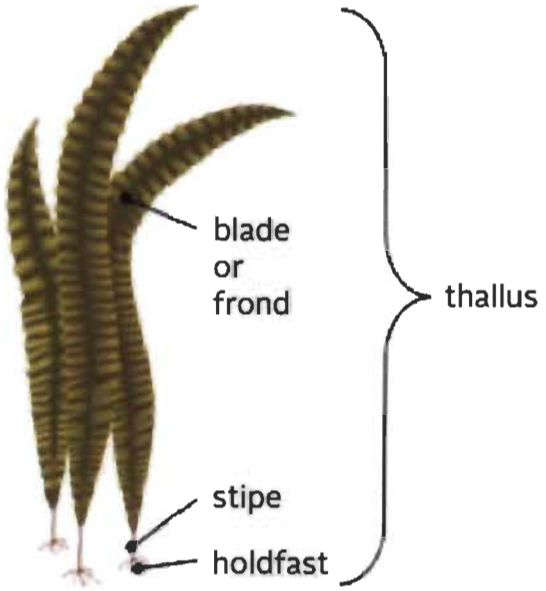

Figure 1.3 Structure of marine macroalgae.

Species of marine macroalgae are grouped in different classes as Phaephyceae, Rhodophyceae, Chlorophyceae, which are also considered brown, red, and green algae, respectively. These three classifications are based on the pigmentation of the algae, however, it also characterizes the active compounds found within them and their usually its size range. The brown algae are typically found in colder waters, where they thrive up to 20°C, and these can also be quite large, ranging from 30 cm to 20 m long. The red seaweeds tend to be smaller, can range from purple to reddish-brown in color, and they may be found in cold, temperate and tropic waters. The green algae are also small in size and are found in various geographical regions (McHugh 2003). The province of Quebec has brown, red and green seaweed species found on the territory (Table 1.1). Brown seaweed dominates the macroalgae found in this province and in the North Atlantic waters, in both species' diversity and biomass weight.

According to the Guide to Commercial Harvesting of Quebec Seaweed prepared and published by Merinov (Côté-Laurin *et al.* 2016), the province of Quebec has a huge potential for harvesting and producing macroalgae. The region is advantageous due to the good water quality, the various climatic conditions, and the extent of shoreline distance.

There are about 20 small-medium enterprises in Quebec harvesting and processing seaweed for a variety of different products (Côté-Laurin *et al.* 2016), however there is currently not an existing business on the Lower North Shore taking advantage of the marine resource.

Table 1.1

The main seaweed species of Québec with commercial value (adapted from Côté-Laurin *et al.* 2016).

Brown Seaweed	Red Seaweed	Green Seaweed
Winged Kelp (*Alaria esculenta)*	Dulse (*Palmaria palmata*)	Sea lettuce (*Ulva sp.*)
Rockweed (*Ascophyllum nodosum*)	Irish moss (*Chondrus crispus*)	
Sea lace (*Chorda filum*)	Laver (*Porphyra sp.*)	
Bladderwrack (*Fucus sp.*)		
Fingered Kelp (*Laminaria digitata*)		
Sugar Kelp (*Saccharina latissima*)		

1.2 Characteristic compounds of brown seaweeds

1.2.1 Nutritional compounds

Approximately 85% of the global seaweed industry is used for producing goods for human consumption, due to its rich nutritional composition. In general, macroalgae species contain "macronutrients such as sodium, calcium, magnesium, potassium, chlorine, sulphur, and phosphorus; micronutrients (iodine, iron, zinc, copper, selenium, molybdenum, fluoride, manganese, boron, nickel, and cobalt); and vitamins (B12, A, K)"

(FAO 2018). The iodine nutrient alone in seaweed food can easily meet the daily adult requirement and can help prevent the impairment of cognitive development in children (FAO 2018). Seaweeds are also known to contain trace amount of fibre and omega-3 fatty acids. Besides the healthy proteins contained in macroalgae that have a good source of essential amino acids, fatty acids, fibre, minerals, and vitamins, seaweed as a food source also consists of sufficient amounts of phenolic compounds (Machu *et al.* 2015).

1.2.2 Phenolic compounds

Within the last few years, society has been putting an increase of demand on more naturally derived products for both consumption and everyday use. This shift in demand has been mostly due to the increasing resistance of bacteria against traditional chemical antibiotics (Aires 2017) and due to the modern research discovering how damaging synthetic materials are on the environment. Consumers are now starting to read labels and ingredients, and checking for certifications, especially in food and health products. The demand has also created an opportunity for scientific researchers to find innovative and effective molecules from natural products to replace synthetic ingredients and antibiotics. Plant-based extracts containing a high concentration of phenolic compounds have comparable results to synthetic antibiotics, due to its high antioxidant capabilities (Balasundram *et al.* 2006).

Antioxidant activity helps the body defend against oxidative damage and free radicals. Oxidative stress is brought upon humans mostly due to external stressors and aging (Machu *et al.* 2015). It is predicted that the oxidative stress impacting the body contributes to many common occurring diseases, such as cardiovascular diseases, neurodegenerative diseases, cancer, diabetes, rheumatoid arthritis, and so on (Machu *et al.* 2015). Therefore, it is becoming very common for plant ingredients containing phenolic compounds with high antioxidant activity to be added to a variety of food, nutraceutical, and cosmeceutical products due to all of its health benefits. The phenolic compounds that are contained within seaweeds, and also found in common foods like strawberries and green tea, have antioxidant capabilities that can be used in anti-aging products (Audibert *et al.* 2010).

1.2.3 Phlorotannins

Brown seaweeds contain high levels of phlorotannins, a type of tannin which represent a type of complex phenolic compound, also known as polyphenols. The phlorotannins compounds are found exclusively in the Phaephyceae class and they are a polymer of phloroglucinol and halogenated oligomers (Mannino *et al.*, 2016). The chemical compounds are produced by the macroalgae and aids the organisms' survival by protecting it from UV radiation damage, deterring grazers, and repairing any wounds (Cruces *et al.* 2015).

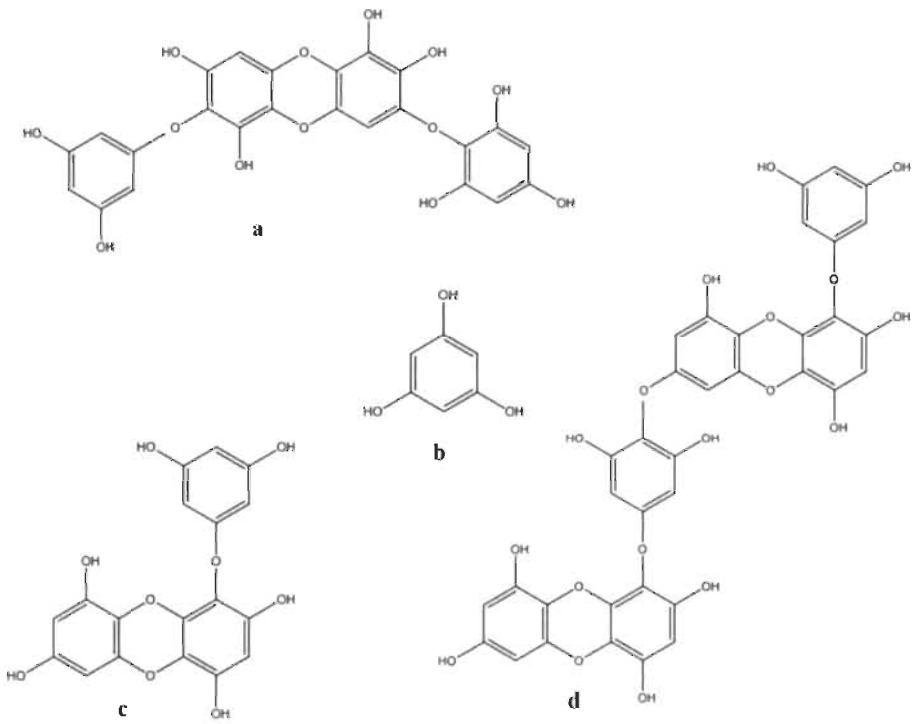

Figure 1.4 Chemical structures of some phlorotannins : (a) diphlorethohydroxycarmalol; (b) phloroglucinol; (c) eckol; (d) dieckol.

In addition to the polyphenol compound having antioxidant capacity, studies analyzing the phlorotannin compounds has shown therapeutic properties, like anti-bacterial, anti-allergic, anti-diabetes, anti-inflammatory, and anti-HIV activities (Machu *et al.* 2015). Some results have even suggested that that phlorotannins contain potential cancer chemopreventative agents that can help fight photocarcinogenesis and other effects of UVB radiation exposure (Thomas & Kim 2010). *In vitro* research has shown

phlorotannins to inhibit the development of matrix metalloproteinases (MMPs) which mostly develop during human skin aging processes causing degradation of dermal collagen (Figure 1.5). Thus recommending that eating or applying the nutrients from brown algae help protect and care for human skin (Thomas & Kim 2010).

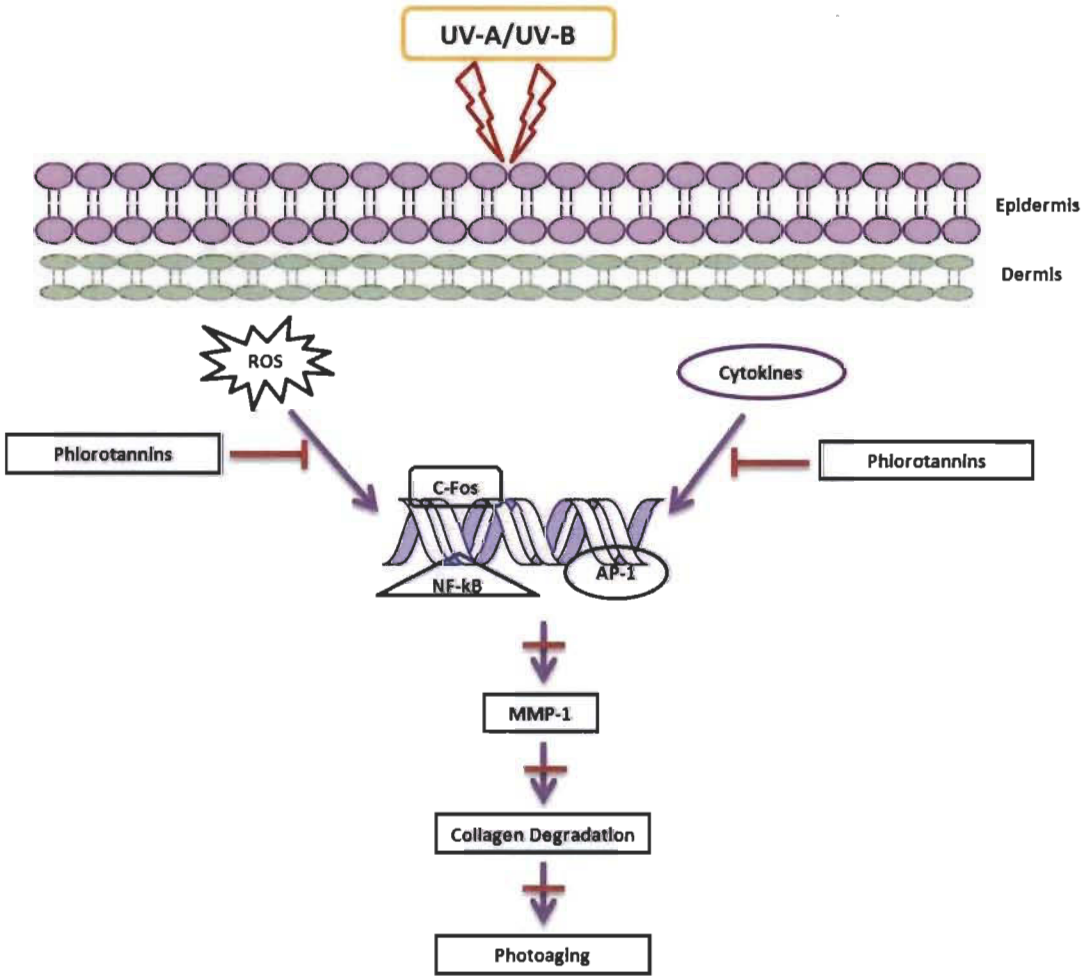

Figure 1.5 Anti-inflammatory effects of phlorotannins via cytokine blockade.

1.3 Rockweed (*Ascophyllum nodosum*)

1.3.1 Biomass and distribution

After studying the macroalgae species diversity, estimating the biomass volumes, and assessing the stock locations on the Lower North Shore coastline in 2014, it was

determined that the species most abundant and dominating the intertidal water in this remote region is *Ascophyllum nodosum*, or commonly known as Rockweed. The report indicated that the harvestable stock of Rockweed in the area was comparable to the volumes of biomass in Nova Scotia. The geographical landscape of the Lower North Shore is favorable for growth of this seaweed, due to its numerous islands and inlets protecting the aquatic plant from rough waters. The study concluded that exploiting *A. nodosum* in this remote region of Quebec was feasible and a profitable industry using this resource could certainly happen (Maloney 2014).

Ascophyllum nodosum is a brown algae that is within the family group Fucaceae. The seaweed is a olive and yellow in color and has long narrow blades with numerous blades branched from the stipe (Figure 1.6). The blades are scattered with air pocket bladders that help the seaweed float towards the sunlight when under water. Rockweed averages 30-60 cm in length but can reach up to 3 m long. The blades of the seaweed averages 3 to 15 years in age and can be measured by counting the number of air bladders found on the main stem. One air pocket bladder on the main stem is considered one year in age, except for the first year (Côté-Laurin *et al.* 2016). The growth of Rockweed is extension of the tip of the blades, which makes the base of the aquatic plant the oldest part. A holdfast of a single *A. nodosum* plant can be up to 40 years in age, making it especially important to keep the holdfast remaining when harvesting.

Figure 1.6 Photo of Rockweed (*Ascophyllum nodosum*) in Québec.

1.3.2 Characteristics

Rockweed is known to have good nutritional compounds, making it an interesting resource for food production. This brown algae species is rich in minerals such as sodium, potassium, magnesium, and calcium and is also a good source of iodine, fiber, and key vitamins (Côté-Laurin *et al.* 2016). *Ascophyllum nodosum* is one of the brown algae species with the highest concentration of phlorotannins (Dutot *et al.* 2012). Many studies have found that the phlorotannin extracts express rich antioxidant levels (Kuda *et al.* 2005, Connan *et al.* 2006) and some have some extracts to contain anti-inflammatory, anti-senescence (Dutot *et al.* 2012) and anti-microbial activity (Jimenez *et al.* 2010). If the extracts from *Ascophyllum nodosum* contain high levels of phenolic compounds and express a high antioxidant efficiency, then it is possible to create ingredients for high-value markets like nutritional products or cosmeceuticals by only harvesting small volumes of seaweeds.

1.3.3 Production possibilities

Based on the chemical and nutrient characteristics of *Ascophyllum nodosum* described previously, the raw material has the potential to be used in a wide variety of industry products. The potential markets include food extracts for thickening materials, feed for animals, agriculture for fertilizing, cosmetics for hair and skin care, nutraceuticals for nutrient supplements, and industrial for stabilizing textiles and paints (Côté-Laurin *et al.* 2016).

Looking on past brown algae industries and scientific literature, it is difficult to make profitable production of *Ascophyllum nodosum* for industrial purposes, such as food additives or fertilizers, because of high harvesting costs and especially high shipping expenses on the Lower North Shore. In order to reach a higher profit, a goal to obtain bioingredients useful for higher market value products, like cosmetics or green materials.

1.4 Seaweed production

1.4.1 Minimizing yield variability

Active compounds, like phlorotannins, vary greatly depending on the stressors acting upon them and the environmental conditions the brown algae are exposed to. Phlorotannin abundance varies within and between species, and the phenolic compound yields can be influenced by the size and age of the seaweed, the type of tissue, the surrounding environmental or biotic factors, and variations between geographical locations (Van Alstyne *et al.* 2001, Stiger *et al.* 2004, Amsler & Fairhead 2006, Jormalainen & Honkanen 2008). The phenolic content will alter if there is a high density of grazers or when there is more exposure to UV radiation, during the summer months. The seasonal variation of the total phenolic yield is specific to the species and location (Connan *et al.* 2004). Many research studies have determined that healthy and stressed brown seaweeds release phenolic compounds into the seawater through exudation or cell damage, where the material is discharged as marine dissolved organic matter (Carlson & Carlson 1984, Jennings & Steinberg 1994, Swanson & Druehl 2002). Studies have found

that factors like temperature and salinity of the surrounding water can influence the total phenolic content found within the seaweed. The phenolic concentration monitored seasonally over a year in one brown algae species found that yields were peaked during the winter and spring months (Mannino *et al.* 2016). Therefore, it is necessary to harvest *A. nodosum* samples from various locations at different times of the year in order to determine the phenolic content and antioxidant level variations within phlorotannin extracts.

Once samples have been harvested, it is essential to prepare them quickly before it they preserved. Since phenolic compounds are very reactive substances and can vary in concentrations based on external conditions (Cruces *et al.* 2015), they should be processed as soon as possible. The algae samples should be preserved within 24 hours of collection (Côté-Laurin *et al.* 2016). Within that time frame, the seaweed must be washed to remove any organisms or sediments that may be attached. The washing is usually done with seawater to keep the algae's color and texture (Côté-Laurin *et al.* 2016). If the algae samples cannot be preserved within 24 hours, it is necessary to keep samples in an aerated tank of saltwater, or in sealed containers in a cold storage room, so they do not dry out (Côté-Laurin *et al.* 2016).

1.4.2 Adapting to remote locations

It is important that the samples are preserved properly until the analysis of compounds can be performed, however, some preservation methods yield in better active compound levels. According to Cruces and colleagues (2015), most algae samples are first frozen instantly using liquid nitrogen to prevent any compounds from changing characteristics. Following this procedure, samples are commonly preserved by freezing or drying, using various methods before being stored until analysis. Each of these procedures vary the phlorotannin content and antioxidant levels. Five different preservation treatments were used, after liquid nitrogen, to find optimal level of activity: frozen at -80°C (control), freeze-dried, silica-dried, oven-dried at 60°C, and air-dried. It was found that freeze-dried samples resulted in the highest phlorotannin content, just above the

frozen samples, but the frozen samples had significantly higher antioxidant activity (Cruces *et al.* 2015). However, these procedures use equipment that are not always accessible in remote locations. On the Lower North Shore in particular, it would not be possible to use the liquid nitrogen before preserving. Therefore, different preservation techniques that can be performed in remote locations need to be tested and analyzed then compared to the phenolic compounds' concentration and antioxidant efficiency of fresh samples. Preservation procedures that are possible and cost-efficient include: shock-freezing at -80°C, dehydrating at 40°C, and air-drying in a greenhouse.

1.4.3 Assuring a natural clean product

In order to release the active compounds of interest out of the seaweed biomass, extractions using solvents are often used. However, because of isolation and expensive shipping costs, it would be difficult to dispose of these organic solvents properly because of a lack of disposal system on the Lower North Shore. The local population takes pride in their pristine environment and they encourage any industrial development to respect it as well. Therefore, it would be necessary to develop other extraction methods that are more environmentally-friendly.

There are many different extraction factors that can influence the efficiency of phenolic content and antioxidant yields to create a favorable *Ascophyllum nodosum* extract. The solvent used, the aqueous mixture within the solvent, and the concentration of sample in solvent volumes, all attribute to the extraction efficiency. The possibilities are endless. Extractions for this project will compare optimal yields of water and organic solvent extractions (a) and any possible innovative extractions without the use of organic solvents (b).

a) Solvent extractions

There are many studies that use the common solvents, such as methanol, ethanol, and acetone. However, Wang *et al.* (2009) found that efficiency of extracts of

phlorotannins lowered when extracted with highly polar organic solvents. This includes solvents such as methanol, ethanol and isopropanol. 70% aqueous acetone solution had shown the highest extracted yield of phlorotannins (Koivikko *et al.* 2005). However, if extracts are to be used in cosmetics, the extractions cannot be done with those solvents, but rather with ones that are cosmetically accepted. The solvents tried for cosmetic materials will include 1,2-propanediol, 1,3-propanediol, and 1,3-dioxolane, since they are not found on the list of substance prohibited for cosmetic products (Buzek & Ask 2009). The above seven solvents will all be considered when determining the optimal yield of phenolic content and antioxidant activity.

b) Solvent-free extractions

Even though extractions using solvents have been shown to be effective by many studies, it is necessary to try to find an alternative extraction method that is environmentally friendly. As mentioned earlier, there are no solutions disposal systems on the Lower North Shore. Therefore, it is necessary to compare the analyzed yields between solvent and solvent-free extracts, to see if the phenolic content or antioxidant activity is sacrificed. However, a study compared water and ethanol extractions in three brown alga species, and results had shown that the total phenolic content and antioxidant levels were significantly higher in the water extraction (Kuda *et al.* 2005). Therefore, using water rather than organic solvents may be the solution to an alternative extraction.

Innovative methods using equipment and technologies differing from the traditional solvent extraction are being used more frequently in recent extraction research. One of these innovative methods include microwave-assisted extraction technology, where a laboratory microwave is used to exert energy and apply heat to extract chemical compounds from sample materials. This technique has been preferred in recent research since extraction time can be reduced to usually 15 to 30 minutes, the use of solvents volumes is minimal, and extract yield tend to increase due to the microwave energy efficiently releasing the interest bioactives (Eskilsson & Bjorklund 2000). Since the

microwave-assisted extraction technology uses little equipment or materials, besides the laboratory microwave itself, and is an ideal extraction method to use in a remote community. Therefore, this method efficiency and extract yield will be compared to the conventional solvent extraction technique to find a solution for preparing seaweed extract high in polyphenol content and antioxidant activity.

1.4.4 Ensuring regrowth and resource sustainability

In order to maintain natural habitat for other organisms and to ensure a sustainable resource for future generations, the brown algae population density must be harvested properly and monitored for proper regrowth. Previous research, however, has found that harvesting of *Ascophyllum nodosum* in Nova Scotia did not show any evidence effect on the fish population after altering their habitat (Black & Miller 1991). Before collection, the best harvesting sites must be selected to ensure there is enough biomass necessary for the project. The harvester must also ensure that the site is geographically accessible, can be reached by foot at low tide, and is in close enough proximity to a processing facility (Côté-Laurin *et al.* 2016). It is also necessary that the harvester receives a permit of consent from the Department of Fisheries and Oceans (DFO) office for that harvesting location. Then, harvesting areas must be delineated before cutting begins. The area of cutting can be no more than 15 m in diameter and there must remain 15 m between each harvesting area, so that there is natural habitat remaining (Côté-Laurin *et al.* 2016). The areas that are cut must remain untouched for at least three years to ensure regrowth. Manual harvesting of *Ascophyllum nodosum*, using a knife or sickle, is currently the only method used in Canada. A Norwegian designed jet-propelled boat was used in Nova Scotia, where rotating cutters suspended in the water cut the *A. nodosum* at high tide. This helped with harvesting efficiency, however, new government restrictions prevented these vessels from operating any longer (McHugh 2003). The Department of Fisheries and Oceans now require cutting the seaweed manually at a minimum of 15 cm above the holdfast. The holdfast must remain attached to the substrate in order for regeneration to be successful (Côté-Laurin *et al.* 2016).

Macroalgae beds are important to marine ecosystems, since they provide other organisms a food source, a habitat and a source of protection from predators. It is important that the algae are harvested sustainably in order not to harm the ecosystem. Therefore, initial monitoring of regrowth is crucial before harvesting mass-producing sized quantities for industries. Once length of time of regrowth is better understood, procedures for harvesting can be written together with the Department of Fisheries and Oceans in order to ensure we are conserving our resource and not harming other resources like fish.

1.5 Objectives of the study

While there is plenty of research quantifying the total phenolic content in brown algae species, including *Ascophyllum nodosum*, the extraction methods being used by scientists are not acceptable in today's modern nutraceutical and cosmeceutical products and not ideal for processing in a remote coastal community. The methodology and suggestion solutions of this research paper keeps in mind a final environmentally friendly product that is efficiently produced using minimal equipment and materials, that can be applied to isolated regions of Québec and Canada in general.

The purpose of this study is to research the possibilities of developing bioingredients from a brown algae species found on the Lower North Shore of Quebec, which could be applied to other remote coastal communities in the North Atlantic. The research specifically focuses on determining whether the interested active compound, phlorotannin, can be isolated into a concentrated extract that could be useful as bioingredients for the nutraceutical or cosmetic industry. The following four objectives will be followed throughout the research project in order to accomplish the main goal, mentioned above. The first objective is to establish best harvesting methods of *Ascophyllum nodosum* on the Lower North Shore, selecting which tool is most effective and which locations are accessible and have enough biomass needed. Also, monitor the regrowth of cut areas to confirm the alga is a renewable resource. This will ensure the

harvesting is not disturbing habitat and the resource will be a sustainable industry for future generations.

The second objective is to determine the preservation technique that conserves total phenolic content and antioxidant activity best. The four preservation techniques that will be compared are freezing, shock-freezing, dehydrating, or greenhouse air-drying and these were chosen based on the equipment available in this remote region. The final yields will be considered as well as that length of time required for preservation and practicality of each method.

The third objective of this research is to find a clean and efficient extraction method of *Ascophyllum nodosum* that will provide optimal yields of total phenolic content and express high antioxidant efficiency. Samples that are dehydrated and milled will undergo two methods of extraction: traditional solvent extraction in 4°C for 24 hours, and temperature-assisted extraction at various temperatures for 20 minutes (Figure 1.7). Extraction efficiency using cosmetic-friendly solvents will be compared to two conventional laboratory organic solvents, ethanol and methanol at different concentrated solutions. Those samples tested using the hot water bath and microwave assisted extraction technology will be extracted in water alone.

The final objective is to detect the variability in phenolic yields between seaweed samples harvested in different locations and at different seasons. The results will determine the influence the surrounding environment has on the phenolic content and well as display the variability an extract yield can have when samples are harvested from different geographical locations. The total phenolic content expressed during different seasons should be able to show which season is suggested to harvest the macroalgae for nutritional and cosmetic purposes.

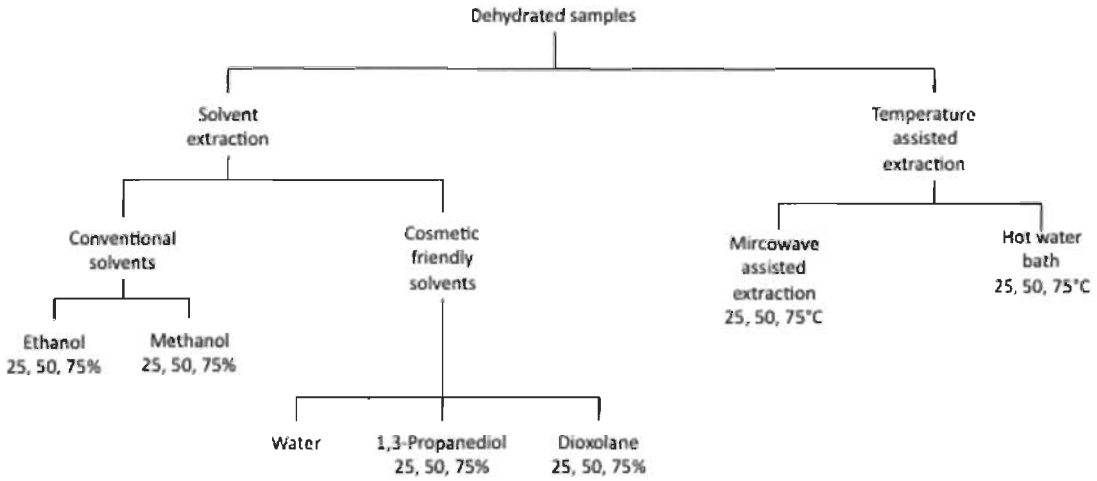

Figure 1.7 Different extraction methods tested to compare extract yield of phenolic compound concentration and antioxidant activity levels.

CHAPTER II

BIOEXTRACTING POLYPHENOLS FROM THE BROWN SEAWEED *ASCOPHYLLUM NODOSUM* FROM QUEBEC'S NORTH SHORE COASTLINE

The content of this chapter is written in the form of a scientific journal article that will be published in the journal "Industrial Biotechnology", in June 2019.

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2.1 Contribution of authors

All of the effort put into completing the research results presented in this article had been conducted by Jessica Poole, with the help of Amadou Diop, a post-doc researcher in the environmental and biotechnology laboratory of Simon Barnabé. The establishment of the concept, the overall problem, and the objectives had been identified and determined by Louis-Charles Rainville (co-director of Master's degree) and Simon Barnabé (director of Master's degree). The writing of this manuscript and the creation of the figures were done by Jessica Poole and the final corrections were made by Amadou Diop, Louis-Charles Rainville, and Simon Barnabé.

2.2 Scientific article

Bioextracting Polyphenols from The Brown Seaweed *Ascophyllum Nodosum* from Quebec's North Shore Coastline

Abstract

Developing innovative industries in rural communities require researching valuable finished products using local natural resources and feasible equipment and technology. Resources like seaweed, are popular in today's global cosmetic ingredient and biotechnology market and are commonly found growing in remote communities, making it an ideal opportunity for rural economic development. The research in this study focuses on the antioxidant rich polyphenol compounds found in the seaweed species *Ascophyllum nodosum*, local to Québec's North Shore coastline. Different processing technologies were compared to optimize polyphenol yields, including different preservation methods as well as bioextraction techniques that are applicable and accessible to remote regions. Analyses of extracts were performed using different colorimetric assays to measure total polyphenols and phlorotannins, as well as to estimate antioxidant activity. Results from the study found that the samples immediately frozen displayed higher polyphenol concentration and the highest antioxidant activity. Analysis also showed that a microwave-assisted extraction method improved polyphenol yield efficiency for water extractions. However, the conventional solvent extraction method using 75% (v/v aq.) 1,3-propandiol solvent resulted in the highest phenolic content, totalling 9.8% (w/w) of its dry weight, and the optimal antioxidant activity.

Keywords: seaweed, macroalgae, bioextraction, polyphenol, antioxidant

Introduction

Seaweed harvesting and processing has been identified as an economic opportunity to establish profitable businesses and improve living conditions for coastal rural communities in developing countries[1]. The idea can also be applied to remote coastal

communities within Québec to diversify and develop the local economy from the primary unpredictable fishing industry. The production of seaweed is promising since the resource is abundant, can be harvested sustainably if well monitored, and has potential for a variety of uses, such as food additives, pet food, fertilizers, biofuel, cosmetics, and medicines[2,3,4]. A lower profit is derived from producing fertilizers and animal food additives[5], due to high volumes of biomass required for a low value product. Some seaweed productions are used for high value ingredients in the cosmeceutical industry, since marine algae contains polyphenol compounds, which are substances that protect the cellular structure from oxidative damage[6].

Polyphenols are commonly found in plants as secondary metabolites, which aid the plant in structural development and react to many biotic and abiotic stressors[7]. Phlorotannin compounds are a class of polyphenols found uniquely in brown algae species as secondary metabolites[8], where it is synthesized under stress since it has the ability to absorb the UV range of radiation, repair wounds and deter herbivore grazers[9]. Phlorotannins have received much interest in recent research because of their bioactive properties[10] showing antioxidant, anti-wrinkling, anti-allergic, anti-cancer, and hair growth promoting abilities[11,12,13]. Many studies have found that the phlorotannin extracts express rich antioxidant levels[11,14], and some research found extracts to contain anti-inflammatory, anti-senescence[15] and anti-microbial properties[13,16]. These characteristics make the brown seaweeds a useful and valuable ingredient for functional food, cosmeceutical, and nutraceutical products[10]. Value-addition ensures investment return, and this will come from innovative products marketed for the benefit of human health[17], and it would thus be beneficial to create a method of processing and extracting a small high-value product that could easily be produced within remote communities.

Ascophyllum nodosum is a brown algae species with the one of the highest concentrations of phlorotannin, a type of polyphenol[15]. The polyphenol content within *Ascophyllum* biomass ranges between 9-14% of its dry weight, depending on seasonal variability[18]. Phlorotannin concentration react to the algae's external conditions, such as light, temperature, and biotic stressors, as well as sample handling and preservation

methods. Studies have shown that phenolic concentration monitored over a year in one brown algae species found that yields differed seasonally[19]. Considering phlorotannins are highly reactive, the method of sample preservation impacts bioactive properties efficiency and a study that compared five different preservation treatments found that freeze-dried samples resulted in the highest phlorotannin content, however the frozen samples had significantly higher antioxidant activity[9]. The preservation procedures commonly used in literature, like freeze drying and liquid nitrogen, are not always accessible in remote locations and other preservation techniques that can be performed on a large scale in remote territories need to be considered.

Polyphenol and antioxidant yields also vary depending on the efficiency of the extraction method. Current seaweed use for nutritional purposes uses raw cultivated seaweed or extracts performed using organic solvents[6]. However, as the consumer demand shifts to more environmentally friendly materials, green technologies and natural ingredients, any possible innovative extraction methods free of organic solvents that also improves yield efficiency will be favored by cosmeceutical industries. In addition, because of isolation and shipping costs in remote coastal communities, there is great difficulty and high expenses to dispose of organic solvents properly. There is a need to find an alternative green extraction method. More recent studies are shifting towards energy efficient methods, such as the microwave-assisted extraction technique, which showed a 70% increase in polyphenol yield in a brown seaweed species compared to the conventional solid-liquid extraction technique using organic solvents[20]. The microwave technique improves the yield by heating the slurry mixture causing ruptures of the cells and releasing soluble compounds, while reducing the volume of solvent necessary and decreasing the extraction time[20]. The aqueous solvent concentration, biomass:solvent ratio, extraction time, temperature, and microwave power are all differing factors that can be tested in order to find an optimal combination leading to the highest polyphenol extraction yield.

Emphasis of research has been done on the extraction, optimization, and identification of phenolic content in brown seaweeds. However, little research considers

the conditions of remote coastal communities and the limited methodology that can be used. With isolation and transportation costs in mind, this study focused on optimizing an extraction and processing technique using biotechnology applicable to isolated regions, which can help boost the bioeconomy in Québec's remote communities. The purpose of this research was to obtain high polyphenol and antioxidant yields from *Ascophyllum nodosum* using environmentally-friendly and cost-efficient preservation and extraction methods. The results are intended to suggest bio-economic development for the remote populations and to offer a clean product to the cosmeceutical industry.

Materials and methods

Raw material harvesting

Fresh *Ascophyllum nodosum* samples were harvested near the processing facility in Bonne-Esperance, QC (51°25.408N, 57°37.121W) during August and September 2017. Samples were cut 15 cm from the holdfast[21] in two sites; within a kilometer and further than three kilometers from a freshwater river outlet. Salinity and temperature measurements were taken at each site using an Onset HOBO U24 saltwater conductivity data logger (Hoskin Scientific, Canada). The algae samples were rinsed and soaked overnight in cold saltwater, to remove organisms and substrates[21] and preserved immediately the following day.

Sample preservation

From one location at one time point, four replicates of five random *Ascophyllum nodosum* plants were preserved using four different preservation techniques to determine the effect of temperature and sun exposure on its polyphenol concentration and antioxidant activity. (i) The first technique consisted of dehydrating seaweed samples for 8 hours at 40°C 2-Zone Commercial Dehydrator (Excalibur, USA). (ii) The second preservation technique consisted of drying samples of seaweed in a conventional solar greenhouse, where the samples were dispersed thinly on mesh netting in a well-ventilated

infrastructure and left to dry for two sunny days. (iii) The third preservation method consisted of freezing fresh macroalgae in a shock freezer for 24 hours at a temperature of -40°C, since this is a common method used to preserve nutritional value of seafood. (iv) The final technique tested was simply freezing the seaweed samples, which was considered the control[9]. The seaweed samples were first patted dry to remove excess moisture. This freezing method was used to compare location and seasonal differences, since it is the most cost and time efficient. All samples were then placed in airtight freezer bags and stored in a freezer at -10°C, until further analysis.

Milling and blending

Dried *Ascophyllum nodosum* samples were grinded (Thomas-Wiley Laboratory Mill, Thomas Scientific, USA) down to 1 and 2 mm sized particles and both sizes were compared for extraction efficiency. Algae samples that were frozen immediately from fresh were blended in an extractor blender (Professional Blender, SharkNinja, USA) to create a slurry. All samples were stored in a freezer at -10°C, if extraction did not begin immediately.

Solvent extraction

Seaweed samples underwent multiple extraction techniques to determine the optimal levels of polyphenolic content and antioxidant activity extraction. To determine the most favorable solvent choice and concentration of solvent, the samples were first extracted in water comparing the solid:liquid ratio over time in order to find the optimal combination[8]. The solid:liquid ratios of 1:100, 1:80, 1:50, 1:30, and 1:10 (dry weight:water content) were extracted in distilled water and kept in the dark at 4°C, and were tested over 24 hours to determine the highest polyphenolic yield. This optimal combination was then used as a standard to compare extractions with two conventional solvents, methanol and ethanol, and two cosmetic-friendly solvents, 1,3-propanediol and 1,3-dioxolane, at 25, 50, and 75% (v/v aq.) solutions.

Temperature-assisted extraction

To test the ability of the microwave to better penetrate cell walls and extract polyphenols, the method used the optimal solid:liquid ratio at varying temperatures and power determined as follows. Water was the only solvent used, since this had previously shown to be the optimal solvent in the micowave-assisted technique[20]. The experiments were performed using an advanced microwave synthesis system (FlexiWave, Milestone Srl, Italy) where biomass was added to 250 ml of water. Extracts were tested at 25, 50 and 75°C, for 20 minutes, with a 5-minute ramp time to reach the desired temperature followed by a 15 minute period at constant temperature. The microwave system was set at a maximum power level of 1000W and sample was stirred at low intensity with a magnetic stir bar. In order to determine that the microwave power was causing a better extract results rather than temperature, dehydrated macroalgae samples were mix in water at the same optimal solid:liquid ratio as the microwave and placed in 25, 50 and 75°C water baths for 20 minutes. Immediately following extraction, all samples were filtered, the supernatants were collected, and were tested for polyphenol, phlorotannin and antioxidant analysis.

Dry content analysis

The dry content (%dw) of all treatment samples, grinded or blended, was determined after drying approximately 5 g of algae at 105°C. Weight measurements were taken daily until weight became consistent for two consecutive days[6].

Phenolic content analysis

The colorimetric assay using Folin-Ciocalteu reagent adapted from Ainsworth and Gillespie[22] was used to measure total phenolic content in *Ascophyllum nodosum* samples and were compared to phloroglucinol standards. 200 µl of diluted extracted samples were mixed with 400 µl of 10% Folin-Ciocalteu reagent with water solution and was mixed thoroughly. Then 1600 µl of 700 mM solution of sodium carbonate in water was added to each mixture. The extract assays were incubated at room temperature for two hours

before measuring the absorbance against the blank at 765 nm (Cary 50Bio UV-visible Spectrophotometer, Varian, USA). The results were expressed as milligrams of phloroglucinol equivalents per gram of sample dry weight (mg PGE/g).

Phlorotannin abundance analysis

The second colorimetric assay using 2,4-dimethoxybenzaldehyde (DMBA) was used to measure phlorotannin compounds in the algae samples[23,24]. This method used a working reagent of 1:1 volume of Stock A (0.5 g DMBA in 25 mL glacial acetic acid) with Stock B (4 mL hydrochloric acid with 21 mL glacial acetic acid) mixed just prior to use. 10 µL of extracted sample was mixed with 2.5 mL of the working reagent and 10 µL of *N-N* dimethylformamide[25]. The mixtures were incubated at 30°C for 60 minutes in a dark incubator (Ecotron, Infors HT, Canada) before measuring the absorbance against the blank at 515 nm (Cary 50Bio UV-visible Spectrophotometer, Varian, USA). The extracts were compared to phloroglucinol standard curve that was prepared using the same method but measured at 494 nm[26]. The results were expressed as milligrams of phloroglucinol equivalents per gram of sample dry weight (mg PGE/g).

DPPH scavenging activity analysis

To determine the antioxidant activity of highest polyphenol and phlorotannin yielding extracts the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay was used[8]. A stock solution of 0.1 mg/ml of DPPH in ethanol was made just prior to testing the colorimetric assay. The sample was prepared by mixing 500 µl of extract with 500 µl DPPH stock solution and was compared to the sample blank, which is a mixture of 500 µl of extract with 500 µl of ethanol. A control was also prepared by mixing 500 µl of DPPH stock solution with 500 µl ethanol and was compared to a control blank, which is 1 ml of ethanol. All mixtures were left in the dark for a 30-minute period at room temperature before the absorbance was measured at 515 nm (Cary 50Bio UV-visible Spectrophotometer, Varian, USA). The free radical activity will be calculated in a percent by:

$$\text{Scavenging effect (\%)} = [1 - (A_{\text{sample}} - A_{\text{sample blank}}) / (A_{\text{control}} - A_{\text{control blank}})] \times 100$$

Finally, the IC_{50} value was calculated based on the concentration (μg/ml) of extract needed to reach 50% of scavenging capability[8]. The treatment with the best antioxidant activity is deemed when 50% of its scavenging activity (IC_{50}) is measured at the lowest concentration.

Statistical analysis

Analysis of variances (ANOVA) were used to compare the treatments for each data set using the JMP Pro 11 Software (SAS, Cary, NC, United States). We considered p value smaller than 0.05 statistically significant.

Results

Site location and seasonality influences

In order to take into account seasonality and site location variability and its influence on polyphenol concentration and antioxidant activity, extracts taken from Site 1 and 2 in August and September were analyzed and compared. Both the location and time at which seaweed samples were harvested had influenced the total phenolic concentration of the frozen samples extracted in 75% ethanol. Site 1, located within one kilometer of the river outlet, resulted in a lower phenolic concentration of 66.94 mg PGE/g in the month of August. Site 2, located approximately four kilometers away from the freshwater source, resulted in an average of 85.13 mg PGE/g in the month of August (Fig. 1).

Conventional and cosmetic-friendly solvent extractions

The total phenolic content based on the Folin-Ciocalteu analysis method showed significant differences between some of the solvent extractions and its different concentrations (Fig. 2). The phenolic content in the dehydrated algae extracts samples ranged from the highest of 98.46 mg PGE/g in the 75% 1,3-propanediol aqueous solution to the lowest abundance of 48.81 mg PGE/g in the 25% methanol aqueous solution. The highest phenolic extraction for the conventional solvents, 75% ethanol aqueous

solution, and for the cosmetic-friendly solvents, 75% 1,3-propanediol aqueous solution, displayed similar high concentration of 95.39 mg PGE/g and 98.46 mg PGE/g, respectively, and were not statistically different ($p>0.05$). Both organic solvents nearly doubled the polyphenol yield when compared to extraction with water alone. As predicted, the solvent extraction expressing the best antioxidant activity corresponded with the highest phenolic content, using a 75% 1,3-propanediol aqueous solution (Fig. 3). The DMBA assay results (Fig. 2) did not show statistically different results in total phlorotannin concentration ($p > 0.05$).

Temperature-assisted extractions

The dehydrated seaweed samples extracted in water decreased in phenolic concentration as temperature increased when using a conventional hot-water bath method (Fig. 4a). Extracts placed in a 75°C hot-water bath for 20 minutes resulted in the lowest phenolic content of 37.1 mg PGE/g, which also corresponded with the decreased antioxidant activity showed by the high IC_{50} value of 429 µg/ml (Fig. 5). However, seaweed samples extracted using the microwave-assisted method displayed a peak in phenolic content (Fig. 4a) when placed in apparatus at 50°C for 20 minutes and yielded 56.4 mg PGE/g. Analysis of polyphenol concentration displayed an overall 36% increase in efficiency when using the microwave-assisted technology over conventional methods. The DMBA assay results (Fig. 4b) did not show statistically different results in total phlorotannin concentration ($p > 0.05$).

Preservation treatments

Analysis of phenolic content when extracted using both water and 75% ethanol displayed the same order of efficiency based on preservation treatments: frozen > dehydrated > greenhouse dried > blast frozen (Fig. 6a). The polyphenol concentration ranged from 89.24 mg PGE/g in the frozen samples down to 67.04 mg PGE/g in the blast frozen samples when extracted in 75% ethanol for 24 hours (Fig. 6a). Statistics proved a significant difference between preservation treatments ($p < 0.05$). The DMBA analysis

resulted in a peak of phlorotannin abundance of 23.3 mg PGE/g when samples were preserved by dehydration and extracted in 75% ethanol for 24 hours (Fig. 6b), which corresponds to 26% of the total polyphenol concentration. DMBA results only displayed a significant difference between preservation treatments when extracted with water for 24 hours ($p < 0.05$).

The preservation treatment with the best antioxidant activity were samples that had been frozen, expressing an IC_{50} value of 108 μg/ml, whereas the samples dried in a greenhouse caused a decrease in antioxidant activity, with an IC_{50} value of 213 μg/ml (Fig. 7).

Discussion

Results from this study show that the preservation technique, extraction solvent, temperature and method of extraction used with *Ascophyllum nodosum* biomass can all have significant impacts on the extracted phenolic yield and antioxidant activity.

Since 75% ethanol extraction expressed higher yields of phenolics in the seaweed samples compared to water, it was decided that this solvent mixture would be used to further compare the preservation techniques in addition to water. As predicted, the frozen samples expressed the highest phenolic yields, since the biomass is not processed following harvesting and frozen as is. However, results had shown the dehydrated samples had expressed similar results as the frozen samples when it was extracted with water alone and with 75% ethanol (v/v aq.). This could be due to the ability to grind the dry materials to a smaller particle size of 1 mm using the laboratory mill compared to the frozen material, which could only be blended to a size of 3-6 mm using a commercial extraction blender. Since the dry material had smaller particles, the solvents could more easily penetrate the surface area to extract the polyphenol compounds. As expected, the samples that were dried in a greenhouse resulted in lower phenolic yields compared to the samples that were dried in the dehydrator, since sun exposure likely causes a degradation of phlorotannins. In addition, previous studies have suggested that drying processes that

require longer time lengths to dehydrate the seaweed samples tended to have a loss in phlorotannins and antioxidant capacity due to degradation and oxidation[9,27]. Both suggestions of the small particle size and minimum time duration of dehydration, likely explains why the dehydrated samples expressed the highest yield of phlorotannins using the DMBA assay.

It was unexpected to have differing best preservation treatment between the two methods, Folin-Ciocalteu and DMBA, which both quantify polyphenol yields. One study had suggested the Folin-Ciocalteu reagent to be a more reliable and precise method since the DMBA reagent is sensitive to time and temperature during the reaction period[28]. In some cases during our study, it was observed that red precipitates would form over the 60 minute incubation period in 30°C, which lowered the colorimetric absorbance read in the spectrophotometer. However, it was expected to differ in quantities since the Folin-Ciocalteu method measures all polyphenolic compounds and DMBA reacts exclusively with 1,3 and 1,3,5-substituted phenols, like phloroglucinol and phlorotannins[26].

The antioxidant activity of the extracts comparing the four different preservation treatments showed the highest scavenging capacity in the frozen samples, as expected. A study looking at the effect of drying temperature on extract characteristics found that increasing drying temperatures negatively affects both the phenol content and the antioxidant activity[6]. This analysis explains how the frozen treatments expressed the highest antioxidant activity where the least amount of oxidation occurred, however it is still unknown as to why the blast frozen samples had the least phenolic content and scavenging capacity. Blast freezing is typically used to preserve foods, especially seafood, since rapid freezing at low temperatures help keep the quality of the nutritional characteristics[28], however the very low temperature seems to have an adverse effect on the phenolic compounds in *Ascophyllum nodosum*. Conventional freezing could have led to crystal formation, which would have broken the cell walls making easier extraction. Whereas the blast freezing may have maintained better cell integrity, hindering extraction efficiency. Further research can look more closely at the impact of a freeze-thaw cycles

on the extraction yield, as this could help increase yields especially for the polyphenols that are bound to the cell wall.

Again, the results had shown that temperature has an adverse effect on the phenolic content, where extreme temperatures are causing phlorotannin degradation. In addition, the extract that was placed in lowest temperature of 4°C, also showed low phenolic content due to the short time period of extraction, over only 20 minutes. As expected, the microwave power had the ability to penetrate the biomass, which slightly increased the phenolic yield. It is suggested that 10-15% of the total pool of polyphenols within the *Ascophyllum nodosum* is bound to the cell-wall, which cannot be separated by the conventional solid-liquid extraction technique[29]. The concentration bound to the cell-wall can become accessible using the microwave-assisted technique[20]. However, results did not show a 70% increase in yields when using the microwave-technique as seen by a previous study[20] likely because the microwave apparatus used in this study did not have the ability to control pressure and reach the same temperatures.

Conclusion

This study suggests that 75% (v/v aq.) 1,3-propanediol extraction solution in water be used as a cosmetic-friendly alternative for those products that cannot use the conventional organic solvents. Out of the four preservation treatments that can be performed within remote regions, we suggested that *Ascopyllum nodosum* seaweed samples be frozen since it expresses high total phenolic content and high antioxidant capacity. We further propose that future studies use wet biomass in 1 mm particles to maximize phenolic extraction efficiency. Finally, we recommend the utilization of microwave-assisted methods when extracts are expected to yield high phenol abundance over a short amount of time. Alternatively, if the microwave approach is not available, we suggest extending the sample extraction over at least 72 hours to achieve similar yields in terms of phenolic content and antioxidant activity.

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Article Figures

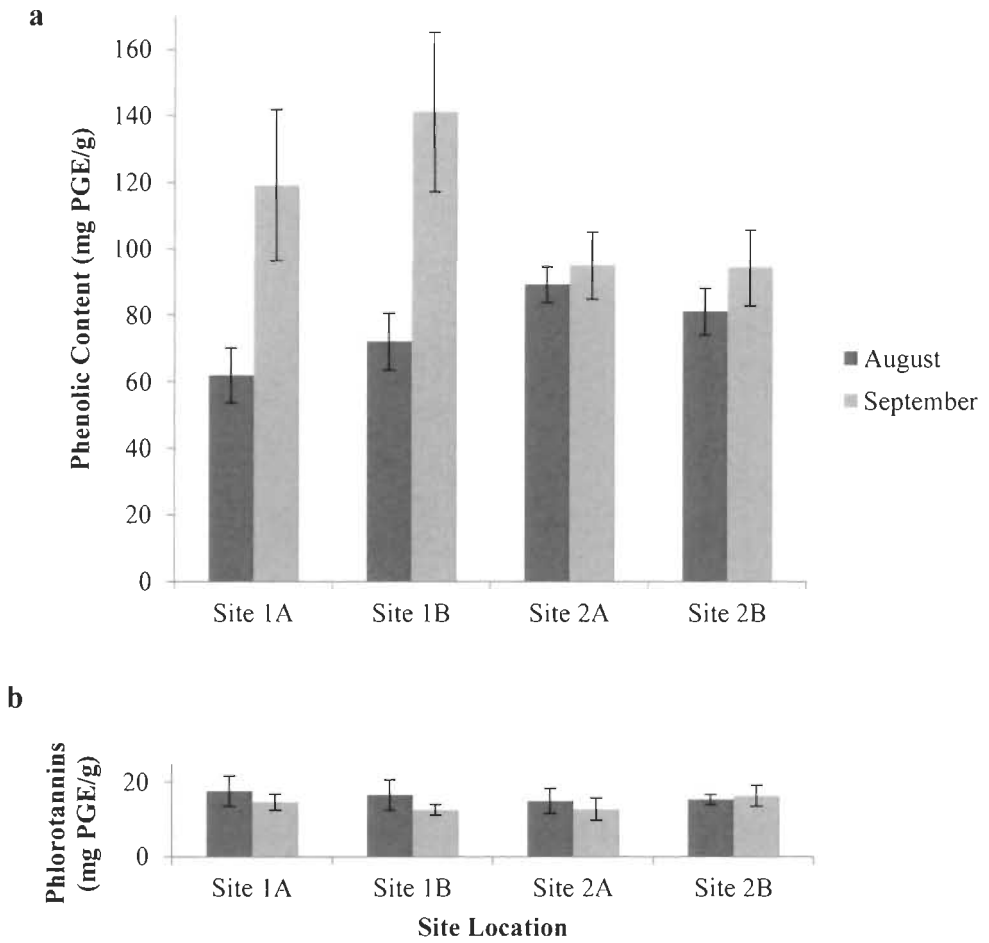

Fig. 1. Comparison of total phenolic content (a) and phlorotannin concentrations (b) of dehydrated *Ascophyllum nodosum* at two site locations in August and September. A and B indicate varying quadrats within the same site. Data are mean ± SD, n = 3.

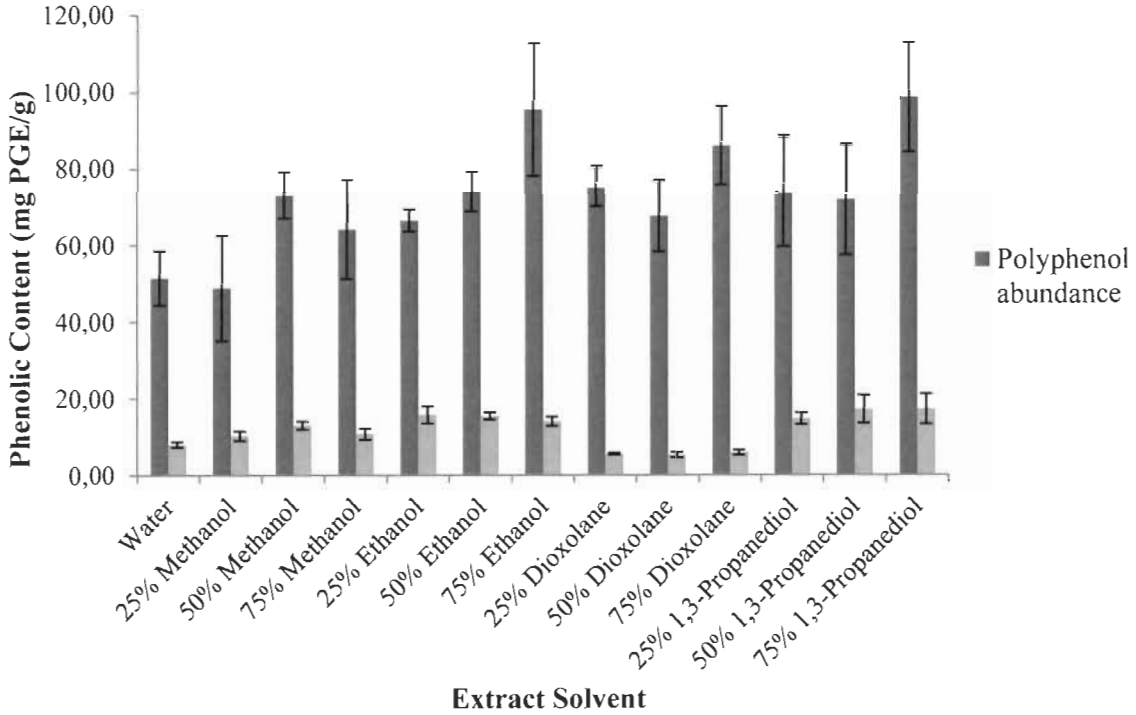

Fig. 2. Comparison of total phenolic content and phlorotannin concentration of dehydrated *Ascophyllum nodosum* when extracted in various solvents at differing aqueous concentrations. Data are mean ± SD, n = 3.

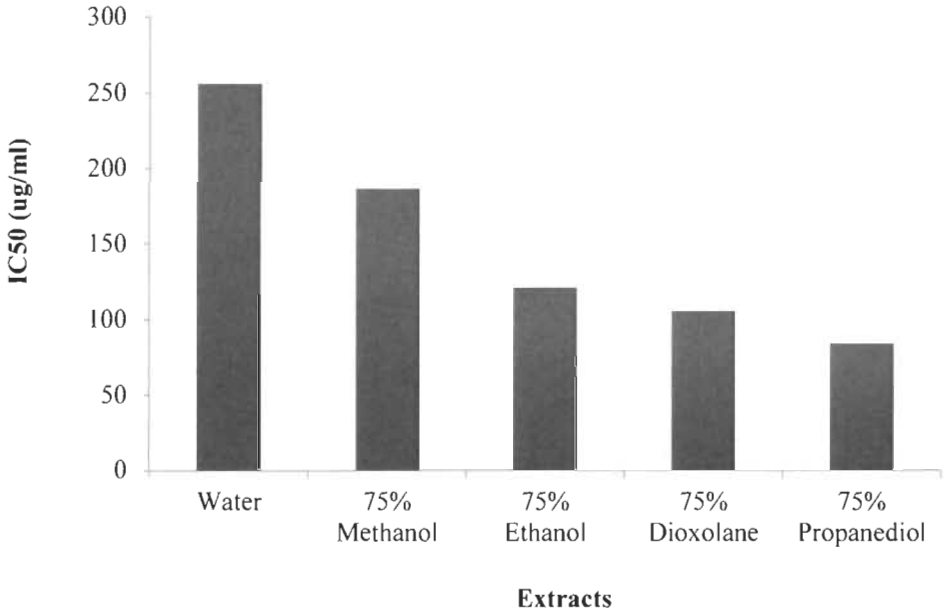

Fig. 3. IC_{50} value result from DPPH radical scavenging activity test in samples of *Ascophyllum nodosum* that undergone traditional solvent extraction using different solvents at 4°C for 24 hours.

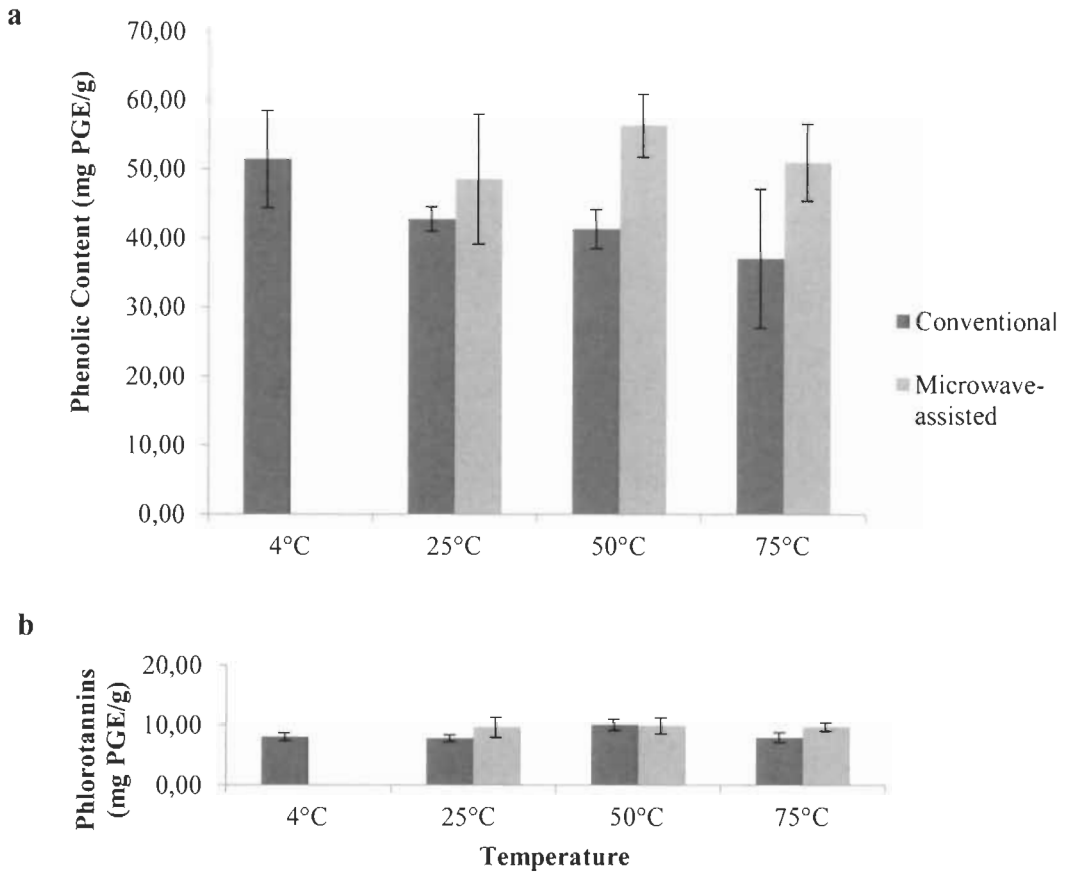

Fig. 4. Comparison of total phenolic content (a) and phlorotannin concentration (b) of dehydrated *Ascophyllum nodosum* when extracted using the conventional extraction technique and the microwave-assisted method at different temperatures. Data are mean ± SD, n = 3.

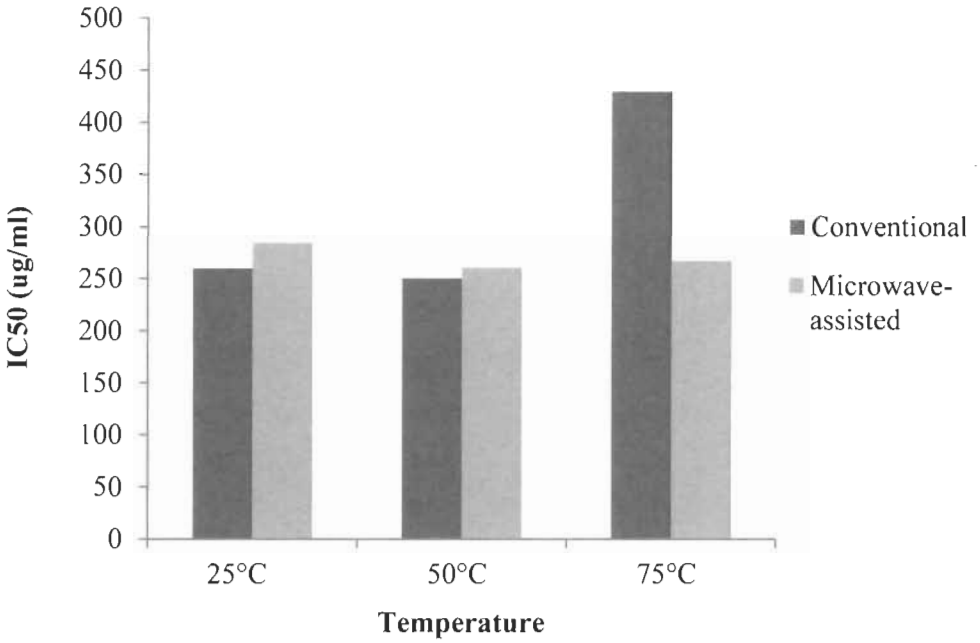

Fig. 5. IC_{50} value result from DPPH radical scavenging activity test in samples of *Ascophyllum nodosum* that undergone different temperature-assisted extraction techniques in distilled water at 1:30 solid:liquid ratio.

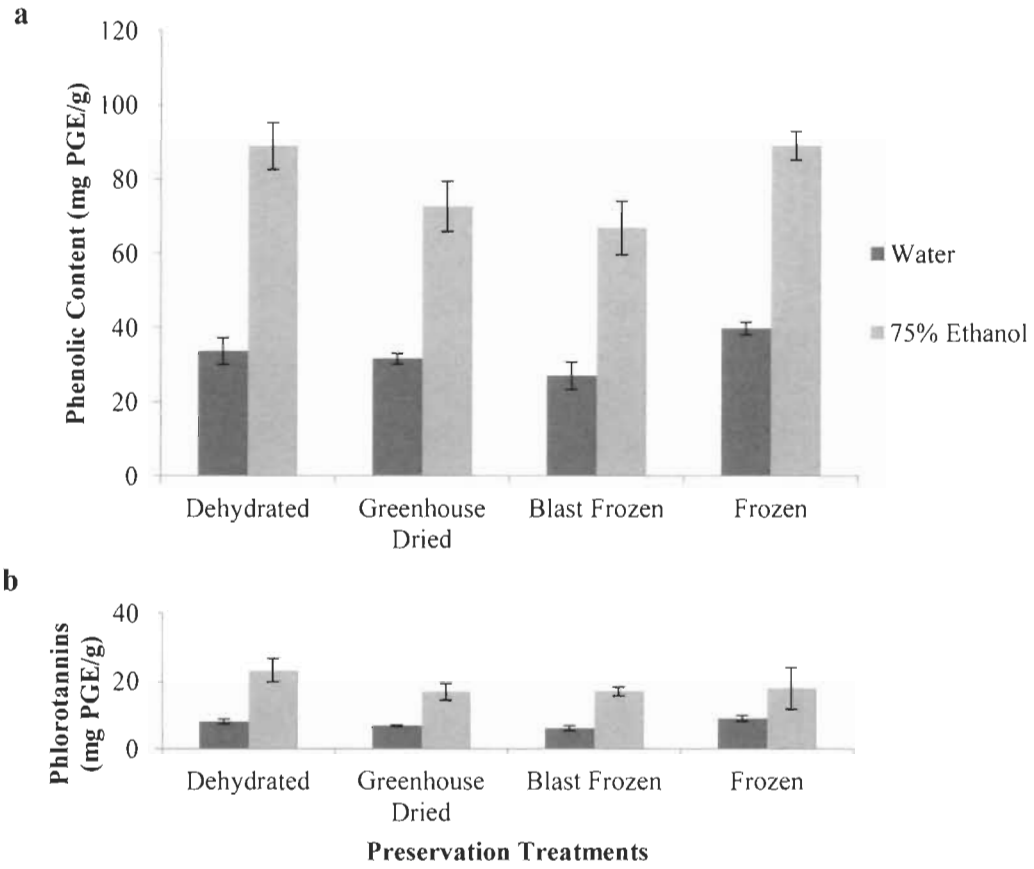

Fig. 6. Comparison of total phenolic content (a) and phlorotannin concentration (b) of *Ascophyllum* nodosum when treated using different preservation methods (frozen, shock frozen, greenhouse dried, and dehydrated) extracted in distilled water and in 75% ethanol. Data are mean ± SD, n = 3.

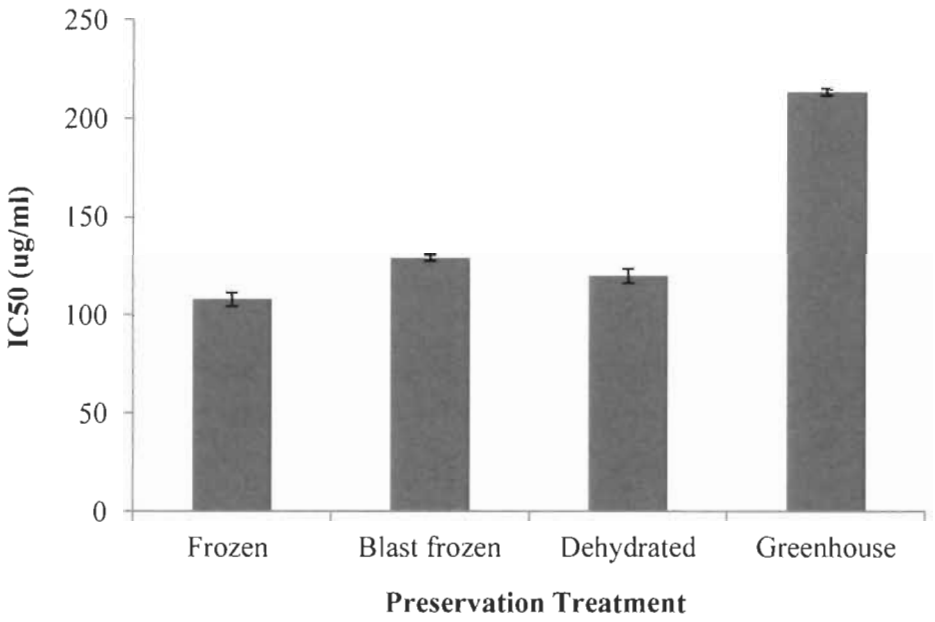

Fig. 7. IC_{50} value result from DPPH radical scavenging activity test in samples of *Ascophyllum nodosum* that undergone four different preservation techniques and extracted in 75% ethanol at 1:30 solid:liquid ratio.

CHAPTER III

DISCUSSION

3.1 Return to the problem and results

As mentioned in the introduction chapter of this thesis paper, there is a marine resource of value and found in abundance along the coastline of remote and isolated regions of Quebec that is not being used to its potential. Seaweeds found along the shoreline of the ocean waters are harvested and produced extensively in other provinces (Ugarte & Sharp 2012) and countries (Zemke-White & Ohno 1999), however Quebec is minimally taking advantage of this industry. Coastal communities in these areas are already predominately fishing villages, relying on unpredictable fish stocks in the waters to make a living. Some of these communities have already felt the hardship of stocks declining and quotas being cut, causing job losses, locals to look externally in urban centers to find employment, and socio-economic struggles within families. It is therefore needed to look further into a sustainable resource on these territories to create business and employment opportunities.

Seaweeds found growing on the shoreline at low tide are not seen as valuable to locals, as it is not a traditional industry like fishing, farming, and forestry. As discussed previously, the global industry of producing and selling macroalgae is worth millions of dollars and the resource itself can be transformed and used in a variety of different products. The aquatic plant is full of nutritional compounds that is valuable in products for human consumption, fertilizing soils, and feeding key nutrients to animals (Dhargalkar & Pereira 2005). Seaweed contain interesting bioactive compounds like polyphenols and hydrocolloids, specifically phlorotannins and alginates in brown algae species (Holdt & Kraan 2011; Gupta & Abu-Ghannam 2011). These actives can be used as extract ingredients in pharmaceutical, nutraceutical, medicinal and cosmeceutical products (Smit 2004). The later industry opportunity using extracts as ingredients has a higher profit

margin and requires less biomass harvested wild from the environment (Côté-Laurin *et al.* 2016). As consumer demand shifts towards more natural ingredients in products that are considered "green" and clean, industries are looking for innovative alternatives to artificial antibiotics and synthetic materials, better for human health and the environment.

Awareness needs to be provided to remote coastal communities on the opportunity of harvesting the macroalgae and processing the resource to be made into a high-value product. The concept of focusing on valuable ingredients for nutraceutical and cosmeceutical industries is to help overcome some of the transportation and research and development costs associated with creating this type of business in an isolated region. However, developing these human health products come with responsibilities. The market requires the scientific evidence to prove the ingredient has high yields, is effective, and is considered a natural product. These preliminary steps can be daunting for an entrepreneur, deciding whether to take on this type of business. This was the reason why this thesis topic was chosen. The research from this study will help educate locals on how to harvest the seaweed, which method of preservation should be used, how to best extract the compound of interest, what phenolic yields to expect, and the level of antioxidant efficiency. Results also suggested a fluctuation in phenolic compounds between the two tested site locations and the month the material was harvested. The wide range of total phenolic content suggests there is yield variability between raw seaweed material harvested. As well, the monitored results of regrowth of the *Ascophyllum nodosum* will suggest how long a harvester should wait to cut the seaweed again in the same location, to ensure a well-managed sustainable resource.

Monitoring the regrowth of the wild macroalgae is important for practicing sustainable use to not over-exploit the resource and is useful information when planning harvesting over numerous years. This step was crucial for being environmentally cautious and why it was the first objective we wished to obtain. A permit for a harvesting business will likely allow them to cut seaweed within a certain geographical area. It is not ideal to harvest all of the material along the shoreline on the first year and have none to harvest the following year. Results from measuring the regrowth of the length and weight of the

harvested area one year after cutting suggested that it should be left fallow for three years. On the fourth year, the algae should be grown back to its original length and weight, if the growth is consistent yearly (Annex B). However, studies have found that cutting all of the resource in one single area leaves an uninhabited spot within the intertidal zone that can be available for new species to begin growing (Mac Monagail *et al.* 2017). Therefore, it is suggested that harvesting be done in sections rather than a whole area and is the reason why it is mandatory to leave at least 15 cm of the plant attach to the substrate to easily initiate regrowth.

When determining the best preservation technique using results to address to the second objective, the highest phenolic content and best antioxidant activity was considered. Analysis had suggested that the frozen samples expressed the highest polyphenol concentration and best antioxidant activity. However, both of these were not significantly different when comparing frozen samples to dehydrated samples. The dehydrated samples even displayed a slightly higher phlorotannin abundance (Fig. 6 – Chapter II). While the two methods are similar in yields, the methods are very different in a production perspective. While the freezing method requires less processing time and no additional equipment besides a freezer, it poses an issue when transporting the heavy raw material to a laboratory to be extracted and takes up more space in the storage freezer. Dehydrating the material eliminates most of the water content and reduces the weight down to approximately 25% of its raw weight. The grinded dry material can then save on shipping expenses and requires less storage space. A dehydrator may be an expensive cost initially; however, it may save the processing business extra expenses over the years.

When comparing the yields of total phenolic content and antioxidant activity from the different extraction methods, it is difficult to recommend a single best extract solution for objective three. Results did show that cosmetic-friendly solvents, in particular 1,3-propanediol, has the capability to extract just as high polyphenol yields as the conventional laboratory ethanol solution. Using just water however, proved to not have enough capability to yield the same results as the polar organic solvents mentioned above. It was also obvious that 1,3-propanediol had the capability to preserve the highest antioxidant

potential out of all of the extraction methods tried, with a low IC_{50} value of 83 µg/ml. Results from the temperature assisted extraction technique proved that increasing temperature while extracting has an adverse effect on the polyphenol concentration. Therefore, the microwave-assisted extraction method did not increase the extract yield of total phenolic content as it was expected. It did increase efficiency compared to the hot water bath when extracted with water at 50°C and it would be interesting to experiment this technology with other solvents besides water.

Concerning objective four, it was interesting to see the variability in the results when comparing extract yields between different two locations and seasons the seaweed samples were harvested. The two sites were approximately three kilometers apart and still displayed a difference in total phenolic content. The water temperature and salinity were also measured at each location during the time leading up to the harvest date and these measurements were compared with the polyphenol concentration (Annex A). Both increasing water temperature and increasing salinity had a negative trend with total phenolic content, even when statistically insignificant. There has only been a slight correlation between water temperature and phenolic concentration ($R^2 = 0.61$). However, for some reason the *A. nodosum* samples harvested near the river outlet in September displayed a significantly higher results than the other harvested samples (Fig. 1 – Chapter II). There are thought to be two possibilities for concluding the increase in polyphenol yield in September. First of all, the water temperature is beginning to drop following the summer heat which causes less exudation of phenolic content by the seaweed since it is under less stress (Mannino *et al.* 2016). And secondly, the stress caused by grazing could have a significant effect on polyphenol concentration. Based on observations made during the harvesting, the species of grazers attached to the macroalgae changed from small mussels in August to periwinkles in September. The specific ecological effect of the grazing community on the seaweed's capability to protect itself is a study that should be looked into further.

When planning and preparing for business that produces extracts rich in bioactive molecules, it is essential to know the range of variability that could result even when

harvesting materials from the same location and processing it using the same technique. The environmental conditions have a huge effect on a living thing's ability to survive, and while the abiotic and biotic conditions change surround the algae, the chemical composition changes as well (Cruces *et al.* 2015). It is good business planning to anticipate the variability that could be encountered. For example, locations 1 doubled in polyphenol concentration only one month apart (Fig. 1 – Chapter II). Therefore, the business should anticipate that one harvest may have half the expected result and will require additional biomass to meet the needs of the extract yield.

3.2 Limitations

While it is predicted that the *Ascophyllum nodosum* will continue to grow back to its original length and weight before it is cut at 15 cm, the study was limited to monitor whether this prediction was true. The field research of this study was only conducted following one year of harvesting and the resource was not able to be monitored after this due to limitations on field work availability. Based on the measurements of regrowth after one year of harvesting, it is only predicted to be grown back to its full potential on the fourth year, but this prediction should be verified before applying to harvesting zoning and planning.

It was also noted that there can be a wide variability in phenolic yield between the two harvested months, August and September, however due to limited time and budget, the seaweed samples were not compared between different seasons. It would be very interesting to compare samples harvested between different months, starting from when the ice is melted in the spring to when it becomes inaccessible to reach the site in the winter months. The results from this research would give a better understanding in to how much sunlight, grazing, and water temperatures influence the concentration of phenolic compounds in the raw material. This comparison would also provide harvesters with a better suggestion to which season would be the optimal time to harvest *Ascophyllum nodosum*.

Also due to limited time to conduct and collect more data in the laboratory, the results from the microwave-assisted extraction is difficult to recommend whether the technique and equipment is worth it. Based on the few results obtained, it is difficult to judge whether the microwave-assisted extraction is an interesting avenue. It would be interesting to compare these results to extraction efficiency undergoing a pressurized microwave system. And it would also be interesting to test the solvents used in this study in the microwave-assisted extraction method, besides only testing water. This information not collected during this research would be ideal studies to conduct in the future.

3.3 Conclusion

To conclude, all of the results obtained throughout the duration of this study does show the potential of developing a bioingredient extract business in remote communities using a brown algae species, *Ascophyllum nodosum*. The resource has interesting active compounds to be easily marketed in the nutritional and cosmeceutical industry. To do this, the research has shown that the seaweed biomass should be preserved using a dehydration method and milled into small particle before extraction. Then the grinded material should be extracted in a 75% aqueous solution of the cosmetic-friendly solvent, 1,3-propanediol to achieve the optimal levels of total phenolic content and the best antioxidant capacity. The results also expressed that there is great variability in phenolic compound yields when samples are harvested, and this should be considered when developing an extract procedure.

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ANNEX A

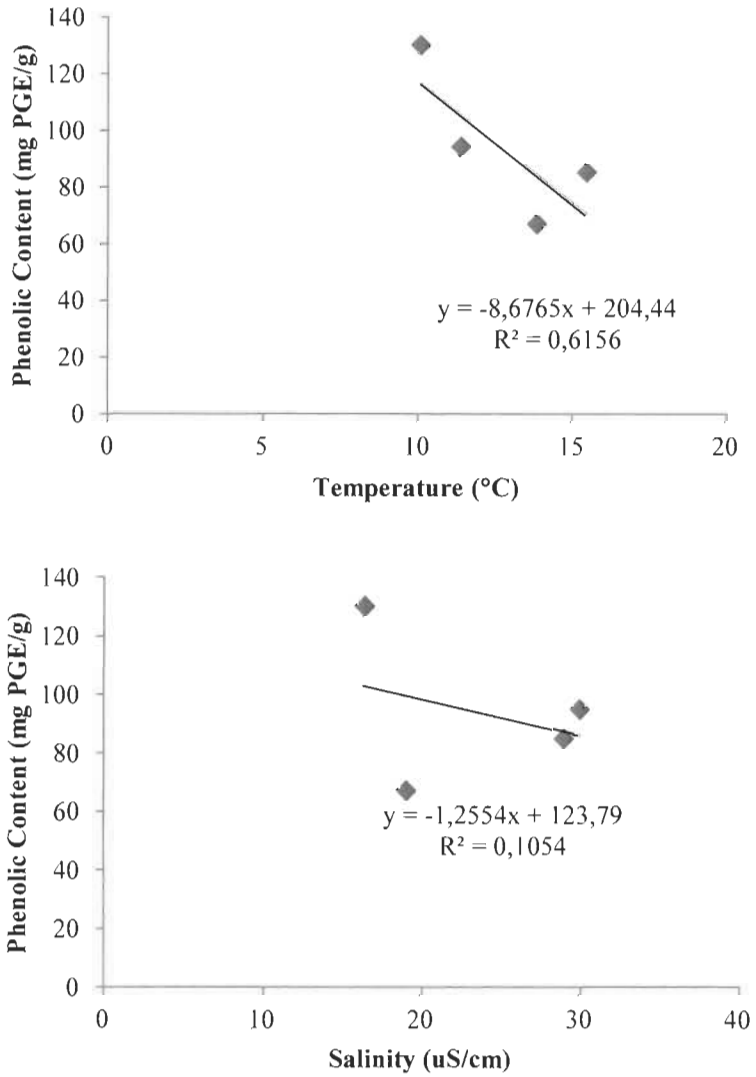

Figure A1 The phenolic content yield of *Ascophyllum nodosum* extracted in 70% ethanol for 24 hours, compared to the water temperature and water salinity when raw material was harvested.

ANNEX B

Table B1

Monitored regrowth of length and weight of *Ascophyllum nodosum* above the cut line of 15 centimeters, one year after harvesting

	2016 Pre-harvest	**2017 1 year of regrowth**	**% of original**
Average length above 15 cm (cm)	39.9	18.8	47.1%
Average weight above 15 cm (g)	268.4	67.6	25.1%