Commercial production of fucoidan from New Zealand *Undaria pinnatifida* (Harvey) Suringar

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Loretta Nicole White
Signed:

Date: 28 May 2015

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Abstract

Undaria pinnatifida is an invasive seaweed species in New Zealand that heavily infests mussel farms throughout the country. Fucoidan, one of the main cell wall polysaccharides of *U. pinnatifida* has numerous proven health benefits and has received increasing attention from researchers and entrepreneurs globally, but no commercial scale extraction of fucoidan from NZ *U. pinnatifida* has been undertaken to date. In 2010 the harvest and farming of this seaweed were permitted by the Biosecurity arm of the Ministry of Primary Industries (MPI), opening up the possibility of commercial scale harvest of the seaweed and production of fucoidan. In this thesis I investigated several aspects of the commercial production of fucoidan.

The objectives of this study were to investigate a) the impact on fucoidan yield and quality of the amount time taken to defrost frozen seaweed prior to fucoidan extraction, b) the optimal extraction time, temperature and seaweed: water ratio, and c) the ability to scale up proven laboratory size extraction methods to a commercial scale.

My results suggest that it is acceptable to leave thawed *U. pinnatifida* at 4°C for up to two weeks before fucoidan extraction, with no loss of total crude fucoidan and no significant changes in several of the "quality" parameters, i.e. fucose and sulphate content of extracted fucoidan. In terms of optimal extraction conditions, neither the length of extraction time (2, 3 and 4 hours) nor the temperatures tested (60, 70 and 80°C) had any significant impact on fucoidan yield or quality. The most influential factor in these experiments was the seaweed-to-water ratio, which had significant impact on both the fucoidan yield and the percentage of the larger than 10 kDa fucoidan fraction, the two most important factors considered in the fucoidan production in this thesis. Essentially the greater the water: seaweed ratio, the more fucoidan was extracted.

Two commercial private sector partners were engaged to a) harvest, chop and transport approximately 12 tonnes of *U. pinnatifida* to a frozen storage facility (this company is

named P1), and b) to carry out pilot fucoidan production using the extraction techniques optimised in experiments above and a 10-kDa membrane to recover the fucoidan from the extract (this company is named P2). While producing fucoidan at a laboratory scale is somewhat routine, there were significant issues with the scale up from a number of angles.

Two pilot scale trials for extraction and collection of fucoidan were run with the other private sector partner (P2). The first Trial was not successful, yielding only 0.72 kg of fucoidan, where the amount of seaweed processed in this trial should have yielded at ~3.9 kg. Following this trial, a number of changes were made: a) increasing the seaweed-to-water ratio, b) "washing" the extracted seaweed in a further 1000 L of water, and c) dewatering the alginates more thoroughly to lower losses at this stage by hanging the wet alginate in a sack overnight.

Even with these modifications, Trial 2 was also unsuccessful in producing the expected amount of fucoidan using the membrane filtration method. However, by taking samples at each step, we found that the extraction time, temperature and seaweed:water ratio were sufficient for good recovery of the fucoidan. The washing step was also successful, delivering an extra 0.41 kg of crude fucoidan. Furthermore, the change to the way the alginates were precipitated in Trial 2 also reduced the loss in fucoidan. The main loss was identified to be in the final 10-kDa membrane filtration step. There was an 85.3% drop from the preceding stage, ending up with a projected 1.08 kg of crude fucoidan, some 92% less than expected.

In summary, the attempt to scale up from the laboratory to a commercial scale to produce fucoidan was unsuccessful in terms of using a 10-kDa membrane to collect the extracted fucoidan. However, the extraction of the fucoidan and precipitation of alginate stages were successful and, if coupled with a different fucoidan collection/precipitation method, will lead to successful production of large quantities of fucoidan.

Chapter 1. Introduction

Undaria pinnatifida is an invasive seaweed species that was accidentally introduced to New Zealand in the 1980s by international shipping (Hay & Luckens, 1987). It has been classified as an unwanted organism by Biosecurity NZ (Biosecurity Act 1993 No.95), and has heavily infested mussel farms throughout New Zealand. From the limited surveys that have taken place on this seaweed in NZ, it appears that there is in excess of 9000 tonnes wet weight of *U. pinnatifida* growing annually on NZ mussel lines (W. W. Chen, 2012). A resource that is currently only used in very few cases. The Ministry of Fisheries (MFISH), Ministry of Agriculture and Forestry (MAF) and the Department of Conservation (DoC) made attempts to eradicate the pest up to the 1990s, but with little success. The Ministry for Primary Industries (MPI) has since allowed the harvest of this alga from artificial structures (e.g. mussel farms) and farming in some limited (highly infested) locations (MAF, 2012). Mussel farm owners and investors have been encouraged to apply for permits and broaden the type and scope of *U. pinnatifida* commercialization.

Native to the cold-temperate coasts of Japan, Korea and China (J. S. Choi *et al.*, 2012), *U. pinnatifida* is farmed extensively in Asia and generates in excess of US$2 billion per annum, primarily through sales as a human food (Zemke-White & Ohno, 1999; McHugh, 2003; W. L. White & Wilson, in press). In the past ten years, there has been a growing interest in developing and incorporating natural bioactive compounds into food (Holdt & Kraan, 2011), this is due to the high rate of obesity and chronic diseases such as cardiovascular diseases, metabolic syndrome and cancer (D'Orazio *et al.*, 2012). Seaweed is well known for its medicinal properties in Asian countries (Jiménez-Escrig, Jiménez-Jiménez, Pulido & Saura-Calixto, 2001; Hong, Hien & Son, 2007; Dhargalkar & Verlecar, 2009; Mohamed, Hashim & Rahman, 2012). Numerous bioactive compounds from brown seaweeds such as fucoidan and fucoxanthin have therapeutic effects (Y. X. Li & Kim, 2011), in fact both of these have numerous clinically proven health benefits and growing markets (Fitton, 2011; S.-K. Kim & Pangestuti, 2011; D'Orazio *et al.*, 2012;

Vo & Kim, 2013). Fucoidan, having easier extraction and storage requirements comparing to fucoxanthin (Kanazawa *et al.*, 2008; Piovan, Seraglia, Bresin, Caniato & Filippini, 2013), seems the most commercially viable product from *U. pinnatifida*. While it is difficult for New Zealand to compete with the enormous scale of *U. pinnatifida* farming and production in Asia, we do have a long coastline of 15,134 km (Metzner, Harte & Leadbitter, 2003) and a reputation of clean, green environment, which could give NZ products a competitive edge in their quality and consumer perception, especially in countries such as China, where pollution and food safety issues have made local products unreliable for consumers.

To date, no commercial scale extraction of fucoidan from New Zealand *U. pinnatifida* has been undertaken in New Zealand. This thesis investigates several aspects of fucoidan production necessary to undertake this on a commercial scale. First, given that harvested seaweed will most likely be stored frozen prior to extraction, this thesis examines the impact of thawing time on fucoidan yield and quality; second, the optimal fucoidan extraction time, temperature and seaweed-to-water ratio are investigated. Finally two commercial scale fucoidan extractions were undertaken and assessed in terms of the quantity and quality of the fucoidan end product. This introductory chapter reviews research on *U. pinnatifida* to date, including its biology, New Zealand specific policies, and quantity availability and harvest information. It then provides a comprehensive literature review on fucoidan structure, health benefits and extraction methods.

General biology/ecology

Undaria pinnatifida is a type of brown kelp commonly referred to as *Wakame* in Japan, *Qundai Cai* in China and *Miyok* in Korea. It is found in sub-tidal zones at depths to 18 m but mainly down to 3 m (Stuart, 2004). *U. pinnatifida* is classified as the following (Guiry and Guiry, 2011):

Empire: *Eukaryota*

Kingdom: *Chromista*
Phylum: *Heterokontophyta*
Class: *Phaeophyceae*
Order: *Laminariales*
Family: *Alariaceae*
Genus: *Undaria*
Species: *pinnatifida*

Undaria pinnatifida is an annual, heteromorphic species with two major life stages, the macroscopic sporophyte stage and the microscopic gametophyte stage (Figure 1), which can remain viable for more than 24 months (Stuart, 2004). In its sporophyte stage it varies from yellowish to dark brown in colour, and grows up to 3 m in length where it occurs in Asia. In New Zealand, it generally reaches between 1 to 2 m in length (Hay & Villuota, 1993). The main stipe (stem) is 1 – 4 cm wide and flattened, extending away from the holdfast to become the midrib of the sporophyte. The stipe of *U. pinnatifida* can extend up to 50 cm long (Hay & Gibbs, 1996; MAF, 2006) and the midribs are only visible on sporophytes that have a blade longer than 5 cm (MAF, 2006). The blades of *U. pinnatifida* are similar to those of *Ecklonia radiata*, but are thinner and more easily torn, with horizontal lobes of 50 – 80 cm long lying in one plane. These blades have smooth toothless margins and tiny hair pits otherwise referred to as cryptostomata scattered across the smooth and glossy surface. They also have characteristic "gland" cells that are microscopic clear or darkened.

The spore-producing sporophylls of *U. pinnatifida* are located close to the base of the alga, on bilateral sides of the flattened edges of the stipe. When the alga is fully mature, sporophylls become interleaved and appear to loop around the base of the stipe. While they appear as one piece, they are in fact two discrete parts (Hay, 1990; MFISH, 2001). The spiral sporophylls and visible distinct midribs are the key structures to distinguish mature *U. pinnatifida* from local species *E. radiata* (MFISH, 2001; MAF, 2006). In the

Marlborough region of New Zealand South Island, most mature plants with sporophylls were found between 1 m to 5 m depth, with almost none found at deeper than 8 m (W. W. Chen, 2012).

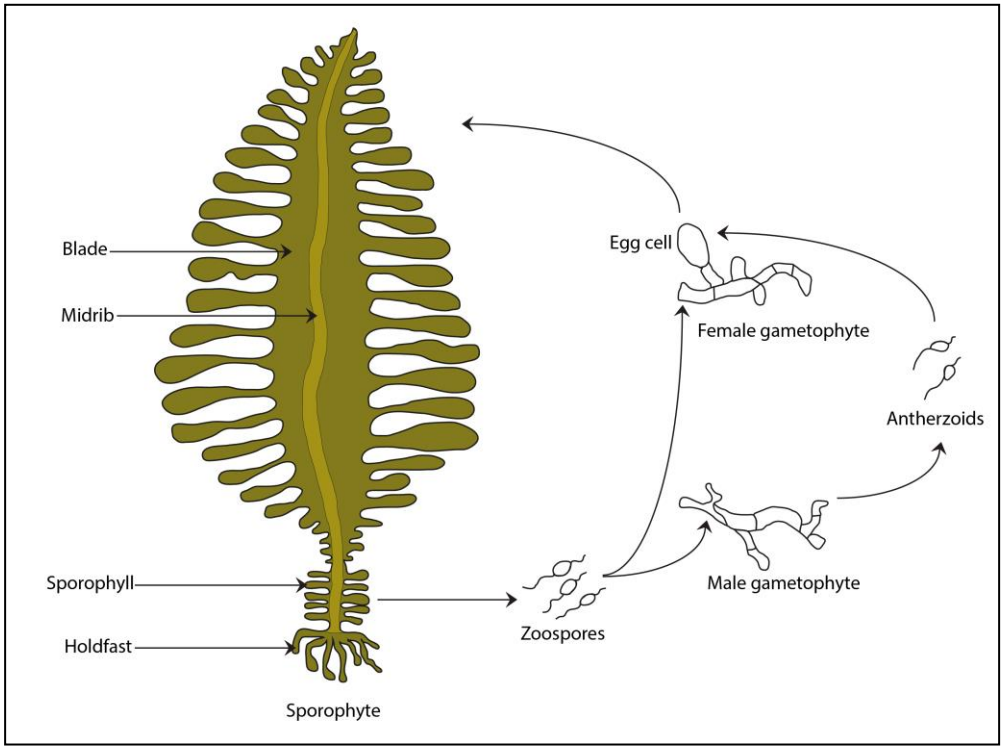

Figure 1. Life cycle of *U. pinnatifida*

The reproduction of *U. pinnatifida* is established by the release of asexual zoospores by the mature sporophylls of the seaweed (Hay & Gibbs, 1996). Millions of the zoospores will drift until they settle at a suitable site. Properly situated and attached, zoospores will germinate into female and male gametophytes (MFISH, 2001). Male gametophytes release mobile sperm into the surrounding water and fertilize the eggs produced by the female gametophytes, which subsequently develop into sporophytes (Parsons, 1994; MFISH, 2001).

Sea surface temperature is the most important and influential factor to the life cycle and ecology of *U. pinnatifida* (Saito, 1975). While sporophyll generation is independent of temperature, the optimal condition for germination of the zoospores to take place is at a range of water temperatures between 17°C to 20°C (Hay & Villuota, 1993). The release of zoospores will not begin without a 10-day period of an average water temperature above 14°C. In Asia, the release of the zoospores occur in spring and summer at water temperature between 17 and 20°C (Saito, 1975; Koh & Shin, 1990; Parsons, 1994). While the germination and the release of zoospores occurs in higher temperatures, the development of sporophytes favours cold water with temperature less than 12°C (MFISH, 2001). They develop quickly between 5°C and 13°C with the optimal temperature being approximately 10°C. the sporophytes degrade to extinction when temperature reaches above 23°C (Hay & Villuota, 1993; MFISH, 2001). In New Zealand, *U. pinnatifida* sporophytes are present throughout the year where the regeneration and the degeneration of some sporophytes happen simultaneously. This is because the annual sea temperature has a narrow range in New Zealand and does not fluctuate as much as that of Asia (Hay & Villuota, 1993; Parsons, 1994).

Undaria pinnatifida is able to settle on surfaces ranging from rocky reef, mudstones, cobbles to shells of abalone, sea grasses and even other seaweeds epiphytically. Human-made structures such as wooden and concrete wharf piles, buoys, pylons, ropes, hulls of ships or boats and pontoons are also highly inhabitable for this alga (Parsons, 1994; MFISH, 2001). Its tendency to colonize artificial surfaces is believed to relate to the long history of rope cultivation in Japan and Korea (Brown & Lamare, 1994). *U. pinnatifida* has been found on sloping artificial rocky shorelines and muddy sea floor in Wellington Harbour and rocks of various sizes as small as 5 cm in diameter in Timaru harbour. Brown and Lamare (1994) observed that while pebbles of 3 – 5 cm in diameter were completely not inhabited by *U. pinnatifida*, it settled well on a steel cable from the same area. Parsons (1994) has suggested that this is related to the frequent turn over movements of the pebbles in the harbour. Hay and Villouta (1993) found *U. pinnatifida*

growing just above the mid low water neap tide level at 7 – 9 m in depth; while in Timaru harbour, most *U. pinnatifida* were growing in about 2 m of depth (Brown & Lamare, 1994).

Impact of the introduction of *U. pinnatifida*

Undaria pinnatifida has proven to be highly invasive and adaptable to new locations. Each fertile alga is able to release millions of zoospores that can be transported by currents up to kilometres away. These spores are able to survive undeveloped for more than two years before they germinate under optimum environmental conditions. As mentioned above, in some parts of the world they can reproduce all year round. They are highly tolerant to both low and high sunlight intensity, as well as very high level of wave exposure (MFISH, 2001).

Undaria pinnatifida tends to form a thick dense canopy that shades the organisms beneath (W. G. Lee, 2001), competing for sunlight and space, which may lead to structural alternations and disrupted balance of the native ecosystem (Walker & Kendrick, 1998). It can decrease the biodiversity of fish (Irigoyen, Eyras & Parma, 2010) and encrusting and sub-canopy sessile communities by displacing certain native algal communities (MFISH, 2001). In New Zealand, the displacement of coralline algae that is directly linked to paua (abalone) recruitment could be a potent impact of *U. pinnatifida*.

As a fouling agent, *U. pinnatifida* also has an economic impact. Raffo, Eyras and Iribarne (2009) suggest that the infestation of *U. pinnatifida* in inter-tidal and sub-tidal areas could interfere with beach users' activities, e.g. snorkelling and scuba diving. Expenditure has increased in labour and harvesting costs for marine farmers, as *U. pinnatifida* fouls fish cages, oyster racks, mussel ropes and scallop bags, restricting water circulation (MAF, 2012). In the Marlborough Sounds, excessive drag caused by *U. pinnatifida* fouling in high current areas has at times led to breakage of mussel farm anchor ropes. Heavy

fouling of *U. pinnatifida* could potentially clog machinery and hulls of boats, which could decrease work efficiency (Stuart, 2004).

The impacts of the introduction of *U. pinnatifida* however, are not fully understood and are likely to vary geographically (Morelissen, 2012). In fact, Castric-Fey, Girard & L'Hardy-Halos (1993) stated that *U. pinnatifida* had relatively mild impact on the native species of the area in Brittany, France; and that *U. pinnatifida* has been cultivated there since 1983, and fits right in the local environment (Floc'h, Pajot & Wallentinus, 1991; Floc'h, Pajot & Mouret, 1996). Schiel and Thompson (2012) also affirmed that *U. pinnatifida* does not seem to compete with native species in New Zealand directly, instead, it grows as a refugee in patches within the native algae canopies, finding habitats that are unsuitable for local dominant algae when they are in their microscopic stage. The native large algae tend to recover to their dominance even when *U. pinnatifida* is the most abundant in spring and winter (Thompson & Schiel, 2012). Until more research is conducted, the ecological and economic impact of *U. pinnatifida* in New Zealand waters cannot be fully understood and described.

NZ Management and Legislation regarding *U. pinnatifida*

The Ministry of Fisheries proposed a National Pest Management Strategy for *U. pinnatifida* in 2001 (MFISH, 2001), in which all commercial harvesting of *U. pinnatifida* was banned. Harvesting of *U. pinnatifida* as a by-product of another activity such as mussel harvesting was allowed in 2004 (MAF, 2009); commercial harvesting of *U. pinnatifida* was permited in 2009 (MAF, 2010) given the inevitable physical contact between the farmers and the seaweed during mussel harvesting. The hope was to turn the pest into commercially viable product that could compensate the loss it caused the marine farmers (MAF, 2010). The changes in policies are summarized in Table 1.

Table 1. Major changes to policies regarding harvesting of *U. pinnatifida* between 2004 and 2010

Activity	2004	2010
Harvesting from artificial structures	No	Yes
Harvesting as a beach cast *U. pinnatifida*, despite of not being part of a control programme or by-product	No	Yes
Farming in heavily infested farming areas with *U. pinnatifida* despite of not being part of a control programme or by-product	No	Yes

Undaria pinnatifida is still classed an unwanted organism under the Biosecurity Act to this day, and permission needs to be obtained for any harvesting activities. This is necessary for national monitoring and control purposes. Harvesting from natural surfaces is still not allowed unless it is part of a control programme. The legal requirements of harvesting *U. pinnatifida* are unchanged, where a permit is required from MPI under section 52 and/or 53 of the Biosecurity Act. Additional permits under Fisheries Act or Resource Management Act 1991 may be required if the harvesting technique is abnormal or the area is close to fishing grounds where specific rules apply. It is an offence under the Biosecurity Act 1993 to breed, knowingly communicate, exhibit, multiply, propagate, release, and sell or offer for sale unwanted organisms without a permit, whether intended for research, exhibition or commercial purposes.

Current NZ harvest status

As mentioned above, the harvest of *U. pinnatifida* is controlled under the Biosecurity Act 1993. Any entity wishing to undertake commercial harvesting must gain a permit from MPI. Unfortunately, there are no requirements under the Act for these permit holders to report the landings of this seaweed, so there is no way to determine the current NZ harvest volume. There are currently there are 17 approved applicants commercially harvesting *U. pinnatifida* in Southland, Otago, Canterbury, the Marlborough Sounds, Wellington and the Coromandel. Their details were acquired by AUT under the Official Information Act 1982 from MPI.

While there is no empirical information on *U. pinnatifida* use, there are some publically known New Zealand stakeholders that have been utilising the seaweed since 2004. S. J. McFarlane, a mussel and paua farmer has created jobs in Marlborough by harvesting from existing mussel farms with divers, with the product made into health products (Bull, 2012). KiwiWakame Ltd. is a food producer based in Invercargill. They hand pick *U. pinnatifida* from Stewart Island and Foveaux Strait and freeze-dry it locally. Roger Beattie, owner of Eyris Blue Pearls Ltd. and NZ Kelp Ltd. has been harvesting giant kelp for Paua feed, and is most enthusiastic about farming of *U. pinnatifida* (Campbell Live, 2012). Auckland-based company Pacific Harvest manufactures seasonings and other innovative food products based on seaweeds including *U. pinnatifida* (Seafood New Zealand, 2006). Owners Louise and Doug Fawcett have been sourcing *U. pinnatifida* from mussel farmers when available. Waikaitu Ltd is a newly founded local business harvesting *U. pinnatifida* by lifting mussel lines and hand picking them in the Marlborough region. They produce both wakame for human consumption and fertiliser.

Research on *U. pinnatifida* specific to NZ

The scientific interest in *U. pinnatifida* has increased over the past ten years as demonstrated by the number of scientific publications with *Undaria pinnatifida* in the title or keywords (Figure 2). Since *U. pinnatifida* made its first appearance in New Zealand in the 1980s, various research programmes have been conducted to investigate its impacts on ecosystems and the possibility of future mariculture. In 2004, Stuart summarized the research to date on *U. pinnatifida* in NZ including its distribution, ecology, chemistry and biology, in attempt to fully understand its impact and the best possible ecological and economic returns. The research at that point had mainly been conducted by: DoC, the Cawthron Institute (CI), and the National Institute of Water and Atmospheric research (NIWA). Local government and stakeholder groups have also participated to control the spread of *U. pinnatifida* at places such as Golden Bay, Nelson Haven and Stewart Island. Stuart (2004) was the last literature review on *U. pinnatifida* in New Zealand.

The earliest report of *U. pinnatifida* in Wellington Harbour was produced by Dr Cameron Hay of the then New Zealand Oceanographic Institute (Hay & Luckens, 1987). It was part of an investigation conducted by DoC, investigating the distribution, abundance and potential impact of *U. pinnatifida.* Several published studies were generated from this project: Hay, 1990; Hay, 1992; Hay & Villouta, 1993. Parsons (1994) prepared a report on the environmental implications of *U. pinnatifida* cultivation in New Zealand for Nelson/Marlborough Conservancy of DoC. When *U. pinnatifida* was discovered in Big Glory Bay by NIWA in 1997, DoC set up a programme that involved manually removing *U. pinnatifida* sporophytes by divers, which significantly reduced the number of sporophytes but failed to eradicate it completely. Several other experiments in the following years were conducted with purpose to sterilise parts of the shoreline by killing the microscopic gametophytes of *U. pinnatifida*, using of Sodium Hypochlorite and brominated oxidizing agents, both of which failed. Webb and Allen (2001) developed an effective treatment for killing gametophytes that involved heating *U. pinnatifida* at 60°C water for five seconds. This technique was tried in the Chatham Islands and Stewart Island. A vessel-monitoring programme was set up in 1999 to evaluate the risk of hull fouling on the dispersal of *U. pinnatifida*. The data collected in a two-year period was collected from this programme and reported to MFISH (Stuart, 2004).

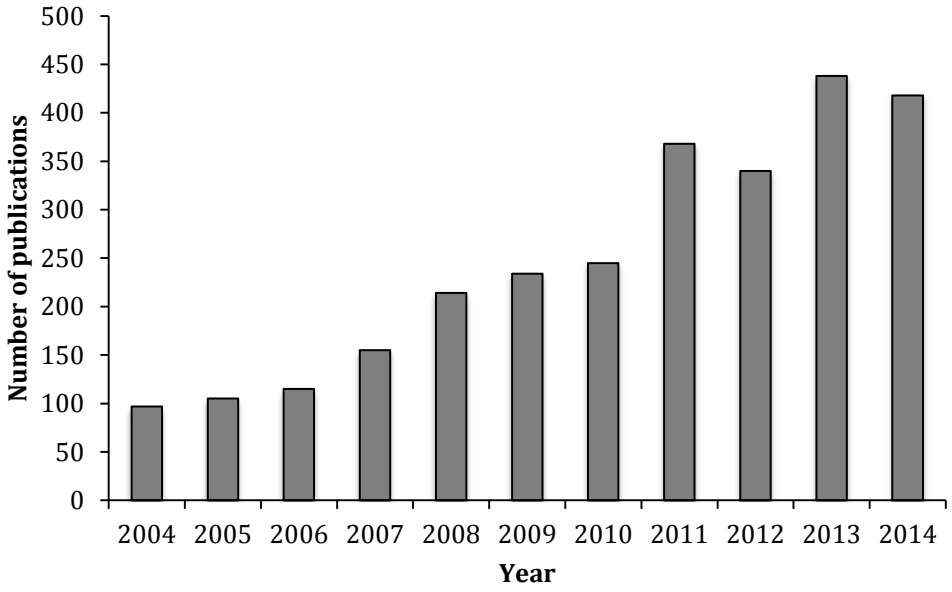

Figure 2. Number of publications with "*U. pinnatifida*" in the title or keywords (Source: Scopus, 2015)

The Cawthron Institute (CI) investigated the possibility of *U. pinnatifida* mariculture in New Zealand (Hay & Gibbs, 1996; Gibbs, Hay & Dodgshun, 1998; Gibbs, Brown, Forrest & Dodgshun, 2000). The programme established the maintenance of gametophyte cultures and carried out growth trials at sea. CI also established the Ballast Water Programme, which investigated the heating and oxygen removal treatment on invasive species including *U. pinnatifida.* They also have a risk-assessment model which estimated the link between transport and establishment of *U. pinnatifida*. In 2000, Jim Sinner of CI prepared a report on management of *U. pinnatifida* in New Zealand, which established the framework of *U. pinnatifida* management strategies (Sinner, Forrest & Taylor, 2000).

Several New Zealand universities have carried out research on *U. pinnatifida*, among which AUT had the most recent contribution. Following the changes in the MPI policy allowing harvest of *U. pinnatifida* from artificial surfaces in 2010, an *Undaria* Research Group was set up in collaboration with Wakatu Inc. (now Kono Seafood Ltd.) at AUT.

Thus far this research group has completed nine Master of Science theses on aspects of *U. pinnatifida* biology, composition and potential commercial uses. These have led to seven journal articles (Mak, Hamid, Liu, Lu & White, 2013; Fung, Hamid & Lu, 2013; Hau, Robertson & White, 2014; Mak *et al.*, 2014; S. Wang, Li, White & Lu, 2014; Zhou, Robertson, Hamid, Ma & Lu, 2014; Balbas *et al.*, 2015) and two technical reports (A. J. Wang, White, Lu, Taylor & White, 2014; L. White, Lu & White, 2014).

Polysaccharide composition

Seaweeds contain high levels of polysaccharides (Up to 71% dry weight) (Darcy-Vrillon, 1993; Jensen, 1993). Cell walls make up the majority of algal cells by dry weight; these are mainly gel-type polysaccharides also called phycocolloids. In the red algae these can be the commercially interesting agar and carrageenan. In brown algae these are alginates and in some cases, fucoidan. All percentages in this chapter are presented by percentage per dry weight seaweed unless otherwise specified.

Storage polysaccharides

The primary storage polysaccharide of brown algae is laminarin. Laminarin is a small polysaccharide of approximately 5 kDa (Nelson & Lewis, 1974; Rioux, Turgeon & Beaulieu, 2007), that is absent in spring and the most abundant in autumn, making up as much as 35% of a brown alga (Rinaudo, 2007) It is made up of long chains of glucose units, with a combination of β-(1→3) and β-(1→6) linkages (Connell, Hirstv & Percival, 1950). It is found in two forms, a highly branched, soluble molecule (soluble laminarin) and an unbranched, insoluble molecule (insoluble laminarin). Generally it is linked with a 3:1 ratio of β-(1→3) and β-(1→6) linkages (Nisizawa, Yamaguchi, Handa, Maeda & Yamazaki, 1963; Lynch, Sweeney, Callan, O'Sullivan & O'Doherty, 2010). Some of the chains (M-chains) end with a mannitol molecule (Lobban & Harrison, 1994), but this depends on the species of seaweed, with some species having more M-chains, which leads to more water-soluble molecules (Peat, Whelan & Lawley, 1958). As well as

variability between species in the number of M-chains, the proportion of insoluble to soluble laminarin also varies.

Laminarin is a good source of dietary fibre and has many proven health benefits. Laminarin is not digestible in the human digestive system, but is fermented by intestinal microflora. It has a modulatory impact on the metabolism in the intestines (Devillé, Damas, Forget, Dandrifosse & Peulen, 2004), as well as a suppressant for the formation of putrefactive compounds responsible for colon cancer (Kuda, Yano, Matsuda & Nishizawa, 2005). Laminarin is also anti-inflammatory by promoting the release of inflammatory substances such as hydrogen peroxide (J. Y. Lee, Kim, Kim, Kim & Park, 2012).

Laminarin can be extracted in water at up to 70°C (Black, Cornhill, Dewar & Woodward, 1951), which makes it an easy beneficial by-product from fucoidan extraction that entails similar procedures. The two polysaccharides can be easily separated by size-specific membranes based on their significant size difference.

Alginic acid and its salts (alginates)

Alginic acid is commercially extracted from the brown algal genera *Macrocystis*, *Laminaria*, *Ascophyllum*, *Ecklonia*, *Eisenia* and *Sargassum* (Glicksman, 1987). It is made up of 1,4 linked □-D-mannuronic acid and □-L-guluronic acids in varying ratios (Margulis, McKhann & Olendzenski, 1993; Ale & Meyer, 2013).

Alginates are used in many food applications: their water-holding capacity make them ideal for maintaining the texture of frozen foods during the freeze-thaw cycle, while their stabilising and emulsifying capabilities are useful in salad dressings, beer, fruit juices, sauces and gravies (Nussinovitch, 1997; Gudmund, Størker, Olav & Kurt Ingar, 2006). One of the useful properties of alginates is their reactivity with calcium to form a rigid

skin. This enables the construction of "fabricated foods". Food pulp is mixed with the alginate and dropped into a soluble-calcium-salt solution where a skin is formed around the surface of the droplet. This method has been used to create of imitation cherries, apples and berries, and to fabricate pimento strips and onion rings (Table 2). Alginic acid/alginates are natural gels and ideal for facemask bases and other cosmetic products (Lesser, 1947; Podkorytova, Vafina, Kovaleva & Mikhailov, 2007).

Alginates and alginic acid have proven to have anticoagulant activity (Fan *et al.*, 2011) and antioxidant properties (Ueno *et al.*, 2012). Alginates are used as wound dressing materials for severe and lasting wounds (Leo, McLoughlin & Malone, 1990; Boateng, Matthews, Stevens & Eccleston, 2008; Brownlee, Seal, Wilcox, Dettmar & Pearson, 2009; Bixler & Porse, 2011; Pawar & Edgar, 2012). They also slow the development of cystic fibrosis (Ramsey & Wozniak, 2005). Alginate gels encapsulate the islets of Langerhans cells in diabetes treatment (Soon-Shiong *et al.*, 1993; K. Y. Lee & Mooney, 2012).

The blades of *U. pinnatifida* have the highest alginic content compared to that in sporophylls and midribs, though the alginate content increases the most during the sporophyll development stage (Skriptsova, Khomenko & Isakov, 2004). The alginic content in blades can vary between 34% (June) to 51% (April) from summer to spring (Skriptsova, Khomenko & Isakov, 2004), while the sporophyll-derived alginic acid showed the opposite variation, peaking in June (Black, Cornhill & Dewar, 1952a). The alginic content in the midrib is between 23 – 25% and does not vary over seasons (Skriptsova *et al.*, 2004; Obluchinskaya, 2008). Black, Dewar and Woodward (1952b) investigated on the composition of several brown seaweeds (whole plant) and found alginate in a range from 10% to 24.4%.

Table 2. Functional properties of phycocolloids used in foods (from Glicksman, 1982)

Function	Example
Binding agent	Pet foods
Bodying agent	Diabetic drinks
Crystalisation inhibitor	Ice cream, frozen foods
Clarifying agent	Beer and wine
Clouding agent	Fruit drinks
Coating agent	Fabricated onion rings
Dietary fibre	Cereals, breads
Emulsifier	Salad dressing
Encapsulating agent	Powdered flavours
Film-former	Sausage casings
Flocculating agent	Wine
Foam stabiliser	Beer
Gelling agent	Deserts, confectionery
Molding agent	Jelly candies
Protective colloid	Flavour emulsions
Stabiliser	Salad dressing, ice cream
Suspending agent	Chocolate milk
Swelling agent	Processed meat products
Syneresis inhibitor	Cheese, frozen foods
Thickening agent	Jams, pie fillings
Whipping agent	Marshmallows

Fucoidan

Fucoidan is a type of sulfated polysaccharide found in some algal cell walls. The structure and composition varies between species and is generally very complex (You, Yang, Lee & Lee, 2010). The generic structure consists of a backbone of L-fucose ($C_6H_{12}O_5$) units branched with characteristic sulfate ester groups that contain various amount of galactose, xylose, mannose, uronic acids and even proteins (Percival & Ross, 1950; Mori, Kamei, Nishide & Nisizawa, 1982; Patankar, Oehninger, Barnett, Williams & Clark, 1993; Chizhov *et al.*, 1999; Nagaoka *et al.*, 1999; Bilan *et al.*, 2002; Pomin, Valente, Pereira & Mourão, 2005; Jiang *et al.*, 2010; B. Li, Lu, Wei & Zhao, 2008; Morya, Kim & Kim, 2012; Bilan *et al.*, 2013; Guo, Liu, Jia, Zhang & Wu, 2013). Fucose

is the main sugar in fucoidan; its content varies from as low as 1.7% in fucoidan from the stipe of some species of seaweed, to 56.7% in the purest form of fucoidan (Black, 1954). For example, in *Fucus vesiculosus*, fucose can make up to 44.1% (Black *et al.*, 1952b; Nishino, Nishioka, Ura & Nagumo, 1994) of the fucoidan from the seaweed; and 43.2% in *U. pinnatifida* (C. Yang, Chung & You, 2008)

The sulfated L-fucose units are either linked by α-(1→3) glycosidic bonds (I); or by alternating α-(1→3) and α-(1→4) glycosidic bonds (II) (Figure 3) (J. B. Lee, Hayashi, Hashimoto, Nakano & Hayashi, 2004; W. J. Kim *et al.*, 2007) The chemical structure and composition of fucoidan molecules can vary significantly between sources, environment the source seaweeds were cultivated in or harvested from, even the time of the year (Quitain, Kai, Sasaki & Goto, 2013). No two isolated fucoidans are exactly the same; they are all unique in their structure and composition and biological activities (Morya *et al.*, 2012; Wijesinghe & Jeon, 2012). The sulphate content of fucoidan from *F. vesiculosus* has been recorded at 26.3% (Nishino *et al.*, 1994), and 10.4% (J. B. Lee *et al.*, 2004). The sulphate from *U. pinnatifida* varies from 9.18% (Synytsya *et al.*, 2010), 10.4% (J. B. Lee *et al.*, 2004), 25% (K. J. Kim, Yoon & Lee, 2012), up to 34.6% (Mak *et al.*, 2013) and 41.5% (C. Yang *et al.*, 2008).

The fucoidan content of *U. pinnatifida* varies between seasons (Skriptsova *et al.*, 2004; Skriptsova, Shevchenko, Zvyagintseva & Imbs, 2010; Mak *et al.*, 2013) and locations (Mak *et al.*, 2013). The content of fucoidan varies from 1.9% (Eluvakkal, Sivakumar & Arunkumar, 2010) to 16% over seasons (Skriptsova *et al.*, 2010), The content of fucoidan peaks when reproductive (Honya, Mori, Anzai, Araki & Nisizawa, 1999; Mak *et al.*, 2013), and is significantly higher in *U. pinnatifida* sporophyll than in the blade, and the maximum content (Skriptsova *et al.*, 2010; Morya *et al.*, 2012; Mak *et al.*, 2013). The fucoidan content of *U. pinnatifida* sporophyll varies between seasons from 8 – 12% (Fitton & Dragar, 2006). Differing significantly from these results, Mak *et al.* (2013)

reported yield from 25.4% to 69.9% in sporophylls and 3.5% to 14% in blades between July and September in New Zealand.

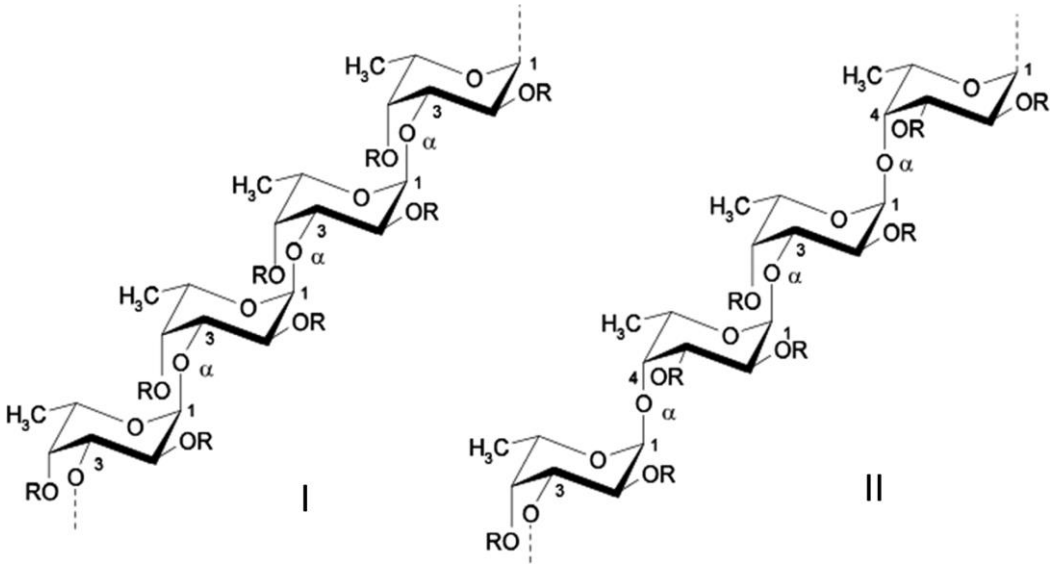

Figure 3. Chemical structure of fucoidan from *U. pinnatifida* (from Quitain *et al.*, 2013) R = α-L-fucopyranoside, α-D-glucuronic acid, sulfuric base, acetyl base. I = α-(1→3); II = α-(1→3), α-(1→4)

Numerous studies have found health benefits of fucoidan, including:

a) Antioxidant (Anggadiredja, Andyani & Hayati, 1997; Rupérez, Ahrazem & Leal, 2002; Heo, Park, Lee & Jeon, 2005; L. Li, Xue, Xue, Li & Fu, 2006; K. Kang, Kim, Kwon & Ha, 2008; J. Wang, Zhang, Zhang & Li, 2008; Zaragozá *et al.*, 2008; Zhao, Xue & Li, 2008; J. Wang *et al.*, 2009a and 2009b; Chattopadhyay *et al.*, 2010; T. Hu, Liu, Chen, Wu & Wang, 2010; H. Y. Luo, Wang, Yu, Qu & Su, 2010; Camara *et al.*, 2011; Costa *et al.*, 2011; Magalhaes *et al.*, 2011; Abu *et al.*, 2013; Balboa, Conde, Moure, Falqué & Domínguez, z2013; Dore *et al.*, 2013; Karaki *et al.*, 2013; Mak *et al.*, 2013; Suresh *et al.*, 2013; Vo & Kim, 2013; Imbs, Skriptsova & Zvyagintseva, 2014; S. J. Lim *et al.*, 2014; Marudhupandi, Ajith Kumar, Lakshmana Senthil & Nanthini Devi, 2014; Sellimi *et al.*, 2014; X. Li, Zhao, Wang, Liang & Jiang, 2015),

b) Anticoagulant (Springer, Wurzel, McNeal, Ansell & Doughty, 1957; Hoffman *et al.*, 1982; Dobashi, Nishino, Fujihara & Nagumo, 1989; Colliec *et al.*, 1991; Nishino, Aizu & Nagumo, 1991a; Nishino, Nagumo, Kiyohara & Yamada, 1991c; Soeda, Sakaguchi, Shimeno & Nagamatsu, 1992; Nardella *et al.*, 1996; Dürig *et al.*, 1997; Chevolot *et al.*, 1999; Mourão & Pereira, 1999; Pereira, Mulloy & Mourão, 1999; Matsubara, Matsuura, Hori & Miyazawa, 2000; Mulloy, Mourão & Gray, 2000; Alban, Schauerte & Franz, 2002; Pereira, Vilela-Silva, Valente & Mourão, 2002b; M. Y. Kim, Varenne, Daniel & Gareil, 2003; Kuznetsova *et al.*, 2003; Albuquerque *et al.*, 2004; Mourão, 2004; Paulo, 2004; Silva *et al.*, 2005; Athukorala, jung, Vasanthan & Jeon, 2006; L. Li *et al.*, 2006; Mao, Zang, Li & Zhang, 2006; Athukorala, Lee, Kim & Jeon, 2007; W. J. Kim *et al.*, 2007; De Zoysa, Nikapitiya, Jeon, Jee & Lee, 2008; Irhimeh, Fitton & Lowenthal, 2009; Mestechkina & Shcherbukhin, 2010; Bianca, Paulo & Vitor, 2013; W. Jin, Zhang, Wang & Zhang, 2013b; Ustyuzhanina *et al.*, 2013; Z. Zhang *et al.*, 2013b, 2015),
c) Antithrombotic (Church, Meade, Treanor & Whinna, 1989; Grauffel, Kloareg, Mabeau, Durand & Jozefonvicz, 1989; Nishino, Aizu & Nagumo, 1991b; Nishino & Nagumo, 1992; Soeda, Ohmagari, Shimeno & Nagamatsu, 1993; Mauray *et al.*, 1995; Trento, Cattaneo, Pescador, Porta & Ferro, 2001; Paulo, 2004; Jung *et al.*, 2007; A. Chen, Zhang, Shi & Zhao, 2012; Min, Kwon, Lee, Park & Kim, 2012; Zhao *et al.*, 2012; Dore *et al.*, 2013),
d) Anti-inflammatory (Semenov *et al.*, 1998; Senni *et al.*, 2006; Cumashi *et al.*, 2007; Ananthi *et al.*, 2010; Jintang *et al.*, 2010; S. M. Kang *et al.*, 2011; C. Li *et al.*, 2011; Siqueira *et al.*, 2011; K. J. Kim & Lee, 2012; K. J. Kim *et al.*, 2012; S. H. Lee *et al.*, 2012; Dore *et al.*, 2013; J. O. Jin & Yu, 2015; Pomin, 2015; Shu, Shi, Nie, & Guan, 2015),
e) Anti-tumour/anti-proliferation/anti-cancer/anti-metastatic (Riou *et al.*, 1996; Logeart *et al.*, 1997; Park, Kim, Kim, Suh & Choi, 2002; Maruyama, Tamauchi, Hashimoto & nakano, 2003; Aisa *et al.*, 2005; Haneji *et al.*, 2005; Ly, Buu, Nhut, Thinh & Van, 2005; Maruyama, Tamauchi, Iizuka & Nakano, 2006; Oomizu, Yanase, Suzuki, Kameyoshi & Hide, 2006; Alekseyenko *et al.*, 2007; Teruya, Konishi, Uechi, Tamaki & Tako, 2007; Kusaykin *et al.*, 2008; N. Y. Lee *et al.*, 2008; Athukorala *et al.*, 2009; Gamal-Eldeen, Ahmed & Abo-Zeid, 2009; Khotimchenko, 2010; Ale, Maruyama, Tamaouchi, Mikkelsen & Meyer, 2011a; Ermakova *et al.*, 2011; Foley, Mulloy & Tuohy, 2011; Vishchuk, Ermakova & Zvyagintseva, 2011; Azuma *et al.*, 2012; Foley, Szegezdi, Mulloy, Samali &

Tuohy, 2012; Boo *et al.*, 2013; Ermakova *et al.*, 2013; Senthilkumar, Manivasagan, Venkatesana & Kim, 2013; Suresh *et al.*, 2013; L. Yang *et al.*, 2013; Dithmer *et al.*, 2014; Moussavou *et al.*, 2014; S. Wang *et al.*, 2014; Zorofchian Moghadamtousi *et al.*, 2014; Delma *et al.*, 2015; Kawaguchi, Hayakawa, Koga & Torimura, 2015; Marudhupandi, Ajith Kumar, Lakshmanasenthil, Suja & Vinothkumar, 2015; Song *et al.*, 2015; Yoshimoto, Higaki, Nanba & Ieguchi, 2015),

f) Anti-virus (Baba, Snoeck, Pauwels & De Clercq, 1988b; Baba *et al.*, 1988a; McClure *et al.*, 1992; Béress *et al.*, 1993; Preeprame, Hayashi, Lee, Sankawa & Hayashi, 2001; Ponce, Pujol, Damonte, Flores & Stortz, 2003; Hemmingson *et al.*, 2006; Mandal *et al.*, 2007; Hayashi, Nakano, Hashimoto, Kanekiyo & Hayashi, 2008; J.-B. Lee, Takeshita, Hayashi & Hayashi, 2011; W. Wang, Wang & Guan, 2012; Prokofjeva *et al.*, 2013; Rabanal, Ponce, Navarro, Gómez & Stortz, 2014; Synytsya *et al.*, 2014; Aguilar-Briseño *et al.*, 2015; Thuy *et al.*, 2015),
g) Inhibitory effect on parasites (J. H. Chen, Lim, Sohn, Choi & Han, 2009);
h) Anti-depression effects (B. Lee, Shim, Lee & Hahm, 2013),
i) Anti-obessity (M. J. Kim, Jeon & Lee, 2014a; Hernández-Corona, Martínez-Abundis & González-Ortiz, 2014)
j) Cholestrol modulation effects (Cuong, Thuy, Huong, Ly & Van, 2014; 2015)
k) Immunostimulatory effects (Itoh, Noda, Amano & Ito, 1995; E. M. Choi, Kim, Kim, Hwang, 2005; Maruyama, Tamauchi, Hashimoto & Nakano, 2005; M. H. Kim & Joo, 2008; Na *et al.*, 2010; Ramberg, Nelson & Sinnott, 2010; Raghavendran, Srinivasan & Rekha, 2011; Negishi, Mori, Mori & Yamori, 2013; Cao, Lee & You, 2014; Cho, Kim & You, 2014; J. O. Jin *et al.*, 2014; Kuznetsova, Besednova, Somova, Plekhova, 2014; Q. Zhang *et al.*, 2015; W. Zhang, Oda, Yu & Jin, 2015),
l) Allergy alleviation effects (Vo & Kim, 2014; Vo, Ngo, Kang, Jung & Kim, 2015)
m) Anti-fatigue effects (Y. M. Chen *et al.*, 2014)
n) Anti-ulcer effects (Nagaoka *et al.*, 2000)
o) Hyperphosphatemia (Renal failure) modulation effects (Katai *et al.*, 2015)
p) Protective effects on diabetes (S. Hu *et al.*, 2014; K. T. Kim, Rioux & Turgeon, 2014; Yu *et al.*, 2014; Vinoth Kumar *et al.*, 2015; Y. Wang *et al.*, 2015; H. Xu *et al.*, 2015)

q) Protective effects on gastric and liver injuries (Shibata *et al.*, 2000; Chale-Dzul, Moo-Puc, Robledo & Freile-Pelegrín, 2014; M. J. Kim, Jeon, Lee & Lee, 2014b; J. D. Lim *et al.*, 2015)
r) Protective effects on the nervous system (Suppiramaniam *et al.*, 2006; D. Luo *et al.*, 2009; W. Jin *et al.*, 2013a; C. Hu, Zhang & Zhao, 2014), and
s) Therapeutic and healing effect in surgery and brain injury (Cumashi *et al.*, 2007; B. Li *et al.*, 2008; D. S. Kim *et al.*, 2014).

The bioactivity of fucoidan is correlated with the degree of sulfation of the molecule, the composition of monosaccharides (Pereira, Melo & Mourão, 2002a) as well as the structure and the molecular weight (Chevolot *et al.*, 1999; Zvyagintseva *et al.*, 1999; Zvyagintseva *et al.*, 2003; B. Li, Wei, Zhao & Zhang, 2006; J. H. Chen *et al.*, 2009; Jiang *et al.*, 2010; You *et al.*, 2010; Wijesinghe & Jeon, 2011; Bilan *et al.*, 2013; Quitain *et al.*, 2013). Numerous studies have found that the anticoagulant (Nishino *et al.*, 1991a, 1991b, 1991c; Nishino & Nagumo, 1992; Soeda *et al.*, 1993; Chevolot *et al.*, 1999; Alban *et al.*, 2002; Silva *et al.*, 2005; Qiu, Amarasekara & Doctor, 2006; W. Jin *et al.*, 2013b) and anticancer (Koyanagi, Tanigawa, Nakagawa, Soeda & Shimeno, 2003) effects of over-sulphated fucoidan molecules were higher than that of normal fucoidans.

The best anticoagulant effects are found in low molecular weight fucoidans (Colliec *et al.*, 1991; Nardella *et al.*, 1996; W. J. Kim *et al.*, 2010; A. Chen *et al.*, 2012; Vishchuk, Ermakova & Zvyagintseva, 2013), whose molecular weight range from 10 to 300 kDa (Nishino & Nagumo, 1992). The optimal anticancer activities are found in similar range of 5 to 300 kDa (Lin *et al.*, 2004; You *et al.*, 2010; Z. Zhang, Teruya, Eto & Shirahata, 2013a). These studies also suggested that the degree of binding efficiency of sulphate groups is the most important factors in regards to the anticancer effects. How the two factors interact is still not fully understood.

Bioactive compounds from seaweeds have gained increasing scientific attention over the last decade. Both the number of publications on fucoidan (Figure 4) and fucoidan from *U. pinnatifida* (Figure 5) has increased dramatically over the past ten years.

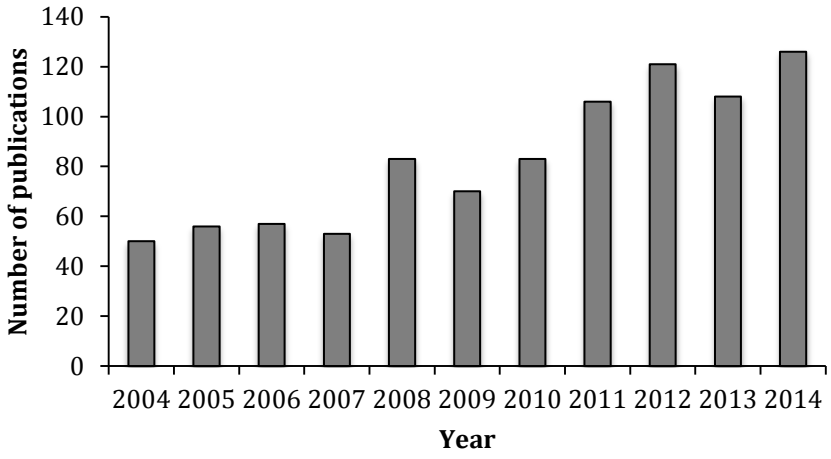

Figure 4. Number of publications with "fucoidan" in the title or keywords between 2004 and 2014 (Source: Scopus, 2015)

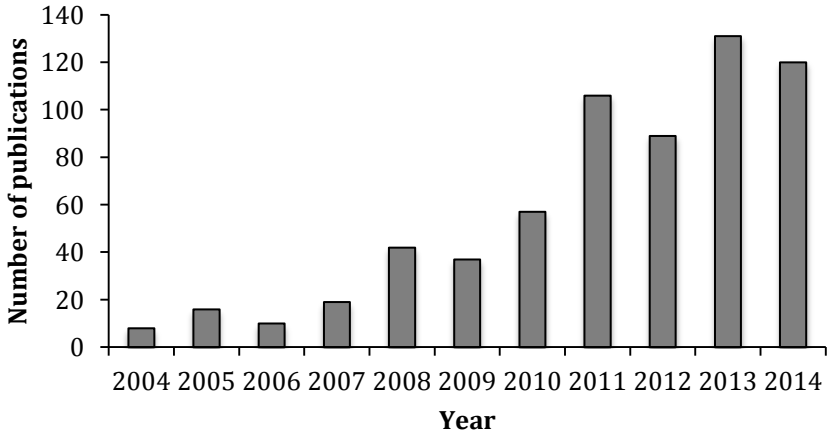

Figure 5. Number of publications with "fucoidan" and "*U. pinnatifida*" in the title or keywords between 2004 and 2014 (Source: Scopus, 2015)

Current fucoidan products on the market

There are numerous fucoidan products on the market today in forms of powder, capsules, tablets and beverages, mostly manufactured in Asian countries Korea, Japan and China, The selling point of the products varies from purity, percentage content, level of anti-oxidant capabilities, as well as the ability to be absorbed in a human body. While no two fucoidans share the same composition and structure, and the definition of purity is somewhat blurred as there is no standard method to measure fucoidan; most literature and business entities agree that the sulphate content and the molecular weight of the compound represent fucoidan purity the best.

The leading product Triple Ace Fuco-Asher by Haerim fucoidan Korea contains 36.018 g of fucoidan per unit (690 mL) and retails at US$300 per unit. They claimed the highest sulphate content of their fucoidan in the current market, and blend the fucoidan with fucoxanthin and curcumin. A Korean company Aviva has a patented processing method that yields 35.7% fucoidan from fresh seaweed with a guarantee of 57% absorbance by the human body, where absorbance is only 7% without their patented method. Their product retails at US$70.99 per unit that contains 57 g of fucoidan The USA and Australia also have a few manufacturers, with "Doctor's Best Fucoidan" by Biovea and Fucoidan-Pro® by Immukare the most popular in America, and the high purity extracts from Australian company Marinova, the closest competitor to potential New Zealand manufacturers.

Marinova claims to use water-based Maritech process and have two main fucoidan products: 1) Maritech Fucoidan Extracts – High purity (>90%), certified organic and without odour; and 2) Maritech Synergy Extracts – Fucoidan combined with marine polyphenols. Both are claimed to have very high antioxidant activity. Their fucoidan from *U. pinnatifida* (>75% purity) retails at Glycomix at US$65.29 per gram and US$186.54 per gram of fucoidan extracted from depyrogenated *U. pinnatifida*.

There are only a couple of New Zealand fucoidan producers: EnzAlg Biosciences and Matakana SuperFood. The former produces fucoidan from imported seaweeds, *Cladosiphon okamuranus* from the Pacific Islands as well as *Fucus vesiculosus* and *Laminaria digitata*. The company is currently the only New Zealand producer that says they can produce fucoidan from NZ *U. pinnatifida*, but according to the owners, the source is both seasonal and scarce, as harvesting is expensive. Their non-NZ fucoidan retails online at NZ$36 per 60 capsules of 500 mg, while Matakana Superfoods fucoidan retails at NZ$90 per 60 capsules. Each capsule contains 130 mg of fucoidan.

General processing methods for fucoidan

Fucoidan is water-soluble and can be extracted from *U. pinnatifida* in a simple hot water extraction. The difficulty is then separating the fucoidan from the other algal products that are also extracted in hot water, e.g. alginates, storage polysaccharides and proteins. A pre-treatment is often employed to eliminate impurities such as pigments, polyphenols, proteins and flavins. The four most common solvents for pre-treatment are either 1) methanol/chloroform/water at a ratio of 4:2:1, 2) formaldehyde in an ethanolic solution (Patankar *et al.*, 1993; Ale, Mikkelsen & Meyer, 2011b). The use of formaldehyde however, leads to potential interactions with polysaccharides such as fucoidan (W. Zhu, Ooi, Chan & Ang Jr, 2003; Bilan & Usov, 2008); 3) acetone (Pereira *et al.*, 1999; Anastyuk, Shevchenko, Nazarenko, Dmitrenok & Zvyagintseva, 2009); 4) ethanol (Ale *et al.*, 2011b). Decoloration can also be achieved by incubating crude fucoidan extract with activated charcoal (Patankar *et al.*, 1993; Berteau & Mulloy, 2003). The activated carbon however can adsorb fucoidan molecules and lead to significant loss; a better alternative is resin (Berteau & Mulloy, 2003).

Most published methods of fucoidan extraction utilise water or aqueous organic solvent extractions (Liu, Yi & We, 2004; Wu, Li, Liu & Mao, 2008; X. Chen, Xing, Yu, Liu & Li, 2012; Wijesinghe & Jeon, 2012). The seaweed (wet or dried) is treated with hot aqueous at 60 – 100°C for several hours or acidic solutions (e.g. HCl) at the same temperature

range for less than an hour (Liu *et al.*, 2004; Pomin *et al.*, 2005; Ale *et al.*, 2011b). The advantage of hot acid extraction is that alginic acid is concurrently precipitated out of the crude fucoidan containing solution (Ale *et al.*, 2011b), this can be achieved by adding Calcium Chloride ($CaCl_2$) in the case of aquaeous extractions (Hahn, Lang, Ulber & Muffler, 2012). The guluronates tend to connect and form blocks, creating voids when the blocks accumulate. The voids are happened to be the right size and dimension to fit Ca^{2+} ions (Ale *et al*., 2011b). Calcium Chloride also helps remove interfering lipids, protein and pigments after organic solvent extractions (Bilan *et al.*, 2002; Liu *et al.*, 2004; Mak *et al.*, 2013).

Fucoidan is then precipitated by organic solvents (Maruyama *et al.*, 2006; Ale *et al.*, 2011a), among which ethanol is the most commonly used (Alexeevna *et al.*, 2004). Ethanol has low dielectric constant and bonds with positively charged molecules such as sulphate ester (fucoidan) to form precipitation, leaving salts and small molecules from other polysaccharides in the solution (Hahn *et al.*, 2012). Tensids such as the cationic surfactant cetyltrimethylammonium bromide (CTAB) is an alternative to ethanol (Semenov *et al.*, 1998; Veena, Josephine, Preetha, Varalakshmi & Sundarapandiyan, 2006; Makarenkova, Deryabln, Lvov, Zvyaglntseva & Besednova, 2010; Z. Zhu *et al.*, 2010).

Given the complexity of the algal cell wall, aqueous extraction can be problematic and inefficient, as the extract will contain other interfering polysaccharides. Alternative methods have been explored; Wijesinghe and Jeon (2012) recommended an enzyme-assisted extraction (EAE) technique (also in Athukorala *et al.*, 2006; Holtkamp, Kelly, Ulber & Lang, 2009; Jiao, Yu, Zhang & Ewart, 2011) for industrial use. Existing patents have successfully increased the efficiency by incorporating ultrasonic waves (Ebringerová & Hromádková, 2010; Hagiwara 2010; Y. Xu *et al.*, 2010; Mason, Chemat & Vinatoru, 2011), chelating agents (Shaklee, Bahr-Davidson, Prasad & Johnson, 2008), selective precipitation, ultrafiltration (Hatano, Nakamoto & Kanetsuki, 2009), and

membrane filtration (Pielesz & Biniaś, 2010; Pielesz & Kulec, 2010; You *et al.*, 2010; Guo *et al.*, 2013). Other novel methods of extracting fucoidan include supercritical Carbon Dioxide (Quitain *et al.*, 2013) and other supercritical fluids (Thinh *et al.*, 2013); microwave-assisted extraction (MAE) (Ru, Zhang, Chen, Pei & Zheng, 2009; Rodriguez-Jasso, Mussatto, Pastrana, Aguilar & Teixeira, 2011; Z. Zhang, Lv, Pan, Shi & Fan, 2011); autohydrolysis (AH) extraction using water and the hydronium-catalyzed reactions of the material fibers proceed through water autoionization at high temperatures (Rodríguez-Jasso *et al.*, 2011).

The crude fucoidan is generally purified with ion-exchange chromatography to yield fractions of fucoidan that have the least interference species (e.g. galactose, xylose, uronic acids) in the structure (Bilan *et al.*, 2002; Gómez-Ordóñez, jénez-Escrig & Rupérez, 2012; Mak *et al.*, 2013). The structure of fraction(s) of interest can be analysed by high field NMR spectroscopy (Bilan *et al.*, 2002; Grachev *et al.*, 2005) and Mass Spectroscopy (MS). Mass spectrometry gives precise molecular mass for sugar units and the sequence of these sugars in the backbone (Jiao *et al.*, 2011).

The fucose content of fucoidan can be determined by the cysteine-sulphuric acid method (Dische & Shettles, 1948) with L-fucose as standard for fucose; the position of the sulphate groups can be determined by comparing the methylated original fucoidan with the methylated desulfated fucoidan (Bilan *et al.*, 2008; Jiao *et al.*, 2011) using the $BaCl_2$-gelatin method (Dodgson & Price, 1962) with K_2SO_4 as standard. The uronic acid content can be determined by carbazole-sulpuric acid borate reaction with D-glucuronic acid and as standard (Bitter & Muir, 1962); while the protein content can be determined by the Bradford assay with albumin as standard (Bradford, 1976). The composition of monosaccharides can be determined by gas chromatography (GC) with sugar standards such as fucose, galactose and glucose (Melton & Smith, 2001). The average molecular weight of fucoidan can be determined by gel permeation chromatography (GPC) (Moore,

1964), gel filtration chromatography (GFC) (Porath & Flodin, 1959), or size-exclusive chromatography (SEC) (Gaborieau & Castignolles, 2011).

Given its future in novel pharmaceuticals, functional foods and nutraceutical developments, attempts to isolate and purify fucoidan at optimum molecular weights and degree of sulphation have been made (B. Li *et al.*, 2008). It is however impossible to find a single formula for the best yield as the composition and complexity of extracted fucoidan varies between species, extraction method and conditions, seasonality of the harvest etc. (Grauffel *et al.*, 1989; Dietrich *et al*., 1995; Jiao *et al.*, 2011). Apart from the work in AUT's Undaria Research Group (Mak *et al.*, 2013; Fung *et al.*, 2013; Hau *et al.*, 2014; Mak *et al.*, 2014; A. J. Wang *et al.*, 2014; S. Wang *et al*., 2014b; L. White *et al.*, 2014; Zhou *et al.*, 2014; Balbas *et al.*, 2015), the extraction and purification of fucoidan from New Zealand *U. pinnatifida* has received little attention and more work needs to be done to acquire a comprehensive understanding of the size dependent bioactivities of New Zealand fucoidan from *U. pinnatifida.* Methods for generating size-specific and fucoidans of low molecular weight such as membrane size selection need to be developed further. In addition, the commercial scale harvest and storage of *U. pinnatifida* and production of fucoidan from that seaweed has not been undertaken and a number of questions around these processes remain to be answered. This study investigates some of these issues, and tests hypotheses around a) the impact on fucoidan of the amount time taken to defrost frozen seaweed prior to fucoidan extraction, this is important as the seaweed is frozen in very large (~1 tonne) blocks, so defrosting the entire block takes a long time, b) the optimal extraction time, temperature and seaweed-to-water ratio, and c) the ability to scale up proven laboratory size extraction methods to a commercial scale.

Chapter 2. Methods

Extraction of fucoidan from *U. pinnatifida* on a lab scale

In order to test for differences in fucoidan yield from the experiments in this thesis, an extraction method established on previous work in the AUT laboratories (sensu A. J. Wang *et al.,* 2014) based on existing literature (Bird & Haas, 1931; Bilan *et al.*, 2013) was developed. The dried or fresh seaweed was extracted in water for 4 hours at 80°C with occasional stirring. The raw material weight to water ratio volume was 1:10 for dried seaweed, and 1:1 for fresh seaweed. The extracted seaweed residue was removed at the end of the 4-hour-extraction by passing the hot extract through a sheet of muslin and squeezing by hand. Calcium Chloride was added to the residue-free extract form a 2% (w/v) solution in order to precipitate the alginic acid from the seaweed. The sticky alginates were either collected by filtering with muslin and vacuum filter paper (Whatman 41) (in the quantitative experiments); or by centrifuging at room temperature for 10 – 20 mins at 4000 rpm (Eppendorf Centrifuge 5810R) (in the qualitative experiments); oven-dried at 55 – 65°C overnight for further analysis or discarded. The alginate-free extract/supernatant was added to 99.6% ethanol to achieve a 70% ethanol solution, stored overnight at 4°C for fucoidan precipitation to develop. The fucoidan was collected by centrifuging at 18°C for 20 mins at 4000 rpm, and freeze-dried for 24 hours (Christ Alpha 2-4 LD). The freeze-dried fucoidan was ground, passed through a sieve of mesh aperture size 106 µm, and stored in a desiccator for further analysis.

Thawing time experiments

The impact of the timing of the thawing process on fucoidan quantity (yield) and quality (fucose, sulphate, fraction molecular weight distribution) was investigated. Essentially, the seaweed was frozen and defrosted at 4°C overnight, over one week or over two weeks. Twelve *U. pinnatifida* plants were collected late November from a mussel farm located in Port Underwood, Marlborough Sounds. Four of these plants were randomly placed into one of three treatments. Group 1 (T1) consisted of Plant 4, 6, 10 and 11; Group 2 (T2) of

2, 5, 9 and 12; Group 3 (T3) of 1, 3, 7 and 8. Each plant was split longitudinally into halves, of which one half was kept separate, intact and frozen for later analysis; the other halves of the same group were homogenised together using a food processor to achieve a particle size of roughly 3 mm. To each homogenate, 100 mL of water was added to aid the blending process. Each homogenate was then divided into three replicates: a, b and c. The replicates of the same code were treated with the same treatments:

1) Thaw overnight at 4 °C before extraction (M1) – T1a, T2a, T3a
2) Thaw and store at 4 °C for one week before extraction (M2) – T1b, T2b, T3b
3) Thaw and store at 4 °C for two week before extraction (M3) – T1c, T2c, T3c

Following the thaw, fucoidan yield was determined after extracted following the method above, at 80°C for 4 hours with seaweed-to-water ratio of 1:2 (g:mL). Once the fucoidan was collected and freeze dried, the fucose and sulphate content was determined as was the molecular weight distribution.

Fucose analysis

The fucose content of the fucoidan was measured by a modified colorimetric assay developed by Dische and Shettles (1948, 1951) for methylpentose quantification. The sulphuric acid solution (H_2SO_4) was prepared by slowly adding 6 parts volume of concentrated sulphuric acid into 1 part volume of deionized (DI) water, while constant cooling with running tap water. The 3% L-cysteine hydrochloride solution (CSOL) was prepared by dissolving 3 g of L-cysteine hydrochloride monohydrate in 100 mL DI water. Each fucoidan sample solution was diluted to 1 mg/mL. Based on previous results (A. J. Wang, 2014), the standard solutions were prepared by dissolving 10 mg L-fucose in 10 mL of DI water, and diluted to a series of concentrations ranged from 5 to 300 μg/mL.

From each sample/standard/blank solution, 268 μL was mixed with 1205 μL of H_2SO_4 and 27 μL of CSOL to make a total volume of 1.5 mL in a 2 mL eppendorf tube. The mixture was vortexed and transferred to a boiling water bath for 10 mins, and then cooled under room temperature. Duplicates of 250 μL of all samples, standards and blanks were

pipetted into one or more 96-well plates. Their absorbance (A) read (as soon as possible) at both 396 nm and 430 nm in a Multiskan™ Go Microplate Spectrophotometer. The absorbances of all were calculated by subtracting A396 nm from A430 nm. This step corrects the interference from the presence of hexoses (Dische & Shettles, 1951).

Sulphate analysis

The sulphate content was determined using a modified assay based on the traditional Barium Chloride ($BaCl_2$)-Gelatin method (Dodgson, 1961; Dodgson & Price, 1962). The gelatin solution was prepared by dissolving 1 g of gelatin powder (Sigma-Aldridge, New Zealand) in 200 mL of DI water at 60 – 70°C and chilled at 4°C overnight. The $BaCl_2$-gelatin reagent was prepared by dissolving 1 g of $BaCl_2$ in 200 mL of gelatin solution prepared the previous day. The mixture was allowed to stand for 2 – 3 hours before being used.

The 1 mg/mL sulphate (SO_4^{2-}) stock solution was prepared by dissolving 1.81 mg of K_2SO_4 in 1 mL of DI water, the standard series were diluted from the stock solution to a range of 5 to 400 μg/mL. The fucoidan samples (3 mg) were hydrolysed in an oven at 105°C for 16 hours, using 3 mL of 1 M HCl in 4 mL Teflon capped amber glass vials. After the samples were cooled to room temperature, the resulting solid and solution were gently mixed with a pipet tip, 1 mL of the homogenate was transferred to a 1.5 mL eppendorf tube to centrifuge (at 5000 rpm, 21°C for 8 mins, Alphatech Z326K). From each tube, 40 μL of the supernatant/standard/blank was mixed with 760 μL 3% (w/v) Trichloroacetic acid (TCA) and 200 μL of $BaCl_2$–gelatin reagent to produce a total volume of 1 mL in another 1.5 mL eppendorf tube. The mixtures were rested at room temperature for 10 mins after vortex. All sample solutions were added in duplicates of 250 μL onto a 96-well plate. The samples were allowed to stand for 10 mins to allow thorough reaction. The absorbances of all were read within an hour at 500 nm (Rupérez *et al.*, 2012)

Molecular weight of extracted fucoidan

Extracted fucoidan (1 g) was dissolved in 45 mL of 80°C water in a water bath with vortexing. The tubes containing this fucoidan were then centrifuged for an hour. The supernatant was collected and the tubes freeze dried and weighed to determine how much fucoidan had remained undissolved. The supernatant was then centrifuged through a series of micro filtration tubes to determine the percentage of each molecular weight class of the extracted fucoidan (Amicon, Merck Millipore, NZ, membrane sizes of 100 kDa, 50 kDa, 30 kDa, 10 kDa and 3 kDa).

Each tube held 15 mL of supernatant at a time, so 15 mL of each crude fucoidan solution started out by being centrifuged in the 100 kDa tubes for up to two hours at 4000 rpm at 18°C (Eppendorf Centrifuge 5810R); the centrifuge time varies between filter sizes. The larger the cut-off size, the longer it took for the entire 15 ml to pass through the membrane. When all of the solution had passed through the membrane and collected at the bottom of the tube, this liquid was transferred into the filter of the next smallest size until the solution had been passed through the smallest filter size (3 kDa).

The membrane filters were removed from the tubes and was washed repeatedly with less than 5 mL of 80°C water using a fine pipet tip until there was no colour observed. This liquid was then freeze-dried and fucoidan collected and weighed.

Extraction conditions experiments

The potential impact of various extraction conditions on the yield of fucoidan, alginates, as well as the percentage of >10-kDa fraction was investigated. The conditions investigated were: 1) the length of extraction (2, 3 and 4 hours); 2) extraction temperature (60, 70 and 80°C); and 3) the ratio of seaweed to water (1:1.5; 1:2 and 1:3) (g:mL). Three replicates of fresh *U. pinnatifida* were extracted under the 27 possible combinations of these conditions.

A total of 8.5 kg of thawed seaweed harvested in early November was roughly chopped in a food processor to a particle size of around 4 mm. 300 mL of water was added to aid the smooth blending. The homogenised seaweed was divided into 81 portions of approximately 100 g for extraction. For logistical reasons, the experiments were carried out over a 3-day period, with samples for days two and three frozen on day one. Wang *et al.* (2014) showed that there are no significant differences in fucoidan quantity or quality following short term freezing. On each of the three days the samples were extracted under the same temperature (temperature randomly assigned to days), with time and ratio varying. The first 27 seaweed samples were extracted shortly after chopping, the rest of the samples were frozen in ziplock bags, and were transferred into the beakers in which the extraction took place when they were still frozen solid to defrost overnight at 4°C. This was done to minimise the loss of seaweed flakes in transportation from the bags to the beakers, and to capture all the liquid from the seaweed during thawing.

The extractions were carried out in three large water baths, with nine extractions in each water bath on each day. The samples were assigned randomly generated numbers to represent their positions (stations) in the water baths. Following the extraction, the extract was poured off and the seaweed residue at the bottom of the beakers was squeezed to collect the remaining extract, using the bottom of another beaker of slightly smaller diameter. The volume of the collected extract was recorded. Two replicates of 40 mL of the extract was subsampled and transferred into two pre-weighed 50 mL Falcon tubes.

To collect alginates, 0.8 g of $CaCl_2$ was added while the extract was still warm. The mixture was allowed to sit in a water bath at 60°C for precipitation of alginates to develop. The tubes were centrifuged at 4000 rpm, 20°C for 20 minutes. The supernatant was collected and its volume measured. The alginate residue was oven dried overnight at 50°C and its weight recorded.

To collect fucoidan, a 15 ml subsample of the supernatant was then added to 35 mL of 99.6% ethanol in a 50 mL Falcon tube, to make up a 70% ethanolic mixture for the fucoidan to precipitate overnight at 4°C. The fucoidan was collected as above.

To determine the amount of this fucoidan that was greater than 10 kDa, a sample of the fucoidan was passed through a 10-kDa microfilter, collected and dried as above.

Commercial scale production of fucoidan

Two private sector partners were engaged to carry out commercial scale production of alginates and fucoidan from fresh *U. pinnatifida*. For confidentiality reasons, and as non-disclosure agreements exist between these companies and AUT, these partners will not be named in this thesis. The first partner (P1) harvested and processed the seaweed and transported it to a commercial cold store. The second private sector partner (P2) was engaged for the use of their premises and equipment to carry out a commercial scale extraction of the seaweed under conditions determined in previous sections of this thesis.

P1 – Seaweed collection and processing

Between 17 and 26 November 2014, P1 collected ~10 tonnes of *U. pinnatifida* from Port Underwood, in the South Island of New Zealand. The seaweed was removed from mussel farm lines by lifting the lines using a mussel harvesting barge, and removing the seaweed by hand. Once landed, the seaweed was ground by passing it through a commercial size wood chipper, placed into plastic bags that lined large plastic pallet-type containers (1.0 m W x 1.2 m L, 1.2 m H) which each held ~500 kg of fresh seaweed. These containers were transported to a commercial cold storage company in Blenhiem and stored at -20°C.

Subsequent testing of collected seaweeds

On one occasion during the collection, there was significant delay in getting the seaweed from the wharf into cold storage. Eight bins of seaweed were left on the wharf (in the sun) for several days as the transport company contracted to collect it was delayed. Then

the seaweed sat on the truck for a further two days before they made it into the freezer. To determine if there had been losses of fucoidan as a result of this mistake, 1 kg samples of each of these bins was transported in a frozen state to Auckland for analysis, as a comparison, 1 kg samples of three bins that had been put into cold storage straight away were also tested. Fucoidan was measured as per the generic method above. Fuocse content was measured as above.

P2 – Commercial scale extraction of fucoidan

While the generic method of fucoidan isolation above is adequate at a laboratory scale, the large amounts of ethanol needed to produce fucoidan on a kilogram scale with this method was thought to be too expensive and would require special facilities to handle large amounts of the dangerous liquid. As outlined above, fucoidan can be collected out of solution by a molecular filter, and previous work (A. J. Wang *et al.*, 2014) demonstrated that the molecules over 10 kDa were mostly fucoidan. So a commercial partner was engaged that had the equipment necessary to pass our extracted liquid through a 10-kDa membrane, to attempt to collect the fucoidan without the need for ethanol precipitation.

Trial 1

On 20th January 2015. Three bins (~1500 kg) of seaweed were delivered frozen to the P2 premises. These were placed into a 3000 L vat with 3000 L water. The water (and later the water and thawed seaweed), were continuously pumped out of the vat and through a heat exchanger (to heat the water) before going back into the vat. The vat was continuously stirred with an electric outboard motor attached to the vat wall.

Once the seaweed was thawed and the solution had reached 60°C, it was maintained at this temperature for a further three hours. The solids form seaweed extract was then separated from the extract using a proprietary piece of equipment. This extract was then

made up to a 3% $CaCl_2$ solution to precipitate out the alginates. The extract was passed through an industrial size, continuous feed centrifuge to separate out the alginate precipitate. This was collected for later analysis.

The resulting supernatant was then passed through a 0.25-μm filter to take out any particulate matter and microbes and passed through a 10-kDa membrane (again using a proprietary piece of equipment. Both the permeate (the liquid that passed through the membrane) and the retentate (the slurry collected on the membrane) were collected for analysis. The alginates, retentate and permeate were transported to the AUT laboratory to determine crude fucoidan yield and fucose content of that yield. Fucoidan yield was measured using the generic ethanol precipitation as above. Fucose was measured as above.

Trial 2

As will be outlined in the results section, the results of Trial 1 were not seen as successful, so some modifications were made for Trial 2. Changes were made to parts of the protocol, samples were collected for analysis at each step of the process and the author oversaw the entire process, instructing the P2 staff on improvements along the way.

For Trial 2, there were two extractions carried out. One (T2a) was carried out during the day and one (T2b) over night by a different operator. These extracts were combined before the alginate precipitation stage. Modifications in the methods from Trial 1 consisted of:

1) T2a used 450 kg of seaweed with a seaweed-to-water ratio of 1:3, T2b used 500 kg of seaweed and a ratio of 1:4
2) The electric outboard motor was placed in a new position to optimise mixing
3) Following each extraction, the seaweed was “rinsed” in another 1000 L of 60°C water an this rinse water combined with the extract

4) The alginate liquid that came out of the centrifuge was further dewatered by hanging in a sack overnight and this water added to the extract

Samples were taken at each stage of the process including:

1) Chopped seaweed for both batches before extraction – 5 kg
2) T2a and T2b extracts before the washing step and before $CaCl_2$ was added – 1 L each
3) T2a and T2b extracts after the washing step and before $CaCl_2$ was added – 1 L each
4) Extracted seaweed residue after the washing step for T2a and T2b (Res) – 800 g each
5) Wet alginates (combined from T2a and T2b) – 500 g
6) The extract following passing through the 0.25-μm filter (combined from T2a and T2b) – 500 mL
7) Retentate (combined from T2a and T2b) – 1 L
8) Permeate (combined from T2a and T2b) – 1 L

These samples were returned to the AUT lab and tested for fucoidan content using the standard ethanol precipitation method. The residue was also then further tested for fucose content, a proxy for fucoidan quality (as above).

Statistical analysis

Analysis of variance (ANOVA) was carried out using Statistica to test for differences between fucoidan/alginate content between treatments, where significant differences were found, Tukey's Honest Significance Difference (HSD) test was employed to investigate where that effect occurred.

Chapter 3. Results

All results in this chapter are presented as percentage dry weight (% dw) or wet weight (% ww). Dry weight percentage was converted to wet weight percentage by multiplying by 10%, and vice versa, as approximately 90% of seaweed is water.

Thawing time experiments

The fucoidan, alginate yield, as well as the fucose, sulphate content of the fucoidans from the thaw time experiments are summarised in Table 3.

Table 3. Summary of the fucoidan, alginate yield, fucose, sulphate content from the three treatments in the thawing experiments (n=3, ±SD)

Treatments	Fucoidan (% ww)	Alginate (% ww)	Fucose (% dw)	Sulphate (% dw)
Thawed overnight	0.50 ± 0.02	0.38 ± 0.02	17.94 ± 3.94	21.23 ± 1.43
Over a week	0.56 ± 0.1	0.35 ± 0.04	15.83 ± 0.95	26.87 ± 8.70
Over two weeks	0.51 ± 0.12	0.35 ± 0.02	14.08 ± 2.55	22.29 ± 5.06

Fucoidan yield

In this section fucoidan yield is presented as a percentage of the wet-weight of the seaweed. Across all treatments fucoidan yield was ~0.52%, and there was no significant differences in the fucoidan content between different treatments (Figure 6, Table 4, p=0.7078).

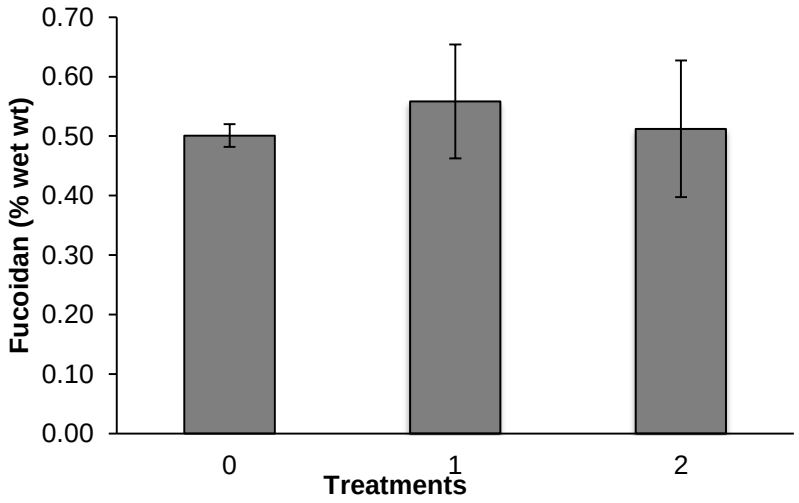

Figure 6. Fucoidan yield of the three treatments. 0=thawing overnight; 1=thawing for 1 week; 2=thawing for 2 weeks. Error bars = SD

Table 4. One-Way ANOVA of fucoidan yield between three treatments in the thawing experiments

Source	Sum of squares	Degrees of freedom	Mean square	F statistic	p-value
Treatment	0.0055	2	0.0028	0.3663	0.7078
Error	0.0455	6	0.0076		
Total	0.0510	8			

Alginate yield

The yield was measured as percentage the wet-weight seaweed in this section. Across all treatments alginate yield was ~0.36%, and there was no significant differences in the alginate content between different treatments (Figure 7, Table 5, p=0.4743).

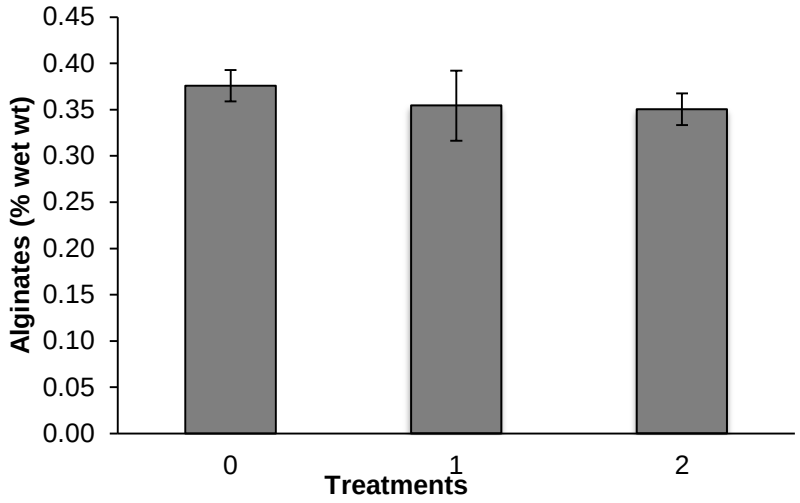

Figure 7. Alginate yield of the three treatments. 0=thawing overnight; 1=thawing for 1 week; 2=thawing for 2 weeks. Error bars = SD

Table 5. One-Way ANOVA of alginates between three treatments in the thawing experiments

Source	Sum of squares	Degrees of freedom	Mean square	F statistic	p-value
Treatment	0.0011	2	0.0006	0.8467	0.4743
Error	0.0040	6	0.0007		
Total	0.0052	8			

Fucose content

The fucose content was measured as percentage the dry-weight crude fucoidan in this section. Across all treatments fucose content was ~16%, and there was no significant differences in the fucose content between different treatments (Figure 8, Table 6, p=0.3009).

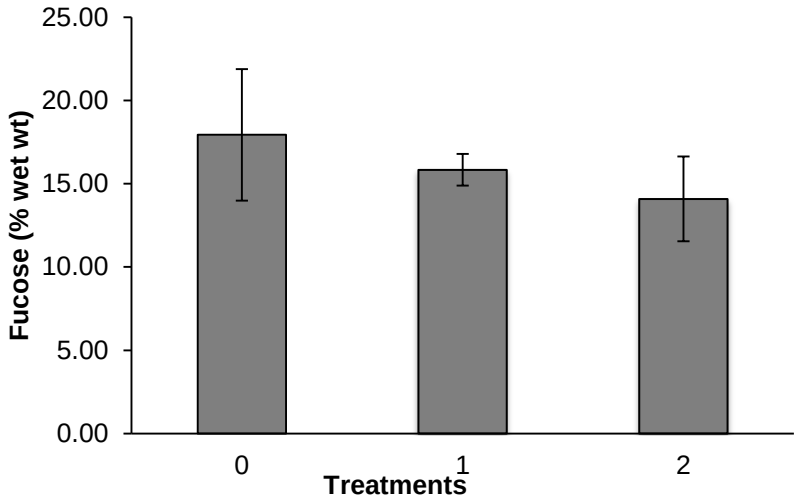

Figure 8. Fucose content of fucoidan extracted from the three treatments. 0=thawing overnight; 1=thawing for 1 week; 2=thawing for 2 weeks. Error bars = SD

Table 6. One-Way ANOVA of fucose between three treatments in the thawing experiments

Source	Sum of squares	Degrees of freedom	Mean square	F statistic	p-value
Treatment	22.4822	2	11.2411	1.4771	0.3009
Error	45.6600	6	7.6100		
Total	68.1422	8			

Sulphate content

The sulphate content was measured as percentage the dry-weight crude fucoidan in this section. Across all treatments sulphate content was ~23.5%, and there was no significant differences in the sulphate content between different treatments (Figure 9, Table 7, p=0.4986).

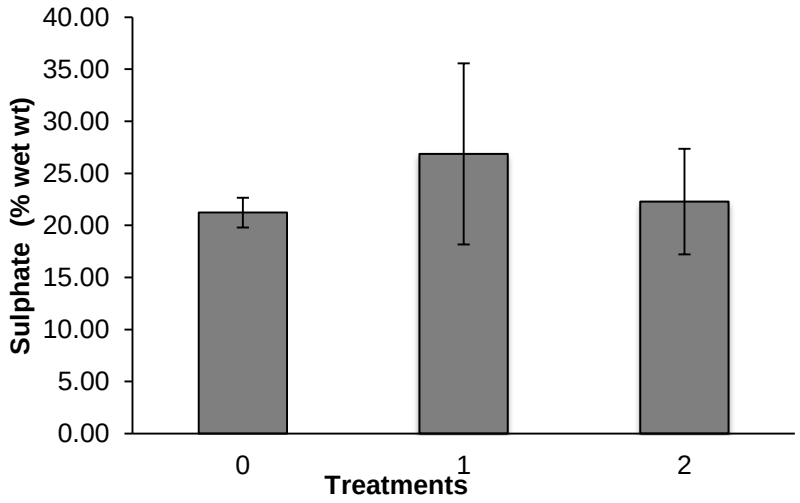

Figure 9. Sulphate content of the fucoidan extracted from the three treatments. 0=thawing overnight; 1=thawing for 1 week; 2=thawing for 2 weeks. Error bars = SD

Table 7. One-Way ANOVA of sulphate content between three treatments in the thawing experiments

Source	Sum of squares SS	Degrees of freedom ν	Mean square MS	F statistic	p-value
Treatment	53.9359	2	26.9680	0.7832	0.4986
Error	206.6032	6	34.4339		
total	260.5391	8			

Fractionation of crude fucoidan

The percentage of different fractions was measured as percentage the dry-weight crude fucoidan in this section. The percentage of different fractions under three treatments is summarised in Table 8. The most abundant fraction is less than 3 kDa, making up more than 68% in all three treatments. The larger than 100-kDa fraction had the second biggest proportion of at least 28% in all treatments.

Table 8. Mean % fucoidan fractions by treatments in the thawing experiments (n=3, ±SD). 0=thawing overnight; 1=thawing for 1 week; 2=thawing for 2 weeks

Treatment	>100 kDa	50-100 kDa	30-50 kDa	10-30 kDa	3-10 kDa	<3 kDa
0	24.868±4.24	2.797±2.29	0.550±0.15	1.905±0.21	1.873±0.39	70.599±8.44
1	22.361±2.23	1.438±0.14	0.457±0.34	1.733±0.40	2.057±0.19	67.934±1.30
2	25.351±5.36	1.195±1.40	0.222±1.82	1.825±0.15	1.946±0.44	68.096±9.68
Average	24.193±3.85	1.810±1.54	0.437±0.24	1.821±0.25	1.959±0.32	68.876±6.58

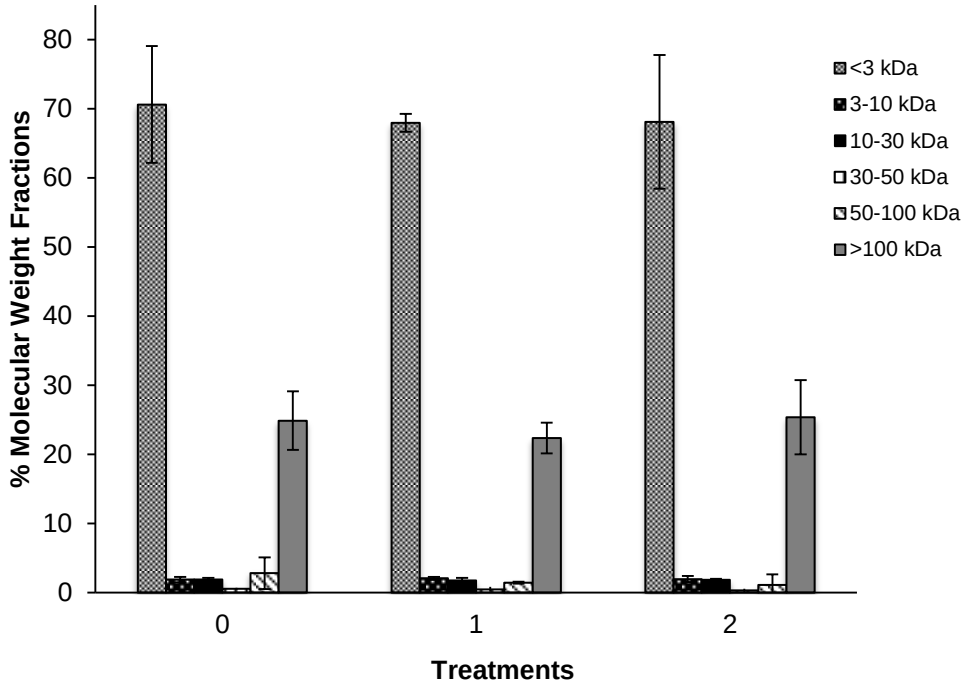

Figure 10. Percentage of different molecular weight fractions from the three treatments. 0=thawing overnight; 1=thawing for 1 week; 2=thawing for 2 weeks. Error bars = SD

Comparing between the treatments for each of the molecular sizes (using one-way ANOVA), showed no statistically significant difference between any of the treatments for any of the molecular sizes (Table 9).

Table 9. Summary of One-Way ANOVA of the fucoidan fractions between the three treatments in the thawing experiments

Source	Sum of squares SS	Mean square MS	F statistic	p-value
>100 kDa	15.4576	7.7288	0.4479	0.6587
50-100 kDa	4.4708	2.2354	0.9254	0.4464
30-50 kDa	0.0913	0.0457	0.7171	0.5257
10-30 kDa	0.0444	0.0222	0.2826	0.7634
3-10-kDa	0.0511	0.0255	0.1976	0.8259
<3 kDa	13.3904	6.6952	0.1204	0.8886

Extraction conditions experiments

The yield of fucoidan and alginate, as well as the percentage of fractions larger than 10-kDa are summarised in Table 10.

Table 10. Fucoidan, alginate and >10-kDa fraction content (±SD) from the extraction conditions experiments

Treatments	Fucoidan (% ww)	Alginates (% ww)	>10-kDa (% dw)
Time (hours)			
2	0.98 ± 0.12	0.57 ± 0.09	85.10 ± 6.26
3	0.96 ± 0.10	2.15 ± 2.44	83.28 ± 6.95
4	0.94 ± 0.11	1.05 ± 0.94	77.02 ± 7.50
Temperature (°C)			
60	0.95 ± 0.12	0.66 ± 0.46	83.69 ± 7.39
70	0.98 ± 0.11	2.30 ± 2.50	81.97 ± 8.97
80	0.96 ± 0.10	0.81 ± 0.17	79.74 ± 6.21
Seaweed:water ratio			
1:1.5	0.85 ± 0.07	0.78 ± 0.65	76.97 ± 6.67
1:2	0.97 ± 0.07	1.48 ± 1.59	81.55 ± 6.70
1:3	1.06 ± 0.06	1.51 ± 2.21	86.88 ± 6.34

Fucoidan yield

The fucoidan yield was measured as percentage the wet-weight seaweed in this section. Across all treatments fucoidan yield was ~0.95% (Table 10), and there was no significant differences in the fucoidan content between different extraction times (Figure 11, Table 11, p=0.4056). There was no significant difference of the fucoidan yield between three extraction temperatures (Figure 12, Table 12, p=0.5269); however, there was a significant difference of fucoidan yield between different seaweed-to-water ratios (Figure 13, Table 13, p=1.1102e16). Tukeys HSD results (Table 14) indicate that the 1:3 ratio produces the most fucoidan (1.06% ± 0.06SD, Table 10).

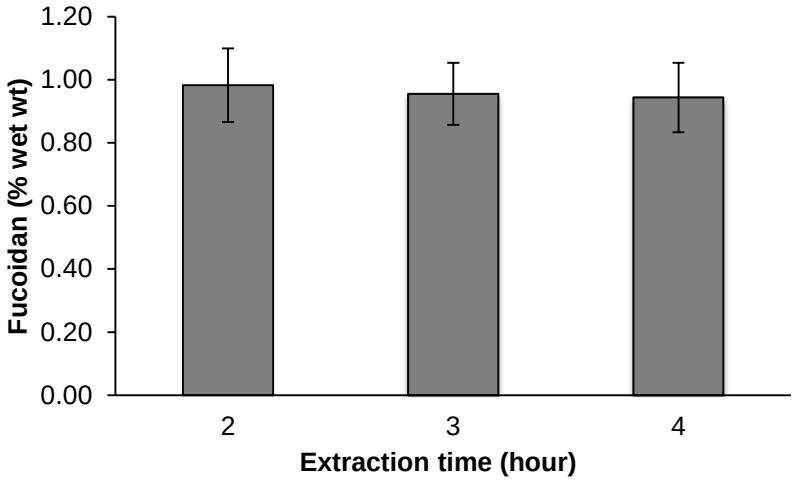

Figure 11. Fucoidan yield at different extraction times

Table 11. One-Way ANOVA of fucoidan yield between different extraction times

Source	Sum of squares	Degrees of freedom	Mean square	F statistic	p-value
Treatment	0.0215	2	0.0108	0.9128	0.4056
Error	0.9198	78	0.0118		
Total	0.9413	80			

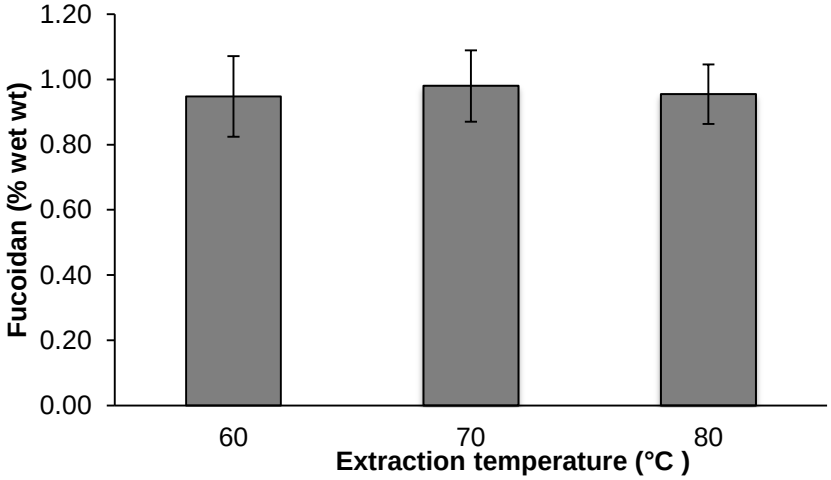

Figure 12. Fucoidan yield at different extraction temperatures

Table 12. One-Way ANOVA of fucoidan yield between different extraction temperatures

Source	Sum of squares	Degrees of freedom	Mean square	F statistic	p-value
Treatment	0.0153	2	0.0077	0.6460	0.5269
Error	0.9260	78	0.0119		
Total	0.9413				

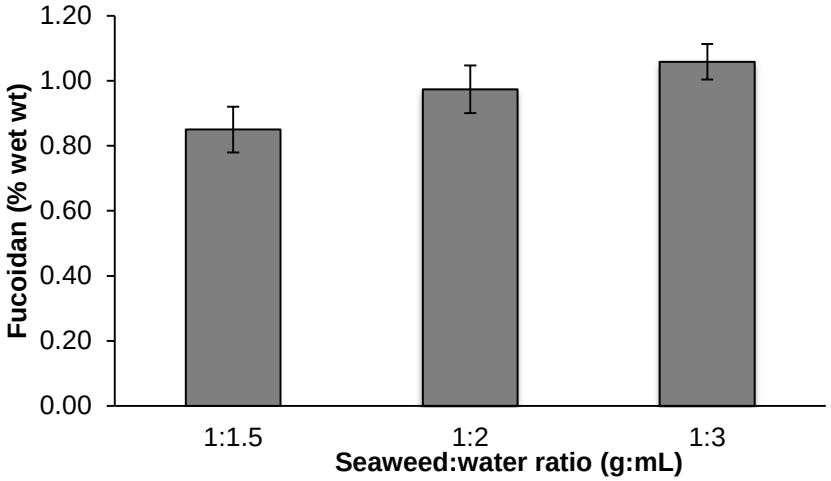

Figure 13. Fucoidan yield at different seaweed-to-water ratios

Table 13. One-Way ANOVA of fucoidan yield between different seaweed-to-water ratios

Source	Sum of squares	Degrees of freedom	Mean square	F statistic	p-value
Treatment	0.7817	2	0.3909	90.6420	1.1102e16
Error	0.3363	78	0.0043		
Total	1.1181	80			

Table 14. Tukeys HSD results of the pairwise comparison of the fucoidan yield between different seaweed-to-water ratios

Treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
1:1.5 vs 1:2	0.0000	0.8999947	insignificant
1:1.5 vs 1:3	16.4902	0.0010053	** p<0.01
1:2 vs 1:3	16.4902	0.0010053	** p<0.01

Alginate yield

The alginate yield was measured as percentage the wet-weight seaweed in this section. Across all treatments alginate yield was ~1.26% (Table 10), and there was a significant difference in alginate yield between different extraction times (Figure 14, Table 15, p=0.0009), with Tukeys HSD (Table 16) indicating that the highest yield was at 3 hours (2.15% ± 2.44SD, Table 10). There was also a significant difference in alginate yield between different extraction temperatures (Figure 15, Table 17, p=0.0001), with Tukeys HSD (Table 18) indicates that the highest yield was at 70°C (2.3% ± 2.5SD, Table 10). There was no significant difference in alginate yield between the different seaweed-to-water ratios (Figure 16, Table 19).

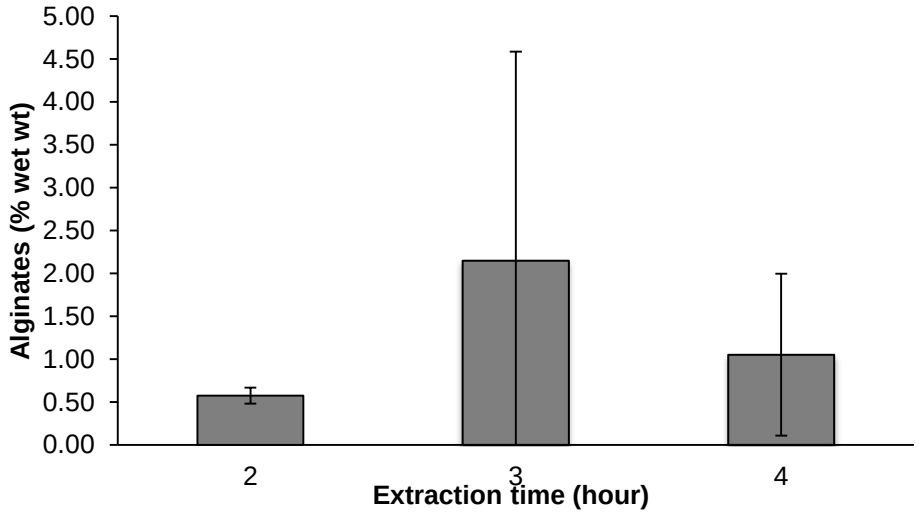

Figure 14. Alginate yield at different extraction times

Table 15. One-Way ANOVA of alginate yield between different extraction times

Source	Sum of squares	Degrees of freedom	Mean square	F statistic	p-value
Treatment	35.1407	2	17.5703	7.6938	0.0009
Error	178.1279	78	2.2837		
Total	213.2686	80			

Table 16. Tukeys HSD results of the pairwise comparison of the alginate yield between different extraction times

Treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
2 vs 3	5.4105	0.0010053	** p<0.01
2 vs 4	1.6438	0.4807869	insignificant
3 vs 4	3.7667	0.0251789	* p<0.05

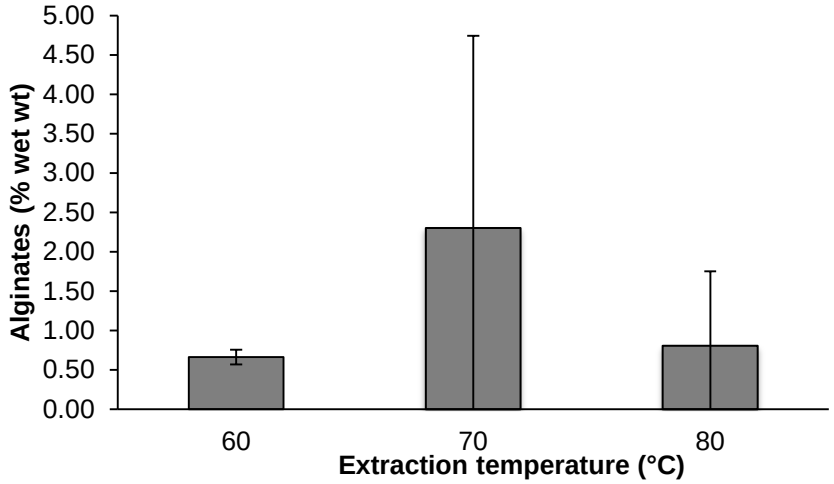

Figure 15. Alginate yield at different extraction temperatures

Table 17. One-way ANOVA of alginate yield between different extraction temperatures

Source	Sum of squares	Degrees of freedom	Mean square	F statistic	p-value
Treatment	44.4418	2	22.2209	10.2663	0.0001
Error	168.8267	78	2.1644		
Total	213.2686	80			

Table 18. Tukeys HSD results of the pairwise comparison of the alginate yield between different extraction temperatures

Treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
60 vs 70	5.7867	0.0010053	** p<0.01
60 vs 80	0.5090	0.8999947	insignificant
70 vs 80	5.2776	0.0010367	** p<0.01

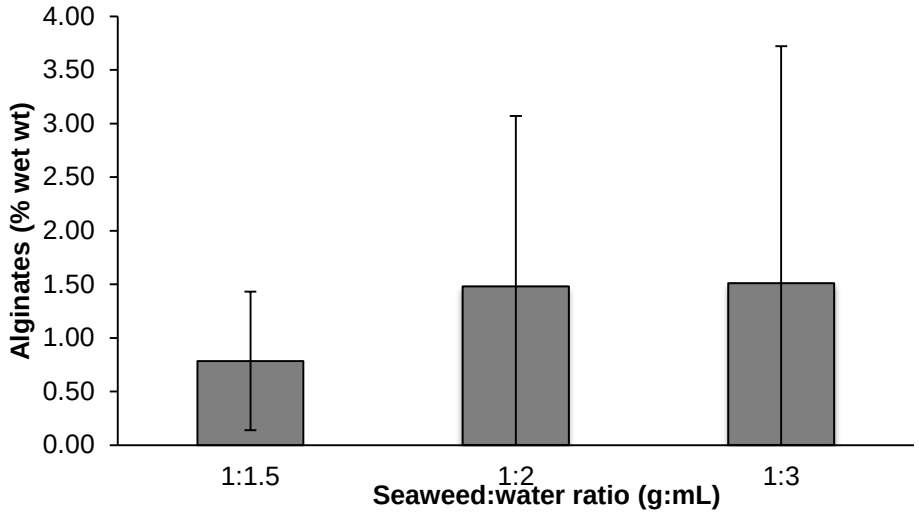

Figure 16. Alginate yield at different seaweed-to-water ratios

Table 19. One-Way ANOVA of alginate yield between different seaweed-to-water ratios

Source	Sum of squares	Degrees of freedom	Mean square	F statistic	p-value
Treatment	9.0665	2	4.5332	1.7316	0.1837
Error	204.2021	78	2.6180		
Total	213.2686	80			

Percentage of >10-kDa fraction

All data in this section is presented as percentage the dry-weight crude fucoidan. Across all treatments percentage >10-kDa was ~81.8% (Table 10), and there was a significant difference of >10-kDa% between different extraction times (Figure 17, Table 20, p=0.0025). Tukeys HSD results (Table 21) indicate that the amount of >10-kDa fraction reduced significantly when extracted for 4 hours, in comparison to 2 and 3 hours. There was no significant difference of >10-kDa% between different extraction temperatures (Figure 18, Table 22, p=0.3040). There was a significant difference in >10-kDa% between different seaweed-to-water ratios (Figure 19, Table 23, p=0.0002). Tukeys HSD results (Table 24) indicate that the highest >10-kDa% was found at the ratio 1:3 (86.88% ± 6.34, Table 10).

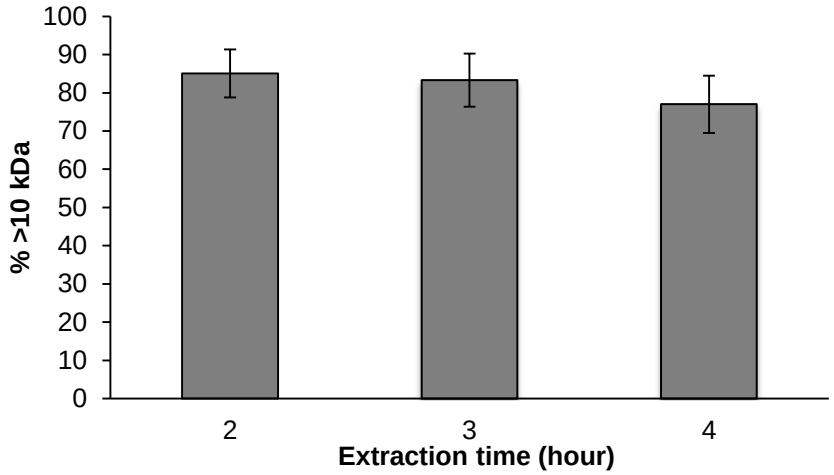

Figure 17. The >10-kDa% at different extraction times

Table 20. One-Way ANOVA of the >10-kDa% between different extraction times

Source	Sum of squares SS	Degrees of freedom ν	Mean square MS	F statistic	p-value
Treatment	646.5597	2	323.2799	6.7457	0.0025
Error	2,444.1143	51	47.9238		
Total	3,090.6740	53			

Table 21. Tukeys HSD results of the pairwise comparison of the >10-kDa% between different extraction times

Treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
2 vs 3	1.1106	0.6973998	insignificant
2 vs 4	4.9499	0.0027575	** p<0.01
3 vs 4	3.8392	0.0241026	* p<0.05

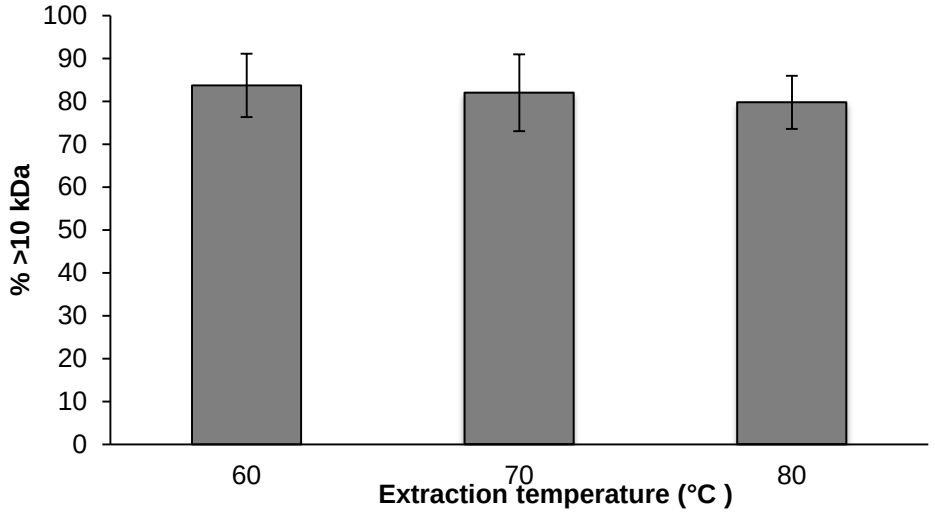

Figure 18. The >10-kDa% at different extraction temperatures

Table 22. One-Way ANOVA of the >10-kDa% between different extraction temperatures

Source	Sum of squares SS	Degrees of freedom ν	Mean square MS	F statistic	p-value
Treatment	140.9868	2	70.4934	1.2188	0.3040
Error	2,949.6872	51	57.8370		
Total	3,090.6740	53			

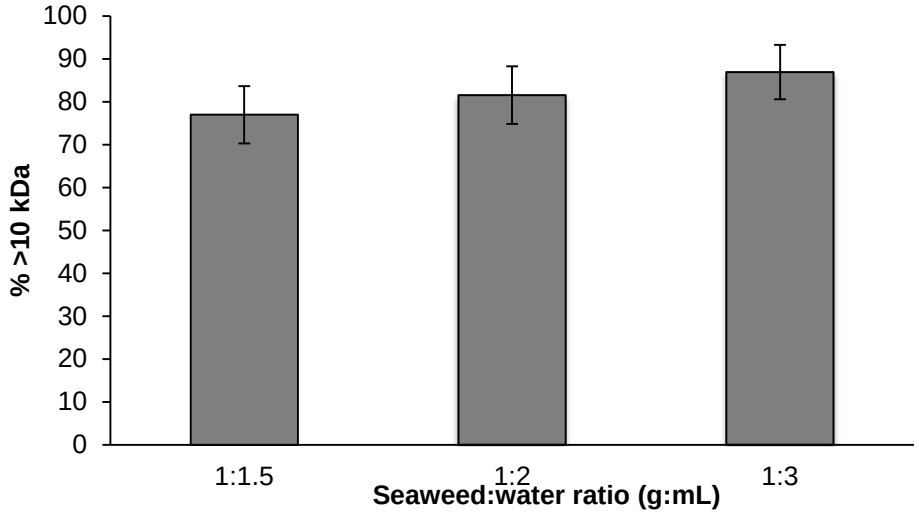

Figure 19. The >10-kDa% at different seaweed-to-water ratios

Table 23. One-Way ANOVA of the >10-kDa% between different seaweed-to-water ratios

Source	Sum of squares SS	Degrees of freedom ν	Mean square MS	F statistic	p-value
Treatment	885.9769	2	442.9885	10.2474	0.0002
Error	2,204.6971	51	43.2294		
Total	3,090.6740	53			

Table 24. Tukeys HSD results of the pairwise comparison of the >10-kDa% between different seaweed-to-water ratios

Treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
1:1.5 vs 1:2	2.9547	0.1021695	insignificant
1:1.5 vs 1:3	6.3961	0.0010053	** p<0.01
1:2 vs 1:3	3.4415	0.0477817	* p<0.05

Commercial scale production of fucoidan

Subsequent testing of collected seaweeds from P1

The eight subsamples of the seaweed that was left on the wharf for three days were slimy and smelled rotten upon arrival. The control samples from the three bins looked and smelled normal. The average fucoidan yield of the control samples was 0.93% ± 0.23SD (ww), approximately 9.3% converted to dry weight; while the delayed samples yielded 0.68% ± 0.15SD (ww) (Figure 20). There was no significant difference between the yield of fucoidan (t-test, p=0.0628). The fucose content of the control samples was 11.13% ± 4.42SD (dw), while the delayed samples had 9.43% ± 1.20SD (dw) (Figure 21). Again there was no significant difference between the two lots of seaweed (t-test, p=0.3118).

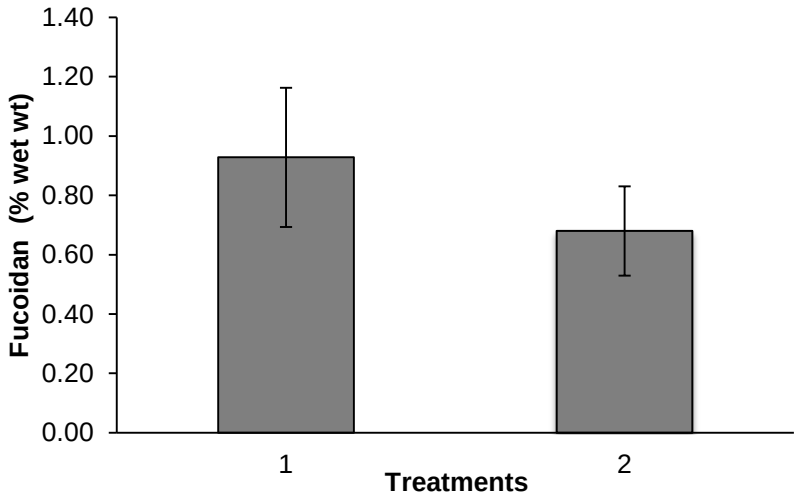

Figure 20. The fucoidan yield of the seaweed from the two treatments. 1=control, freshly harvested seaweed from the 3 bins; 2=delayed, 8 bins of seaweed left on the wharf for three days

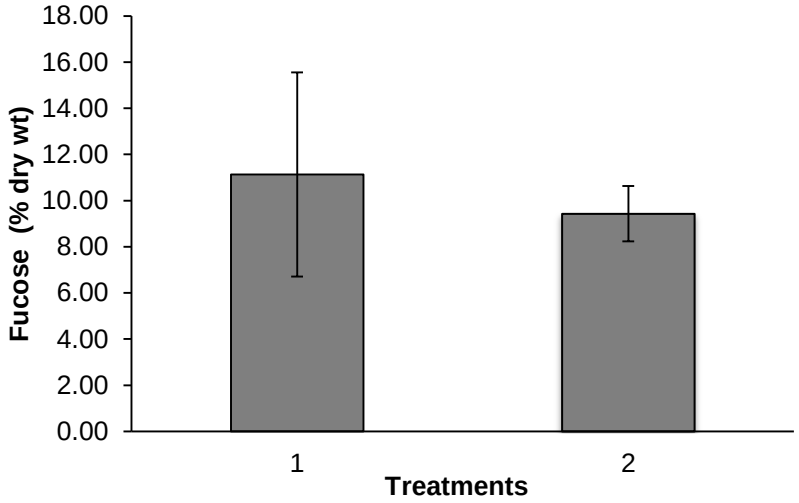

Figure 21. The fucose content of the crude fucoidan from the two treatments. 1=control, freshly harvested seaweed from the 3 bins; 2=delayed, 8 bins of seaweed left on the wharf for three days

P2 – Commercial scale extraction of fucoidan

The yield of the major fractions from both trials is summarised in Table 25. The total retentate yield between the two trials was similar, though 582 kg more seaweed was used in Trial 1. Both retentate and permeate yield increased in Trial 2, although not significant. The alginate yield was significantly lower in Trial 2 compared with Trial 1. The extract passed through the 0.25-μm filter was not measured or analysed from Trial 1.

Table 25. Product yield of the two commercial trials (wet weight). Filter=extract passed through the 0.25-μm filter

Seaweed extracted		Product	Yield	
Trial 1	Trial 2		Trial 1	Trial 2
1532 kg	950 kg	Retentate	54 kg	56 kg
		Permeate	3200 L	3983 L
		Alginate	540 kg	143 kg
		Filter	N/A	4428 L

Table 26 summarises the yields of the major fractions from Trial 1. “Total solids” were determined by freeze-drying a subsample. This is presented as both the percentage of total solids per the wet weight seaweed (% ww) and then extrapolated (from Table 25) to the total solids in each fraction, presented as Total kg. “Crude fucoidan” was determined following ethanol precipitation and freeze-drying a subsample. This is again presented both as the percentage of the fraction as well as extrapolated to the total amount in each fraction. Fucose was also measured in each fraction.

The fucoidan yield was presented as percentage per the wet-weight seaweed. The fucoidan yield from the treated retentate (0.047%) was approximately half of the total solid yield (0.076%) from freeze-dried retentate; while the fucoidan yield from treated permeate (0.161%) also decreased significantly (92%) in comparison to the solid yield of permeate (1.999%). The fucoidan yield of the supernatant from centrifuged wet alginate was 0.064%, only 5% (dw) of the total solid alginate.

The fucose content was presented as percentage per the dry-weight total solid or crude fucoidan. The fucose content of the total solids was in general lower than that of the crude fucoidan. The fucose content of the fucoidan from treated retentate (4.609%) was doubled comparing to that of the freeze-dried retentate (2.119%). The fucose content of treated permeate (0.722%) was eight times higher than that of the freeze-dried permeate (0.082%). The alginate had 5.416% fucose (Table 26), higher than that of the main product retentate.

Table 26. Fucoidan and fucose content from Trial 1. Alginate=supernatant from wet alginate after high-speed centrifuging

	Total solids			Crude fucoidan		
	% ww	Fucose (%)	Total (kg)	% ww	Fucose (%)	Total (kg)
Retentate	0.076	2.119	1.16	0.047	4.609	0.71
Permeate	1.999	0.082	30.62	0.161	0.722	2.47
Alginate	1.165	–	17.85	0.064	5.416	0.90

The fucoidan yield (via ethanol precipitation) from each step of the process in Trial 2 is summarised in Table 27, along with the projected total product calculated in conjunction with the data from Table 25, following which, the percentage loss in each step and the consecutive steps was calculated.

Table 27. Actual fucoidan yield from Trial 2 and the projected total fucoidan yield. Seaweed=combined chopped seaweed from two batches; Extract=extract before the washing step and before CaCl2 was added; Wash=extracts after the washing step and before CaCl2 was added; E+W=combined extracts before and after the washing step; Filter=extract passed through the 0.25-µm filter; Residue=seaweed residue after the extraction

	EtOH precipitate (% ww)			Total product (kg)			Loss (%)	Fucose (%)
	T2a	T2b	T2a+T2b	T2a	T2b	T2a+T2b		
Seaweed			0.823			7.82		7.54
Extract	0.607	0.249		2.73	1.30			
Wash	0.032	0.054		0.14	0.23			
E+W	0.639	0.348	0.463	2.88	1.74	4.62	40.9	
Filter			0.416			3.95	14.5	4.60
Retentate			0.061			0.58	85.3	3.18
Permeate			0.113			1.08		0
Alginate			0.009			0.09		0
Residue	0.077	0.045		0.347	0.225			10.035^{T2a} 6.131^{T2b}

Table 27 summarises the yields from each extraction step in Trial 2. "EtOH precipitate" was determined following ethanol precipitation and freeze-drying a subsample. This is presented both as the percentage of the fraction per the wet-weight seaweed, as well as extrapolated (from Table 25) to the total amount in each fraction (Total product (kg)). The loss between each step is presented as percentage the dry-weight product. Fucose was also measured in major fractions, and presented as percentage the dry-weight crude fucoidan.

The "seaweed" sample was a combination of subsamples from both batches. The crude fucoidan extracted by the generic method in our laboratory from the sample was 0.823%

and the total product 7.82 kg. The extracts of the two batches (before $CaCl_2$ was added and the washing step) yielded 0.607% and 0.249% respectively, while the fucoidan yield of the extracts following the washing step were 0.032% and 0.054% respectively. The actual total fucoidan yield from the combined extracts was 0.463%, projecting 4.62 kg of product from 950 kg seaweed, 40.9% less in comparison to the projected total product from the seaweed subsamples extracted in our laboratory. Following the filtering step through the 0.25-μm filter, the projected total product was decreased by 14.5% from the previous step. The biggest loss occurred at the final membrane filter stage, a steep 85.3% drop following the filtering step. The total projected product from permeate is more than all other products. Very little fucoidan was discovered in alginate (0.009%), and approximately 0.06% from the seaweed residue from both batches.

Chapter 4. Discussion

Placing the results in this thesis into a wider context using existing literature is challenging, as very little informaiotn has been published on commercial scale production of fucoidan. While fucoidan production is well established in the Asia-Pacific region, with companies such as Haerim Fucoidan (Korea) and Marinova (Australia) producing fucoidan of high purity and bioactivity, their techniques are held confidential and therefore not accessible. There is no existing literature on the commercial-scale production of fucoidan; nor did any of the current patents on fucoidan extraction (Nardella *et al.*, 2000; Shaklee *et al.*, 2008; Hatano *et al.*, 2009; Hagiwara, 2010) explicitly state whether their methods were applicable on a commercial scale. Information on the impact of storage/extraction conditions on fucoidan quality and quantity is also scarce. The only recent study was carried out by Wu *et al.* (2008), in which the impact of extraction time, temperature and material to water ratio on fucoidan was investigated using fresh *U. pinnatifida*. Black *et al.* (1952b) were the pioneers that examined the impact of the above plus the pH value, they compared aqueous extraction with acidic extraction and recommended the optimal extraction to be with hydrochloric acid at 70°C for 1 hour at pH 2.5.

In the present study however, aqueous extraction was used to 1) avoid potential degradation of fucoidan by incomplete hydrolysis (Black *et al.*, 1952b); 2) create clean natural market perspective; 3) reduce cost by eliminating large volume of organic solvents. Black *et al.* (1952b) suggested that aqueous extraction can be efficient if the seaweed-to-water ratio is sufficiently large and the extraction time long. Increasing the number of extractions also helps getting better yield. Pilot studies (using ethanol precipitation) at AUT have established an expected range of 0.2% – 1.5% (ww) for crude fucoidan, of which 30 – 50% (dw) is the >10-kDa fraction (A. J. Wang, 2014; A. J. Wang *et al.*, 2014). These were used as guidelines in the present study. Table 28 summarises the crude fucoidan, alginate, fucose and >10-kDa fraction yield from selected experiment in the present study, in comparison of established values from existing literature.

Table 28. Selected average crude fucoidan, alginate, fucose and % >10-kDa fucoidan fraction across all experiments in the present study, in comparison to the established values in existing literature

Percentage content	Fucoidan yield (% ww)	Alginate yield (% ww)	Fucose content (% dw)	>10-kDa% (dw)
Established values	0.19-1.6[a]	1-5.1[b]	1.7-56.7[c]	30-50[d]
Experiments				
Thawing	0.52	0.36	16	>28
Extraction conditions	0.96	1.26	-	8
P1 (control samples only)	0.93	-	11.13	-
P2 Trial 2 seaweed	0.82	0.39	7.54	-

[a]Eluvakkal *et al.*, 2010; Skriptsova *et al.*, 2010; Mak *et al.*, 2014; A. J. Wang, 2014; A. J. Wang *et al.*, 2014

[b]Black *et al.*, 1952a; Skriptsova *et al.*, 2004

[c]Black, 1954

[d]A. J. Wang, 2014

Thawing experiments

Leaving thawed *U. pinnatifida* at 4°C for up to two weeks following thawing, did not make a significant difference to the yield (p=0.7078), or quality (fucose (p=0.3009), sulphate (p=0.4986)). There was also no difference in alginate yield (p=0.4743). The average fucoidan yield from these experiments was 5.2% (dw), which was well within the range found in the existing literature (1.9% - 16% dw, Table 28), and close enough to the 9% (dw) yield found by previous AUT studies (A. J. Wang, 2014; A. J. Wang *et al.*, 2014). The yield was on the lower end of the range, which might be the result of the time of year the seaweed was harvested. The abundance of *U. pinnatifida* peaks between September and October (New Zealand spring) (W. W. Chen, 2014), while the fucoidan content decreases after September (Mak *et al.*, 2014). The seaweed in this study was collected in late November, when lower yields of fucoidan were expected. Personal observations showed that there was more fluid present at the end of one-week and two-week thaw in comparison to overnight thaw. It is safe to assume that the freezing and thawing process promotes better cell wall breakage, releasing fucoidan before the actual

extraction; hence within a reasonable amount of thaw time, the amount of fucoidan in the liquid shouldn't fluctuate significantly.

The average alginate yield across all treatments was 3.6% (dw), The average fucose content of the fucoidan from these experiments was 16% (dw), well within the range of 1.7% to 56.7% (Black, 1954, Table 28), this is also comparable to the previous studies at AUT of up to 20% from seaweed harvested in September and October (A. J. Wang, 2014; A. J. Wang *et al.*, 2014). The average sulphate content from the present experiments was 23.5% (dw), similar to that of the fucoidan from *F. vesiculosus* at 26.3% (Nishino *et al.*, 1994), and 25% (K. J. Kim, Yoon & Lee, 2012) from *U. pinnatifida*.

The distribution of the molecular weight fractions from the crude fucoidan was as expected and followed generally previous finding from the same laboratory (A. J. Wang, 2014), most of the molecules being either <10 kDa or >100 kDa, and little in between. But unlike previous studies, the larger molecules did not make up the majority, rather the smaller molecules did. None of the three treatments had any significant impact on the molecular weight composition of the crude fucoidan and the > 10-kDa fraction made up around 29% of the crude fucoidan across all treatments, close to the lower end of the 30-50% range found by A. J. Wang (2014). This, along with the fucoidan yield being at the lower end in comparison to the same study, confirmed that the seaweed harvested was past September and has less fucoidan, and thus less of the >10-kDa fraction.

It has been shown that freezing doesn't have significant impact on fucoidan quantity and quality following a previous study where seaweed was frozen for three months and thawed overnight for extraction (A. J. Wang *et al.*, 2014). However, it is not possible to rule out decay in the fucoidan and alginate content when the seaweed was left to thaw for longer than two weeks. It is therefore necessary for future studies to establish a boundary on how long the thaw length could stretch before fucoidan yield and quality were compromised.

Extraction condition experiments

Neither the length of extraction times (p=0.4056) nor the temperatures (p=0.5269) tested in this thesis had any significant impact on fucoidan yield. The former confirmed Black *et al.* (1952b)'s finding that an aqueous extraction between 3 to 7.5 hours does not impact on fucoidan yield significantly; the latter also confirmed Black *et al.* (1952b)'s recommendation of doing aqueous extractions by boiling the seaweed for up to 7.5 hours. The extraction time however, had significant impact on the alginate yield (p=0.0009) and the percentage of >10-kDa fucoidan fractions (p=0.0025). The alginate content was the lowest at 2 hours (5.7% dw), peaked at 3 hours (21.5% dw) and reduced significantly to 10.5% (dw) at the end of a 4 hour-extraction. The lowest alginate yield in this set of experiments was higher in comparison to the highest alginate yield from the thawing experiments (3.8% dw); however was still lower when compared to the 10 – 51% (dw) range in existing literature (Table 28). On the other hand, the alginate yield from 3 and 4 hour-treatments were within the established range. Given the relatively large standard deviation, further experiments are recommended to confirm these results.

The average fucoidan yield across all treatments was 9.5% (dw), which is well within the established range in existing literature (1.9% – 16% dw) (Table 28), exceeding the 9.3% (dw) yield of seaweed control samples from P1 (Table 28), almost doubled the average yield of 5.2% (dw) from the thawing experiments. Evidently the fucoidan content from seaweed harvested in early November is significantly higher than that of the seaweed from late November. A decrease was observed in the >10-kDa% as the extraction time increased. The highest percentage occurred when the extraction was 2-hour-long (85.1% ± 6.26SD dw), the decrease was moderate up to 3 hours (83.28% ± 6.95SD dw), then a quick drop of over 10% at the end of 4 hours (77.02% ± 7.5SD dw). Given the >10-kDa fraction is what considered to be the "true" fucoidan, and any molecules less than 10 kDa were mostly likely be free sugars and other polysaccharides such as laminarin, keeping the extraction longer than 3 hours is not recommended. This agreed with the optimal

extraction time of 2.5 hours using *U. pinnatifida* and similar aqueous extraction protocol recommended by Wu *et al.* (2008).

There were a few recommendations of what the optimal temperature for aqueous extraction of fucoidan should be. Black *et al.* (1952b) recommended boiling seaweed at 100°C for 3 to 7.5 hours in aqueous extraction, and 70°C in acid extractions; Wu *et al.* (2008) suggested 76°C for 2.5 hours using fresh *U. pinnatifida*. In the present study, 100°C was not used as there was reported loss in other polysaccharide by-products such as laminarin when the extraction temperature exceeded 70°C (Zha *et al.*, 2012). With Wu *et al.* (2008)'s recommendation in mind, we tested the impact of three extraction temperatures: 60, 70 and 80°C. As mentioned above, none had any significant impact on the fucoidan yield, nor the >10-kDa% ($p=0.3040$). The temperatures however did have a significant impact on the alginate yield ($p=0.0001$). The lowest alginate yield was found at 60°C (6.6% dw), following a similar pattern in the extraction time experiment, the alginate yield was the highest at 70°C (23% dw) and decreased at 80°C (8.1% dw), which confirmed Zha *et al.* (2012)'s observation, that deterioration of certain polysaccharides occurs when temperature exceeds 70°C. It is likely that the optimal extraction temperature for alginate is 70°C. However, since temperature doesn't seem to have any significant impact on the yield of fucoidan, the principal product; and that higher temperature raises the production cost on a commercial scale, a temperature range of 60 – 65°C is perfectly adequate for fucoidan production on a commercial scale.

The ratio between fresh seaweed and water did not have any significant impact on the alginate content ($p=0.1837$), however had the greatest impact on fucoidan yield ($p<0.0001$) and the percentage of >10-kDa fraction ($p=0.0002$), which contradicts Wu *et al.* (2008)'s findings, that ratio was not as important a factor than time and temperature, and that increasing the seaweed-to-water ratio doesn't necessarily increase the yield of fucoidan. Their recommendation of the optimal ratio being 1:6.5 (fresh seaweed to water) was also different significantly from Black *et al.* (1952b), in which 1:10 (dried seaweed

to water, equivalent to 1:1 fresh seaweed to water) was recommended as the optimal ratio. Based on these findings, I tested the impact of three seaweed-to-water ratios: 1:1.5, 1:2 and 1:3. I used fresh seaweed in all the experiments, for in commercial scale production, the seaweed is more likely to be fresh or thawed to reduce the cost from drying. It was when the extraction was performed at the largest ratio 1:3, that the most fucoidan (10.6% dw) and the >10-kDa% (86.88% dw) were found, the two most important factors we consider for a successful production. The difference was significant in both cases. Increasing the ratio to 1:6.5 as Wu *et al.* (2008) recommended might increase the yield even more, however heating significantly larger volume will increase the production cost exponentially. It is worth mentioning that the >10-kDa% (~80%) in these experiments was significantly higher than that from the thawing experiments (>28%) and previous studies at AUT (30-50%). Fucoidan content from seaweed harvested in early November is significantly higher than that from seaweed harvested in late November in this set of experiments, perhaps explaining the high >10-kDa% as well, as no previous studies have examined *U. pinnatifida* harvested in November in New Zealand.

Commercial scale production of fucoidan

Subsequent testing of collected seaweeds from P1

Although the eight delayed subsamples might have been expected to be substandard, as they arrived with rotten smell and unpleasant texture, there was no significant decrease (t-test, p=0.0628) in fucoidan yield between these samples and the control samples, which had been frozen straight after harvest, and smelled normal. The average fucoidan yield of the control samples was approximately 9.3% (dw), while the delayed samples yielded 6.8% (dw). Both were within the acceptable range of 1.9 – 16% (Table 28), this result also agreed with the fucoidan yield from extraction condition experiments, where 9.5% (dw) fucoidan was produced on average.

The fucose content did not differ significantly either (t-test, p=0.3118) between control samples (11.13% ± 4.42SD dw) and the delayed samples (9.43% ± 1.20SD dw). However, the fucose content was on the lower end of the scale of 1.7 – 56.7%, which was suggested by Black (1954), as well as the 20% suggested by a previous study at AUT (A. J. Wang, 2014). The low fucose content can be explained by its natural fluctuation over seasons (Black, 1954), the seaweed used in present study was harvested later in the year past the reproductive stage, hence the low fucose level.

P2 – Commercial scale extraction of fucoidan

Trial 1 was not successful in producing the amount of fucoidan that was expected from the amount of algae extracted. While the actual fucoidan content of the seaweed was not measured at the start of the trial, assuming a 0.9% (ww) crude fucoidan yield (as established in P1) it is reasonable to expect that at least 3.9 kg of fucoidan could have been extracted from this seaweed. Instead only 0.72 kg of fucoidan was collected in the retentate, an 81.5% loss. Following this lack of success, a number of changes were made; the most important was actually being at the plant while the process was taking place. The changes made for Trial 2 were a) increasing the water-to-seaweed ratio and b) "washing" the cooked seaweed in a further 1000 L of water and c) dewatering the alginates more thoroughly to lower losses at this stage by hanging the wet alginate in a sack overnight.

Even with these modifications, Trial 2 was also unsuccessful in producing the expected amount of fucoidan from the membrane filter (found in the retentate). However, by taking samples at each step along the way, it was possible to determine exactly where the losses were occurring. In terms of quantity, the actual fucoidan content of the seaweed extracted in T2 was measured in our laboratory (0.823% ww, Table 27), and extrapolating from this, at least 7.8 kg of fucoidan was expected to be extracted from the total 950 kg of seaweed. Following the process through, it is clear that there were major differences

between the first extraction (T2a) and the second one (T2b), with much more fucoidan in the extract of the former. This was due to (suspected) operator error (discussed in more detail below). Extrapolating from the fucoidan found in the T2a extract to the entire 950 kg of seaweed, this would have yielded ~6.07 kg crude fucoidan, not that much less that the 7.8 kg from the lab scale extraction, and potentially an acceptable loss due to the scale up factors. So the extraction time, temperature and seaweed-to-water ratio were sufficient for good recovery of the fucoidan.

The wash steps were also successful, delivering a potential 0.41 kg of crude fucoidan between both T2a and T2b. The changes to the way the alginates were precipitated in Trial 2 (further dewatering of the alginates overnight) was very successful, in that the alginate water (this was the supernatant from the alginate residue centrifuged at high speed in the laboratory) in Trial 2 contained 0.009% fucoidan as opposed to 0.064% in Trial 1. While there were some further losses of fucoidan in the filtration step (~14.5% loss from preceding step), the main loss was when this final extract was passed through the final 10 kDa membrane. At this stage there was an 85.3% drop from the preceding stage, ending up with a projected 1.08 kg of crude fucoidan, some 92% less than expected from the total 950 kg of seaweed.

The very low yield from the T2b trial is most likely because of the thawing process. I was not on-site to supervise the second extraction as it was undertaken overnight, but the following morning the operator told me he had changed some of the parameters to make the seaweed thaw more quickly. In order to both thaw and extract the seaweed, the seaweed/water mixture was drawn into a hose and circulated through a boiler before coming back into the extraction vessel. To kick-start the defrosting process, the operator had set the boiler temperature to 100°C, and gradually dialled it down to the optimal extraction temperature 60 – 65°C. While this accelerated the defrosting process, the seaweed was not heated as a homogenate. Seaweed that was defrosted earlier passed through the boiler at high temperatures repeatedly for unknown amount of time, this has

led to fucoidan yield decay, as been proven by the results from T2b, in which the majority of the seaweed went through a defrosting process that was actually a extraction process at well over 80°C overnight.

Conclusions

In this thesis, I examined several aspects of fucoidan extraction that needed to be investigated before large-scale commercial production of this important polysaccharide is undertaken. First, I found that fucoidan is very resilient, with little change in the yield or quality even after being frozen, thawed and stored at 5°C for up to two weeks. This is an important finding if the seaweed is to be harvested and stored frozen prior to extraction. Second, I found that extraction time and temperature had little impact on the yield or fucoidan, but that, at least when using non-dried seaweed, the yield increased with an increase in the water: seaweed ratio up to a ratio of 1:3. Further experiments need to be carried out to see if this trend continues with even more water and then this needs to be modelled against the cost of heating more water in relation to the extra fucoidan produced. Finally, I attempted to scale up from a lab scale to a commercial scale to produce fucoidan. While this was unsuccessful in terms of producing the fucoidan by attempting to collect the fucoidan on a membrane, the extraction of the fucoidan and precipitation of alginate stages were successful and, if coupled with a different fucoidan collection/precipitation method, will lead to successful production of large quantities of fucoidan.

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