Advanced and integrated technologies for the valorization of South Australia's unique seaweed resources

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Contents

Executive summary

Australia's southern coastline is a biodiversity hotspot for seaweed, with up to 1,500 described species, of which approximately 62 % are endemic to the region. South Australia also has close trading ties with Asia, and strong technological capabilities, and is therefore well-placed for the advanced manufacturing of seaweed-derived products. However, the seaweed industry in South Australia is currently limited to the small-scale manufacture of agricultural commodities from beach-cast and imported algae. We set out to assess the potential of South Australia's seaweed resources as feedstocks for higher-value products, and to develop advanced and integrated processing technologies for their valorization.

Ecklonia radiata was selected as the model feedstock due to its abundance, potential for aquaculture, and possession of compounds of commercial interest. The primary aim was the extraction of fucoidans due to their high commercial value. When the kinetics of a classical extraction process were studied, it was found that only 22% of the total available fucoidan was extracted from *E. radiata*, accompanied by a gradual reduction in purity, cleavage of sulfate groups, and rapid depolymerization

The pre-treatment of algae was trialed using enzymes and ionic liquids, with the aim of disassociating the fucoidans from other components that may hinder their extraction. However, no significant improvements in yield were observed. It was thus hypothesized that a biorefinery approach may be necessary for the comprehensive valorization of seaweed biomass.

A sequential extraction process was devised, based around the acidic extraction of fucoidans and the sodium carbonate extraction of alginates. The acidic treatment was considered to be a critical step, as it served as both an extractant for fucoidan and an important pre-treatment for alginate extraction. Therefore, response surface methodology and desirability functions were used to predict the best overall process for improved fucoidan yield and the high-yielding sequential extraction of high molecular weight alginates.

The optimized process was applied to three other brown algae: *Durvillaea potatorum*, *Seirococcus axillaris*, and *Macrocystis pyrifera*, and the products were assessed for key indicators of value. The fucoidans from *E. radiata* demonstrated the ability to stimulate the proliferation of human skin fibroblasts; the alginates from

S. axillaris had strong gel-forming capacity; and the alginate extract of *M. pyrifera* was lightly colored and highly viscous.

Finally, a techno-economic analysis was performed to assess the potential industrial production of fertilizers, fucoidans and alginates in South Australia. The integrated production of fucoidans and fertilizers from *M. pyrifera* was predicted to be the most profitable option. In a scenario of limited biomass availability, the project could break even if a minimum of 140 dry tonnes of feedstock could be accessed annually.

The studies outlined in this thesis are expected to guide decision making, and facilitate the development of sustainable, yet profitable marine-based industries in South Australia and elsewhere. Hopefully, this will stimulate investment in regional communities, and allow them to participate internationally in the emerging “blue economy”.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed.....

Date.....

Acknowledgements

This thesis has been pieced together with the invaluable help and support of a multitude of dedicated peers, from my professional and personal networks alike. It is with the most grateful appreciation that I acknowledge their various contributions here. (Please note that I will describe the specific involvement of co-authors in the contextual statements at the beginning of each chapter.)

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Thank you.

1. Introduction

This introductory chapter which contains a review of the literature was published as a review article in the Journal of Applied Phycology (Vol. 25(3), pp. 717-732; first page shown in Appendix 1.1). This journal has proven to be the most relevant source of literature in the field of study outlined in this thesis. Since publication, the article has accrued 9 citations to date, and has been heavily referenced in a South Australian Government report entitled, *Development of a marine macroalgae industry in South Australia*.

The article was published in a species issue of the journal, dedicated to covering the research presented at the 8th Asia-Pacific Conference on Algal Biotechnology, held in Adelaide, Australia, in July of 2012. At that conference, I gave an oral presentation in which I presented some of the key information outlined in this article.

Author contributions: W.Z. and R.T both played advisory roles, and helped edit the draft before and during the journal peer-review process. I performed the literature search and analysis, and wrote all of the primary content.

Potential products from the highly diverse and endemic macroalgae of Southern Australia and pathways for their sustainable production

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1.1 Abstract

Macroalgae provide a substantial and renewable resource that can be sustainably utilized for economic and social benefit. A US$7 billion global industry already exists for macroalgae, but the huge majority of this is based on the production of species belonging to approximately six genera, within eight countries, for the manufacture of foods, industrial biomaterials and agricultural products. However, seaweed-derived functional products spanning numerous chemical classes have been identified with valuable therapeutic and industrial applications.

This review focuses on the breadth of valuable bioproducts that could be produced from the seaweeds of Southern Australian – a hotspot for seaweed diversity, and the pathways available for their sustainable commercial production.

This region contains among the highest level of recorded macroalgal diversity and endemism in the world, with approximately 1200 described species, of which 62% are considered endemic. Whilst a number of these species have been shown to be rich sources of higher-value functional products, and most of them still await exploration in this field, the seaweed industry of Southern Australia is largely limited to the harvest of beach-cast biomass for the manufacture of lower-value commodities such as fertilizer and animal feed.

There is potential for the development of a substantial industry based on human functional products from seaweeds in Southern Australia. However, a number of challenges and knowledge gaps – including environmental, technological, agronomic, political, and cultural factors – are identified in this review, which must be addressed before sustainable expansion can be achieved. Furthermore, numerous strategic approaches and areas of suggested foci are underscored for

research bodies and industry alike. Particular emphasis is given to the need for comprehensive surveying and bioprospecting of the resource; a focus on advanced downstream processing capabilities for improving production efficiency and enhancing product value; the use of biorefinery approaches to improve utilization efficiency; and pursuing means of improving the sustainability of supply chains.

Keywords

Seaweed, bioprocessing, biodiscovery, commercialization, aquaculture, biorefinery

1.2 Introduction

Marine macroalgae, or "seaweeds", provide an attractive opportunity for utilization by humans. Their fast growth rates (up to half a metre per day in some species), coastal habitat and capacity for aquaculture both in the ocean and in land-based systems mean they need not compete with terrestrial crops for ever-diminishing land, fresh water or nutrient resources, making the commercialization of macroalgal products very appealing.

Southern Australia has been touted a macroalgal biodiversity hotspot (Phillips, 2001). This stretch of coastline (the Great Australian Bight) is home to approximately 1200 described species of seaweed, 62% of which appear to be endemic to the region, meaning it has the highest degree of recorded species richness and endemism on the planet (Phillips, 2001; Womersley, 1990).

Aided by a favorable climate and clean, nutrient-rich waters, the marine plants of Southern Australia grow in abundance. Meanwhile, the turbulent seas generated by frequent storms cause their regular detachment from substrate, and carriage onto beaches as “beach-cast wrack”. The harvest of attached and beach-cast seaweed has led to the development of several modest industries in Southern Australia, based on the production of hydrocolloids (initial processing only – extraction performed abroad), agricultural products such as fertilizers and animal feeds, and health products containing seaweed derived bioactive compounds (Lee, 2010).

However, Australia remains a net importer of seaweed products, with 5,000 tonnes valued at over A$17 million imported during the 2008/09 financial year (Lee, 2010). Furthermore, the huge majority of that harvested locally is being underutilized, processed only into low-value commodities. Little has been published assessing the true commercial potential of Southern Australia’s macroalgal resources, but with the

annual value of global seaweed production being estimated at approximately US$7 billion (Budarin et al., 2011), a lucrative industrial opportunity may exist.

Comprehensive taxonomic analyses have been conducted on the marine benthic flora of Southern Australia, most notably by the late Bryan Womersley who dedicated a lifetime of work to this field (1994; 1996; 1987a; 1987b) and developed a substantial collection of voucher specimens, now standing at over 90,000, held at the State Herbarium of South Australia. However, data regarding the biomass and annual production of specific species in ecosystems along the coastline, and perhaps more importantly from a commercial viewpoint, the availability of each species within the beach-cast wrack at sites of current or potential harvest, is rather scarce. Also lacking is an assessment of chemical and bioprocessing techniques available for the efficient production of higher-value products, and an analysis of the true production potential of the resources, given available means of supply.

This review aims to discuss the valuable products that could potentially be generated from Southern Australia's macroalgal resources, and to make recommendations regarding the best targets and most efficient pathways for the sustainable development and expansion of such an industry.

1.3 Macroalgal biodiversity and endemism in Southern Australia

The macroalgae of Southern Australia have been well documented and described, and the region is of principle worldwide interest in the field of phycology due to its apparent vast and diverse natural resources. Kerswell (2006) has demonstrated that according to current taxonomic data, the coastline of Southern Australia appears to be home to among the greatest number of seaweed genera in a single region in the world, as well as the highest degree of endemism.

This could be attributed to a combination of factors. Not only is it the longest Southern-facing coastline in the world, but more importantly, is the point of convergence for east and west-flowing currents, as well as the Great South Australian Coastal Upwelling System. The Leeuwin Current transports warm tropical waters from the Timor Sea east along the Southern coastline, where it is met by the cold, nutrient-richer waters of the Flinders Current, moving from east to west and inducing an upwelling (Kerswell, 2006). These flows disperse macroalgal species into the region, whilst preventing their migration out, thus resulting in their genetic isolation (Phillips, 2001). Finally, the region has been stable for millions of years,

with no recent mass extinctions (Phillips, 2001).

Of the three major classes of macroalgae, the red algae show particularly high diversity and endemism in Southern Australia. Womersley (1990) observed that of the 4010 species of red algae described worldwide at the time, over 800, or 20% were found in Southern Australia. Furthermore, of those species, 75% were considered endemic to the region. Relatively high diversity and endemism was also noted within the brown algae (231 species, 57% of which were considered endemic), although the trend does not continue within the green algae (124 species, 30% of which were considered endemic), except within the genera *Caulerpa* and *Codium* (Womersley, 1990). Particularly well represented orders are listed in Table 1.1.

Table 1.1. Particularly well represented orders of macroalgae in Southern Australia (Womersley 1990)

Class	Order	Genera	Species
Chlorophyta	Bryopsidales	Caulerpa	19
		Codium	16
Phaeophyta	Fucales	15	61
	Chordariales	27	44
	Dictyotales	15	41
	Sphacelariales	6	26
	Ectocarpales	13	21
	Sporochnales	8	12
Rhodophyta	Ceramiaceae	53	199
	Rhodomelaceae	59	167
	Gigartinales	46	122
	Cryptonemiales	49	105
	Nemaliales	21	62
	Delesseriaceae	24	50
	Rhodymeniales	17	40
	Dasyaceae	3	32
	Gelidiales	5	9

From a commercialization point of view, Southern Australia's high species diversity makes it an attractive location for bioprospecting, and promising species could be utilized at an industrial scale once their cultivation can be established in Southern Australian aquaculture systems. However, in terms of more immediate applications, it is important to pay particular attention to the most abundant species, for which "resource management" (sustainable wild harvest) utilization, or initial pilot-scale processing, could be developed. Although the red seaweeds show the highest level

of biodiversity in Southern Australia, the brown algae account for the largest fraction of surveyed biomass productivity due to their large morphology, fast growth rates and canopy-forming nature that enables domination of benthic regions.

Meanwhile, beach-cast wrack may provide opportunities for low-cost harvesting, as it can be obtained without the use of marine vessels and machinery. Its composition varies with location, time of year, and even the depth of swell leading to its detachment from the substrate at any given time. An appropriate wrack to consider, however, is that at Rivoli Bay, Beachport, SA, as significant volumes of macroalgae are deposited throughout the year, and it is easily accessible and already commercially harvested under license for the production of low value products such as fertilizer and animal feeds. According to quantitative surveys conducted by the author's group (not published) from June through October of 2011, the most prolific species were *Ecklonia radiata* (C.Agardh) J.Agardh*, Scytothalia dorycarpa* (Turner) Greville*, Cystophora spp*. (including *C. moniliformis* (Esper) Womersley & Nizamuddin and *C. platylobium* (Mertens) J.Agardh), *Durvillaea potatorum* (Labillardière) Areschoug and *Macrocystis angustifolia* Bory de Saint-Vincent. Of the red algae, which made up an estimated average proportion of approximately 7% of the wrack, the most abundant species included *Callophyllis lambertii* (Turner) Kützing and *Hymenena affinis* (Harvey) Kylin. Whilst green algae were encountered, usually belonging to the genera *Codium* or *Caulerpa*, they accounted for a negligible proportion (<1%) of the wrack, once again probably due to their small morphology and scarcity in the nearshore waters.

The prospects for the commercialization of each of these resources (genetic, attached, and beach-cast) will be assessed later in the article.

1.4 Higher-value products potentially sourced from Southern Australian macroalgae

Edible whole seaweeds for human consumption are estimated to account for about 80% of the US$7 billion a year global seaweed industry (Lee, 2008; McHugh, 2003). Meanwhile, macroalgae have also long been used in the agricultural industry to aid in the growth and health of plants and animals alike. However, extensive descriptions of macroalgal food products and their health benefits (Fleurence, 1999; Holdt & Kraan, 2011; Kumari et al., 2010; Mabeau & Fleurence, 1993) and discussions of the benefits conferred to plants and livestock by seaweed products (Craigie, 2011; Khan et al., 2009) have been provided in the past.

These whole-biomass food and agricultural products, however, are generally lower-value commodities. Industrialized countries (particularly those with high labor costs) like Australia are typically more competitive in the manufacture of higher-value products (Winberg et al., 2009), through which advanced downstream processing capabilities can be utilized, which will therefore be the main focus of this review.

The extraction and purification of functional polysaccharides, which make up the bulk of seaweed biomass, has thus far enjoyed the most interest in the field of advanced products manufacturing, but some proteins and secondary metabolites have also shown promising commercial prospects, usually due to their diverse range of biological activities. Ioannou and Roussis (2009), who have provided one of several extensive discussions of bioactive compounds from macroalgae (Holdt & Kraan, 2011; Stengel et al., 2011), stated as recently as 2009 that seaweed metabolites accounted for approximately 30% of all marine natural products isolated, with 3,280 structures reported at that time. Discussed herein are the major groups of seaweed-derived compounds that have been successfully commercialized, or appear to be good candidates for future commercialization. Southern Australian species which have been proven to be valuable sources of those compounds, or are closely related to such species, have been summarized in Table 1.2.

Table 1.2. Documented and potential Southern Australian sources of macroalgal products with current or anticipated commercial significance

Compound	Applications	Well documented sources in Southern Australia	Potential Southern Australian sources
Fucoidan	Anti-coagulant Anti-cancer Anti-aging Contraceptive Microbicidal Anti-inflammatory	*Scytosiphon lomentaria* *Macrocystis pyrifera* *Undaria pinnatifida* *Durvilleae antarctica*	Most brown algae, most notably: *Chordaria cladosiphon* 2 *Cladosiphon* spp. *Macrocystis angustifolia* *Durvilleae potatorum* Many *Sargassum* spp. *Ecklonia radiata*
Alginate	Thickener and emulsifier Fire and water proofing Paper and textile sizing Drug delivery systems	*Durvilleae potatorum* *Durvilleae antarctica*	Various kelp, most notably: *Ecklonia radiata* *Macrocystis angustifolia*
Carrageenan	Thickener and emulsifier Lubricant Anti-viral		Various red algae, most notably: >6 *Gigartina* spp.
Agar/agarose	Gelling agent Thickener and emulsifier	*Gracilaria chilensis* *Pterocladia lucida*	Certain red algae, most notably: ~*6 Gelidium* spp. ~7 other *Gracilaria* spp. 2 other *Pterocladia* spp.
Ulvan	Anti-viral Anti-coagulant Anti-hyperlipidemic Immuno-stimulating Anti-cancer	*Ulva lactuca* *Ulva rigida*	Ulvales, including: ~10 other *Ulva* spp.
Phycoerythrin	Fluorescence	*Corallina officinalis*	Various red algae, most notably: >2 other *Corallina* spp. >7 *Gracilaria* spp.
Haloperoxidases	Halogenating organic compounds	*Ulva compressa*	Diverse algae, most notably: ~10 other *Ulva* spp. Several *Corallina* spp.
Terpenoids	Anti-cancer Anti-fouling agents Pest-controlling agents Anti-viral		Diverse algae, most notably: ~14 genera belonging to Dictyotaceae >7 *Laurencia* spp.
Polyphenols	Anti-fouling agent Anti-cancer Anti-bacterial Skin-whitening		Various algae, especially brown algae, most notably: Many *Cystophora* spp. *E. radiata*
Fucoxanthin	Anti-obesity Anti-aging		Many brown algae
Halogenated compounds	Anti-fouling agent Anti-cancer Anti-bacterial	*Sargassum fallax*	Mostly red, some brown, few green algae, most notably: >7 *Laurencia* spp. 2 *Dictyopteris* spp.
Mycosprine-like amino acids	UV protection		Various red algae inhabiting the eulittoral zone

Table 1.3. Compounds or crude extracts isolated from endemic and cosmopolitan algae collected in Southern Australia
Distibution according to AlgaeBase (Guiry & Guiry, 2012). N.d. denotes 'not determined'.

Taxonomic class	Species	Distribution	Compound	Application	Reference
Brown algae	*Phyllospora comosa*	South Eastern Aus.	Fatty acid extract	Protein kinase A inhibitors	(Zivanovic & Skropeta, 2012)
	Sargassum sp.	N.d.	Fatty acid extract	Protein kinase A inhibitors	(Zivanovic & Skropeta, 2012)
	Notheia anomala	Southern Aus. & N.Z.	A new *cis*-dihydroxytetrahydrofuran	Inhibitor of the larval development of parasitic nematodes	(Capon et al., 1998)
	Perithalia caudata	Southern Aus.	New isoprenylated phenols	N.d.	(Blackman et al., 1988; Rochfort & Capon, 1994)
Red algae	*Caloglossa leprieurii*	Cosmopolitan	MAA's (especially high in Porphyra-334)	Ultraviolet sunscreen compounds	(Karsten et al., 2000)
	Laurencia filiformis	Cosmopolitan	Five new pargueranes (brominated diterpenes)	Anticancer	(Rochfort & Capon, 1996)
	Prionitis linearis	Cosmopolitan	Fatty acid extract	Protein kinase A inhibitors	(Zivanovic & Skropeta, 2012)
	Corallina vancouveriensis	Cosmopolitan	Fatty acid extract	Protein kinase A inhibitors	(Zivanovic & Skropeta, 2012)
	Plocamium cartilagineum	Cosmopolitan	Two new polyhalogenated monoterpenes	Toxic to brine shrimp	(Jongaramruong & Blackman, 2000)
	Plocamium costatum	Southern Aus., N.Z. & Philippines	New and known polyhalogenated monoterpenes, and a phytol derivative	Deterrence of barnacle larvae settlement	(König et al., 1999)
	Phacelocarpus peperocarpos	Southern Aus. & N.Z.	Ten new macrocyclic or halogenated y- and a-pyrones	N.d.	(Blackman et al., 1990; Kazlauskas et al., 1982; Murray et al., 1995; Shin et al., 1986)
Green algae	*Caulerpa trifaria*	Southern Aus.	Trifarin, a new terpene	N.d.	(Blackman & Wells, 1978)
	Caulerpa flexilis	Southern Aus.	New terpenoids	N.d.	(Blackman & Wells, 1978; Capon et al., 1981)
	Caulerpa brownii	Southern Aus. & N.Z.	New and known diterpenoids	N.d.	(Blackman & Wells, 1976; Handley & Blackman, 2005)
	Codium dimorphum	Southern Aus., N.Z. & Chile	Fatty acid extract	Protein kinase A inhibitors	(Zivanovic & Skropeta, 2012)
	Ulva lactuca	Cosmopolitan	Fatty acid extract	Protein kinase A inhibitors	(Zivanovic & Skropeta, 2012)
	Dictyosphaeria sericea	Southern Aus.	A new bicyclic lipid, dictyosphaerin	N.d.	(Rochfort et al., 1996)

One of the most exciting opportunities presented by the diverse and endemic macroalgae of Southern Australia, however, is the potential discovery of novel functional compound classes and applications. Biodiscovery investigations using algae collected from – and many of them endemic to – Southern Australian are listed in Table 1.3. Research in this field of biodiscovery should be actively pursued, as the greatest competitive advantage will be attained through the commercialization of a natural product not found in any other region.

1.4.1 Polysaccharides

Seaweed-derived hydrocolloids, referred to as phycocolloids, had a global worth of over US$1 billion in 2009, accounting for more than half of the market for non-whole food macroalgal products (Bixler & Porse, 2011). However, several other exciting new industrial applications for algal polysaccharides are now emerging.

Phycocolloids are high molecular weight structural polysaccharides found within the cell walls of macroalgae, which form viscous solutions when dissolved in water. Three commercially significant types exist, including the sulfated galactans agar and carrageenan from red algae, as well as alginate from brown algae, with a certain level of heterogeneity existing within each class (Stengel et al., 2011).

Agar is extracted primarily from members of the red algae genera *Gelidium* and *Gracilaria*, and occasionally *Pterocladia*, and may account for up to 35% of dry weight (Lemus et al., 1991; Marinho-Soriano & Bourret, 2003). Native agar molecules typically have a small amount of sulfate esterification (<6% w/w) (Cardozo et al., 2007) – the degree of which having profound effects on the rheological properties of the colloid formed with water (Lahaye & Rochas, 1991). In general, the lower the level of sulfate substitution, the stronger the gel formed, with those compounds completely devoid of sulfate being termed agarose. Other physicochemical factors affecting the gelling properties of agar include molecular weight, and pyruvate and methoxyl substitution (Lahaye & Rochas, 1991). Low-value agars are used as thickeners, emulsifiers, and gelling agents in foods, whilst higher-value agars (those with a more idealized physicochemical structure) are used to make solid culture media for plant and bacterial propagation, and gels for the separation and analysis of molecules in molecular biology research (Selby & Whistler, 1993).

Carrageenans, on the other hand, on occasion represent more than 60% of the plant's dry weight (Fuller & Mathieson, 1972), and have a significantly higher degree of sulfation, with sulfate typically accounting for around 20% of the polymer's weight (Li et al., 2008a). *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva*, Eucheuma spinosum* J.Agardh and *Gigartina spp*. are the most commonly used species for commercial production due to the nature and content of their carrageenans along with other factors such as their capacity for aquaculture (McHugh, 2003).

Carrageenans are the most commonly used phycocolloids in the food industry, commonly employed as stabilizers and emulsifiers. They are also often used in cosmetics, such as shampoos, skin creams and toothpastes, and a range of other gelatinous commodities including air fresheners and personal lubricants (McHugh, 2003). Aside from their rheological properties, carrageenans and other red algal galactans have also been shown to possess significant antiviral activity *in vivo* against sexually transmitted infections such as HSV-2 (Zeitlin et al., 1997), which suggests their potential application as microbicidal personal lubricants (Smit, 2004).

Given the large morphology and canopy-forming nature of many brown algae, their polysaccharides are of particular commercial interest within the natural seaweed resources of Southern Australia. Alginate, or alginic acid, the phycocolloid derived from brown algae, is a structural polymer with a backbone consisting of D-mannuronic acid and L-guluronic acid residues, often accounting for 10-30% of the plant's dry weight (Cardozo et al., 2007). It is most commonly extracted from species of the genera *Saccharina* (formally *Laminaria*)*, Macrocystis, Ascophyllum, Durvillaea* and *Ecklonia*, with the latter two (now only *Durvillaea*) being commercially harvested for this purpose in King Island, Tasmania, Australia (Lee, 2010).

As with the other phycocolloids, alginates form viscous solutions and gels when dissolved in water. They also absorb water particularly quickly, and have the ability to chelate metal ions (Cardozo et al., 2007). This broad range of characteristics lend themselves to a wider spectrum of industrial applications, including the sizing of paper and textiles, water- and fire-proofing of fabrics, an additive to dehydrated products, a thickening agent in ice creams, jellies, soups, beverages and cosmetics and also as a slow-release vehicle for drugs in the pharmaceutical industry (Lee, 2010).

Whilst currently nowhere near as commercially important as those hydrocolloidal

polysaccharides, there is a growing market for fucoidans – the heterologous family of polysaccharides typically dominated by L-fucose monomers and sulfate (and occasionally acetate) esters (Li et al., 2008a) – due to their diverse biological activities. The fucoidan content of brown algae varies between about 2-20% of dry weight (Ito & Hori, 1989). Anticoagulant and antithrombotic activities have thus far proven the most common and well-studied, since first being reported in the late 1950s (Springer et al., 1957). Two studies, led by Nishino (1987) and Cumashi (2007), each screened fucoidans from nine species of brown algae, and between them all except one preparation exhibited some anticoagulant activity. Mechanisms of action seem to vary, and may include direct thrombin inhibition; activation of thrombin inhibitors, most notably heparin cofactor II, but occasionally antithrombin; and the competitive binding of fibrinogen to block thrombin's action (Kuznetsova et al., 2003; Nishino et al., 1991).

Fucoidans from a very wide range of brown seaweeds have also been shown to possess antiviral, anticancer, immunomodulatory, anti-inflammatory, anti-lipidemic and other activities, all of which have been comprehensively reviewed by Li (2008a) and Jiao (2011) and their respective colleagues. Also discussed are the structural determinants of these activities, of which sulfation (content and positioning) and molecular weight tend to play the most significant roles, with monomeric composition, linkage and branching also contributing.

Similarly, interest in the laminarins from brown algae, and more-so the ulvans of green algae, has also recently increased due to their biological activities. Laminarins are the β-1→3 linked d-glucans (Read et al., 1996), usually accounting for less than 5% of the dry weight of the brown algae (Ito & Hori, 1989), whilst ulvans are sulfated heteropolysaccharide composed of uronic acids alternating with neutral sugar monomers, typically accounting for 18-29% of carbohydrate in species of the order Ulvales (Alves et al., 2012). Alves and colleagues (2012) have provided a good review of ulvan research, listing anti-viral, anti-coagulant, anti-hyperlipidemic, immunostimulating, anti-cancer and antioxidant effects, again often being accredited to the molecules' sulfation.

1.4.2 Proteins

Fleurence (1999) has provided one review discussing the composition and potential uses of seaweed proteins. In terms of protein content, that of brown algae is generally quite low (3-15% dry weight) compared with the red and green seaweeds

(10-47% dry weight) (Fleurence, 1999).

In terms of higher-value products, the proteins of macroalgae are generally of little commercial significance. However, some exceptions exist, and functional macroalgal proteins, peptides and amino acids have been extensively reviewed by Harnedy and FitzGerald (2011). Perhaps the most noteworthy use of seaweed proteins comes from the research industry's utilization of phycobiliproteins – the light-harvesting cellular machinery involved in the photosynthetic pathways of red algae and cyanobacteria. In many red algae the most common phycobiliprotein is known as R-phycoerythrin, which can account for the majority of soluble protein in the cells of some species when grown under optimal conditions (Glazer, 1994). This highly fluorescent complex serves as a fluorescent tag with applications in flow cytometry, cell sorting, histochemistry and other fields, all of which are discussed in detail in the review by Glazer (1994). Phycoerythrin has also been explored as a food dye, but this application is limited by its relative instability to heat (Fleurence, 1999).

Meanwhile, several useful enzymes have been isolated from seaweeds. Haloperoxidases are rare enzymes that catalyze the oxidation of halides in the presence of hydrogen peroxide to form hypohalous acids, which in turn react readily with a variety of organic compounds to form halogenated products (Butler & Sandy, 2009). These enzymes can be used to enhance the bioactivity of organic compounds, and thus synthesize useful pharmaceutical and industrial products. Haloperoxidases have been detected within all three major taxonomic groups of macroalgae, which use this pathway to produce volatile halogenated compounds, most likely as part of a defence and antifouling system based on their antimicrobial properties (Wever & Hemrika, 2001). Mehrtens (1994) assayed twenty one species of arctic brown, red and green macroalgae for haloperoxidase activity, detecting the highest iodoperoxidase activity in the green algae *Acrosiphonia sonderi* (Kützing) Kornmann and *Ulva compressa* Linnaeus, and the highest bromoperoxidase activity in the brown algae *Saccharina latissima* (Linnaeus) C. E. Lane, C. Mayes, Druehl & G.W.Saunders. The latter, however, is known to be particularly abundant in the red algae genus *Corallina* (common in Southern Australia), which together with the enzyme's heat resistant nature and the development of a simple and highly efficient purification process, makes commercial extraction an attractive prospect (Zhang et al., 2011a).

1.4.3 Terpenoids

Terpenoids account for many of the macroalgal cytotoxic metabolites isolated. Dehydrothyrsiferol, for instance, from the red algae *Laurencia viridis* Gil-Rodríguez & Haroun of the Canary Islands is a triterpenoid polyether that was shown in preclinical studies to induce apoptosis in human breast cancer cell lines (Pec et al., 2003). Meanwhile the sesquiterpene caulerpenyne, which is the major secondary metabolite of *Caulerpa taxifolia* (M.Vahl) C.Agardh (a genera particularly well represented in Southern Australia), exhibited antiproliferative activity against a broad range of human cancer cell lines (Barbier et al., 2001; Fischel et al., 1995).

Given their biological functions as feeding deterrents and antifouling agents, terpenoids also display toxicity against a number of organisms – both marine and terrestrial. This includes mosquito larvae, cockroaches, epiphytes, and parasitic worms, pointing towards their potential for use in household and aquaculture pest controlling agents (Smit, 2004). A number of novel terpenoids (and compounds belonging to other chemical classes) isolated from Southern Australian macroalgae have exhibited this activity (Table 1.3).

A tetraterpenoid pigment gaining particular recent interest is the brown algal carotenoid fucoxanthin, whose dietary administration has been shown to prevent and treat obesity and metabolic syndromes, thus lowering the risk of Type II diabetes. Fucoxanthin, perhaps through the increased expression of fat-catabolising protein UCPI in white adipose tissue (Miyashita & Hosokawa, 2008), has been shown to promote weight loss; body and liver fat reduction; and an increase in resting energy expenditure in mice, rats and obese women (Abidov et al., 2010; Maeda et al., 2005).

Meanwhile, fucoxanthin and other algal carotenoids have been reported to inhibit proliferation and induce apoptosis in a range of human cancer cell lines, including leukaemia, prostate cancer, colon cancer and tumor cells (Narayan et al., 2008). Furthermore, the topical application of fucoxanthin on UV-B exposed hairless mice was shown to prevent wrinkle formation and reduce epidermal hypertrophy (skin thickening, which causes wrinkle formation), pointing toward its potential application in cosmeceuticals such as skin creams (Urikura et al., 2011).

1.4.4 Halogenated compounds

Many macroalgal secondary metabolites (including many terpenoids) are halogenated, which can result in especially interesting and potent biological

activities, particularly when bromine or chlorine account for the incorporated halogens. In red algae this may be the case in up to 90% of secondary metabolites, followed by around 7% in green algae and less than 1% in brown algae (perhaps due to the preferential incorporation of iodine for halogenation) (Cabrita et al., 2010).

Cytotoxic activities are common in these compounds. One example is the polyhalogenated monoterpene, halomon, from the red alga *Portieria hornemannii* (Lyngbye) P.C.Silva was shown to produce an interesting cytotoxicity profile when screened against a range of tumor cell lines, showing preferential activity against brain, renal and colon tumor cells (Fuller et al., 1992). Meanwhile, a host of halogenated compounds with antimicrobial properties have been isolated from macroalgae – most notably those from various *Laurencia* species reported by Vairappan's group, including the particularly potent elatol and iso-obtusol from *L. majuscula* (Harvey) A.H.S.Lucas (Vairappan, 2003; Vairappan et al., 2001a; Vairappan et al., 2004; Vairappan et al., 2001b).

Another interesting application of algal halogenated compounds is for use as natural anti-fouling agents for coating boats and marine structures. An Australian group based in Sydney isolated halogenated furanones or fimbrolides from the red algae *Delisea pulchra* (Greville) Montagne (common along the South coast), which act as specific antagonists of molecules involved in quorum sensing recognition systems in bacteria, thus preventing their colonization through a non-toxic and natural mechanism of action (De Nys et al., 1995). These compounds and others like them have since been shown to also inhibit the settlement of fouling invertebrates such as barnacles (Nylund & Pavia, 2003) as well as fouling algal species (De Nys et al., 1995). Cabrita et al. (2010) have provided an extensive review of the bioactive halogenated compounds of macroalgae.

1.4.5 Polyphenols/phlorotannins

Polyphenolic compounds, particularly those of the brown algae (referred to as phlorotannins), have shown a great deal of promise as functional compounds. Whilst *Cystophora* has been the most common source of isolation, phlorotannins are also widespread in kelps such as *Ecklonia* – these being predominant genera of brown algae found along the Southern coastline of Australia.

Antibacterial activity is particularly common in the phlorotannins. The bactericidal activity of phlorotannins from *Ecklonia kurome* Okamura against food borne pathogens including methicillin-resistant *Staphylococcus aureus* Rosenbach has

been documented by Nagayama's group (2002), who observed no harmful effects in mice, illustrating the compounds' potential as a new class of antibiotic.

Meanwhile, an interesting cosmeceutical application of phlorotannins lies in their ability to induce depigmentation or whitening of the skin. This is achieved through the inhibition of melanogenesis, which is the process by which melanin is produced by cells known as melanocytes in the bottom epidermal layer of the skin, regulated by the enzyme tyrosinase (Wijesinghe & Jeon, 2011). A number of phlorotannins have been found to effectively inhibit tyrosinase, including 7-phloroeckol and dieckol from *Ecklonia cava* Kjellman (Heo et al., 2009; Yoon et al., 2009) and phloroglucinol derivatives from *Ecklonia stolonifera* Okamura (Kang et al., 2004). The potential for use of such compounds in cosmeceuticals was demonstrated *in vivo* by Cha and associates (2011), who successfully reduced pigmentation in zebra fish.

The phlorotannins have also shown promise as antifouling agents, as was demonstrated by Jennings and Steinberg (1997) who inhibited the settlement and growth of the gametes of the fouling green alga *Ulva lactuca* Kjellman with phlorotannins from *E. radiata*, which is particularly common along the Southern Australian coastline.

1.4.6 Mycosprine-like amino acids

Mycosporine-like amino acids (MAAs) are intracellular structures containing a cyclohexenimine, or less commonly a cyclohexenone ring, conjugated to one or two amino acids, that absorb UV light and thus protect aquatic organisms from solar radiation (Bandaranayake, 1998). These compounds are synthesized by a wide range of algae, particularly red algae inhabiting the eulittoral zone (Karsten et al., 1998), and have been intensively explored, along with their synthetic analogues, as topical products for protection against sunlight exposure (Bandaranayake, 1998). An MAA from the red alga *Porphyra umbilicalis* Kützing was used in the commercially available skincare product Helioguard® 365 (Cardozo et al., 2007), whilst a sample of the epiphytic red algae *Caloglossa leprieurii* (Montagne) J. Agardh collected from mangroves in Southern Australia expressed high concentrations of the MAA Porphyra-334.

1.5 Process development and value enhancing

Ideally, in order to maximize the efficiency of resource utilization, a portfolio of products should be developed ranging from high value commodities that require

complex downstream processing, purification and quality control, to those that enjoy high yields and little need for refinement, but which typically command a relatively low market price. Industrial by-products from the manufacture of the former product type (e.g. residual biomass from which higher-value metabolites have been extracted), can therefore be funnelled into the production pathway for the latter. Production efficiency can be further improved when products that share processing steps (usually at the initial stages) can be co-processed together. This can be referred to as the biorefinery concept, with a hypothetical example depicted in Table 1.4. Such an approach was exemplified by Zhang's group, who demonstrated the biorefinery extraction of agar, R-phycoerythrin and bromoperoxidase from *Gracilariopsis lemaneiformis* (Bory de Saint-Vincent) E.Y.Dawson, Acleto & Foldvik, to achieve yields of 19.1%, 0.32%, and 5.67 U per gram, respectively (Li, 2008; Li et al., 2008b; Li et al., 2008c; Li et al., 2009).

Table 1.4. Hypothetical representation of some of the value-adding pathways associated with macroalgal whole-biomass and carbohydrate products

	Harvest	Wash	Dry	Mill	Digest	Extract/ purify	Desulfate/ oversulfate	Depolymerize
Whole biomass products	Aquafeed							
	Salads							
	Preserved healthy snacks							
	Food seasonings Fertilizer Soil conditioners Animal feed							
	Liquid fertilizer							
Functional polysaccharides	Phycocolloids Bioactives							
Enhanced polysaccharides	Strong gels Agarose Hyper-activated bioactives							
Functional oligosaccharides	Plant growth & defense elicitors Bacterial growth promoters Bioactive oligosaccharides							

As mentioned earlier, the major polysaccharides of brown algae, including fucoidan, alginate, and laminarin, are all water soluble (at least in certain forms), as are agar and carrageenan of the red species. Many of them can therefore be co-extracted in water, although they are usually selectively extracted and isolated using dilute acid (for fucoidan and laminarin) or alkali (for alginate) solutions, which can be achieved sequentially (Usov & Smirnova, 2003). In either case, the cell must be disrupted, which, in the absence of strong aqueous extractants, is generally achieved with prolonged heating and often high pressures (Ale et al., 2012; Knutsen et al., 1995;

Lai & Lii, 1998; Torres et al., 2007).

Recent improvements in technology though, have led to reduced extraction times and energy requirements. For instance, microwave energy has recently been employed to great effect. Morais and associates (2010) achieved a higher yield of agar from *Gracilaria vermiculophylla* (Ohmi) Papenfuss (13.5% compared with 8.5%) using a ten minute microwave-assisted extraction compared with a two hour extraction using conventional heating. The quality of agar also compared favorably using the microwave based approach. Similarly, Rodriguez-Jasso's group (2011) used microwaves to extract fucoidan from *Fucus vesiculosis* Linnaeus with the impressive yield of 18.22% with only one minute of irradiation. The potential for the scale-up of this technology for industrial processes has been demonstrated using a continuous-flow microwave to extract carrageenan from two species of red seaweed (Uy et al., 2005).

Ultrasound has also been used to extract algal polysaccharides (Zhang et al., 2011b), along with other valuable metabolites such as the phycobiliprotein phycoerythrin (Zhu et al., 2008), an approach that has been patented in the United States (Hagiwara, 2009). Whilst the extraction times and yields achieved in these studies have not been as impressive as those using microwave irradiation, they have the advantage of maintaining relatively low temperatures, which may reduce degradation of heat labile molecules. However, a comparative study demonstrated that the carotenoid astaxanthin was less stable under ultrasound irradiation compared with that of microwaves (Zhao et al., 2006), suggesting that similar effects may result with macroalgae derived carotenoids such as fucoxanthin. Meanwhile, the degradation of polysaccharides by ultrasonic waves has been reported by Zhou and Ma (2006).

Heo (Heo et al., 2003) investigated enzymatic means of extracting fucoidans from dry algal biomass, whereby five carbohydrases and five proteases were separately applied to seven species of brown algae (belonging to the genera *Ecklonia, Sargassum, Scytosiphon and Ishige*), in order to digest unwanted materials. Whilst some of the enzymes performed very well (the carbohydrases Viscozyme, Celluclast and AMG 300L, and the protease Alcalase each digested between 27% and 43% of biomass in four or more species), scale-up of such an approach would be difficult due to the costs and operating conditions associated with such enzymes.

Following extraction, the subsequent recovery of polysaccharides is conventionally performed through their selective precipitation. For alginate, this is usually performed by adding an acid or calcium salt (McHugh, 2003), whilst fucoidan and laminarin can be precipitated with the addition of ethanol (Rodriguez-Jasso et al., 2011). However, a stepwise method for separating all three of these polysaccharides from a single source extract based on hydrophobic chromatography has also been developed (Zvyagintseva et al., 1999).

The functionality of macroalgal metabolites depends largely on their molecular structure. In the case of the polysaccharides, their monomeric composition, surface functional groups, and molecular weight are particularly important. These parameters can often be altered using processes carried out prior to, during, or after extraction, using physical, chemical or enzymatic approaches, which has been achieved in the studies listed in Table 1.5.

Table 1.5. Structural modification of algal polysaccharides yielding products with new or improved applications

Modification	Compound	Method	Potential application	Reference
Depolymerization	Agarose, carrageenan, alginate and fucoidan	Enzymatic	Anti-inflammatory and anti-oxidant	(Adachi & Vallee, 2002)
	Agars	Enzymatic	Anti-oxidant	(Wu et al., 2005)
	Agarose	Enzymatic	Prebiotic	(Hu et al., 2006)
	Sodium alginate	Chemical	Bacterial expression elicitor	(Murphy, 2011)
	Sodium alginate	Enzymatic	Plant growth elicitor	(Yonemoto et al., 1993)
	Fucoidan	Free-radical exposure	Anti-inflammatory	(Deux et al., 2002)
	Fucoidan	Enzymatic	Plant defence elicitor	(Klarzynski et al., 2003)
	Agar	Microwave irradiation	Soft gel	(Sousa et al., 2012)
	Fucoidan and laminarin	Gamma irradiation	Anti-oxidant	(Choi et al., 2009)
	Laminarin	Enzymatic	Anti-apoptotic	(Kim et al., 2006)
Hypersulfation	Fucoidan	Chemical	Anti-coagulant	(Nishino & Nagumo, 1992)
	Fucoidan	Chemical	Anti-cancer	(Cho et al., 2010)
	Carrageenan	Chemical	Anti-coagulant	(Opoku et al., 2006)
	Laminarin	Chemical	Plant defence elicitor	(Ménard et al., 2005)
Desulfation	Carrageenans, an agar-like corallinan, and a fucoidan	Chemical (microwave intensified)	Various	(Navarro et al., 2007)
	Agar	Enzymatic	Strong gel	(Shukla et al., 2011)
	Agarans and carrageenan	Chemical	Strong gel	(Kolender & Matulewicz, 2004)

As an example, the degree of sulfation plays an important role in the functionality of many seaweed derived polysaccharides. The strength of the gels formed by phycocolloids is improved with reduced sulfation (Murano, 1995), whereas the bioactivity of many fucoidans is enhanced with increased sulfate (Li et al., 2008a). Physical alteration has been demonstrated by Navarro's group (Navarro et al., 2007) who desulfated the pyridium salts of carrageenan, agar and fucoidan using microwave irradiation, whilst Nishino and Nagumo (1992) used a chemical approach to achieve the over-sulfation of fucoidan.

Meanwhile, in the case of molecular weight, enzymatic depolymerization has been used to great effect for the highly controlled production of more functional oligomers from macroalgal polysaccharides (Wang et al., 2006; Yonemoto et al., 1993). Chemical and physical methods have also yielded active compounds (refer Table

1.5), but these approaches tends to be less reproducible. An interesting new prospect lies in the microwave intensification of enzymatic modification, made possible by recent improvements in microwave technology, and the development of heat-stabilized enzymes (Rejasse et al., 2007), although such an approach has yet to be applied to macroalgal polysaccharides.

1.6 Production potential and challenges of Southern Australian macroalgae

With such a vast natural renewable resource, the wild harvest of South Australian macroalgae for the production of higher-value algal products seems attractive, but significant barriers may restrict the commercial viability of such an endeavor.

Most importantly, seaweeds are protected by legislation to varying degrees in most Southern Australian waters due to their important ecological functions, whether attached plants, unattached drift, or beach-cast wrack. In each case, they provide habitat and food (either directly or as habitat for prey) for a broad range of different organisms, including epiphytes, molluscs, crustaceans, fish, shorebirds and marine mammals, whilst the unattached forms also play an important role in providing recycled nutrients and detritus to support further primary production (Goldberg & Collings, 2006; Kirkman & Kendrick, 1997).

The commercial harvest of attached seaweed is therefore prohibited by law, with one interesting exception. In Tasmania, the commercial exploitation of the introduced brown algae *Undaria pinnatifida* (Harvey) Suringar has been allowed, due to its status as an invasive species. Its harvest has provided not only protection to native ecosystems, but a valuable source of biomass for the extraction of fucoidan by the local marine bioactives company Marinova Pty Ltd (Lee, 2008). This exemplifies one clever approach for making resource management eco-friendly.

Licences for the harvest of beach-cast wrack have been granted in more instances. *D. potatorum* is collected in King Island, Tasmania, where it is dried and milled, then primarily shipped to the UK for alginate extraction, and deposits of beach-cast macroalgae (mixed species) are harvested by a handful of small operations along the Southern coast of the mainland for the production of fertilizers, soil conditioners and animal feeds (Lee, 2008). In each case only a small percentage of deposited seaweed is generally taken, environmental impacts are carefully monitored and strict regulations are in place (PIRSA, 2003).

The redirection of the already harvested resource toward the manufacture of higher-value products is of course desirable. However, in most cases (with the exception of King Island) the beach cast wrack contains a wide variety of species in a densely entangled mass – a major impediment when one considers that most higher-value compounds appear within a relatively narrow gene pool. In such situations, this means the seaweeds would either need to be sorted prior to extraction, or comprehensive downstream fractionation would be required to remove the mass of unwanted co-extracts – each being laborious and expensive tasks.

In order to overcome such a production barrier, careful quantitative and qualitative surveying of the resource is imperative in order to allow for the identification of species and target products best suited to commercialization. A species' aptness for utilization will be improved if it has a high affinity for isolation or target harvesting (those that make up a high proportion of the biomass, have a large morphology, or deposit onto the beach at a different time or under different conditions to other species); or capacity for biorefinery-type extraction of multiple valuable compounds. Meanwhile, target compounds will show greater promise if present in a relatively broad range of taxa, reducing the need for isolation during harvesting and allowing the industry to be less sensitive to changes in wrack composition, due to habitat migration resulting from global warming for instance.

Efforts to produce certain higher-value compounds (e.g. drugs, nutraceuticals and cosmeceuticals) from beach-cast seaweeds may be further marred by concerns regarding product traceability and reproducibility, and the potential for contamination, which may cause government administrations to be wary of awarding product approvals. In some cases, very regular and thorough product analysis may suffice. Alternatively, it may be wise to avoid such added costs by producing commodities with more relaxed safety requirements, such as value-added agricultural products and industrial metabolites.

In any event, environmental impacts should be held paramount, and there is a significant possibility that wild resources would not withstand large scale industry expansion. It should therefore be pre-empted that wild resources are utilized – with the utmost scrutiny with regard to their ecological importance – only for small scale operations and initial industry start-up and development processes (e.g. verification of production and scale-up processes and market characterization). Significant expansion should be accompanied by a shift toward the use of more sustainable

materials, such as cultured biomass, particularly with regard to those products manufactured from a narrow genetic resource. An additional attribute to consider when determining the most viable product to be manufactured from a wild resource should therefore be the source species' aptitude for cultivation.

Whilst Australia's production of seaweed is currently based solely on its collection from the wild, the global situation is in stark contrast, with wild catch accounting for a mere 4.5% of the 19 million tonnes produced in 2010 (FAO, 2012). That said, 98.9% of the cultivated biomass was accounted for by just a handful of taxa (*Saccharina japonica* (Areschoug) C.E.Lane, C.Mayes, Druehl & G.W.Saunders*, K. alvarezi, Eucheuma spp., Gracilaria spp., Porphyra spp., U. pinnatifida*, and unconfirmed species – mostly from China), and 99.6% was grown in just eight countries (China, 58.4%; Indonesia, 20.6%; the Philippines, 9.5%; the Republic of Korea, 4.7%; Democratic People's Republic of Korea, 2.3%; Japan, 2.3%; Malaysia, 1.1%; and the United Republic of Tanzania, 0.7%) (FAO, 2012).

The vast majority of globally cultivated seaweed is grown in the ocean, where it must be able to withstand a host of environmental challenges, including weather and water movement, competitive algae and epiphytes, and grazing animals (Titlyanov & Titlyanova, 2010). Seaweeds cultivated in this way must therefore be exceptionally robust, and the narrow range of species suited to such mass production has been identified as the most significant limitation to the development of diverse functional products from macroalgae, as has been achieved with terrestrial plants (Hafting et al., 2012).

In situ seaweed farms must be substantial in size and dispersed throughout several locations in order to be economically viable and to mitigate the risks associated with environmental crop damage, which conflicts with tourism, recreation and environmental priorities of Western developed countries (Hafting et al., 2012), and Australian legislation has therefore limited such cultivation close to the coast (Lee, 2010). Turbulent offshore seas and high labor rates further limit the viability of sea-based cultivation in Southern Australia.

Recent reports in Australia have therefore suggested that on-land seaweed cultivation in tanks or ponds is more feasible (Lee, 2010; Lee, 2008; Winberg et al., 2009; Winberg et al., 2011). This obviously has the huge advantages of consistency and controllability with respect to a full range of abiotic (temperature, salinity, pH,

UV, nutrients and minerals) and biotic (epiphytes, grazing animals, symbionts) factors. Meanwhile, the modular arrangement of land-based facilities allows for easy access and harvesting, and the application of different systems and conditions optimized to suit various species or life cycles at the one site. This makes possible the cultivation of less robust species that cannot be successfully cultivated in the open sea, and initial product development using species that aren't already commercially cultivated (i.e. can start small while a market is developed). Furthermore, quality control and traceability can be better ensured for products for human consumption (Hafting et al., 2012).

Large scale land-based commercial cultivation, however, has only truly been realized so far for *Ulva, Caulerpa, Chondrus* and *Palmaria*, whilst pilot-scale operations have shown promise for a handful of other red and green algae (Hafting et al., 2012; Lee, 2008). Brown algae have been cultivated as juveniles in land-based ponds in Asia, but typically transferred to sea-based systems prior to maturation due to their large physiology, and the existence of previously established infrastructure.

This limited commercial-scale success can be attributed mainly to the high costs associated with waterfront land (all successful operations have used marine seawater), infrastructure and energy. Seeing as its principle advantage is for the cultivation of species which are not yet produced in mass in the sea, for the manufacture of higher value products which therefore do not yet have established markets, these costs can limit profitability and form a significant barrier to initial developers (Hafting et al., 2012).

An interesting prospect lies in the potential for integrated multi-trophic aquaculture (IMTA), whereby the by-products from the cultivation of one organism can be utilized as inputs for another. The most common model for such a system involves fed finfish, whose organic and inorganic culture effluents are extracted and utilized by shellfish and seaweeds, respectively (Chopin et al., 2002). Herbivorous animals such as abalone may also be included, whereby the algal biomass is used as a feed source (Neori et al., 1998). This philosophy can be applied to both land and sea based systems, and can enable the division of infrastructure and running costs and mitigate environmental impacts (Neori et al., 2000). Whilst a lack of detailed understanding in key aspects of algal biofiltration, and difficulties in achieving true synergy between multiple complex systems have thus far prevented the widespread

commercial success of IMTA (Neori et al., 1998; Titlyanov & Titlyanova, 2010), the approach has been touted as an essential avenue for the sustainable expansion of world aquaculture (Neori, 2008). Given Southern Australia's status as a key fisheries and aquaculture region of Australia with growing industry, the existing infrastructure for complementary integrated multi-trophic aquaculture should not be ignored.

It is generally accepted that seaweeds are best suited to the seawater from their endemic environment (Lee, 2008), indicating that in order to commercialize higher-value products from Southern Australia's vast range of unique species, cultivation efforts should be undertaken locally.

1.7 Conclusion

According to current taxonomic distribution records, the macroalgae of Southern Australia display among the greatest species diversity and endemism in the world, yet the industry for algal products in the region is limited to a few small operations producing mainly lower-value fertilizers and animal feeds.

By comparative studies with literature, a broad range of the species present along the Southern Australian coastline have been identified as potential sources of metabolites that could be utilized as industrial biomaterials, functional foods and cosmetics, and pharmaceutical agents. However, when compared to those locations with an existing large-scale seaweed industry, Southern Australia - like most other industrialized Western regions - faces a very different set of challenges for industry development. Due to high costs of labour and environmental priorities, advanced processing capabilities must be developed to improve production efficiency, minimize environmental and ecological impacts, and produce higher-value products. Innovative technologies such as microwaves, ultrasound and enzymes can enable the rapid and controlled extraction and functional modification of algal metabolites, with reduced energy, water and chemical inputs. Meanwhile, biorefinery production of multiple products from the same biomass can improve resource utilization efficiency.

Furthermore, the acquisition of algal biomass must be administered carefully in order to protect delicate natural ecosystems as well as peripheral societies. Therefore, the scope for the wild harvest of attached macroalgae appears limited unless resource management is an option, or invasive species can be targeted.

Beach-cast seaweed may provide opportunities for low-cost harvesting if handling

and traceability issues can be managed, however resource management remains important due to the roles played by the wrack in shore ecosystems and nutrient cycling. Therefore, the scale of such harvesting operations should be limited, with large-scale expansion being met by a gradual shift toward more sustainable biomass production.

Ideally, such a shift would involve the development of seaweed aquaculture initiatives. Whilst large scale sea farms have been most successful in other regions of the world, tourism and recreation priorities, environmental concerns, and logistical problems associated with the deep, turbulent coastal waters of Southern Australia would need to be resolved. Offshore aquaculture, with carbon and waste assimilating potential could provide one solution.

Cultivation in land-based tanks and ponds could see the commercialization of a vast range of species, including those that are neither prolific in wild fisheries nor robust enough for *in situ* sea farming, thus allowing Southern Australia to benefit from its genetic resources. Meanwhile, integrated aquaculture of seaweeds with marine animals has the potential to resolve problems associated with the monoculture of both classes, whether in the sea or on land, which may be facilitated by a better understanding of biotic interactions, as well as cooperation between research, industry and government institutions.

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1.10 Appendix 1.1. First page of published article

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Potential products from the highly diverse and endemic macroalgae of Southern Australia and pathways for their sustainable production

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Abstract Macroalgae provide a substantial and renewable resource that can be sustainably utilized for economic and social benefit. A US$7 billion global industry already exists for macroalgae, but the huge majority of this is based on the production of species belonging to approximately six genera, within eight countries, for the manufacture of foods, industrial biomaterials and agricultural products. However, seaweed-derived functional products spanning numerous chemical classes have been identified with valuable therapeutic and industrial applications. This review focuses on the breadth of valuable bioproducts that could be produced from the seaweeds of Southern Australia—a hotspot for seaweed diversity, and the pathways available for their sustainable commercial production. This region contains among the highest level of recorded macroalgal diversity and endemism in the world, with approximately 1,200 described species, of which 62 % are considered endemic. Whilst a number of these species have been shown to be rich sources of higher-value functional products, and most of them still await exploration in this field, the seaweed industry of Southern Australia is largely limited to the harvest of beach-cast biomass for the manufacture of lower-value commodities such as fertilizer and animal feed. There is potential for the development of a substantial industry based on human functional products from seaweeds in Southern Australia. However, a number of challenges and knowledge gaps—including environmental, technological, agronomic, political, and cultural factors—are identified in this review, which must be addressed before sustainable expansion can be achieved. Furthermore, numerous strategic approaches and areas of suggested foci are underscored for research bodies and industry alike. Particular emphasis is given to the need for comprehensive surveying and bioprospecting of the resource; a focus on advanced downstream processing capabilities for improving production efficiency and enhancing product value; the use of biorefinery approaches to improve utilisation efficiency; and pursuing means of improving the sustainability of supply chains.

Keywords Seaweed · Bioprocessing · Biodiscovery · Commercialisation · Aquaculture · Biorefinery

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Introduction

Marine macroalgae, or "seaweeds", provide an attractive opportunity for utilisation by humans. Their fast growth rates (up to half a metre per day in some species), coastal habitat and capacity for aquaculture both in the ocean and in land-based systems mean they need not compete with terrestrial crops for ever diminishing land, fresh water or nutrient resources, making the commercialisation of macroalgal products very appealing.

Southern Australia has been touted a macroalgal biodiversity hotspot (Phillips 2001). This stretch of coastline (the Great Australian Bight) is home to approximately 1,200 described species of seaweed, 62 % of which appear to be endemic to the region, meaning it has the highest degree of recorded species richness and endemism on the planet (Womersley 1990; Phillips 2001).

Aided by a favourable climate and clean, nutrient-rich waters, the marine plants of Southern Australia grow in abundance. Meanwhile, the turbulent seas generated by

2 Bound sugar composition of ten common brown algae from Beachport, South Australia

For now, the beach-cast wrack in the south-east of South Australia remains the state's only commercial seaweed resource.

When this project began, a small proportion of the wrack was being harvested and processed into agricultural commodities by Australian Kelp Products Pty Ltd (AKP) – a family-run business in Beachport, South Australia. They commissioned our research group to perform a scoping project, in order to determine whether the resource could be utilized more effectively. We therefore performed a range of bioprospecting activities, some of which are outlined within this chapter, and were also used to produce a commercial report for AKP.

The findings presented within that report, and our mutual involvement with both companies, helped facilitate the acquisition of AKP by Qingdao Gather Great Ocean Seaweed Industry Co. Ltd, and their wholly owned subsidiary for Australian operations, Gather Australian Treasure Shareholding Co. Ltd, was created.

Author contributions: WZ was involved in the design of the experimental work and he and CMMF assisted in the editing of this chapter; PS, DL and WZ helped with sample collection and communications with industry partners; and CFDG performed the taxonomic assessments of the specimens collected. I collected specimens, prepared feedstock for laboratory experiments, wrote the protocols and provided training and informational resources for the beach survey, communicated with surveyors, performed the bound sugar analysis, analyzed all data and wrote all of the content.

Bound sugar composition of ten common brown algae from Beachport, South Australia

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2.1 Abstract

South Australia is a world hotspot for seaweed diversity, with approximately 1,500 species reported. However, its seaweed industry is currently limited to the manufacture of agricultural commodities from beach-cast biomass harvested in the south-east of the state. The aim of this study was to collect biomass samples belonging to some of the more common species of brown algae found within the beach-cast wrack at Rivoli Bay – the main commercial harvesting site in South Australia – and to analyze their bound sugar profiles as a preliminary assessment of commercial polysaccharide content and their scales of variability. Seventeen biomass samples belonging to ten species – six being endemic to the region – were collected over five site visits. The sugar analysis revealed significant intra- and inter-species variability with regard to the content of fucoidan- and alginate-derived monosaccharides. Given the high variability and low sampling replication, no significant differences could be identified with regard to the content of alginates and fucoidans between species. However, there was less intra-species variability in the ratio of guluronic to mannuronic acids, which allowed for the identification of species containing alginates likely to possess strong gel-forming capabilities. The prospects for the development of an industry based on the extraction of polysaccharides from the resource are discussed, and recommendations for further studies are made.

Keywords

Fucoidan; alginate; brown algae; carbohydrates; screening.

2.2 Introduction

Southern Australia has been identified as a global hotspot for seaweed diversity and endemicity (Kerswell, 2006; Phillips, 2001). Of the three major taxonomic groups, it has been estimated that southern Australia is home to approximately 800 species of red algae, 231 species of brown algae, and 124 species of green algae, with 77 %, 59 %, and 40 % being endemic to the region, respectively (Phillips, 2001; Womersley, 1990). Newer articles suggest that the total number of seaweed species reported in southern Australia has risen to approximately 1,500 (Waters et al., 2010).

Southern Australia also has favorable growing conditions for its seaweeds, with converging currents and upwellings providing nutrient streams to relatively unpolluted and sunny coastal habitats (Phillips, 2001). This, along with powerful swells and occasional storm surges, results in the growth and subsequent detachment and carriage onto the beach of large volumes of macroalgae (Figure 2.1). These deposits are referred to as beach-cast wrack, and are particularly voluminous in the south-east of South Australia, from Cape Jaffa toward the border with Victoria (PIRSA, 2014).

Figure 2.1. A photograph of the beach-cast seaweed at Rivoli Bay, Beachport, taken May 2011.

A miscellaneous fisheries license was issued for the harvesting of beach-cast seaweed by a single operator from Cape Jaffa to Rivoli Bay in 2003 (PIRSA, 2014). In practice, this operator, Australian Kelp Products Pty Ltd (AKP), conducted most of their harvesting operations at Rivoli Bay, Beachport, due to ease of access and biomass abundance. As no commercial aquaculture or harvesting of attached seaweed is conducted in South Australia, the beach-cast wrack of Beachport remains the only commercialized seaweed resource in the state.

Despite red algae having far greater diversity in Southern Australia, it was observed during site visits and through communications with AKP that the brown algae – particularly *Ecklonia radiata*, *Durvillaea potatorum*, *Macrocystis pyrifera*, and various *Cystophora* species – make up the bulk of the harvestable beach-cast biomass at Rivoli Bay (Mills, 2011). This can be explained, in part, by their morphologies, which are typically far larger than that of the red or green macroalgae. It probably also reflects the composition of the near-shore algal assemblages specific to Rivoli Bay. Furthermore, some brown algae are canopy-forming (e.g. *E. radiata*) and extremely fast-growing (e.g. *M. pyrifera* and *D. potatorum*), which can make them more productive and competitive in certain habitats. Meanwhile, some brown algae shed biomass seasonally (e.g. *M. pyrifera*), form free-floating rafts (e.g. *Sargassum* spp.),

grow in areas of high surge and swell (e.g. *D. potatorum*), and experience more drag due to their large morphologies and higher position in the water column, all of which makes their carriage onto the beach more likely (Collings & Cheshire, 1998).

After harvesting, AKP processes the biomass into a number of agricultural commodities including liquid fertilizer and feed for terrestrial and marine animals. Liquid fertilizers produced from brown algae have been shown to contain a complex cocktail of plant hormones, buffering molecules, active polysaccharides, minerals, and trace elements, which confer a broad range of benefits to crops, including the elicitation of plant growth and resistance to pests, diseases, and environmental shock (Arioli et al., 2015). As animal feed, macroalgae can be used as the primary source of nutrition for some farmed marine animals, such as abalone, in some cases achieving improved growth, health and nutritional profiles compared with formulated terrestrial feeds (Mulvaney et al., 2015; Mulvaney et al., 2013). Meanwhile, as a livestock feed, some seaweeds have shown potential as a supplementary source of protein and minerals, while also conferring health benefits through prebiotic effects in the gut, and biologically active compounds (Machado et al., 2014; Makkar et al., 2015; Charoensiddhi et al., 2016). Interestingly, some species have also demonstrated the potential to inhibit methanogenesis in ruminants, when incorporated into their feed (Kinley et al., 2016).

However, macroalgae can alternatively be used as a feedstock for the manufacture of higher-value products. In particular, seaweed polysaccharides, which account for the bulk of macroalgal biomass, dominate the market for non-whole food products derived from seaweeds (Lorbeer et al., 2013). The most commercially important are the phycocolloids – industrial gums including alginate from brown algae, as well as agar and carrageenan from red algae (Bixler & Porse, 2011). However, as a result of their interesting biological properties, as well as broader growth in the previously overlooked medical research field of glycobiology, the sulfated polysaccharides of seaweeds – most notably the fucoidans of brown algae – are now the focus of a large body of research and an emerging market based on health products (Winberg et al., 2014).

Alginates are structural polymers composed of mannuronic acid (M) and guluronic acid (G) residues, arranged in an irregular sequence, typically accounting for up to 40 % (w/w) of brown algae on a dry weight basis (Draget et al., 2005). Alginates are valued for their unique rheological properties, which include the ability to viscosify

solutions, and form heat-stable solid gels in the presence of polyvalent cations, such as calcium (Draget et al., 2005). The physical nature of alginate gels is determined primarily by the monomeric sequence, with alginates richer in G forming stronger, more rigid gels. This has led to brown algae species containing high amounts of extractable G-rich alginates commanding the greatest demand and value as feedstocks by the global alginate industry (Bixler & Porse, 2011).

Fucoidans, on the other hand, are a structurally diverse family of sulfated polysaccharides containing fucose as a major monomer, usually along with other monosaccharides, which may include galactose, xylose, arabinose, mannose, glucose, or glucuronic acid (Morya et al., 2012; Winberg et al., 2014). Biological activities that have been reported for fucoidan include antioxidant, immune modulation, anticoagulant, antiviral, anti-inflammatory and anti-cancer effects (Winberg et al., 2014). Various factors, including sulfation, molecular weight, and monosaccharide composition, may contribute to the biological properties of fucoidans (Morya et al., 2012). While some progress has been made with regard to characterizing the specific effects of sulfation and molecular weight on certain fucoidan bioactivities, the effects of sugar composition have been more difficult to establish (Ale et al., 2011).

The other major polysaccharides present in brown algae include cellulose and laminarin. Cellulose is a high molecular weight structural β-1,4 glucan, generally accounting for less than 10% of the dry weight of brown algae (Siddhanta et al., 2009), whilst laminarin is a low molecular weight (~5 kDa) storage β-1,3 glucan (Iwao et al., 2008). As a secondary photosynthate, it typically accumulates during summer, before being expended during maturation processes in autumn (Stewart et al., 1961)

Here we identified and collected biomass samples of ten of the more abundant species of brown algae in the beach-cast wrack at Beachport, South Australia. We then analyzed their bound sugar composition in order to gain early insights into the content, nature and variability of polysaccharides contained within the whole tissues, which would be used as feedstocks for extractive studies into fucoidans and alginates, as detailed in other chapters of this thesis.

2.3 Materials and methods

2.3.1 Specimen collection and preparation

Seventeen biomass samples belonging to ten species of Phaeophyceae were collected from the freshly-deposited beach-cast wrack at Rivoli Bay, Beachport, South Australia (37°30’55”S 140°4’17”E) during five site visits, as detailed in Table 2.1. Replicate samples were only collected for *E. radiata*, *S. axillaris*, *D. potatorum*, and *M. pyrifera* due to their abundance/availability in the resource, and interest generated from preliminary data.

Biomass samples were immediately rinsed in fresh water and cleaned of any visible surface contaminants, such as sand, epiphytes and mollusks. Voucher specimens were preserved in 4 % (v/v) formaldehyde in seawater, and identified at the State Herbarium of South Australia. Tissues for bound sugar analysis were dried in an oven at 45 °C for three days, milled with a blender, sieved to a particle size of less than 250 µm (to allow for adequate contact with acid during hydrolysis), and stored in a desiccator. The moisture content of the samples was determined (by drying aliquots at 105 °C to constant weight) immediately prior to chemical analysis, and the data was reported in dry weight terms.

Table 2.1. Species identified and sampled from the beach-cast wrack at Rivoli Bay, Beachport

Order	Family	Genus	Species	Distribution	Authority	n=	Collection dates
Fucales	Sargassaceae	*Cystophora*	*C. platylobium*	Southern Aus and NZ	(Mertens) J.Agardh	1	Aug-13
			C. moniliformis	Southern Aus	(Esper) Womersley & Nizamuddin	1	Aug-13
			C. monilifera	Southern Aus and NZ	J.Agardh	1	Aug-13
		Sargassum	sp.			1	Aug-13
		Carpoglossum	*C. confluens*	Southern Aus and NZ	(R.Brown ex Turner) Kützing	1	Aug-13
	Seirococcaceae	*Seirococcus*	*S. axillaris*	Southern Aus and NZ	(R.Brown ex Turner) Greville	3	May-11, Aug-13, Jul-14
		Phyllospora	*P. comosa*	Southern Aus and NZ	(Labillardière) C.Agardh	1	Aug-13
	Durvillaeaceae	*Durvillaea*	*D. potatorum*	Southern Australia, NZ and Chile	(Labillardière) Areschoug	2	May-11, Mar-13,
Laminariales	Lessoniaceae	*Ecklonia*	*E. radiata*	Cosmopolitan	(C.Agardh) J.Agardh	4	May-11, Mar-13, Jul-14, Mar-14
	Laminariaceae	*Macrocystis*	*M. pyrifera*	Cosmopolitan	(Linnaeus) C.Agardh	2	May-11, Jul-14

n= the number of biomass samples collected.
Taxonomic and distributional information from Algaebase (Guiry & Guiry, 2015).

2.3.2 Bound-sugar analysis

Whole biomass samples were washed three times with 70% ethanol in order to remove free sugars. The insoluble fractions were then dried in a centrifugal evaporator (Labconco, USA). Samples were then hydrolyzed using a two-step acid hydrolysis process, similar to that demonstrated by Haug and Larsen (1962) to achieve the best yield of uronic acids from alginate, and later used by Malihan et al., (2012) to estimate the polysaccharide composition of brown algae. This involved the samples first being placed in 72 % sulphuric acid at room temperature for one hour with intermittent shaking, and then in 2 N (5.3 %) sulphuric acid in sealed tubes at 100°C for three hours.

Liberated monosaccharides were then derivatised with 1-phenyl-3-methyl-5-pyrazolone, and quantified using RP-HPLC (system: Prominence UFLC XR, Shimadzu; column: Kinetex 2.6u C18 100A, 100×3 mm, Phenomenex; detection: Prominence SPD-20A UV-VIS Detector, Shimadzu; flow rate: 0.8 mL/min; eluents: (A) 10 % acetonitrile and 40 mM ammonium acetate, and (B) 70 % acetonitrile; gradient: 8 to 16% eluent B over 12 min) as reported previously (Comino et al., 2013).

Peaks were identified and quantified using a set of ten standard monosaccharides (mannose, ribose, rhamnose, glucose, galactose, xylose, arabinose, fucose,

glucuronic acid, and galacturonic acid). As pure mannuronic acid and guluronic acid preparations were not commercially available, alginate was quantified by calibrating with the hydrolysate of a commercial alginate preparation (Sigma). Individual uronic acid peaks were identified by separately analyzing hydrolysates of polyguluronic acid and polymannuronic acid (Carbosynth, UK). Data were adjusted for the hydrolysis recovery rate of individual monosaccharides. The recovery rates reported by Haug and Larsen (1962) were used for the calculation of alginate-derived uronic acids.

All chemicals and reagents were purchased from Merck or Sigma, with the exception of the polyguluronic and polymannuronic acid standards from Carbosynth, UK.

2.3.3 Statistical analysis

The bound-sugar analysis was conducted in triplicate for each biomass sample. For the species n=1, the data presented represent the mean values of the analysis. For species with n≥2, the data presented represent the mean of all replicate biomass samples ± standard deviation, and an ANOVA with post-hoc Tukey test was used to determine statistically significant differences (significance, $p \leq 0.05$). As the group sizes (n) were unequal, a harmonic mean sample size of 2.53 was applied.

2.4 Results and discussion

2.4.1 Alginate-derived uronic acids

Figure 2.2 shows the total alginate detected within the seaweed samples, along with their ratios of guluronic and mannuronic acids (G/M ratio). From the graph, considerable intraspecies variability is evident with regard to total alginate content. For instance, the standard deviation between the *E. radiata* samples was 37 % of the mean value. This was due to the *E. radiata* sample collected in July of 2014 containing nearly twice as much alginate (47 %) compared with the other three samples (22-27 %) (individual data not shown). For this reason, differences between the mean alginate contents of the four species with replicate samples failed to reach statistical significance. However, there was considerably less intraspecies variation with regard to the G/M ratio.

Of the biomass samples analyzed, those belonging to *D. potatorum* exhibited the highest alginate content, with the two samples having an average of 57±13 % alginate by dry weight. However, the *D. potatorum* alginates had the lowest G/M

ratio (0.34±0.01) of all samples (significantly lower than the other species with replicate samples), indicating a low gel-strength capacity in the presence of polyvalent cations (Haug et al., 1974). The biomass samples belonging to the endemic species *S. axillaris* consistently contained high quantities of alginate (41±5 % w/w), with high G/M ratios (1.7±0.17).

An industry currently exists based on the harvest of *D. potatorum* (4,500 tonnes p.a., dry weight, as of 2009) from the beach in King Island, Tasmania, for alginate extraction in Europe, despite higher global demand for high G feedstocks (Bixler & Porse, 2011). *M. pyrifera* has been harvested extensively for commercial alginate production in the Americas in the past (Bixler & Porse, 2011), while *E. radiata* has received interest in Australia and New Zealand for this purpose, but has not been used commercially. Another member of the same genus, *E. maxima*, is harvested for its alginates in South Africa (Schiel & Nelson, 1990).

Aside from yield and G/M ratio, the viscosity and color of alginate extracts are also key determinants of value, but extraction studies are required in order to assess those attributes.

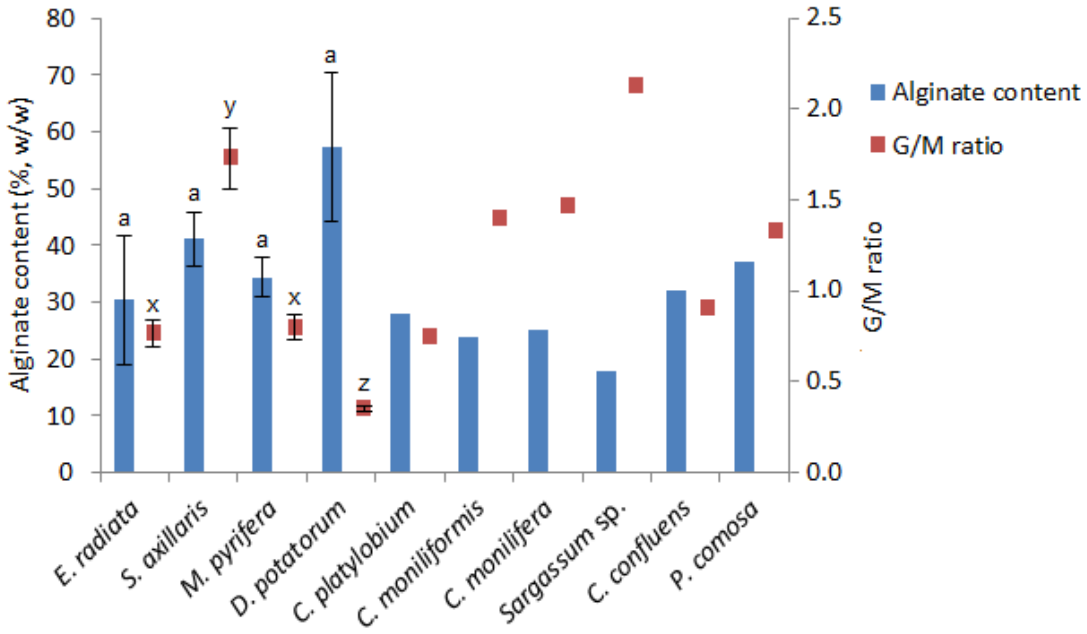

Figure 2.2. Alginate content of dry seaweeds and ratio of guluronic and mannuronic acids
Number of samples (n) per species: *E. radiata,* n=4; *S. axillaris,* n=3; *M. pyrifera* and *D. potatorum,* n=2; all other species, n=1.
For species with n≥2, mean values are shown with error bars indicating standard deviation between replicate samples.
Letters above bars/points indicate homogeneous subsets according to ANOVA with post-hoc Tukey test (significance, p ≤ 0.05). The statistical analyses could not be conducted for the species with n=1. For the species with n≥2, as the group sizes were unequal, a harmonic mean sample size of 2.53 was applied.

2.4.2 Bound neutral sugars

Following the alginate-derived uronic acids, glucose accounted for the greatest proportion of the bound-sugars in all samples tested (data shown in Figure 2.3). While it is reported to be an occasional minor inclusion in fucoidans (Ale & Meyer, 2013; Li et al., 2008), the majority of the detected glucose was likely to have come from cellulose and laminarin, which are composed principally of glucose, and can account for up to 10 and 30 % of algal dry weight, respectively (Kraan, 2012).

The remainder of the bound-sugar fractions of all species contained fucose (1.5-4.7 % w/w), and generally smaller proportions of galactose, xylose, and mannose – all of which are common components of fucoidans (Ale & Meyer, 2013; Fitton et al., 2015; Li et al., 2008). While the incidence of these sugars in whole seaweed tissue could be used as a rough preliminary screening for fucoidan content, no significant differences could be identified in this study, partly due to the low sampling replication and substantial intraspecies variability.

In addition to content, other important determinants of fucoidan value include extractability and biological activity, which cannot be determined prior to the extraction of the intact polymers. Extractability can vary markedly between different seaweed species, perhaps due to associations with other components of the cell wall such as polyphenols and celluloses (Deniaud-Bouët et al., 2014). Meanwhile, the potential applications of a fucoidan cannot be predicted prior to its extraction and purification, due to complex and various structural determinants of activity, and interference from other seaweed metabolites (Fitton et al., 2015).

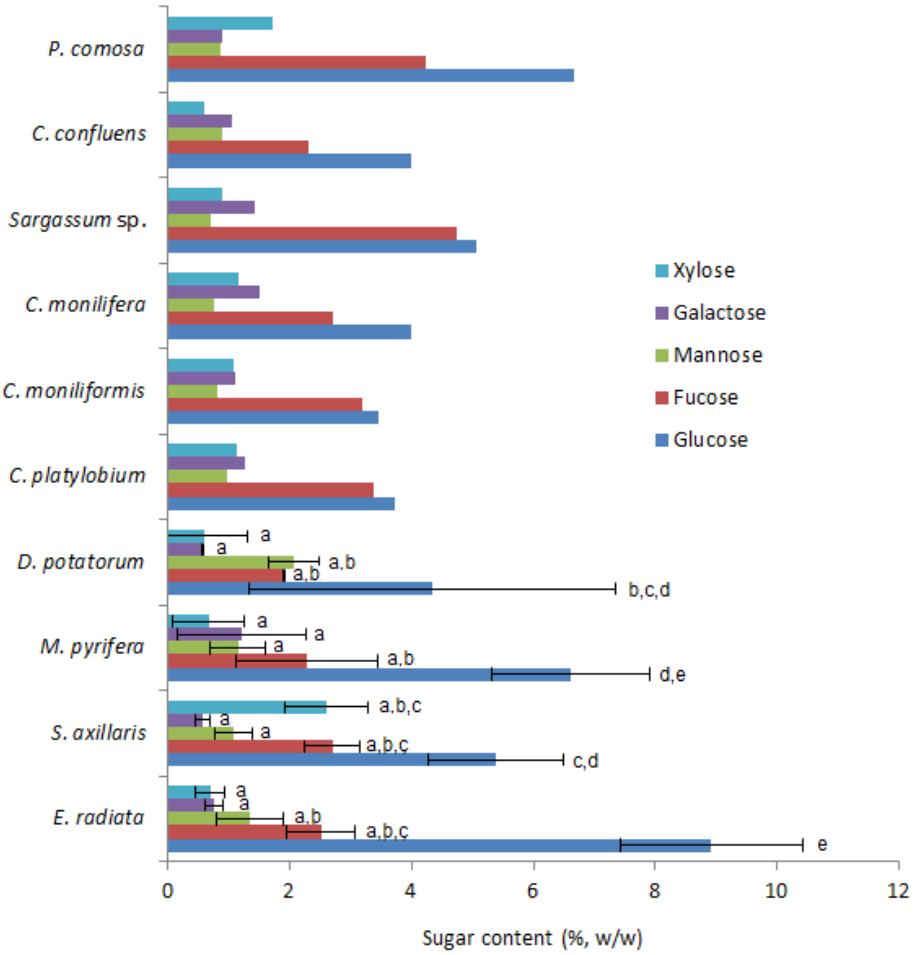

Figure 2.3. Content of selected bound neutral sugars
Number of samples (n) per species: *E. radiata,* n=4; *S. axillaris,* n=3; *M. pyrifera* and *D. potatorum,* n=2; all other species, n=1.
For species with n≥2, mean values are shown with error bars indicating standard deviation between replicate samples.
Very small peaks consistent with arabinose and glucuronic acid were discernible in the chromatograms of most samples, but were typically below the calibration range.
Letters next to bars indicate homogeneous subsets according to ANOVA with post-hoc Tukey test (significance, $p \leq 0.05$). The statistical analyses could not be conducted for the species with n=1. For the species with n≥2, as the group sizes were unequal, a harmonic mean sample size of 2.53 was applied.

2.4.3 Prospects for seaweed industry development in South Australia based on the extraction of valuable polysaccharides

In their assessment of the ecological significance and commercial harvesting of beach-cast wracks in Australia, Kirkman and Kendrick (1997) noted that in general, these resources "… are patchily distributed on beaches and their biomass highly variable over all time scales, thus they do not generally represent a dependable resource base for a harvesting industry. In exceptional locations, like […] King Island, Tasmania, where ocean conditions and the aspect of the coastline result in dependable long-term harvesting, these industries should be fostered, and aided through research and management for sustainability".

One could certainly argue that the seaweed fishery in the south-east of South Australia also represents an exceptional resource, at least in terms of the biomass available. However, unlike King Island, where the beach-cast seaweed is dominated by *D. potatorum*, the wrack around Rivoli Bay has substantial taxonomic diversity. The mixed-species biomass could only be utilized for relatively low-value applications without sorting, such as animal feed and soil conditioners, and even then a lack of product consistency may cause issues. For that reason, AKP used only *D. potatorum* in the manufacture of its liquid fertilizer, which it target-harvested locally, and supplemented with imported biomass from King Island (Mills, 2011). For higher-value applications, where consistency and traceability are priorities, a well-defined resource would be required. This is certainly the case for commercial alginate and fucoidan preparations.

As documented by Bixler and Porse (2011), the global alginate industry – particularly in the Western hemisphere – has undergone comprehensive consolidation this century, which has led to a relatively small number of large-scale producers supplying most of the market. This resulted in the cessation of alginate production in the USA (where *M. pyrifera* had once been harvested), and two-thirds of Europe's annual production being accounted for by a single facility – FMC's facility in Norway. There, large quantities of *Laminaria hyperborea* are available, which is considered among the most attractive resources for producing high-grade, guluronate-rich alginates (Bixler & Porse, 2011). Through economies of scale, and supplying the highest value market segment, FMC have maintained market share despite the high costs of production in Europe. At Beachport, on the other hand, the most abundant algae in the beach-cast wrack – *E. radiata*, *D. potatorum*, and *M. pyrifera* – were shown to contain alginates with G/M ratios of 0.85 or lower (Figure 2.2). This, along with the limited quantities available for harvesting, high labor costs, and high barriers to market entry, make onshore alginate production appear difficult.

Perhaps it would be more feasible for a rural South Australian operator to focus on a very high-value product, with an emerging market supplied by small producers, such as fucoidan. In 2008, annual global fucoidan production was estimated to be approximately 250 metric tons (mt), at a value of US$125 million (Lee, 2008), which equates to an average selling price of US$500 per kilogram. Furthermore, one of the key determinants of price is the country of origin, with Australia supplying the high-end of the market (Lee, 2008).

Marinova is currently Australia's only commercial fucoidan producer. Initially, their primary feedstock was invasive *Undaria pinnatifida*, wild-harvested from Tasmanian waters at an annual rate of 200 mt (fresh weight). But, following expansion, they now import a significant proportion of their raw material (Lee, 2008). At Beachport, the harvest ranged from zero to 305 mt (fresh weight) per year between 2000 and 2014, with an average of 79.5 mt (PIRSA, 2014). As this was the mixed-species wrack, the harvest of any single brown algae species would have been much lower. However, from personal communications with AKP, this represented only a very small proportion of the total available biomass (Mills, 2011).

However, in order to confidently predict whether it would be economically feasible to utilize the beach-cast wrack at Beachport for the commercial production of polysaccharides, a comprehensive survey of the resource, in terms of its magnitude, taxonomic composition, and variability over various time scales would be essential, in order to understand the limitations with regard to the scale of potential production. Furthermore, extraction studies would be required to assess the quality of the products, and a comprehensive techno-economic analysis would need to be conducted, comparing the various valorization strategies available.

2.5 Conclusions

This preliminary sampling and sugar analysis of brown algae commonly found within the beach-cast wrack at Beachport, South Australia, provided early insights into the contents and monosaccharide profiles of their commercial polysaccharides. This may assist in the planning and execution of comprehensive screening and surveying studies of the resource in the future.

2.6 Acknowledgements

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3 Kinetics of conventional and microwave-assisted fucoidan extractions from the brown alga, Ecklonia radiata

After it was determined that brown algae constituted the bulk of the harvestable biomass in South Australia, fucoidans were identified as a key candidate for production due to their interesting biological activities and high commercial value.

While reviewing the literature, it was noted that lengthy treatments in hot, dilute acid were most commonly used to extract fucoidans (or cited as a benchmark when reporting novel approaches), perhaps with the goal of maximizing yields. But, there appeared to be a knowledge gap in exactly how this treatment affected the purity, structure, and composition of fucoidans.

This led to an investigation into the kinetics of a conventional and microwave-assisted extraction process. The findings were presented orally at the 5th Congress of the International Society for Applied Phycology, held in Sydney during June 2014, and later published in the conference special issue of the Journal of Applied Phycology (Vol. 27(5), pp. 2079-2087). The article is included in this chapter, and the first page of the published version can be seen in Appendix 3.3.

Please note that in the pie chart showing the biochemical composition of the feedstock (Figure 3.1), the ‘alginate’ designation refers only to the carbohydrate fraction of the alginates (the uronic acids), whilst the associated metal ions contribute to the ‘ash’ designation. In later chapters, the alginate content is given as the total weight of alginate, in its sodium salt form.

Author contributions: JL and PS helped conduct chromatographic analyses and training on the use of the equipment and sample preparation; GF provided advice on experimental design and analytical capabilities; WZ provided valuable advice on research directions; and all of the co-authors helped with the article editing process. I designed the experiment, performed the extractions, performed the sulfate assay, prepared the samples for chromatography and ran the chromatography with assistance from JL and PS, analyzed all data, and wrote all of the content.

Kinetics of conventional and microwave-assisted fucoidan extractions from the brown alga, *Ecklonia radiata*

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3.1 Abstract

Fucoidans – the bioactive and structurally diverse polysaccharides from brown algae – have potential applications in the health industry, but development has been hampered by difficulties in controlling the structure, purity, and consistency of the product. Importantly, the extraction technique affects the nature of the product by preferentially extracting differing fucoidan compounds, whilst simultaneously altering their structure. Extractions are typically performed using hot, dilute acid, but the kinetics of extraction are poorly understood, especially with regard to trade-offs between yield, purity and structural integrity.

Here, the extraction kinetics of fucoidan from the brown alga *Ecklonia radiata* under classical conditions (hydrochloric acid at 60°C, pH 2) have been investigated, using both convective and microwave heating in open-vessels for up to three hours. The yield, purity and key structural characteristics of the fucoidan extracts were assessed

Only modest gains in yield were achieved beyond six minutes of extraction. Extended extraction was accompanied by undesirable declines in fucose and sulfate content, a substantial increase in the co-extraction of the contaminant laminarin, and significant reductions in the molecular weight of fucoidans.

The results demonstrate that maximising fucoidan yield often comes at the cost of purity and structural integrity. It is important to tailor an extraction technique to best suit the desired applications of the final products. Open-vessel microwaves offer a highly efficient and controllable alternative to convective heating, and merit further investigation, but techniques that better disrupt the cell wall complex, whilst preserving fucoidan structure must also be pursued.

Keywords: Fucan; Fucose; Hydrolysis; Laminarin; Seaweed; Sulfated

polysaccharides

3.2 **Introduction**

Aside from their already popular applications in the food, agriculture and biomaterials industries, as well as their potential for use in biofuel production, seaweeds are becoming increasingly recognised for their biologically active compounds, which have a range of potential applications in the health sector.

One family of such compounds is referred to as the fucoidans, the sulfated polysaccharides found in nature almost exclusively in brown algae. These are complex, high molecular weight polymers, typically dominated by L-fucosyl residues and sulfate esters, but also occasionally containing mannose, galactose, xylose, uronic acid, and acetate (Li et al., 2008).

A broad range of biological activities have been reported for fucoidans and derivatives; most notably anticoagulant and antithrombotic, antiviral, anticancer, immunomodulating, anti-inflammatory, anti-lipidemic and anti-fertilisation effects (Morya et al., 2012). This breadth of activities can be explained by the structural diversity of fucoidans, with substantial variations having been reported in their monomeric composition, structural configuration (e.g. branching), molecular weight and sulfation pattern, with each of these aspects, particularly the last two, having significant effects on the compounds' functionality (Morya et al., 2012). Whilst a relatively high level of sulfation is generally desired, the issue of molecular weight is somewhat more application specific (Ale et al., 2011). On the one hand, some activities, such as anticoagulant and antithrombotic, appear to benefit from a high molecular weight, and researchers aiming to characterise native fucoidans can only do so if the structure is conserved during extraction (Berteau & Mulloy, 2003; Li et al., 2008). Others, however, have suggested that therapeutic applications are hampered by the high molecular weight and viscosity of native fucoidans, and some activities have been enhanced by its partial hydrolysis (Hahn et al., 2012; Morya et al., 2012).

The structure of fucoidans differ with species, growth conditions, anatomical region, and indeed the method of isolation and analysis (Yang et al., 2008). Ale and Meyer (2013) have provided an excellent review of the range of structures reported and extraction techniques applied. However, there is currently no widely used and well understood standard extraction and purification procedure for fucoidan (Ale et al., 2011), making it difficult to reliably compare fucoidans between species, and to benchmark novel processing techniques.

Most extraction techniques involve the aqueous extraction of fucoidan over several hours, usually with hot acid (Ale & Meyer, 2013). The advantage of using acid lies in its ability to disrupt the hydrogen bonding between polysaccharides, whilst simultaneously precipitating the potentially contaminating polysaccharide, alginate (Hahn et al., 2012). Whilst these conditions have been selected with the chief aim of maximising yield (Black et al., 1952), the detailed kinetics of classical extraction, with regard to its effects on the purity and structure of fucoidan, are not well understood. This limits the ability of producers to tailor fucoidan preparations to better fit their desired applications.

Recent publications have reported a variety of alternative extraction technologies, involving the use of ultrasound, enzymes, pressurized liquids, and microwaves, all with vastly different associated operational conditions (Athukorala et al., 2006; Hahn et al., 2012; Rodriguez-Jasso et al., 2012; Rodriguez-Jasso et al., 2011; Santoyo et al., 2011).

Microwave-assisted extraction, which enables the rapid and controlled heating of polar solvents through direct ionic conduction and dipole rotation, can be classed as either closed- or open-vessel systems. The former operates with a limited capacity, allowing the solvent to be heated well past its natural boiling point, accompanied by an increase in pressure. The latter operates at atmospheric pressure, meaning the operating temperature is limited by the boiling point of the solvent (Sparr Eskilsson & Björklund, 2000).

Microwave-assisted fucoidan processing has shown promise with regard to its efficiency, yield, and propensity for the simultaneous controlled modification of the polymers, potentially bearing derivatives with enhanced activity (Quitain et al., 2013; Rodriguez-Jasso et al., 2011). Those studies have utilized closed-vessels, with high operating temperatures and pressures, but as Pereza et al. (2014) stated when they set out to extract phenolic compounds from brown algae, open-vessel microwave systems have the advantages of increased scalability, milder conditions, lower costs and improved safety.

Here, we therefore aim to directly compare the kinetics of an open-vessel fucoidan extraction using microwave and conventional (convective) heating, with regard to yield, purity and key structural characteristics. *Ecklonia radiata* (C. Agardh) J. Agardh has been selected as the experimental species, given its abundance along the southern coastline of Australia and potential for aquaculture and valorization (Loo et al., 2011; Lorbeer et al., 2013).

3.3 Materials and methods

3.3.1 Chemicals

All chemicals used were purchased from Merck and Sigma, with the exceptions of the polyguluronic and polymannuronic acids (Carbosynth, UK), and the β-glucan molecular weight standards (Megazyme, Ireland).

3.3.2 Alga collection, identification, characterization and preparation

Algae (confirmed to be *Ecklonia radiata* by the State Herbarium of South Australia) were collected from the freshly deposited beach-cast seaweed in Rivoli Bay, Beachport, South Australia in March 2013.

They were immediately rinsed in fresh water, removed of any visible surface contaminants, and placed on mesh racks to dry. The whole plants were then milled with a blender (Blendtec, USA), sieved to a particle size of 250-1400 µm, and further dried in an oven at 45°C prior to storage.

The National Measurement Institute determined the protein, fat, carbohydrate, and free sugar content of the prepared tissue. Cellulose was estimated using the method of Mihranyan et al. (2004), whilst key polysaccharides were estimated by monosaccharide analysis, as described later.

3.3.3 Extraction and fractionation

3g of dried alga was extracted with 90mL of pre-heated aqueous hydrochloric acid solution (pH 2, prior to addition of algae) in open-vessel, with temperature maintained at 60±2°C for up to three hours (six durations tested), using either a hotplate or a microwave – each with magnetic mechanical stirring. A time = 0 minutes control extraction without heating from either mechanism was included, whereby the extraction was halted immediately after commencement. All extractions were replicated three times, in random order.

Open-vessel microwave method

A StartSYNTH Microwave Synthesis Labstation (Milestone Inc., USA) was used, with stirring speed set to 70%, and the temperature controlled using an infrared sensor and automatic power adjustment.

Hotplate method

A pre-heated hotplate stirrer (S.E.M. Pty Ltd, Australia) was used, with stirring set to a speed roughly equal to that used for the microwave-assisted extraction, and the temperature was monitored using a thermometer placed within the extraction mixture.

Post-extraction

Immediately following the extraction, the mixtures were cooled in an ice-slurry for two minutes, and residual biomass was removed by vacuum filtration. Ethanol was added to a concentration of 20% (v/v), along with calcium chloride to 0.5% (w/v) to precipitate alginate at 4°C overnight. The precipitate was removed with centrifugation. More ethanol was then added to the supernatant to a concentration of 67% (v/v), and the remaining polysaccharides were precipitated at 4°C overnight, then collected with centrifugation. The fucoidan-rich extract was washed twice with ethanol (67%, v/v), before being freeze-dried.

3.3.4 Characterisation

Turbidimetric sulfate assay

The sulfate content of the fucoidan-rich extracts was determined turbidimetrically, after hydrolysis in 1N hydrochloric acid at 105°C for two hours, using K_2SO_4 as a standard (Dodgson & Price, 1962).

Monosaccharide analysis

Seaweed tissue and extracts were removed of unbound sugars by washing with 70% ethanol three times. Residues were then submitted to Saeman hydrolysis (72% sulfuric acid, room temperature for one hour, then 1M sulfuric acid, 100°C for three hours), before being derivatized with 1-phenyl-3-methyl-5-pyrazolone, as previously reported (Comino et al., 2013). The derivatized monosaccharides were then quantified using HPLC (system: Prominence UFLC XR, Shimadzu; column: Kinetex 2.6u C18 100A, 100 x 3mm, Fenomenex; detection: Prominence SPD-20A UV-VIS Detector, Shimadzu).

Peaks were identified and quantified using a set of standard monosaccharides (D-mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, D-galactose, D-xylose, L-arabinose, L-fucose, D-glucose, and the internal standard 2-deoxyglucose). Values were adjusted for recovery, by including a set of the standards in the hydrolysis protocol.

Polysaccharide estimation

Fucoidan was estimated from the fucose concentration of the samples, assuming the polymers had a fucose content of 39% (w/w), which is in the centre of the typical range of 34-44% (Holtkamp et al., 2009). Laminarin was estimated from the glucose

content, with the assumption that there was negligible cellulose in the extracts or glucose in the extracted fucoidans. Alginate was roughly estimated by the sum of mannuronic and guluronic acid. Pure samples of those uronic acids were not commercially available. Therefore, their peaks were identified by comparison with hydrolysates of commercial samples of polyguluronic and polymannuronic acids, and roughly quantified using the average extinction coefficient and recovery rate of other uronic acids (glucuronic and galacturonic acid).

Rate of hydrolysis

The relative molecular weight profiles of the extracts were estimated by size-exclusion HPLC (Agilent), using in-line PolySep GFC-P4000 and PolySep-GFC-P5000 columns (Phenomenex), with ELSD detection (Alltech), in 0.1M NH_4HCO_3. (1,3;1,4)-β-glucans were used as molecular weight standards, with peak molecular weights of 33.6, 67.1, 160, 247, 375, and 667 kDa.

3.3.5 Statistical analysis

All analyses were performed in triplicate, with results expressed as mean values. An unpaired student t-test was used to determine whether values of interest were significantly different, which was defined as $p<0.05$.

3.3.6 Supplementary investigations

Given the low yields achieved using classical approaches to extraction, it was hypothesized that tight associations between the fucoidans and other components of the cell wall may inhibit their dissolution. Therefore, two methods of feedstock pre-treatment were trialed involving the use of ionic liquids and catalytic enzymes. Those investigations yielded negative results, with regard to the aim of improving fucoidan extractability, but are documented in Appendix 3.1 and Appendix 3.2.

3.4 Results

3.4.1 Alga characterization

Analysis of the starting material used in this investigation indicated that polymeric carbohydrates accounted for the bulk of its dry weight. Judging from its monosaccharide profile, fucoidan appeared to account for approximately 11% of the alga's dry weight, whilst the potential contaminating compounds laminarin, alginate and protein contributed to 11%, 19% and 9% of the dry weight, respectively (Figure 3.1).

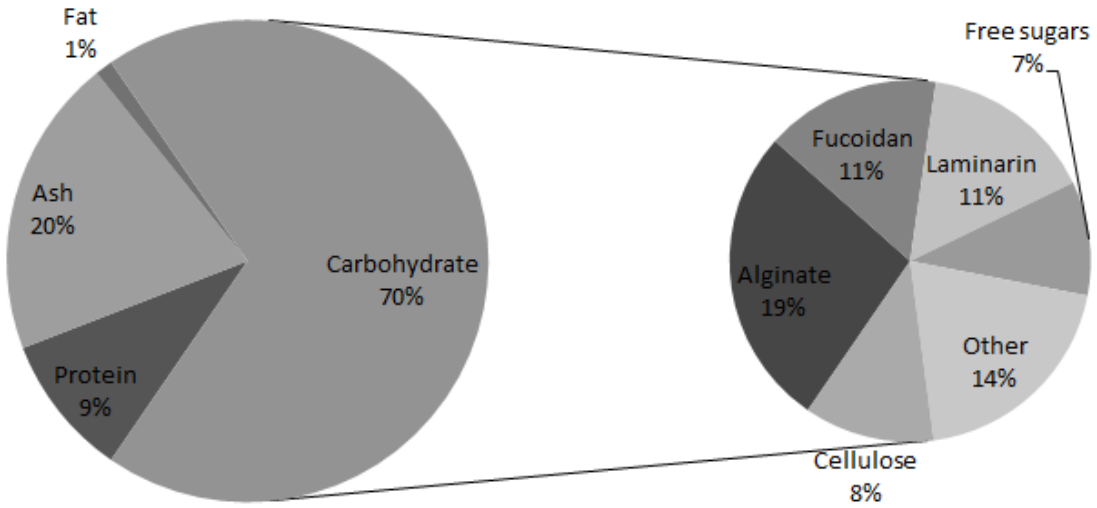

Figure 3.1. Estimated chemical composition of the dried and ground E. radiata tissue
Proximates were determined by the National Measurement Institute and polysaccharides were estimated by calculation from monosaccharides detected after the acid hydrolysation of the tissue.

3.4.2 Yield of fucoidan-rich extracts

The yield of the fucoidan-rich extract had a logarithmic relationship with the extraction duration, increasing rapidly initially, but with diminishing growth as the extraction proceeded. After 180 minutes, the yields achieved by the microwave and hotplate extractions were not significantly different, at 4.8% and 4.7%, respectively (Figure 3.2). During the first six minutes of the extractions, there was a more distinct difference between the performance of the two techniques, with the microwave achieving a significantly higher yield at the end of that period (Figure 3.2, inlay). The average yield at the twenty minute time-point, however, was actually lower for the microwave, and this was the only point at which the hotplate appeared to perform better, albeit not by a significant margin. Judging by the shape of the time-course curve for the microwave, those differences may have been caused by an anomaly.

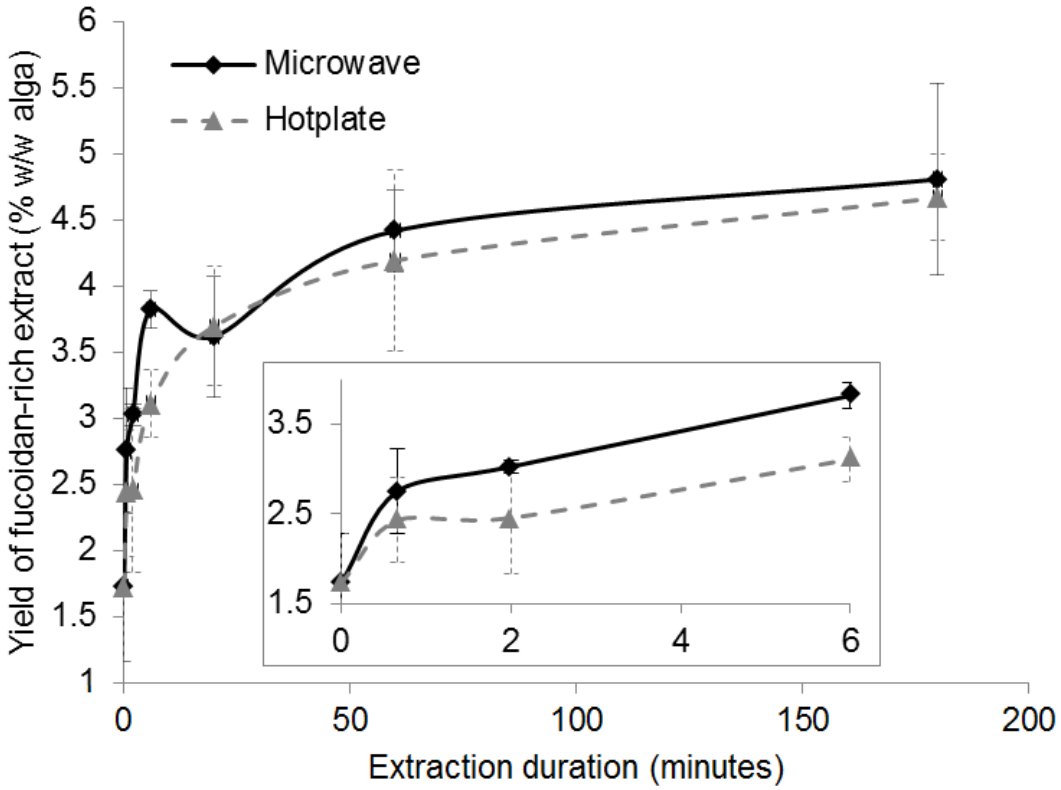

Figure 3.2. The yield of fucoidan-rich extract achieved using the microwave and hotplate throughout the 180 minutes extractions. And inlay: the same plot, zoomed in to better show the 0-6 minute period. Error bars indicate standard deviation (n=3).

3.4.3 Chemical composition

Figure 3.3 shows the concentration of key monosaccharides in the fucoidan-rich extracts, for which a number of trends can be observed as the extraction duration increased. No statistically significant differences existed between the two extraction systems, with regard to the monosaccharide composition of the products.

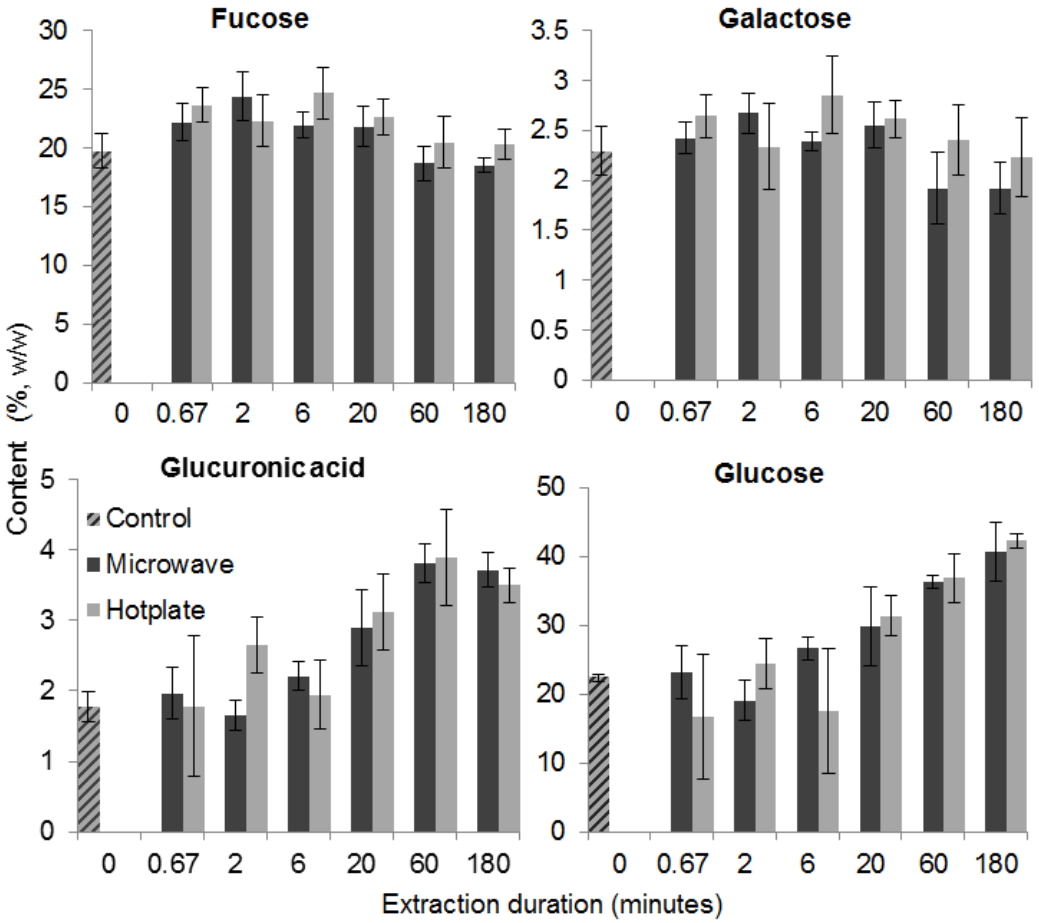

Figure 3.3. The content of key monomers in the fucoidan-rich extracts, as determined by the analysis of monosaccharides liberated with acid hydrolysis
Error bars indicate standard deviation (n=3).

The fucose content of the fucoidan-rich extracts, which may reflect the fucoidan purity of the extracts, and/or the fucose content of the fucoidan molecules themselves, appeared to increase or remain stable during the first six minutes of extractions, and gradually drop thereafter. Meanwhile, galactose, whilst at a far lower concentration (2-3% of the weight of the extracts), followed a strikingly similar general trend to the fucose.

Conversely, the content of glucuronic acid – another minor component – increased with extraction duration, whilst xylose and mannose, each present at concentrations of less than 1%, remained relatively constant between both extraction systems at all time-points, with no obvious correlations with extraction duration. No alginate uronic acids were detected in any of the fucoidan-rich extracts.

Glucose, which was a major component of the fucoidan-rich polysaccharide fractions, can be taken as an indicator of laminarin contamination due to the fact that this is the only major polysaccharide likely to have been extracted with substantial

glucose content (cellulose is water-insoluble). The glucose content of the fucoidan-rich extracts gradually increased with the extraction time, from an initial value of 22.4% to just over 40% after 180 minutes of extraction.

Finally, the sulfate content of the fucoidan-rich extracts (Figure 3.4) followed a similar trend to that of the fucose, which could be anticipated as both groups would be expected to be present mainly within the same polymers – the fucoidans. However, the ratio of sulfate to fucosyl residues gradually decreased during the extraction, suggesting that as the extraction progressed, either sulfate esters were cleaved or the fucoidans preferentially extracted had lower sulfate content. Once again, no significant difference was observed between the two extraction systems.

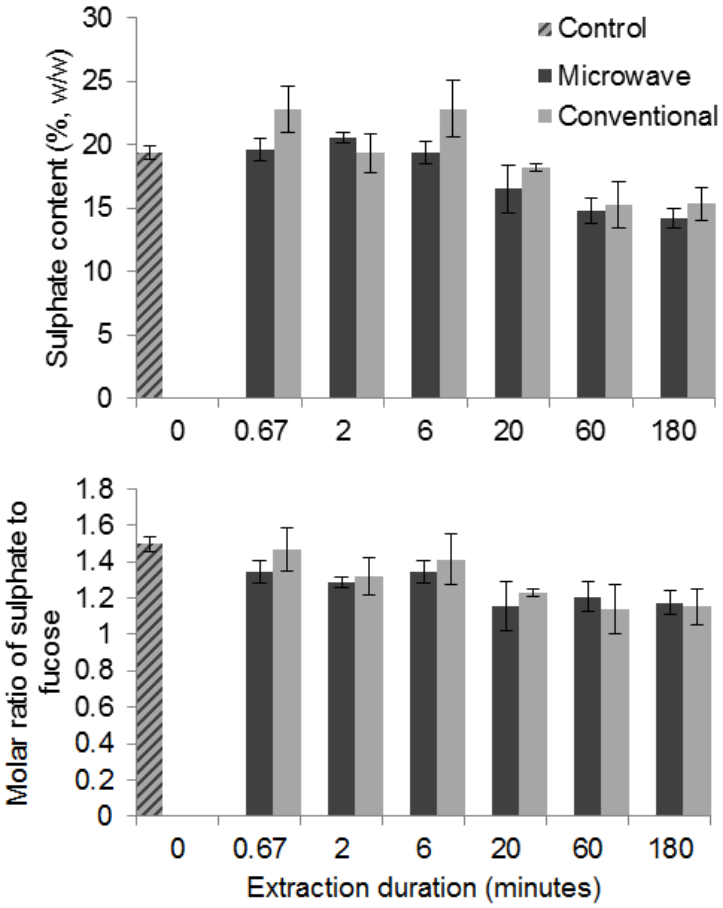

Figure 3.4. The sulfate content of the fucoidan-rich extracts, by weight (top), and by molar ratio to fucose (bottom)
Error bars indicate standard deviation (n=3).

3.4.4 Yield of extract components

The amounts of actual fucoidan and laminarin obtained throughout the extractions were estimated using the gravimetric yield of the fucoidan-rich extracts, together with their fucose and glucose contents, respectively (with the assumption that the fucose content of the extracted fucoidans remained relatively stable) (Figure 3.5). It

appears as though the amount of fucoidan extracted per unit time diminished exponentially as the extraction progressed. In fact, after the initial six minutes of extraction, a three-fold increase in duration only achieved an increase of 10% or less in fucoidan yield. During those later stages of the extractions, the modest gains observed in the yield of fucoidan-rich extract were mostly accounted for by increasing laminarin contamination. Indeed, during that period the yield of laminarin increased more than ten times faster than that of the fucoidan.

3.4.5 Apparent hydrolysis

All fucoidan-rich extracts exhibited a similar elution profile using size exclusion HPLC, showing two major peaks, and a later-eluting minor peak that was not examined further. Monosaccharide analyses were conducted on the fractions of five separate elutions. In each case, fucose was only detected in the first major peak, and glucose was only detected in the second (Figure 3.5), suggesting that they contained the fucoidan and laminarin, respectively. This supports the assumption that most of the glucose detected in the fucoidan-rich extracts did indeed belong to the laminarin, as opposed to the fucose-containing polysaccharides.

β-glucans were used to establish a correlation between retention time and molecular weight, as fucoidan molecular weight standards were not commercially available. Whilst this allowed for the rate of hydrolysis during the extractions to be estimated, the real molecular weights of the extracted compounds could not be determined due to inevitable differences in the hydrodynamic behaviour of the β-glucans and the algal polymers, and because none of the extracted compounds had been purified.

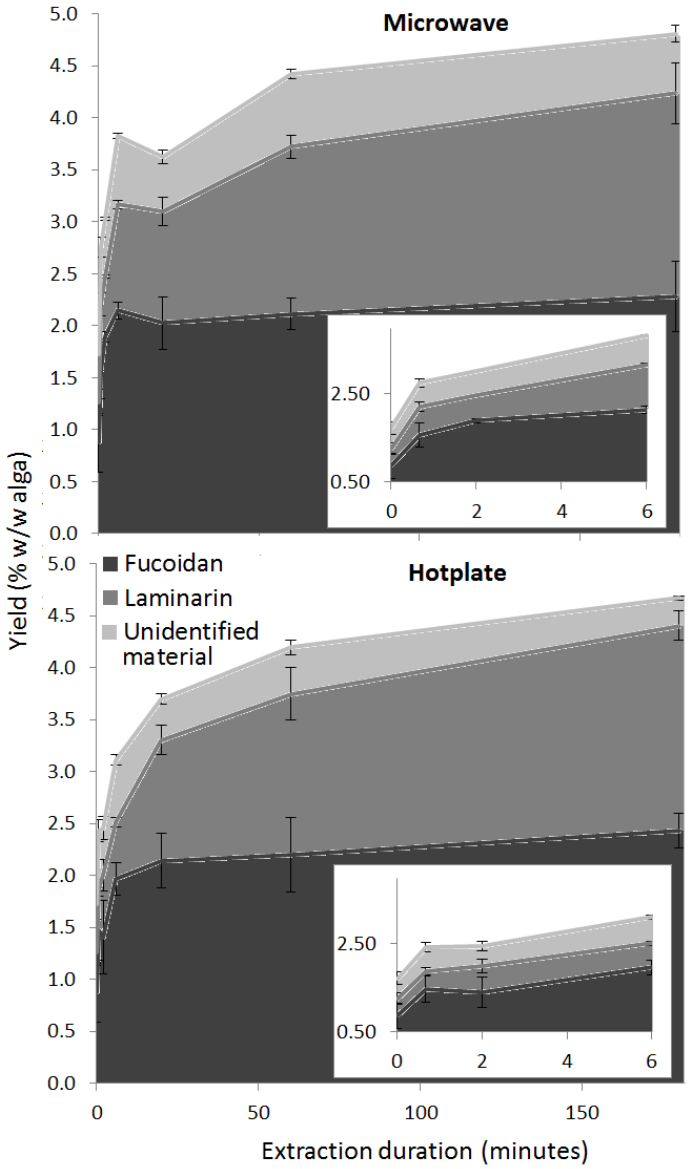

Figure 3.5. The time course of the two extraction processes (microwave: top, hotplate: bottom), showing the estimated proportion of fucoidan and laminarin in the fucoidan-rich extracts
Error bars indicate standard deviation (n=3).

The retention time of the glucose-containing peak remained constant at approximately 17.30 minutes for all extracts, indicating that those polymers had a significantly lower hydrodynamic volume than the smallest β-glucan standard (Mp = 33.6 kDa, retention time = 15.45 minutes), and that the extraction duration had no effect on their molecular weight at the conditions tested. The retention time of the fucoidan-containing peak, however, increased with the duration of both extractions. This most likely indicates hydrolysis; however, it could otherwise represent some other change in the chemistry of the polymers that affected their hydrodynamic volume in the column.

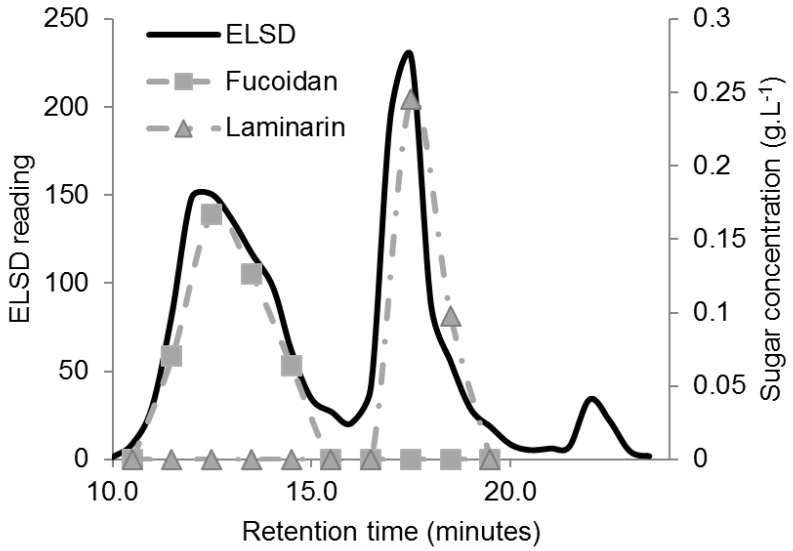

Figure 3.6. Chromatogram obtained with size exclusion HPLC analysis of a fucoidan-rich extract (microwave extraction system, twenty minute duration) using ELSD detection (left vertical axis), showing the estimated fucoidan and laminarin content of fractions collected every minute (right vertical axis)

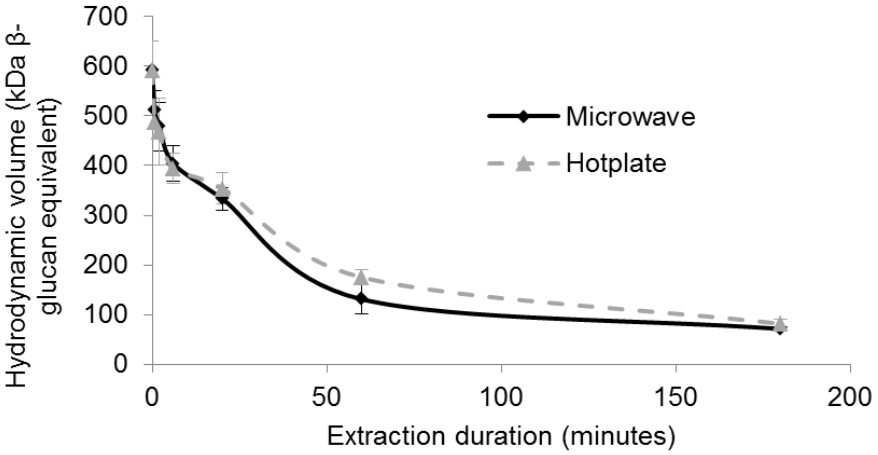

Figure 3.7. Apparent reduction in the peak hydrodynamic volume of fucoidans with extraction time, estimated using size exclusion HPLC, using β-glucan molecular weight standards for reference

Error bars indicate standard deviation (n=3).

The peak molecular weight of the fucoidans appeared to decay exponentially (perhaps in a second-order manner), as the extraction duration increased (Figure 3.7), with the apparent hydrolysis proceeding very quickly initially, but slowing as the extraction proceeded. For both extractions, the peak molecular weight of the fucoidans appeared to reduce by just over 30% in the first six minutes, and by 86-88% after 180 minute of extraction. No significant difference was observed between the two systems at any time-point.

3.5 Discussion

The *E. radiata* tissue used in this investigation contained relatively high proportions of fucoidan, alginate and laminarin (Figure 3.1). The latter two polysaccharides, whilst considered here to be potential contaminants, each have the capacity for commercial utilisation as industrial hydrocolloids, or plant and bacterial growth elicitors, respectively (Aziz et al., 2003; O'Sullivan et al., 2010). This, along with *E. radiata*'s abundance along Australia's southern coastline and potential for aquaculture, may suggest the possibility for its valorization in a biorefinery production process (Lorbeer et al., 2013).

Table 3.1. General trends observed during both the microwave and hotplate extractions

	0-6 minutes	6-180 minutes
Yield of fucoidan-rich extracts	Increasing rapidly	Increasing slowly
Fucose and galactose content of extracts	Increasing	Decreasing
Glucuronic acid content of extracts	Increasing	Increasing
Ratio of sulfate to sugar residues (excl. glucose)	Decreasing	Decreasing
Laminarin contamination of extracts	Increasing	Increasing
Peak molecular weight of fucoidan	Decreasing rapidly	Decreasing slowly

As illustrated by Table 3.1, the kinetics of both fucoidan extraction techniques can be summarised by dividing the process into two stages: the initial zero to six minute period, and the progressing six to 180 minute period of the extraction.

The yield of fucoidan-rich extract achieved after the 180 minutes using each process was just under 5% (w/w), which is fairly consistent with other studies using classical operating conditions after a single extraction step (Kim et al., 2007; Rabanal et al., 2014; Rioux et al., 2007). The extraction initially proceeded rapidly, but diminishing returns were achieved with increasing extraction time after about six minutes. The returns in actual fucoidan – estimated using the fucose content of the fucoidan-rich extracts – appeared to diminish even more severely with extended extraction time, as the fucose content of the extracts began to decrease after six minutes. A similar reduction in the fucose content of extracted polysaccharides with increasing duration, temperature, and acid concentration has been demonstrated previously (Ale et al., 2012; Ponce et al., 2003; Rabanal et al., 2014). Here, it could indicate an increase in the co-extraction of other compounds, and/or a change in the structure of the fucoidans extracted. The accompanying increase in glucose content and subsequent separation of the main fucose- and glucose-containing compounds with size exclusion HPLC certainly supports the first explanation.

Similarly, the sulfate content of the fucoidan-rich extracts also decreased as the

extractions proceeded, both in terms of total sulfate by weight of extract (after the six minute mark) and the ratio of sulfate to fucosyl residues (Figure 3.4). This may indicate the gradual cleavage of some of the sulfate groups from the extracted fucoidans, as has been reported previously (Ale et al., 2012; Rabanal et al., 2014). More peculiar though, is the unusually high proportion of sulfate to monosaccharide moieties in the extracted fucoidans (again, disregarding the glucose with the assumption that it mostly belonged to unsulfated laminarins). Fucoidans usually contain one sulfate group per backbone sugar or less (Ale & Meyer, 2013). However, a sulfation level of greater than one ester per monosaccharide, as it appears could have existed here, has been reported for some species, such as *Ascophyllum nodosum* (Chevolot et al., 1999).

Galactosyl and glucuronic acid residues were observed in all fucoidan-rich extracts at concentrations of about 2-4% (w/w). Their presence within fucoidan molecules has been widely reported, and has led to the occasional recognition of two distinct classes of fucoidans, occasionally both present within the one alga. These are the galactofucans, containing mainly fucosyl, galactosyl and sulfate moieties; and the uronofucoidans, with high fucose and uronic acid content, less sulfate, and often some mannosyl and xylosyl residues (Duarte et al., 2001; Ponce et al., 2003). In this investigation, it was observed that as the extraction duration increased, the galactose content of the fucoidan-rich extracts decreased whilst the glucuronic acid increased, suggesting a gradual shift in the types of fucoidans mostly being extracted as the duration progressed. However, this would need to be confirmed with the analysis of purified compounds.

Fucose remains the best indicator of total fucoidan extraction, and it was noted that after the entire 180 minute duration of each extraction, only 21-22% of the available fucose had been extracted from the tissue. This was on the lower side of that achieved by Black et al. (1952), who extracted fucoidan from four different species using hydrochloric acid (pH 2-2.5) for one hour at 70°C, three times (*Laminaria cloustoni* 20%, *Ascophyllum nodusum* 54%, *Fucus vesiculosus* 62%, and *Pelvetia canaliculata* 76% of total fucose). Whilst reporting the % of total fucose extracted seems like a good tool for comparing the success of extraction techniques or the extractability of different alga, it does not appear to have been utilized elsewhere.

The result obtained here points toward an unsatisfactory dissolution of the fucoidan-containing cell wall matrix, however further increases in extraction time appear unlikely to see much improvement. More aggressive techniques involving

higher temperatures and pressures, such as closed-vessel microwave-assisted extraction and autohydrolysis could be expected to achieve a higher yield, but they have been shown to cause a high degree of fucoidan hydrolysis, even in the absence of acid (Balboa et al., 2013; Quitain et al., 2013). Ultrasound-assisted approaches may provide a mild temperature and pressure alternative, but it has not yet been demonstrated whether this alone can achieve a satisfactory yield, and it too has been shown to rapidly hydrolyze fucoidan (albeit one from a sea cucumber) (Guo et al., 2014; Ye et al., 2008).

Indeed, even at the relatively moderate extraction conditions trialled here, a large degree of fucoidan hydrolysis was observed. Rabanal et al. (2014) also observed a reduction in molecular weight with duration when extracting into dilute acid. In that study, significant hydrolysis was only observed after four lengthy sequential extractions, but any rapid initial hydrolysis may not have been noticed since the shortest time-point was seven hours.

Where conservation of structure is considered more important than extraction yield and rapidity, one might suggest the use of water as opposed to acid as the extractant, or a substantially lower extraction temperature. Peculiarly, however, both Ponce et al. (2003) and Rioux et al. (2007) obtained higher molecular weight fucoidans from various species when extracting with acid than with water or calcium chloride solution, respectively. In such cases, the preferential extraction of different fucoidans, as opposed to the structural alteration of identical compounds during extraction, seems more likely to account for those observations. The former study, however, did obtain significantly larger fucoidans with both solvents when extracting at room temperature than at 70°C, but their yields were almost five times lower.

One method that may enable both a high yield and a well-preserved natural product, is the use of a well-defined enzyme formulation to disassemble the cell wall complex without degrading target polysaccharides. It has recently been suggested that fucoidans tend to be closely associated with cellulose and proteins in the cell wall, which may inhibit their dissolution, so a preparation of proteases and cellulases could be proposed (Deniaud-Bouët et al., 2014). Whilst enzymes can be expensive, comparatively slow acting, and require an additional de-activation step, they may avoid costs associated with infrastructure and its maintenance when compared with other techniques utilising acid or high operating pressures, for instance.

Of further importance when assessing an extraction protocol, is the co-extraction of unwanted or contaminating compounds. The more exclusively selective an extraction protocol is the less effort is likely to be required for downstream

purification processes. Classical fucoidan extractions may yield a crude extract containing small compounds, polyphenols, some proteins and the polysaccharides laminarin and alginate. Typically, ethanol can be used to remove most of the small compounds, polyphenols and proteins, and the co-extraction of alginate can be avoided or subsequently dealt with by conversion to insoluble alginic acid or calcium alginate. Laminarin, however, is more likely to persist to the fucoidan-rich polysaccharide fraction, and so has been quantified here.

For each of the extraction systems tested, the degree of apparent laminarin contamination increased as the extraction progressed. This may further discourage the commonly-held tendency to select optimal extraction conditions as those maximising fucoidan yield. Imbs et al. (2015) also observed substantial laminarin contamination when extracting with hot acid for three hours, but this was avoided when the solvent was changed to a 2 % aqueous CaCl2 solution. Alternatively, laminarin can be separated from fucoidan according to differences in charge, for example through the binding of fucoidan with cationic surfactants or other positively charged binding agents, or through the use of anion exchange chromatography (Chen et al., 2012; Marais & Joseleau, 2001). Although, binding agents can be difficult to subsequently remove and may affect the activity of the fucoidan, whilst anion exchange chromatography suffers from relatively low efficiency and capacity, so minimising contamination during the initial extraction is desirable (Hahn et al., 2012).

During this investigation, few significant differences were observed in response to the use of microwave or convective heating. However, for the purpose of keeping all conditions constant for a direct comparison between the two systems, several advantages of microwave processing were not maximally exploited, such as its capacity for rapid heating and highly-controlled temperature programming (the solvent is heated directly, so the temperature-response lag caused by heating vessel surfaces in convective systems is avoided). Nonetheless, it has been shown that open-vessel microwave processing does offer a suitable alternative to conventional fucoidan extraction systems.

3.6 Conclusions

The heated dilute acid extraction of fucoidan investigated here resulted in crude fucoidan-rich products, which had decreasing levels of fucose, galactose and sulfate, and increasing proportions of glucuronic acid and laminarin, as the extraction progressed. Especially after approximately six minutes of heating,

relatively minor gains in fucoidan yield were accompanied by substantial losses in purity and structural integrity, so for most applications, a relatively short extraction period using these conditions would be suggested. However, a rapid reduction in the molecular weight of the fucoidans was apparent during the initial stages of the process, which may or may not be desirable, depending on the intended applications of the products.

These results highlight the need for novel extraction techniques that can better disaggregate the cell wall complex, whilst conserving the structure of fucoidan. Furthermore, they emphasise the importance and complexity of optimising extraction conditions according to the desired characteristics of the products, rather than simply maximising fucoidan yield.

Finally, at the conditions tested, the use of open-vessel microwave heating was shown to provide an equivalent alternative to conventional systems for the extraction of fucoidan. However, the inherent advantages of rapid and highly controlled heating, and the potential for up-scaling, encourage further investigation to be conducted into the use of open-vessel microwaves.

3.7 Acknowledgements

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3.9 Appendix 3.1. Ionic liquid pre-treatment for fucoidan extraction

3.9.1 Aim

To test whether a deep eutectic solvent formed between urea and choline chloride could disassociate fucoidan from other cell wall components that may be inhibiting extraction.

3.9.2 Introduction

Room temperature ionic liquids (IL) are salts that are liquids at room temperature (in the absence of solvent), and generally possess high solvating capacity for polar compounds (Handy, 2003). The deep eutectic solvent formed between urea and choline chloride is a type of IL. It was chosen for this investigation because it is made from cheap, renewable, and biodegradable ingredients with low-toxicity; can be recovered and reused; and is capable of dissolving "highly ionic or strongly hydrogen bonding compounds" (Handy, 2003). Its effect on the dissolution of the brown algal cell wall complex was determined.

3.9.3 Method

1. Solid urea and choline chloride were mixed at a molar ratio of 2:1, to form a deep eutectic melt at room temperature
2. It was then mixed with 2 g of oven dried *E. radiata* (milled to a particle size of 250-1,400 µm) at the desired weight-to-weight ratio, and incubated at the desired temperatures and duration in an incubator with shaking (speed 5), according to the Box-Behnken experimental design shown in Table 3.2
3. pH 3 HCl was added to a final volume of 60 mL, and the mixture was extracted at 98 °C for 10 minutes with stirring using an open-vessel microwave (Milestone Inc., USA)
4. The slurry was immediately cooled in an ice bath, and centrifuged at 5000 *g* for 15 min at 4°C to remove the residual biomass
5. The filtrate was neutralized with the addition of 1 M NaOH, and calcium chloride was added to 1 % (w/v) to precipitate alginates. The mixture was left at 4 °C overnight, and then centrifuged at 5000 *g* for 15 min at 4°C
6. The supernatant was recovered, two volumes of ethanol absolute were added to precipitate the fucoidan-rich polysaccharide fraction (FRPF), and the mixture was left at 4 °C overnight
7. It was then centrifuged at 5000 *g* for 15 min at 4°C, and the pellet was washed three time in 70 % ethanol, and then oven dried at 60 °C
8. The gravimetric crude yields of the FRPFs were assessed as a % proportion of the weight of the starting material (oven-dried alga, adjusted for residual moisture content)

A control was also conducted in triplicate, whereby the microwave-assisted extraction was conducted without any pre-treatment of the dry alga.

The data were analyzed using response surface methodology, using the 'rsm' package on the R project software platform, using the methodology and statistical analysis described in the following chapter (Lorbeer et al., 2015).

3.9.4 Results

The yields of the FRPFs are presented in Table 3.2. It appears as though the yields achieved after the IL pre-treatment were not significantly different from those obtained without the pre-treatment. Furthermore, no correlation (with an adequate

fit) could be found between the pre-treatment conditions, and the yield of FRPF.

Table 3.2. Experimental design and yield of fucoidan-rich polysaccharide fractions

IL to alga ratio (w/w)	Temperature (°C)	Duration (hr)	Yield (% w/w alga)
3	42	4	4.67
3	42	4	5.00
3	42	4	4.67
2	42	1	4.33
2	42	7	4.33
4	42	1	5.00
4	42	7	5.33
3	24	1	5.00
3	24	7	4.00
3	60	1	5.00
3	60	7	5.33
4	24	4	4.67
4	60	4	5.00
2	24	4	5.00
2	60	4	5.00
	Control 1		5.00
	Control 2		4.33
	Control 3		5.00

3.9.5 Discussion

- If the IL pre-treatment had any effect on the extractability of the fucoidans, it was not statistically significant. Therefore, it is apparent that at the tested conditions the pre-treatment did not substantially improve the yield of FRPFs. And, given the range of temperatures, durations and IL: alga ratios tested, it seems unlikely that further modifying those parameters will result in substantial improvements.
- Furthermore, the IL was quite difficult to fully remove, necessitating several washing steps. This could hamper its desirability for industrial applications, due to high water requirements and the potential persistence of IL residues in product streams.
- Future investigations could trial different types of ILs. Although, given the intended applications of fucoidans as human health products, it would be crucial that the ILs could be completely removed, and exhibit very low toxicity.
- Integrating the IL with a complementary technology may be able to intensify its solvating action. For example, Han et al. (2011) used an ultrasonic bath assist with the IL extraction of phenolic compounds from brown algae

3.10 Appendix 3.2. Enzymatic pre-treatment for sequential extraction

3.10.1 Aim

To test whether commercial enzyme preparations could aid in the disaggregation of the cell wall complex by digesting structural components (e.g. cellulose and proteins) that may inhibit the extraction of fucoidans and other valuable compounds.

3.10.2 Method

Processing method

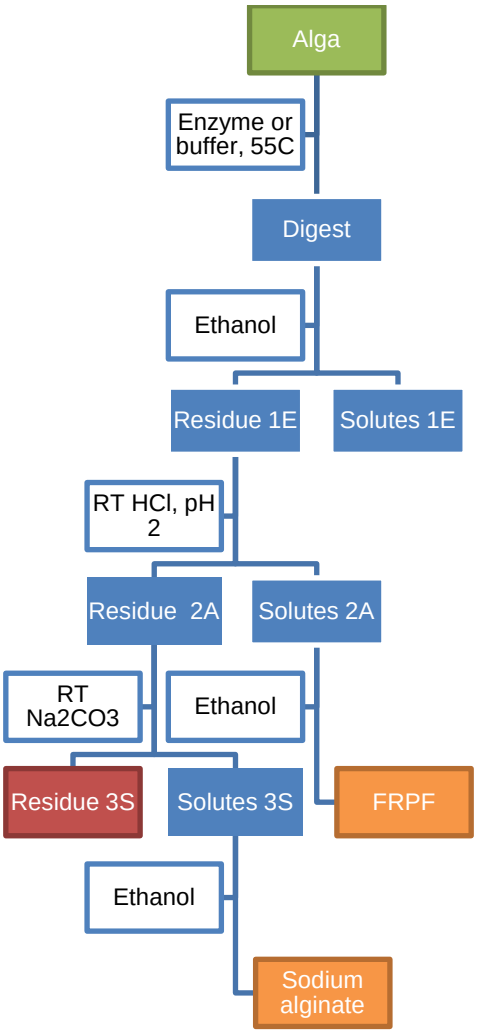

Figure 3.8. Flow chart of the enzyme treatment and sequential extraction process

The enzyme treatment and sequential extraction is represented in Figure 3.8. In more detail, the steps were as follows:

1. 150 mL of water was pre-heated to 50 °C in a 250 mL conical flask, in an incubator
2. 7.5 g of oven-dried *E. radiata* (milled to 250-1400 µm particle size) was added, and the mixture was returned to the incubator for 10 minutes with shaking

3. The pH was adjusted to 7 (the optimal working pH of the enzymes) with the drop-wise addition of 1 M NaOH or HCl
4. 750 µL of the commercial enzyme suspension was added (when both enzyme preparations were used, 375 µL of each was added)
5. The flask was covered with aluminum foil and incubated for 6hr at 50C in the incubator with shaking (speed 7)
6. The flask was removed from the incubator, cooled in an ice slurry, and two volumes of ethanol absolute were added. The mixture was shaken and left at 4 °C overnight
7. The mixture was centrifuge at 5000 *g* for 15 minutes, at 4 °C
8. The supernatant (Solutes 1E) was kept for other investigations, and 150mL of 0.01M HCl was added to the Residue 1E. The pH was adjusted to 2±0.1 with the addition of 2M HCl
9. The flask was incubated for 1 hour with vigorous shaking (speed 9) at room temperature
10. The slurry was centrifuged at 5000xg for 15min at 4C
11. The supernatant (Solutes 2A) was removed, neutralized with the addition of 2M NaOH, and 2 volumes of ethanol absolute were added to precipitate the fucoidans and other extracted polysaccharides
12. It was left overnight at 4 °C, then centrifuged at 5000xg for 15min at 4 °C
13. The supernatant was discarded, and the pellet (fucoidan-rich polysaccharide fraction (FRPF)) was washed twice with 70 % ethanol, and dried under vacuum
14. To the Residue 2A, 150 mL of 0.2 M sodium carbonate solution was added, and the pH was adjusted to 10 with 2 M sodium carbonate
15. The mixture was placed in a 55 °C incubator with shaking (speed 7) for 2 hours
16. The slurry was diluted with 600 mL of cold water, and centrifuged at 5000xg for 15min at 4 °C
17. The supernatant (Solutes 3S) was recovered, 1 volume of ethanol absolute was added, and the mixture was shaken well to precipitate the sodium alginates. It was then centrifuge at 5000xg for 10min to recover the sodium alginate pellet, which was washed twice with 50 % ethanol, and dried under vacuum

The enzyme preparations trialed were Ultraflo (a multi-active β-glucanase), Flavourzyme (a protease containing endo- and exo- catalytic activity), and a mixture of both. They were compared against a 'no enzyme' control, where all steps were kept constant, except 750 µL of water were added instead of enzyme preparation. All treatments were conducted in triplicate.

Analysis

1. The gravimetric crude yields of the FRPFs and sodium alginates were assessed as a % proportion of the weight of the starting material (oven-dried alga, adjusted for residual moisture content)
2. The sugar composition of the FRPFs was analyzed using the same method described earlier in this chapter
3. Data are presented as mean values ± standard deviation.

3.10.3 Results

Figure 3.9, 3.10, and 3.11 show that no significant differences were observed in the FRPFs and sodium alginates obtained after different enzymatic pre-treatments, in terms of their yield, composition, or molecular weight.

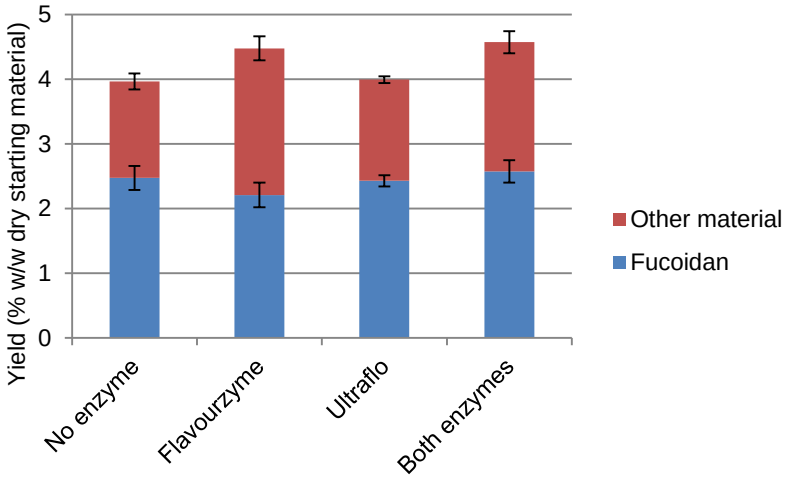

Figure 3.9. The yield and composition of the fucoidan-rich polysaccharide fractions

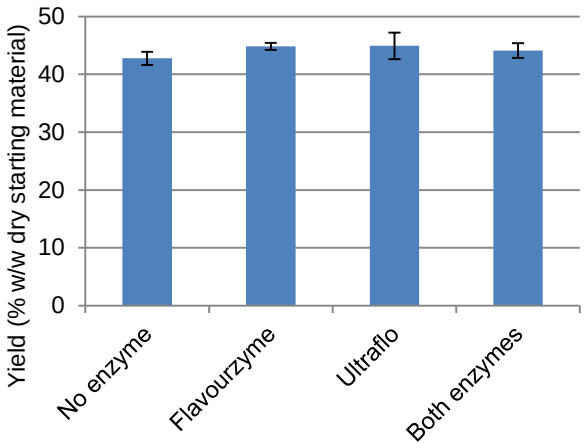

Figure 3.10. The yield of the sodium alginate extracts

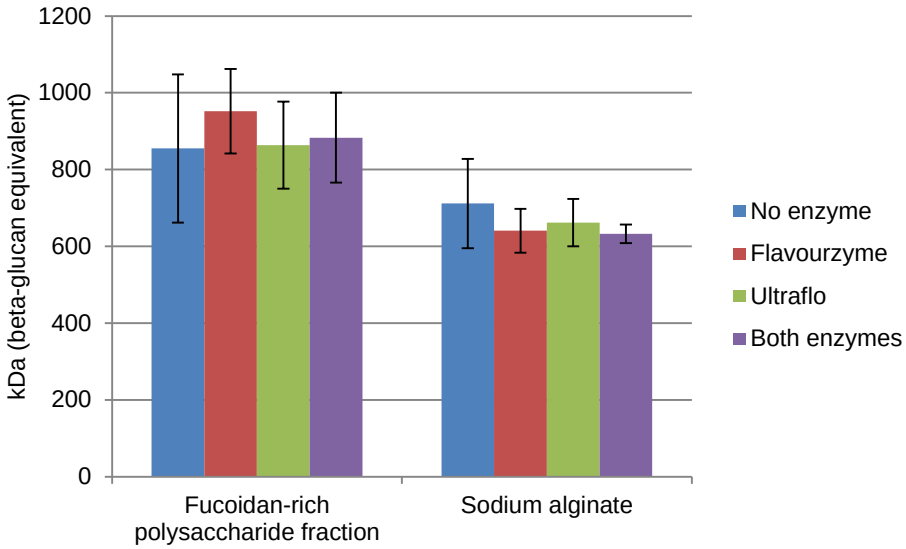

Figure 3.11. The peak molecular weight of the polymers eluting in the main peak during size exclusion chromatography of the sequential extraction products

3.10.4 Discussion

- Possible reasons why the enzymes did not improve the disaggregation of the cell wall complex:
    - The molecular environment of the brown algal cell wall is different to that of terrestrial plant cell walls, which may affect the specific activity of terrestrial enzymes (Baldan et al., 2001). For example, Leskinen et al. (2015) showed that the presence of wood sterols improves the activity of cellulase on a cellulosic substrate.
    - The alginate-dominated mucilage might obstruct the access of enzymes to minor structural components of the cell wall, such as cellulose and certain proteins (Baldan et al., 2001).
    - The enzymes used may not possess specific activity against the cell wall components responsible for inhibiting the extraction of fucoidans. For example, some fucoidans may be tightly associated with insoluble polyphenols (Deniaud-Bouët et al., 2014)
    - Algal metabolites, such as proteases, may have inhibited the enzymes.
- Potential directions:
    - It would be interesting to isolate cellulose from brown algae, and then use it as a substrate to test the activity of commercial cellulases. If none of them work, it may suggest that the crystal structure of brown algal cellulose is resistant to terrestrial cellulases. If they work well, it may suggest that their access to the cellulose is being blocked by other components of the cell wall and mucilage, such as the alginates.
    - Cellular labelling and microscopy could be used before and after various enzymatic treatments to assess whether components of interest have been digested of disassociated from each other.
    - The experiment could be repeated, but with the starting material first being treated with boiling ethanol to extract and denature proteases and other enzyme inhibitors. Also, the particle size of the starting material could be reduced to a fine powder to improve enzyme access to substrates. However, if either of these treatments are necessary for the enzymatic digestion to work effectively, it may hinder industrial-scale utilization.

3.11 Appendix 3.3. First page of the published article

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Kinetics of conventional and microwave-assisted fucoidan extractions from the brown alga, *Ecklonia radiata*

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Abstract Fucoidans—the bioactive and structurally diverse polysaccharides from brown algae—have potential applications in the health industry, but development has been hampered by difficulties in controlling the structure, purity, and consistency of the product. Importantly, the extraction technique affects the nature of the product by preferentially extracting differing fucoidan compounds, whilst simultaneously altering their structure. Extractions are typically performed using hot, dilute acid, but the kinetics of extraction are poorly understood, especially with regard to trade-offs between yield, purity and structural integrity. Here, the extraction kinetics of fucoidan from the brown alga *Ecklonia radiata* under classical conditions (hydrochloric acid at 60 °C, pH 2) have been investigated, using both convective and microwave heating in open vessels for up to 3 h. The yield, purity and key structural characteristics of the fucoidan extracts were assessed. Only modest gains in yield were achieved beyond 6 min of extraction. Extended extraction was accompanied by undesirable declines in fucose and sulphate content, a substantial increase in the co-extraction of the contaminant laminarin, and significant reductions in the molecular weight of fucoidans. The results demonstrate that maximising fucoidan yield often comes at the cost of purity and structural integrity. It is important to tailor an extraction technique to best suit the desired applications of the final products. Open-vessel microwaves offer a highly efficient and controllable alternative to convective heating and merit further investigation, but techniques that better disrupt the cell wall complex whilst preserving fucoidan structure must also be pursued.

Keywords Fucan · Fucose · Hydrolysis · Laminarin · Seaweed · Sulphated polysaccharides

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Introduction

Aside from their already popular applications in the food, agriculture and biomaterials industries, as well as their potential for use in biofuel production, seaweeds are becoming increasingly recognised for their biologically active compounds, which have a range of potential applications in the health sector. One family of such compounds is referred to as the fucoidans, the sulphated polysaccharides found in nature almost exclusively in brown algae. These are complex, high molecular weight polymers, typically dominated by L-fucosyl residues and sulphate esters but also occasionally containing mannose, galactose, xylose, uronic acid and acetate (Li et al. 2008).

A broad range of biological activities has been reported for fucoidans and their derivatives; most notably anticoagulant and antithrombotic, antiviral, anticancer, immunomodulating, anti-inflammatory, anti-lipidemic and anti-fertilisation effects (Morya et al. 2012). This breadth of activities can be explained by the structural diversity of fucoidans, with substantial variations having been reported in their monomeric composition, structural configuration (e.g. branching), molecular weight and sulfation pattern, with each of these aspects, particularly

4 Multiple-response optimization of the acidic treatment of the brown alga *Ecklonia radiata* for the sequential extraction of fucoidan and alginate

This chapter comprises an article that was published in the academic journal Bioresource Technology (Vol. 197, pp. 302-309). The first page of the published version can be seen in Appendix 4.1.

Author contributions: VB provided advice on the conduct and analysis of high-performance size exclusion chromatography (HP-SEC), JL provided technical assistance for HP-SEC and helped operate the instrumentation, TN provided training for the use of the 'rsm' software package, WZ assisted with the experimental design, and all authors helped edit the article throughout the publication process. I designed the experiment, performed the extractions, conducted all sample analyses (with the assistance of JL for the HP-SEC), performed the RSM and desirability experiments, analyzed all data and wrote all original content.

Multiple-response optimization of the acidic treatment of the brown alga *Ecklonia radiata* for the sequential extraction of fucoidan and alginate

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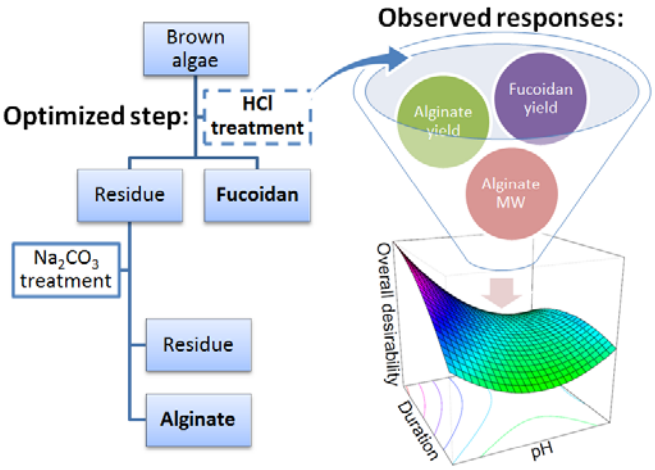
Graphical abstract



4.1 Abstract

The aim of this study was to optimize the acidic treatment of the brown alga *Ecklonia radiata* in order to extract fucoidan and facilitate the efficient sequential extraction of alginates. Response surface methodology was used to determine the effects of the temperature, pH, and duration of the acidic treatment on fucoidan yield, alginate extractability, and the molecular weight of sequentially extracted alginates. Desirability functions were then used to predict the best overall combinations of responses. The most desirable compromise allowed for the recovery of a fucoidan-rich fraction with a yield of 3.75% (w/w of alga) and the sequential extraction of alginates having an average molecular weight of 730 kDa at

a yield of 44% (w/w of alga), with low cross-contamination between the products. The optimized acidic treatment could form the basis of an industrial biorefinery process for the production of both fucoidan and alginate.

Keywords:

Response surface methodology, desirability function, experimental design, biorefinery, sulfated polysaccharides.

4.2 Introduction

Seaweeds, also known as marine macroalgae, are becoming an increasingly attractive resource for human utilization due to their high growth rates, non-requisite of fresh water and arable land, ability to clean seawater polluted by industrial effluents, and carbon capturing potential.

While these traits lend themselves favorably to the prospect of bioenergy production, it is the functional polysaccharides that seaweeds produce that currently dominate the market for non-wholefood seaweed-derived products. This owes mostly to the phycocolloids – hygroscopic algal gums with thickening, emulsifying, stabilizing and gel-forming properties – which include alginates from brown algae, and carrageenans and agars from red algae (McHugh, 2003). In recent years, however, the sulfated polysaccharides of seaweeds have received increasing attention due to their specific biological activities and unique properties; the most noteworthy being fucoidans from brown algae, ulvans from green algae, and the carrageenans from red algae (Winberg et al., 2014).

Current industrial extraction operations from seaweeds often suffer from low efficiencies of biomass utilization, and the generation of waste streams. The development of integrated biorefinery processes for the production of multiple products from the starting material may mitigate these issues, while also improving the economic viability of the seaweed processing industry. Jung et al. (2013) provided an excellent discussion regarding the prospects for seaweed biorefinery at an industrial scale, in which they predicted the rapid development of the industry in the near future, aided by some technological advancements.

Brown algae are amenable to such biorefinery processing as they contain a suite of metabolites with existing or potential applications, including the extracellular matrix polysaccharides alginates and fucoidans, the storage compounds laminarins and mannitol, the pigment fucoxanthin, and the often-bioactive polyphenols, terpenoids and halogenated compounds (Lorbeer et al., 2013). A number of

biorefinery strategies have been devised for brown algae, involving the use of new technologies such as supercritical CO_2 extraction, autohydrolysis and ultrafiltration (Balboa et al., 2013; González-López et al., 2012; Pérez-López et al., 2014; Quitain et al., 2013). Such approaches have the potential to extract difficult-to access compounds, reduce chemical and water use, and valorize post-extraction residues. However, industry may be slow to adopt these techniques due to the need for comprehensive and expensive infrastructure upgrades and difficulties associated with their scaling-up.

In the meantime, parallels between existing processing technologies for different products reveal immediate opportunities for a progression toward the seaweed biorefinery. For instance, dilute acid treatments are commonly used in both fucoidan and alginate extraction processes. For fucoidan extraction, acid treatments are used to disrupt hydrogen bonds between polysaccharides, thus liberating fucoidans, whilst simultaneously preventing alginate contamination by converting it into insoluble alginic acid (Hahn et al., 2012). In conventional alginate production processes, dilute acid is used as a pre-treatment to extraction in order to remove potential contaminants (including fucoidans, laminarins and polyphenols), while converting insoluble alginate salts into alginic acid, which is then more easily converted into soluble sodium alginate upon treatment with sodium carbonate (Hahn et al., 2011). Therefore, if all of these functions could be achieved by an acidic treatment simultaneously, it could form the basis of a sequential biorefinery process as a fucoidan extractant and alginate pre-treatment.

Several analytical studies have employed the acidic treatment for the sequential extraction of fucoidan and alginate to allow for their characterization, but process development was not pursued from an industrial biorefinery perspective (Chattopadhyay et al., 2010; Rioux et al., 2007; Shyamali et al., 1988). Therefore, the precise conditions necessary to achieve an adequate yield and quality of both products remains unknown. If the conditions are too mild, product yields may suffer, whereas if they are too aggressive, the alginates may degrade, resulting in a low viscosity product (Gomez et al., 2009; Hernández-Carmona et al., 1998). Here we have analyzed the effects of pH, temperature and duration of the acidic treatment on the yield of fucoidans, and the extractability and molecular weight (MW) of alginates, in order to optimize this critical step and assess its potential as the basis for seaweed biorefinery processes.

4.3 Materials and Methods

4.3.1 Materials and reagents

All chemicals were purchased from Merck and Sigma, with the exception of the β-glucan MW standards, which were from Megazyme (Ireland).

Ecklonia radiata (C. Agardh) J. Agardh biomass (identified by the State Herbarium of South Australia) was collected from the freshly deposited beach-cast seaweed in Rivoli Bay, Beachport, South Australia in July 2014. It was immediately rinsed in fresh water, removed of any visible surface contaminants, and placed on mesh racks to dry. The whole plants were then milled with a blender (Blendtec, USA), sieved to a particle size of 250-1400 µm, and further dried in an oven at 45°C prior to storage in a desiccator. *E. radiata* was chosen as the feedstock for this investigation due to its abundance throughout the region of Southern Australia, its potential for aquaculture, and its potential for valorization due to a high alginate content and interest in its fucoidans and other potential bioactives (Charoensiddhi et al., 2014; Loo et al., 2011; Lorbeer et al., 2013).

4.3.2 Sequential biorefinery extraction process

Figure 4.1 summarizes the sequential biorefinery process used in this study. For each treatment, 7.5 g of the dried and milled alga was first extracted twice with 75 mL of anhydrous ethanol with constant stirring for three hours to remove some unwanted components (including phenolic compounds and proteins), and dried overnight at 40°C.

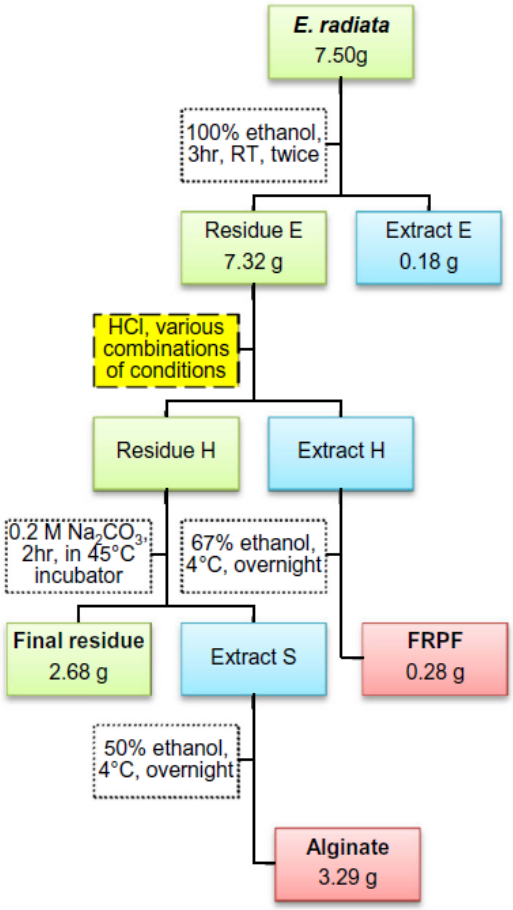


Figure 4.1. Overview of the sequential biorefinery extraction process.
The HCl treatment step was optimized in this study using experimental design, while every other step was held constant. The average mass of the quantified fractions is listed, as observed when using the optimal conditions predicted within the expanded domain (duration = 159 minutes, pH = 1, temperature = 42°C).

Extract E was discarded and the defatted tissue (Residue E) was placed in 150 mL of pre-heated aqueous HCl solution and extracted in a shaking incubator, with the temperature, pH (prior to the addition of alga) and duration determined by the experimental design. Immediately after extraction, the mixture was cooled in an ice slurry and neutralized by the drop-wise addition of 2M NaOH. The residual biomass (Residue H) was then removed by vacuum filtration, and stored at -20°C for further use. Two volumes of anhydrous ethanol were added to the extract liquor (Extract H), which was then left overnight at 4°C to allow for the precipitation of polysaccharides. The precipitate was then removed by centrifugation (7000 x g, 15 minutes, 4°C), washed twice with 70% ethanol, and freeze-dried to obtain a fucoidan-rich polysaccharide fraction (FRPF).

Residue H was subsequently thawed, placed in 150 mL of 0.2 M sodium carbonate solution, and extracted in a 45°C shaking incubator for two hours. The mixture was then immediately cooled in an ice slurry and diluted with water to a total volume of 600 mL. Residual biomass (Final residue) was removed by low-speed

centrifugation, washed with water, and freeze-dried for further analysis. Meanwhile, one volume of anhydrous ethanol was added to the extract liquor (Extract S) and left at 4°C overnight to precipitate the sodium alginates. The precipitate was collected by centrifugation, washed twice with 50% ethanol, and freeze-dried.

4.3.3 Optimization strategy

A Box-Behnken design with three replicates at the centre point was used to test the combined effects of three variables (temperature, pH and duration) in the acid treatment step, each at three levels, on three different responses: FRPF yield, alginate extractability (in terms of alginate yield after the sodium carbonate extraction step), and the MW of the extracted alginate. The experimental domain was defined around conditions that had previously been reported to have performed well (Gomez et al., 2009; Hernández-Carmona et al., 1998). The experimental design and responses are presented in Table 4.1.

Response surface methodology (RSM) was then applied using the 'rsm' package on the R Project software platform to fit second-order models to the experimental data and generate surface plots to visualize the effects of the variables on each response individually. This software was also used to conduct statistical tests for each model (significance, lack of fit, and R^2 correlation coefficient, shown in Table 4.2) and to determine the probability of each factor having the estimated effect on each response (significance codes in Table 4.3). A model of this nature is generally considered acceptable if it reaches 95% significance ($P_S < 0.05$), its lack of fit is insignificant ($P_L > 0.05$), and its correlation coefficient is sufficiently high ($R^2 \geq 0.8$ for chemical data) (Lundstedt et al., 1998; Sousa et al., 2010).

Table 4.1. Experimental design of the acid treatment conditions, and the observed responses

Temperature[a] (°C)	x_1[b]	pH[a]	x_2[b]	Duration[a] (min)	x_3[b]	FRPF yield[c] (%)	Alginate yield[d] (%)	Alginate MW[e] (kDa)
35	0	3.5	0	60	0	3.19	33.7	709
35	0	3.5	0	60	0	3.05	33.2	719
35	0	3.5	0	60	0	2.98	34.7	746
25	-1	3.5	0	10	-1	2.31	32.2	736
25	-1	3.5	0	110	1	3.10	33.3	734
45	1	3.5	0	10	-1	2.69	33.1	728
45	1	3.5	0	110	1	3.00	34.5	798
35	0	2	-1	10	-1	2.65	35.0	672
35	0	2	-1	110	1	3.03	38.0	663
35	0	5	1	10	-1	2.92	35.0	677
35	0	5	1	110	1	3.09	33.7	693
25	-1	2	-1	60	0	2.86	36.4	514
25	-1	5	1	60	0	2.93	33.1	616
45	1	2	-1	60	0	3.03	38.3	690
45	1	5	1	60	0	2.95	35.1	586

[a]Real values.
[b]Coded values.
[c]Amount of crude fucoidan extract (with the weight of co-extracted alginate removed) obtained during the acid treatment, as a percentage of the weight of dry alga used as a starting material.
[d]Amount of alginate extract obtained by extracting the acid pre-treated residue using sodium carbonate, as a percentage of the weight of dry alga starting material.
[e]Given in terms of the equivalent hydrodynamic volume of β-glucan MW standards.

Table 4.2. The quality of fit of the second-order functions

Function	Model significance (P_S)	Lack of fit (P_L)	Correlation coefficient (R^2)
FRPF yield	0.057	0.415	0.889
Alginate yield	0.008	0.659	0.952
Alginate MW	0.004	0.424	0.966

Table 4.3. The quadratic models fitted to the three responses, with significance codes

Y	a	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}	b_{11}	b_{22}	b_{33}
FRPF yield (%)	3.07***	0.06	0.04	0.21**	-0.04	-0.12	-0.05	-0.14^	0.01	-0.16^
Alginate yield (%)	33.9***	0.74*	-1.33**	0.52^	0.00	0.06	-1.08*	-0.18	1.98**	-0.47
Alginate MW (kDa)	724***	25.2*	4.12	9.37	-51.5**	18.0	6.25	-25.2^	-97.9***	49.5**

The equations are of the type: $Y = a+b_1x_1+b_2x_2+b_3x_3+b_{12}x_1.x_2+b_{13}x_1.x_3+b_{23}x_2.x_3+b_{11}x_1^2+b_{22}x_2^2+b_{33}x_3^2$
Significance codes: *** = P<0.001, ** = 0.001<P<0.01, * = 0.01<P<0.05, ^ = 0.05<P<0.1
Responses are in real values, whilst variables are in coded values.

The Solver Add-In on Microsoft Excel was used in conjunction with the fitted equations and response surfaces to identify local maxima and simulate various parameter scenarios. Where optimum conditions were not predicted within the experimental domain, an expanded domain was used for extrapolative predictions, according to what might be practical in a commercial setting, and extending no

further than twice the distance from the centre-point of the experimental domain. The expanded domain used limited the temperature from 25 to 55°C, the pH from 1 to 6.5, and the duration from 1 to 160 minutes.

After ascertaining that the optimal conditions varied significantly for each response, the 'desirability' package on the R platform was used to apply desirability functions in order to find the best possible compromise of responses within the experimental domain and the expanded domain. To do this, minimally and maximally desirable outcomes on a common scale of zero to one were assigned to each response according to the minimum and maximum values predicted by the corresponding second-order models. Overall desirability was calculated as the geometric mean of the three responses predicted at any given set of conditions. The model was confirmed experimentally by performing extractions in triplicate using the predicted optimal conditions.

4.3.4 Analysis of fractions

For the analysis of bound sugars in the starting material, extracts and residue, unbound sugars were removed with 70% ethanol, and polysaccharides were hydrolyzed using a two-step acid hydrolysis (72 % sulfuric acid, room temperature for 1 hour; then 1M sulfuric acid, 100 °C for 3 hours). The liberated monosaccharides were then derivatized with 1-phenyl-3-methyl-5-pyrazolone using the method of Comino et al. (2013), prior to HPLC fractionation. Peaks were identified and quantified using a set of standard monosaccharides (D-mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, D-galactose, D-xylose, L-arabinose, L-fucose, D-glucose) and the internal standard 2-deoxyglucose. The quantitative data were adjusted for acid degradation by including a set of standards in the hydrolysis protocol. Sodium alginate (Sigma) was used to identify alginate-derived uronic acids and quantify total alginate (in equivalent mass terms of sodium-alginate).

The relative MW profiles of the alginate extracts were estimated by size-exclusion chromatography on an Agilent HPLC, using PolySep GFC-P5000 and PolySep GFC-P6000 columns in series (Phenomenex), with refractive index detection (Shimadzu), in a mobile phase of 0.1 M aqueous sodium nitrate. Two sets of MW standards were used for calibration, including (1,3;1,4)-β-glucans (peak MW of 67.1, 160, 247, 375, and 667 kDa) and branched dextrans (peak MW of 65, 195, 400, and 1050 kDa). Where necessary, fractions were collected and their sugar profiles were analyzed in order to identify the main components of the peaks.

4.4 Results and discussion

Fucoidan yield

The gravimetric yield of the FRPF was first analyzed, and a maximum of 3.42% was predicted just outside of the experimental domain (34.6°C, pH 5.44, 86 minutes) using a second-order equation that was deemed to have an adequate fit (P_S = 0.043, P_L = 0.389, R^2=0.903). However, sugar analysis revealed that the observed trends in the FRPF yield were affected by the co-extraction of alginates, which increased with the pH, duration and temperature of the treatment.

This was therefore an undesirable response to maximize, as the co-extraction of alginates reduced both the purity of the extracted fucoidans and the quantity of alginates remaining in Residue H for subsequent extraction with sodium carbonate. The alginate content of the FRPFs was therefore subtracted from their total weight, which eliminated the variation in the fucose content of the FRPFs (an indicator of fucoidan purity). With fucoidan purity stabilized, the alginate-corrected FRPF yield was deemed a suitable response for optimization. Unfortunately, the second-order model fit to the resulting data had a slightly less adequate fit (Table 4.2), but it was used for further process optimization in lieu of more suitable alternatives.

Within the conditions tested, the pH actually had very little influence on the yield of the FRPF, whereas the duration exerted a significant positive linear effect (Figure 4.2), and the temperature and duration both exerted significant quadratic effects (at the 90% confidence level) (Table 4.3). Within the experimental domain, the maximum achievable yield of FRPF (correcting for alginate) was predicted to be 3.15%, which was not significantly improved when extrapolating within the expanded domain (Table 4.4).

Our results therefore support the role of acids proposed by Hahn et al. (2012) in extracting fucoidans while preventing the co-extraction of alginates, although their ability to also improve fucoidan yields by disrupting hydrogen bonds was not observed. In fact, the predicted optima occurred at the lower limit of the experimental domain and the upper limit of the expanded domain. Rabanal et al. (2014) also observed a reduction in crude polysaccharide yield accompanied by an increase in fucose content (indicative of fucoidan concentration) with increased extractant acidity within a broader domain of 0 to 0.2 M HCl (except when at high temperature and duration, perhaps due to the hydrolysis of fucoidans). We had observed a positive relationship between the duration of the acid treatment and the yield of fucoidan-rich polysaccharides in a previous investigation (Lorbeer et al., 2015). However, in this earlier work it was found that the co-extraction of laminarin

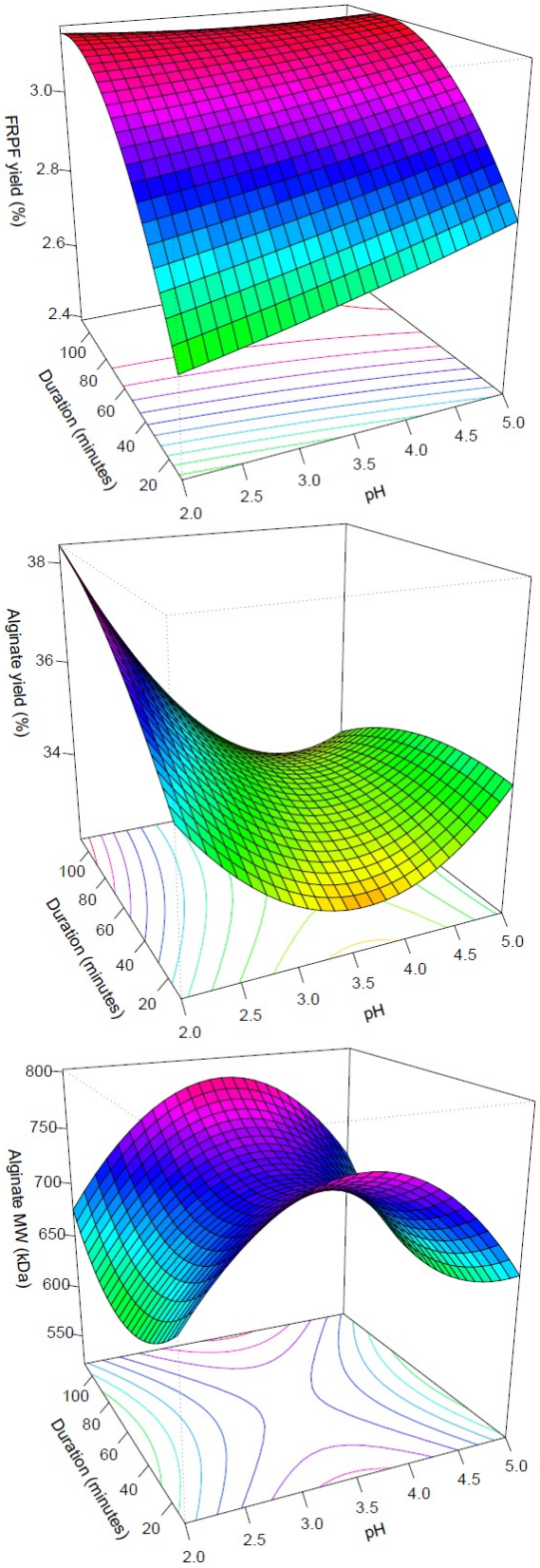


Figure 4.2. Individual response surfaces showing the effects of pH and duration of the acid treatment on each of the three responses (temperature maintained at 35°C).

accounted for an increasing proportion of the fraction as the duration increased. Here, fucose accounted for 73-78% of the sugars detected in all FRPFs, whilst glucose (indicative of laminarin) remained constant at 2-3% (data not shown) due to the very low laminarin content of the starting material (probably due to the season of collection).

4.4.1 Alginate extractability

Following the extraction of fucoidans, the acid-treated residues (Residue H) were heated in a sodium carbonate solution at constant conditions to facilitate the extraction of alginates, in order to investigate how the temperature, pH and duration of the preceding acid treatment affected extractability. According to the sugar analysis, there were only slight variations in the content of alginate-derived uronic acids between the alginate extracts, and no correlation was observed between purity of the product and the acidic treatment conditions. Therefore, the yield of the alginate extract was deemed a suitable response for optimization.

The model fitted to the data had a very high quality of fit (Table 4.2), and suggested that all three variables exerted significant linear effects, while the pH also displayed a very strong quadratic effect and a significant interaction with the duration of the acid treatment (Table 4.3). Within the experimental domain, the extractability of alginates was generally seen to improve with more acidic conditions, especially when at elevated durations and temperatures (Figure 4.2). As such, a maximum alginate yield of 39% was predicted to occur following an acid treatment at a pH of 2 and a temperature of 45°C for 110 minutes, but this was substantially increased to 45% when operating at the limits of the expanded domain with a pH of 1 (Table 4.4).

This can be explained by three phenomena. Firstly, the heat-assisted conversion of naturally occurring alginate salts – including the insoluble calcium and magnesium forms, and the soluble sodium salts – to insoluble alginic acid under acidic conditions. Alginic acid is then more easily converted to (and extracted as) sodium alginate upon subsequent treatment with sodium carbonate, compared to the direct conversion of calcium or magnesium alginates (Hahn et al., 2011). Secondly, if the naturally occurring sodium alginate was not converted to alginic acid, then a proportion of it would be co-extracted with the fucoidans during the pre-treatment, leaving less available for extraction at the sodium carbonate treatment step, as was observed here when more neutral solvents were used during the acid treatment step. Finally, acid treatments can remove phenolic compounds, which may otherwise remain associated with alginates and inhibit their extraction

(Deniaud-Bouët et al., 2014; Haug, 1964). Hernández-Carmona et al. (1998) and Jayasanker (1993) also observed improved alginate yields after pre-treatment with 0.1M HCl compared with a pH 4 solution or water, which the first group demonstrated to be accompanied by increased Ca/H exchange.

Table 4.4. Estimated optimal conditions and responses within the experimental and expanded domains, and the overall desirability of each combination of responses.

Domain	Optimised response	Temperature (°C)	pH	Duration (min)	Fucoidan yield (%)	Alginate yield (%)	Alginate MW (kDa)	[c]Overall desirability
[a]Experimental	FRPF yield	35	2.0	101	**3.15**	38.2	657	0.66
	Alginate yield	45	2.0	110	2.98	**39.0**	745	0.69
	Alginate MW	45	3.2	110	2.93	35.2	**806**	0.55
	Overall desirability	42	2.0	110	3.05	38.8	731	**0.69**
[b]Expanded	FRPF yield	33	6.5	80	**3.21**	38.2	373	0.54
	Alginate yield	55	1.0	160	2.19	**45.4**	834	0.65
	Alginate MW	50	3.0	160	2.28	35.3	**986**	0.47
	Overall desirability	42	1.0	159	2.82	45.0	735	**0.81**

[a]Experimental domain defined as a temperature between 25 and 45°C, a pH between 2 and 5, and a duration between 10 and 110 minutes

[b]Expanded domain defined as a Temperature between 25 and 55°C, a pH between 1 and 6.5, and a duration between 1 and 160 minutes

[c]Overall desirability is equal to the geometric mean of the individual desirabilities of the three responses, each on a scale of 0 to 1, corresponding to the minimum and maximum predicted responses within the expanded domain

Bold text highlights the value of the optimized response

4.4.2 Molecular weight of the extracted alginates

The alginate extracts were then subjected to size exclusion HPLC in order to determine the effects of the conditions used during the acid treatment on the MW of the sequentially extracted alginates. The chromatograms obtained all displayed a single, regularly-shaped peak, and sugar analysis suggested that all of the alginate extracts were approximately 90% pure. Therefore, the retention times of the peaks could be used to estimate the MW of the extracted alginates, as a function of their hydrodynamic volume relative to that of commercial MW standards. Both β-glucans and dextrans were used for calibration. However, the relationship between their hydrodynamic volume and MW varies markedly due to substantial differences in their conformations. Therefore, the β-glucans were used to estimate the MW of the alginates, as both molecules exist mainly as extended chains, whereas branched dextrans are similar to globular coils, and therefore better suited to sizing fucoidans (Draget et al., 2005; Patankar et al., 1993).

The model generated had an excellent fit with the data (Table 4.2) and showed a significant positive correlation between the temperature and the MW of the extracted

alginates within the experimental domain, as well as an interaction between temperature and pH, and quadratic effects of all three variables (Table 4.3).

The quadratic effect of the pH used during the acid treatment was the most significant determinant of the MW of the sequentially extracted alginates. Relatively high MW alginates were extracted following an acid treatment at a pH close to 3.5, whilst the MW reduced sharply as the pH approached either 2 or 5. It has been widely reported that the acid pre-treatment step in alginate production processes can hydrolyze the molecules if the pH is too low (Gomez et al., 2009; Hernández-Carmona et al., 1998; Jayasankar, 1993). However, the reduction in MW observed as the pH approached neutrality seems more peculiar. It may be caused by the less effective removal of phenolic compounds, whose presence can drastically exacerbate the cleavage of alginate during the alkali extraction step (Smidsrod et al., 1963; Wedlock & Fasihuddin, 1990). Another plausible explanation is that without the comprehensive removal of calcium and magnesium ions from alginates using a sufficiently strong acid treatment, the larger molecules would retain a greater number of calcium and magnesium ions, and thus remain more resistant to extraction relative to the smaller molecules. Jayasankar (1993) also reported an increase in alginate MW (as indicated by viscosity) when an acidic pre-treatment had been used compared to a neutral one (water).

A maximal alginate MW of 806 kDa (β-glucan equivalent) was predicted within the experimental domain after treatment with acid at pH 3.2 and 45°C for 110 minutes. The apparent MW could be increased to 986 kDa by increasing the duration of the treatment to 160 minutes, without substantially changing the other parameters. This is in good agreement with the findings of Hernández-Carmona et al. (1998), but the optimal pH is slightly lower than the pH of 4 suggested by Gomez et al. (2009) (although this may simply reflect the fact that the pH reported here refers to the HCl solution before the addition of alga, whereas their group adjusted the pH after).

4.4.3 Multiple-response optimization

The data suggests that the treatment of brown algae with an acid solution of sufficiently low pH does indeed extract fucoidans while preventing the co-extraction of alginates, while simultaneously improving the sequential extractability of alginates. However, it was observed that the three measured responses were maximized with different treatment conditions. Therefore, compromise was necessary in order to identify the overall most desirable treatment conditions.

This was achieved using desirability functions, whereby the desirability of each response at a given set of conditions was determined on linear gradients from least desirable (0) to most desirable (1), which were defined as the minimum and maximum achievable values within the expanded domain, as predicted by the three individual second-order models. The overall desirability was then calculated as the geometric mean of the three individual desirabilities.

As shown in Figure 4.3, the overall desirability within the experimental domain rose toward 0.7 as the temperature, duration, and acidity of the treatment increased. The desirability was fairly low (<0.5) at pH higher than 3.5, owing mostly to a low yield of alginates relative to that achievable within the expanded domain. Meanwhile, it was predicted that the overall desirability could be increased to 0.8 by reducing the pH to 1 and increasing the duration to 159 minutes (Table 4.4). This was predicted to substantially increase the sequential alginate yield from 39% to 45% (individual desirability of 0.51 to 0.96), at the cost of a reduction in FRPF yield from 3.05% to 2.82% (individual desirability of 0.89 to 0.74), which was deemed a favorable trade-off using the desirability directives. A similar alginate MW was predicted in both cases.

Extractions were performed at the optimal conditions predicted within both the experimental and expanded domains. All predicted outcomes were satisfied for the experimental domain (alginate-corrected FRPF yield of 3.16±0.12%; alginate yield of 38.6±1.6%; alginate peak MW of 755±22kDa). However, for the expanded domain, the predicted alginate yield and MW were confirmed (44.0±0.15% and 731±15kDa, respectively), but the observed alginate-removed FRPF yield of 3.75±0.09% was actually considerably higher than predicted. This was explained by the fact that the algae swelled less and therefore retained significantly less fluid when using the pH 1 solution, allowing for a greater volume of Extract H to be recovered by centrifugation after the treatment. This phenomenon was not observed when using acids of pH 2-5, so it was not predicted by the experimental design model, which exemplifies the risks involved when extrapolating beyond the experimental domain. An average of 85% of Extract H's volume was recovered at pH 1, compared to 66% at pH 2-5. If the extract recovery for the pH 1 treatment had been the same as the experimental design treatments, the yield would have been 3.16±0.14%, as predicted by the model.

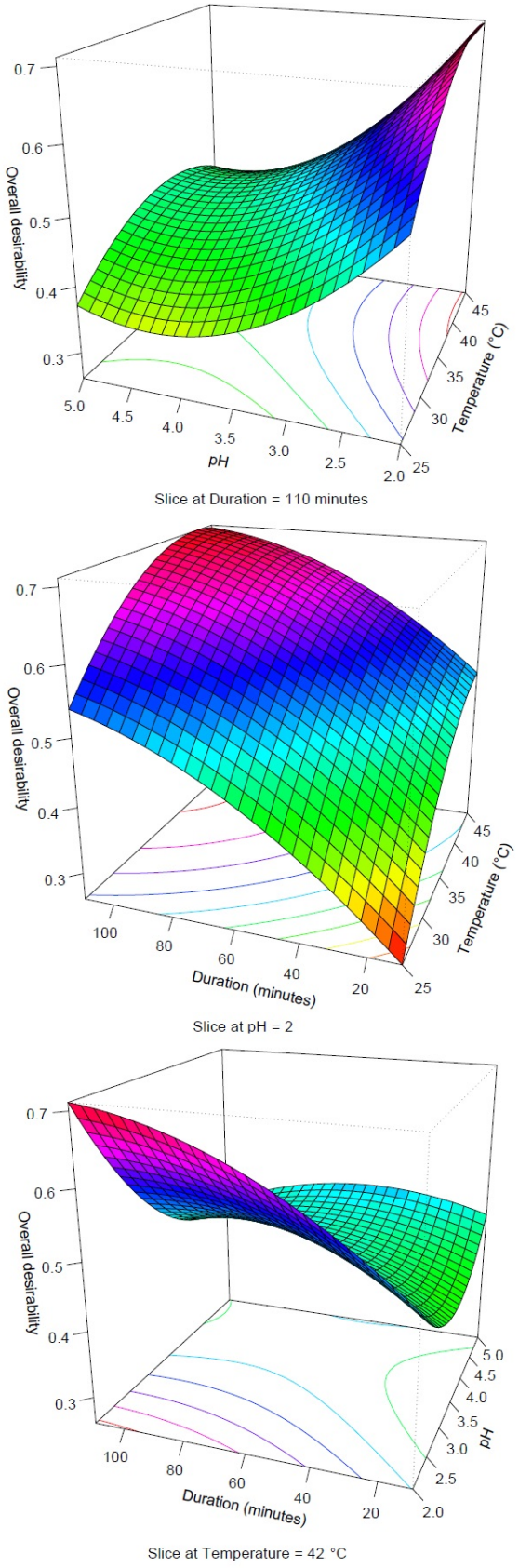


Figure 4.3. Response surfaces showing the effects of the acid treatment conditions on the overall desirability of the observed responses.
The omitted variable in each case is held at its optimal value (duration = 110 minutes, pH = 2, temperature = 42°C) within the experimental domain.

The data presented in Figure 4.4 demonstrate the improved selectivity of the sequential extraction process for both products when operating at the optimal conditions of the expanded domain. For example, alginate contamination in the FRPF was reduced 4.9-fold (1.04% compared with 5.13% w/w) and fucoidan contamination in the alginate extract was reduced 1.7-fold (as indicated by a fucose content of 0.69% compared with 1.21% w/w) when using the optimal conditions within the expanded domain, compared with that of the experimental domain.

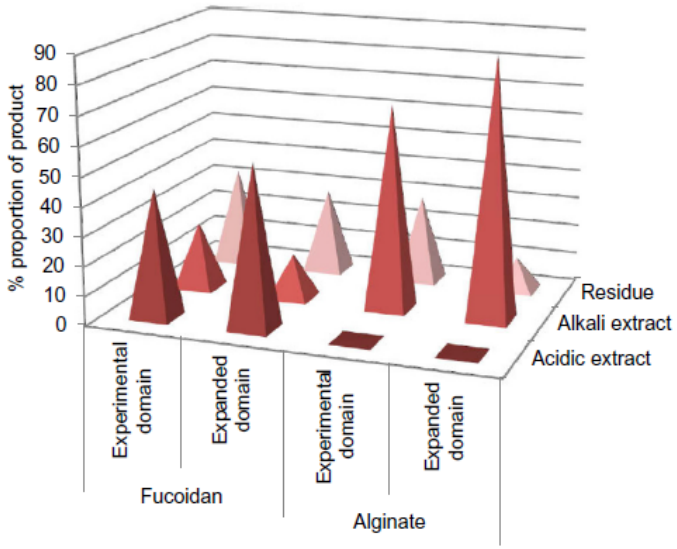


Figure 4.4. The proportion of alginates and fucoidans (as indicated by sugar composition) occurring in the acidic extract (FRPF), alkali extract (crude sodium alginate), and the final residue, after the sequential extraction process using the optimal conditions predicted for the acid treatment step within the experimental and expanded modelling domains.

While the relatively high MW achieved for alginate at the optimal conditions suggests high viscosity applications, it should be noted that we have analyzed a crude product, whereas commercial alginate processing generally involves a post-extraction precipitation with acid or calcium to remove color-causing contaminants. This inevitably further reduces the alginate MW (Gomez et al., 2009). However, very high MW alginate preparations are less stable, so manufacturers often opt instead to produce moderate MW alginates, and add calcium to enhance viscosity (McHugh, 1987).

Although the MW of the extracted fucoidans was not a focus of this investigation, it was observed that it appeared to be significantly more acid sensitive than that of the alginates. When submitting the FRPFs to size exclusion HPLC, the main peaks from all extracts obtained when operating within the experimental domain indicated a peak hydrodynamic volume far greater than that of the largest dextran standard (1050 kDa), whereas the FRPF obtained with the expanded domain optimal conditions was seen to have a hydrodynamic volume of only 115±21 kDa (dextran

equivalent). The viscosity associated with very high MW fucoidans impedes therapeutic applications, so the observed hydrolysis may be advantageous (Hahn et al., 2012; Morya et al., 2012).

From the results, one cannot rule out the possibility of further improving the overall desirability by reducing the pH below 1. However, no further significant increases in the alginate yield are possible, as very little remained in the final residue after the sequential extraction process using the optimal conditions predicted for the expanded domain (Figure 4.4). On the other hand, a large proportion of fucoidan resisted extraction during the process. But it is unlikely that stronger acids would resolve the issue, as they would likely increase the degradation of the products and may prove impractical in a commercial setting due to safety considerations, corrosion of infrastructure and increased generation of hazardous wastes.

Impressive yields of fucoidan have been achieved using very high temperatures and pressures, such as with microwave-assisted extraction and autohydrolysis (Rodriguez-Jasso et al., 2012; Rodriguez-Jasso et al., 2011), but those techniques are obviously not suitable prior to alginate extraction. They could, however, be applied to the final residue to liberate the remaining fucoidans. Deniaud-Bouët et al. (2014) suggested that the extraction of fucoidans might be inhibited by their close association with insoluble cellulose and proteins, which might explain the persistence of the fucoidans (Figure 4.4). A well-defined enzyme-assisted extraction approach might therefore seem an attractive option for disrupting the cell wall matrix while avoiding the degradation of the target polymers.

4.5 Conclusions

The acidic treatment of *E. radiata* was optimized to facilitate the sequential extraction of fucoidans and alginates. The use of solvent with a pH of 1 improved the yield of fucoidans by increasing the volume of extract recoverable from the algae. It also improved the sequential extractability of alginates, and resulted in less cross-contamination between products. The MW of the fucoidans was substantially reduced, but a set of conditions was found that avoided excessive degradation of the alginates. The results suggest that the process devised could form the basis for a biorefinery system.

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4.8 Appendix 4.1. First page of the published article

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Multiple-response optimization of the acidic treatment of the brown alga *Ecklonia radiata* for the sequential extraction of fucoidan and alginate

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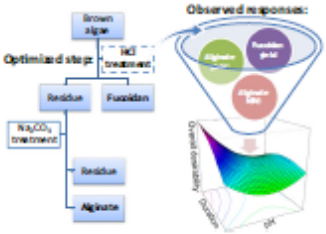
HIGHLIGHTS

- Desirability functions were used to find the best compromise of responses.
- A high yield and molecular weight were achieved for alginate.
- The fucoidans were partially hydrolyzed during acidic extraction.
- Selectivity of sequential extractions improved with a lower pH acid treatment.
- A greater volume of extract could be recovered from the algae when using a lower pH.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this study was to optimize the acidic treatment of the brown alga *Ecklonia radiata* in order to extract fucoidan and facilitate the efficient sequential extraction of alginates. Response surface methodology was used to determine the effects of the temperature, pH, and duration of the acidic treatment on fucoidan yield, alginate extractability, and the molecular weight of sequentially extracted alginates. Desirability functions were then used to predict the best overall combinations of responses. The most desirable compromise allowed for the recovery of a fucoidan-rich fraction with a yield of 3.75% (w/w of alga) and the sequential extraction of alginates having an average molecular weight of 730 kDa at a yield of 44% (w/w of alga), with low cross-contamination between the products. The optimized acidic treatment could form the basis of an industrial biorefinery process for the production of both fucoidan and alginate.

1. Introduction

Seaweeds, also known as marine macroalgae, are becoming an increasingly attractive resource for human utilization due to their high growth rates, non-requisite of fresh water and arable land, ability to clean seawater polluted by industrial effluents, and carbon capturing potential.

While these traits lend themselves favorably to the prospect of bioenergy production, it is the functional polysaccharides that seaweeds produce that currently dominate the market for non-wholefood seaweed-derived products. This owes mostly to the phycocolloids – hygroscopic algal gums with thickening, emulsifying, stabilizing and gel-forming properties – which include algi-

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5 The sequential extraction and potential applications of fucoidans and alginates from four species of brown algae common in Southern Australia

After developing the optimized sequential extraction process, it was important to assess whether it would work effectively when applied to other brown algae. Furthermore, the examination of the yield and quality of the products that could be obtained from common South Australian seaweeds was required to build an understanding of their potential value as feedstocks. Therefore, the data presented within this chapter have broad significance to the overarching aims of the project.

This chapter has been accepted for publication in the Journal of Applied Phycology, pending minor revisions.

Author contributions: CL conducted the activity assays with advice and training from SC and JJW; JL and VB assisted with the use of instrumentation for the size exclusion chromatography; and CMMF and WZ assisted with the editing of the article. I designed the experiment, performed the extractions, conducted all sample analyses (with the exception of the activity assays), analyzed all data and wrote all of the content.

Sequential extraction and characterization of fucoidans and alginates from *Ecklonia radiata*, *Macrocystis pyrifera*, *Durvillaea potatorum*, and *Seirococcus axillaris*

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5.1 Abstract

In a previous study, we optimized the acidic treatment of brown algae to facilitate the efficient sequential extraction of fucoidans and alginates, using a sample of *Ecklonia radiata*. Here, we aimed to apply the optimized process to other species of brown algae from South Australia, in order to assess their potential for valorization, and to determine whether the process was effective when using different feedstocks. The starting materials included samples of *Macrocystis pyrifera*, *Durvillaea potatorum*, *Seirococcus axillaris,* and two more samples of *E. radiata* collected from different sites and at different periods. The feedstock sample used for optimization was also included for comparison. The yield of alginate achieved with these species ranged from 29 to 55 % (w/w dry alga). The quality of the alginate varied, with *M. pyrifera* yielding the most viscous (599 mPa s) and colorless alginates, whereas the alginates from *S. axillaris* had the highest guluronic to mannuronic acid ratio (1.83). It was estimated that 30 to 40 % of the total available fucoidan was recovered from the feedstock samples during the acidic extraction, with the exception of *S. axillaris*, where the recovery was as low as 5.4 %. The remainder of the fucoidan either resisted extraction or was hydrolysed by the acid treatment. The fucoidan extracts had crude yields of 1.2 to 7.6 %, sulfate contents of 10 to 30 % by weight, and exhibited antioxidant potential.

Keywords:

Alginates; fucoidans; biorefinery; integrated process; seaweed; macroalgae

5.2 Introduction

It has been well documented that southern Australia is a global hotspot for seaweed diversity and endemism (Kerswell, 2006; Phillips, 2001). These unique natural resources, along with clean waters, a well-developed seafood aquaculture industry, and advanced processing capabilities, suggest that South Australia is well positioned for the manufacture of value-added seaweed-derived products (Lee, 2010; Lorbeer et al., 2013).

However, South Australia's seaweed industry is currently limited to the production of agricultural commodities such as fertilizers from beach-cast wrack and imported biomass. In contrast, the company Marinova Pty Ltd in Tasmania produces very high-value fucoidan products from local and imported brown algae. Other companies in Tasmania, e.g., Kelp Industries Pty Ltd and TAS Kelp Pty Ltd, export beach-cast brown algae to Europe for alginate extraction or produce seaweed products for human consumption (Kai Ho 'Oceans Treasure' Sea Vegetables and Sea Shanti).

In order to facilitate industry development, research is needed to assess the potential value and applications of the local algal species. Processing strategies must also be devised for their valorization, while meeting stringent local health and environment regulations.

A number of brown algae of Southern Australia hold potential for valorization due to their rapid growth rates, abundance within the currently available resource, potential for aquaculture, and the suite of valuable compounds they produce (Lorbeer et al., 2013). Of those compounds, the polysaccharides – particularly alginates and fucoidans – are of commercial and research interest.

Alginates are composed of mannuronic and guluronic acid monomers, in varying proportions and sequences. They are used as an industrial gum given their unique rheological properties, including their capacity to increase viscosity in solutions and form gels in the presence of polyvalent cations. Low-grade alginates are generally used in textiles and printing, whilst alginates with more favorable physical properties (high viscosity, strong gel-forming capability, and near-absence of color) are employed extensively in the food and beverage industries. High-grade and specialty alginates are also used in drug delivery systems and for cell immobilization

applications (Lee & Mooney, 2012). The viscosity and gel strength of alginates depend largely on their molecular weight and uronic acid composition and sequence, while the color depends on the presence of pigments such as polyphenols and fucoxanthin (Donati & Paoletti, 2009).

Fucoidans, on the other hand, are a structurally diverse family of sulfated polysaccharides containing fucose as a major monomer, usually along with other monosaccharides, which may include galactose, xylose, arabinose, mannose, glucose, or glucuronic acid (Morya et al., 2012; Winberg et al., 2014). Fucoidans are attracting increasing interest for their broad range of biological activities, in particular antioxidant, immune modulation, anticoagulant, antiviral, anti-inflammatory and anti-cancer effects (Winberg et al., 2014). Various factors, including sulfation, molecular weight, and monosaccharide composition, may contribute to the biological properties of fucoidans (Morya et al., 2012).

A dilute acid treatment of brown algal biomass is often used to extract fucoidans, and as a pre-treatment to alginate extraction. For fucoidans, acids are used to disassociate polymers from the cell wall matrix and solubilize the fucoidans, while simultaneously preventing the co-extraction of alginates by converting soluble forms (e.g. sodium alginate) into insoluble alginic acid (Hahn et al., 2012). Acid pre-treatment is utilized in alginate production processes to remove potential contaminants, including fucoidans, laminarins, proteins and polyphenols, and convert insoluble alginate salts, e.g., calcium and magnesium alginates, into alginic acid, which is subsequently more easily converted into soluble sodium alginate (Hahn et al., 2011). This occurs through sequential ion exchange reactions whereby the cations of the alginate salts are first displaced by protons from the acid, which are subsequently displaced by sodium ions during the following treatment with sodium carbonate (McHugh, 1987).

In a previous study, we established a set of conditions for the acid treatment of *Ecklonia radiata* (C. Agardh) J. Agardh, which performed both sets of functions simultaneously and effectively, thus facilitating the sequential extraction of fucoidans and alginates in an industrially-relevant context (Lorbeer et al., 2015a). However, it needs to be determined whether the optimized process can be applied to other brown seaweed feedstocks of commercial relevance, to yield products with valuable applications.

With this goal in mind, we applied the sequential extraction on six samples of brown algae collected in Southern Australia, and assessed the products for key indicators of quality and value. Three of the samples were *E. radiata*, including the feedstock sample used in the previous study, harvested from two different locations and at two different times of the year, in order to assess the effects of intraspecies variation on the extractive process. *E. radiata* has shown potential for aquaculture, and is one of the most prolific species along Australia's southern coastline (Loo et al., 2011). One sample belonged to *Durvillaea potatorum* (Labillardière) Areschoug, which is the principal species harvested in Tasmania for export to European alginate producers (Guiry & Guiry, 2015). It is also used extensively in Australia for the production of seaweed-based fertilizers. A sample of *Macrocystis pyrifera* (Linnaeus) C. Agardh was also included. In 1999, this species – harvested from the U.S.A., Mexico, and Chile – was the main feedstock used for commercial alginate extraction (Bixler & Porse, 2011). The South Australian *Macrocystis*, which is yet to be exploited for commercial polysaccharide extraction, was originally thought to belong to a distinct species – *M. angustifolia* – due to a high degree of morphological plasticity. However, the genus has recently been synonymized into a single species (Macaya & Zuccarello, 2010). Finally, a sample of *Seirococcus axillaris* (R. Brown ex Turner) Greville was included, which is endemic to Southern Australia (Guiry & Guiry, 2015). The fucoidans generated during the sequential extraction process were assessed for their antioxidant activity, which is of interest for functional food applications, in which it may improve product stability and healthfulness.

5.3 Materials and methods

5.3.1 Materials and reagents

All chemicals and reagents were purchased from Merck (Germany) or Sigma (USA), with the exception of the β-glucan MW standards (Megazyme, Ireland), the polyguluronic and polymannuronic acid standards (Carbosynth, UK), and commercial fucoidans (Marinova Pty Ltd, Australia).

All seaweed samples, each containing several plants, were collected during March or July of 2013 and 2014. Five samples were collected from the freshly deposited beach-cast wrack at Rivoli Bay, Beachport, South Australia, while one *E. radiata* sample was taken at O'Sullivan Beach, South Australia (~350 km north-west of Beachport), while in an attached and submerged state. Details regarding the taxonomy and time of collection of the samples are presented in Table 1. Visible

surface contaminants were removed and the algae were rinsed with fresh water and dried, either on mesh racks if collected from Beachport or in a 45°C oven if from O'Sullivan Beach. The plants were then milled with a blender (Blendtec, USA), sieved to a particle size of 250–1400 µm, and further dried in an oven at 45°C prior to storage in a desiccator. The species were identified at the South Australian State Herbarium (Table 1). The *E. radiata* sample collected from Beachport in 2014 was the feedstock used in the previous optimization study (Lorbeer et al., 2015a).

Table 1. Taxonomy and collection details of feedstock samples

Taxonomy			Collection		
Order	Family	Species	Location	Year	Month
Laminariales	Lessoniaceae	*Ecklonia radiata*	Beachport	2013	March
			Beachport	2014	July
			O'Sullivan Beach	2014	March
	Laminariaceae	*Macrocystis pyrifera*	Beachport	2014	July
Fucales	Durvillaeaceae	*Durvillaea potatorum*	Beachport	2013	March
	Seirococcaceae	*Seirococcus axillaris*	Beachport	2014	July

5.3.2 Sequential extraction and fractionation process

All feedstock samples were treated using the optimal sequential extraction conditions reported previously (Lorbeer et al., 2015a), as summarized in Figure 1. Following the sequential extraction process, the fucoidan-rich polysaccharide fractions (FRPF), crude sodium alginates, and final residues were freeze-dried before analysis.

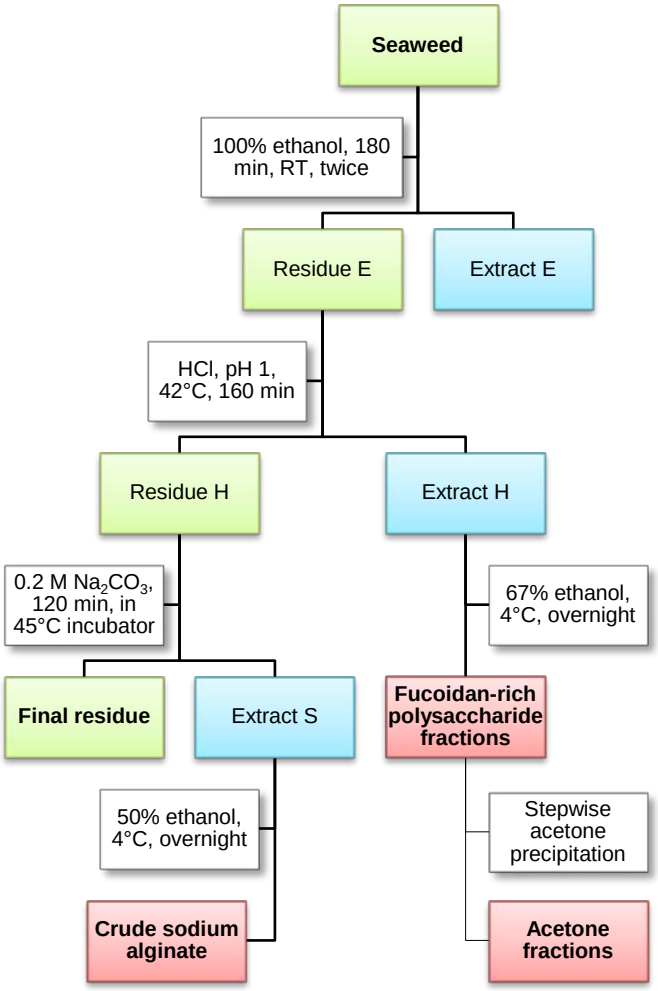

Figure 1. Overview of the sequential biorefinery extraction process
RT: Room temperature.

A fractional precipitation protocol similar to those reported previously (Castro et al., 2015; Castro et al., 2014; Silva et al., 2005) was used to enrich the fucoidans in the FRPFs, by separating them from alginates and other contaminants. Briefly, 180 mg of each FRPF was dissolved in 18 mL of water at 4 °C overnight, with shaking for the first three hours. The samples were then centrifuged at 6000 *g* for 20 minutes at 4 °C, and the pellets were isolated and dried under vacuum to produce the fractions referred to as AF0. Half a volume of cold acetone was added to the supernatants, which were then subjected to the same cold dissolution and centrifugation protocol to make AF0.5. This was repeated five more times, with acetone-to-water ratios of 0.75, 1, 1.25, 1.5, and 2, to make fractions AF0.75 through to AF2, respectively. The final supernatants were also dried under vacuum for analysis, and labelled AFS. The AF fractions with >15% yield by weight from FRPFs were further analyzed.

5.3.3 Analysis of samples

The sugar composition of the starting materials, FRPFs, major AF fractions, crude sodium alginates, and final residues were determined by HPLC analysis of the acid hydrolysates with pre-column derivatization, as described previously (Comino et al., 2013; Lorbeer et al., 2015a). Additionally, in order to determine the guluronic to mannuronic acid ratios (G/M) of the crude sodium alginate fractions, guluronic and mannuronic acid peaks were identified by comparison with hydrolysates of commercial samples of polyguluronic and polymannuronic acids, and adjusted for acid degradation using the correction factors provided by Haug and Larsen (1962).

The molecular weight (MW) profiles of the FRPFs, AFSs (which contained the bulk of the fucoidans), and crude sodium alginates were determined using size-exclusion chromatography (SEC), as described previously (Lorbeer et al., 2015a), but with PolySep GFC-P4000 and PolySep GFC-P5000 columns (Phenomenex), in series.

The crude sodium alginate samples were then re-solubilized in water at a concentration of 1 % (w/v) for color and viscosity measurements. Aliquots were centrifuged at 16,000*g* for ten minutes to remove turbidity and the percentage transmittance was measured in a spectrophotometer at 510 nm as an indication of relative clarity (Hernández-Carmona et al., 1998; McHugh et al., 2001).

The viscosity of the solutions was measured at 22 °C using an NDJ-8S Digital Rotary Viscometer (Rinch Industrial Co. Ltd., China) with the appropriate spindle attached. The viscosity of each sample was determined at two rotation speeds that returned readings within the acceptable detection range of the spindle (these speeds varied depending on the sample viscosity), and the average of the readings was used. Sodium hexametaphosphate was then added at 0.5 % (w/v) in order to sequester calcium ions, and the viscosity was measured again. The viscosity after calcium sequestration gives the best indication of the effective viscosity of the extracted alginates, while the reduction in viscosity (RV) after sequestration can be used to assess how much calcium remained bound to the alginates following the sequential treatment (McHugh et al., 2001).

The sulfate content of the FRPFs and AFSs was determined using the turbidimetric sulfate assay, after hydrolysis in 1N hydrochloric acid at 105 °C for two hours, using K_2SO_4 as a standard (Dodgson and Price 1962).

5.3.4 Antioxidant assays

The antioxidant activities of the FRPFs (with the exception of the *S. axillaris* extract,

due to its low yield and purity) were assessed using the 'Ferric reducing ability of plasma' (FRAP) assay, as described by Benzie and Strain (1996), and the 'Oxygen radical absorbance capacity' (ORAC) assay (Huang et al., 2002) adapted to seaweed extracts by Charoensiddhi et al. (2014). FRAP values were expressed in ferrous equivalent terms, i.e., the number of mmol of ferrous ion produced during the reaction per gram of extract (as determined by comparison with standard solutions of ferrous sulfate after addition of the FRAP reagent). ORAC values were expressed in Trolox equivalent terms, i.e., the number of µmol of Trolox (a water-soluble analog of vitamin E) required to produce a reaction of equal magnitude to that produced by one gram of extract.

5.3.5 Statistical analysis

All extractions were performed in triplicate (with the exception of the acetone precipitation), and sample analysis was conducted on all replicates, with data expressed as mean values ± standard deviation. A one-way ANOVA paired with Tukey's test was used to determine whether antioxidant assay results were significantly different at the 95 % confidence level ($p<0.05$).

5.4 Results

5.4.1 Distribution of products after the sequential extraction process

Figure 2 shows the distribution of fucoidan and alginate between the acidic extract (FRPF), sodium carbonate extract (crude sodium alginate), and final residue fractions, following the sequential extraction process. Only 52% (*S. axillaris*) to 75% *(E. radiata* from O'Sullivan Beach) of the total bound fucose detected within the starting materials was recovered within these three fractions, with the rest seemingly lost during the sequential extraction process. In general, the FRPFs contained 40-64% of the recovered fucoidan (as reflected by fucose content), with 13-25% co-extracting into the crude sodium alginate fractions, and 13-45% persisting to the final residue. *S. axillaris* was the noticeable exception, for which only 10% of the recovered fucoidan was found in the acidic extract, compared with 23% in the sodium carbonate extract and 66% in the final residue.

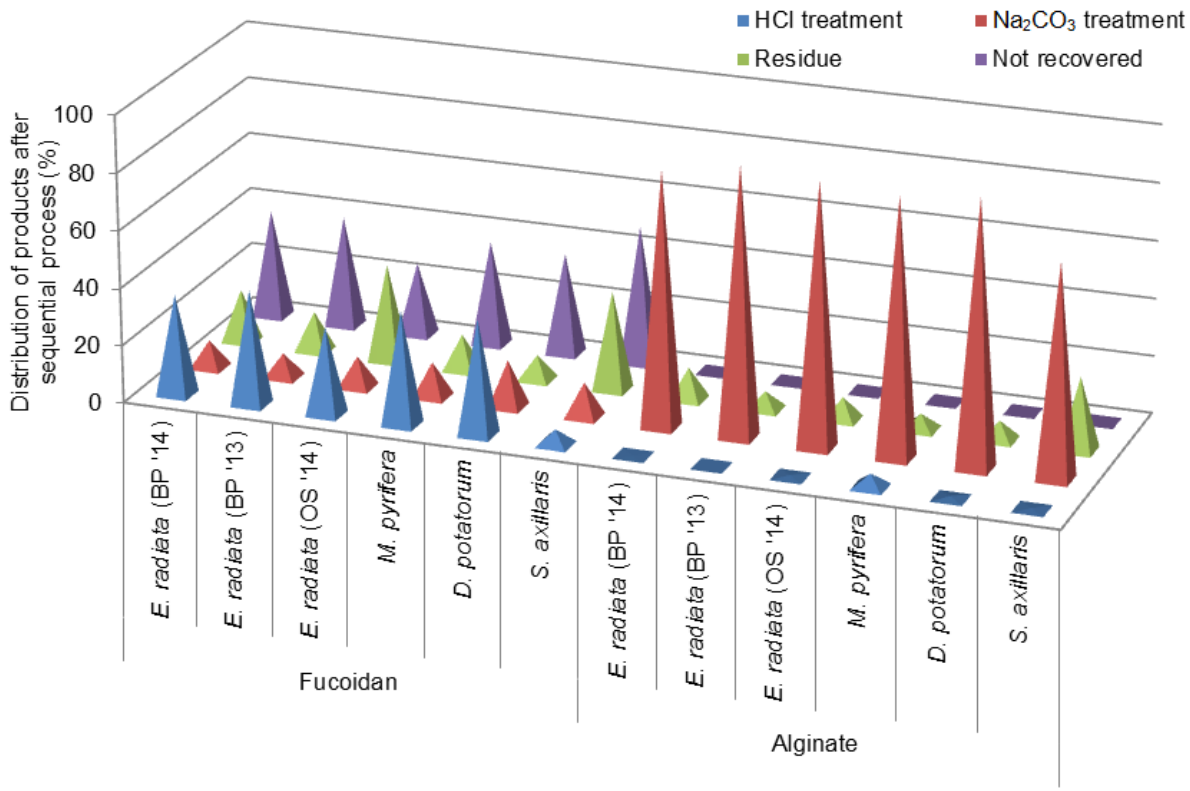

Figure 2. Distribution of fucoidan and alginate (as inferred from fucose and alginate-derived uronic acid contents, respectively) between the acidic extract, sodium carbonate extract, and final residue fractions, following the sequential extraction process; and proportion not recovered from the starting material in any of those fractions.
BP and OS indicate the site of collection (Beachport and O'Sullivan Beach, respectively), followed by the year of collection.

Conversely, all of the alginate detected within the starting materials was recovered in the extracts or residual biomass. The sodium carbonate treatment was effective for the extraction of alginates, with 88-94 % of the alginates being detected within the crude sodium alginate fraction for all feedstock samples apart from *S. axillaris*, for which the recovery at this step was slightly lower (74 %).

5.4.2 Yield and composition of the fucoidan-rich fractions

Table 2 shows the yield and chemical composition of the FRPFs and AFSs (which contained the highest yield of fucoidan of the AF fractions) from each feedstock sample, along with that of commercial fucoidan extracts for comparison. All fractions listed contained substantial proportions of fucoidan, as indicated by the presence of fucose and sulfate. However, the crude yields of the FRPFs were not particularly high, ranging from 1.2 % of the weight of the starting material for *S. axillaris*, to 7.6 % for *M. pyrifera*; this being reduced further in the AFSs (0.63 to 5.1 %). The FRPF extracted from the *E. radiata* feedstock sample collected from Beachport in 2014 had a sulfate content roughly equal to or higher than that of all three of the commercial fucoidan samples, despite having a significantly lower carbohydrate

content than two of them. However, the FRPFs from the other two *E. radiata* feedstock samples had significantly lower sulfate contents, perhaps indicating lower fucoidan purity. All fractions (including the commercial preparations) also contained some galactose, and most contained some xylose and trace amounts of mannose and glucuronic acid. Trace amounts of xylose and mannose were detected in one of the three commercial preparations, but glucuronic acid was not.

Table 2. Yield and chemical composition of the fucoidan-rich fractions and fucoidan enriched fractions

Feedstock sample	Fraction	Yield (%)	SO_3 (%)	Carbohydrate (%)	Fuc (%)	Molar ratio to fucose (fuc = 100)					
						Gal	Xyl	Man	GlcA	Glc	G+M
E. radiata (BP '14)	FRPF	3.79 ±0.09	30.3 ±2.3	37.2 ±0.5	29.7	7	4	3	3	3	2
	AFS	3.26	27.9 ±0.5	34.8	28.1	9	4	3	3	3	0
E. radiata (BP '13)	FRPF	5.42 ±0.40	15.6 ±0.4	52.7 ±0.8	18.2	10	4	4	4	149	0
	AFS	4.28	20.0 ±0.3	46.3	20.6	10	3	3	4	93	0
E. radiata (OS '14)	FRPF	3.04 ±0.01	19.9 ±0.1	36.4 ±1.8	18.6	15	9	8	10	25	19
	AFS	2.50	20.6 ±0.7	36.5	19.8	15	8	7	9	21	14
D. potatorum	FRPF	6.28 ±0.25	12.4 ±1.4	55.5 ±0.5	12.2	14	8	0	3	256	37
	AFS	3.52	13.0 ±1.1	57.8	17.2	14	0	0	0	199	0
S. axillaris	FRPF	1.24 ±0.18	9.79 ±2.5	37.9 ±2.7	13.4	23	42	8	10	3	86
	AFS	0.63	20.2 ±1.4	41.1	23.1	23	26	5	7	3	10
M. pyrifera	FRPF	7.59 ±0.95	18.6 ±0.6	41.2 ±2.0	16.2	15	23	4	4	3	86
	AFS	5.10	24.2 ±1.0	35.4	20.9	15	13	3	2	2	26
M. pyrifera	CFE		24.1 ±0.8	32.5	26.0	21	0	4	0	0	0
U. pinnatifida	CFE		30.9 ±2.4	59.1 ±0.8	30.9 ±0.6	92	0	0	0	0	0
F. vesiculosis	CFE		26.4 ±0.5	57.8	51.6	7	5	0	0	0	0

BP and OS indicate the place of collection (Beachport and O'Sullivan Beach, respectively), followed by the year of collection.
Abbreviations: CFE = commercial fucoidan extract; Fuc = fucose; Gal = galactose; Xyl = xylose; Man = mannose; GlcA = glucuronic acid; Glc = glucose, G+M = sum of guluronic and mannuronic acids.
Yield, sulfate and carbohydrate contents given as % w/w of extract.
Where no standard deviation is given, analyses were not replicated (except for molar ratios, which were determined along with the carbohydrate content during sugar analysis).

The presence of the guluronic and mannuronic acids in most of the FRPFs indicates contamination with alginates (highest for the species other than *E. radiata*). Much of the alginate appears to have been removed by the acetone fractionation step in the AFSs. Some FRPFs also contained high proportions of glucose, which may point toward the co-extraction of laminarins. In fact, in the extracts from *D. potatorum* and

the *E. radiata* from Beachport 2013, there was significantly more glucose than fucose. In those samples, the proportion of glucose to fucose dropped significantly in the corresponding AFSs. This, along with secondary peaks on the HP-SEC chromatograms (not shown) corresponding to MWs of 4-7 kDa, supports the inference that at least some of the glucose was from laminarins. None of the commercial fucoidan preparations contained any glucose or alginate-derived uronic acids.

It is also worth noting that the ratio of galactose to fucose did not vary within all FRPFs and their corresponding AFSs, whereas the xylose to fucose ratio decreased for all feedstock samples containing species other than *E. radiata*. In those cases, the remainder of xylose was detected in the AF0.5 fractions, which also contained the bulk of the alginates, along with some fucose (data not shown).

Between the three *E. radiata* samples, the most noticeable differences between the FRPFs are the sulfate content (highest in the Beachport 2014 sample, at 30 % w/w, and lowest in the Beachport 2013 sample, at 16 % w/w) and glucose content, which have the opposite trend. This could be explained by a significantly higher proportion of laminarin in the 2013 sample, reducing the purity of the sulfated fucoidans. As laminarins consist exclusively of unmodified glucose, this could also explain the higher carbohydrate content within the extracts from the sample harvested in 2013.

5.4.3 Yield and nature of extracted alginates

Some key characteristics of the crude sodium alginates (all conservatively estimated to be more than 90% pure) are shown in Table 3. Yields of 29 % (w/w of starting material) or above were achieved using all feedstock samples. *D. potatorum* had the highest alginate yield, at 55% of the weight of the starting material, but among the lowest G/M ratio, MW and viscosity, indicating that the extracted alginate would be expected to form a relatively soft-gel in the presence of calcium, and a low viscosity solution, respectively. The alginates from *S. axillaris* had a very high G/M ratio, indicating the ability to form very strong and rigid gels in the presence of polyvalent cations, accompanied by a low MW and viscosity, suggesting a low viscosifying capacity. The alginates extracted from the *E. radiata* and *M. pyrifera* samples had medium to high viscosities and MWs, and moderate G/M ratios.

Most of the extracted alginates had relatively low RV values (with the exception of those from *D. potatorum* and *S. axillaris*), indicating that little residual calcium remained associated with the extracted polymers following the sequential extraction

process.

Table 3. Characteristics of the crude sodium alginate extracts

Feedstock sample	Yield (%)	G/M	MW (kDa)	Viscosity (mPa s)	RV (%)	Transmittance (%)
E. radiata (BP '14)	44.0±0.15	0.63±0.01	731±15	368±32	8.99±1.4	74.4±0.8
E. radiata (BP '13)	29.3±0.05	0.81±0.02	534±50	344±21	6.48±4.4	73.5±1.6
E. radiata (OS '14)	29.8±0.29	0.85±0.01	508±13	320±72	9.98±1.1	42.2±0.9
M. pyrifera	38.9±0.45	0.94±0.04	606±25	599±84	14.8±9.1	62.7±19
D. potatorum	55.2±0.51	0.40±0.00	373±16	120±7.3	34.0±3.5	60.6±1.0
S. axillaris	41.3±0.66	1.83±0.07	402±2.7	118±15	47.8±3.4	40.9±1.9

BP and OS indicate the site of collection (Beachport and O'Sullivan Beach, respectively), followed by the year of collection.
MWs expressed in equivalent terms to (1,3;1,4)-β-glucan MW standards.
RV denotes reduction in viscosity after the sequestration of calcium ions.
Yield given as % w/w of dry starting material.

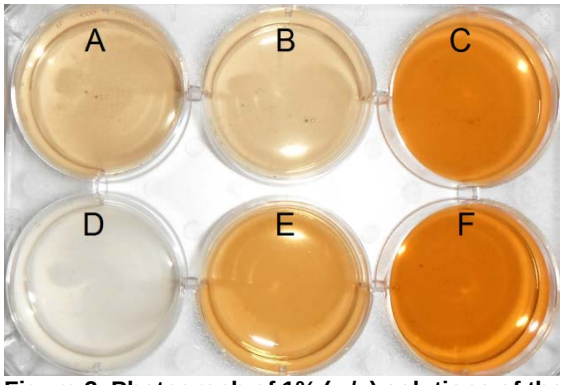

Figure 3. Photograph of 1% (w/v) solutions of the crude sodium alginate extracts, showing their different levels of pigmentation
(A) *E. radiata* (Beachport 2014); (B) *E. radiata* (Beachport 2013); (C) *E. radiata* (O'Sullivan Beach 2014); (D) *M. pyrifera*; (E) *D. potatorum*; (F) *S. axillaris*

The transmittance of the solutions was variable (even within the same species), with the alginate extracts from both of the *E. radiata* samples from Beachport having transmittances of 74%, compared with only 41-42% for the alginate solutions from the *S. axillaris* and the *E. radiata* from O'Sullivan Beach. As seen in Figure 3, most of these transmittance values are consistent with the color intensity of the alginate solutions. However, the alginate solution from *M. pyrifera* was significantly less pigmented than that from the Beachport *E. radiata* feedstock samples, yet had lower transmittance. This was due to turbidity (which was much stronger in one of the replicates than the others, which explains the standard deviation of 19), which could not be removed by centrifugation. Interestingly, the turbidity was reduced quite rapidly with the addition of sodium hexametaphosphate during the RV determination, resulting in consistent and high transmittance values for the *M. pyrifera* alginates of 80.1±2.5%. The color intensity of the FRPFs did not correlate with that of the alginate extracts. For instance, *M. pyrifera* produced a brown-colored

FRPF (in solid and aqueous states) and a colorless crude alginate fraction, whilst *D. potatorum* produced a colorless FRPF and a brown alginate extract. Meanwhile, *E. radiata* (O'Sullivan Beach) consistently produced the darkest colored extracts of both varieties.

5.4.4 Antioxidant activity of the fucoidan-rich extracts

Antioxidant assays were conducted on the FRPF extracts, as well as the commercial fucoidan preparations. As shown in Table 4, the antioxidant activity trends indicated by the FRAP and ORAC assays were in good agreement for the FRPF fractions, with the FRPF from *E. radiata* (O'Sullivan Beach 2014) displaying the strongest activity (FRAP: 217 mmol.g^{-1} Fe^{2+} equivalent; ORAC: 150 µmol.g^{-1} Trolox equivalent), and the FRPF from *D. potatorum* showing the weakest (FRAP: 141 mmol.g^{-1} Fe^{2+} equivalent; ORAC: 58.7 µmol.g^{-1} Trolox equivalent). Conversely, the pure (>95%) commercial fucoidan preparations showed FRAP results that were significantly higher (305-319 mmol.g^{-1} Fe^{2+} equivalent) and ORAC results that were significantly lower than that of the FRPFs (5.0-10.3 µmol.g^{-1} Trolox equivalent). Meanwhile, the commercial *M. pyrifera* extract, which contained fucoidans along with polyphenols, responded to both assays similarly to the FRPFs, with no significant difference to the *D. potatorum* sample (FRAP: 159 mmol.g^{-1} Fe^{2+} equivalent; ORAC: 53.7 µmol.g^{-1} Trolox equivalent).

Table 4. Antioxidant activities of tested samples

Sample	FRAP (mmol/g)	ORAC (µmol/g)
E. radiata (BP '14) FRPF	182±9.1^{b,c}	96.3±12^{b}
E. radiata (BP '13) FRPF	177±16^{b,c}	91.7±1.2^{b,c}
E. radiata (OS '14) FRPF	217±12^{b}	150±14^{a}
D. potatorum FRPF	141±16^{c}	58.7±6.7^{d,e}
M. pyrifera FRPF	170±4.2^{b,c}	75.0±6.1^{c,d}
M. pyrifera extract*	159±7.0^{b,c}	53.7±2.9^{e}
U. pinnatifida fucoidan*	305±46^{a}	5.00±3.5^{f}
F. vesiculosis fucoidan*	319±54^{a}	10.3±0.6^{f}

BP and OS indicate the site of collection (Beachport and O'Sullivan Beach, respectively), followed by the year of collection.
FRAP activity expressed in Fe^{2+} equivalent terms.
ORAC expressed in Trolox equivalent terms.
$^{a-f}$Values with different letters are statistically different (P<0.05)
*Commercial preparations. The *U. pinnatifida* and *F. vesiculosis* fucoidans are both >95% pure, while the *M. pyrifera* extract contains >90% fucoidan, along with polyphenols.

5.5 Discussion

5.5.1 Extraction of fucoidans during the acidic treatment

The yields and sugar profiles of the FRPFs indicated that typically only 30-40 % of the total available fucoidans (as inferred from fucose content) could be extracted from the different seaweed feedstock samples by acidic treatments. In the case of *S. axillaris*, however, this value was as low as 5.4 %. Deniaud-Bouët et al. (2014) also demonstrated variability between species with regard to the extractability of fucoidans using various extraction conditions, and suggested that this might reflect the associations occurring between the fucoidans and other cell wall components. With the exception of *S. axillaris*, the yields achieved here were in good agreement with those reported by Black et al. (1952) (*Laminaria cloustoni* 20 %, *Ascophyllum nodusum* 54 %, *Fucus vesiculosus* 62 % and *Pelvetia canaliculata* 76 % of total available fucose), considering they repeated their acidic extraction three times at 70 °C compared to a single treatment at 42 °C in our protocol. The *E. radiata* sample that was collected from Beachport during 2013 and referred to in this study is the same material that was used for our previous investigation looking into the kinetics of a classical fucoidan extraction strategy (pH 2 HCl, 60 °C, 3 hours) (Lorbeer et al., 2015b). In the current study, 40 % of the total available bound fucose was recovered during the acidic treatment, compared to 22 % during that previous study, suggesting that the conditions used here were more effective than those classical conditions for the extraction of fucoidan from that feedstock sample.

The gravimetric yields of the FRPFs, in terms of their weights relative to that of the starting materials, ranged from 1.2 % for *S. axillaris* to 7.6 % for *M. pyrifera.* As these fractions contained some laminarins, alginates, and other contaminants (e.g. phenolic compounds, proteins, etc.), the actual yield of fucoidan was lower. Crude fucoidan yields reported in the literature typically vary between 1 and 20% (Foley et al., 2011); however their relative purities differ, so this figure does not necessarily reflect the true yield of fucoidan, or the potential value of the feedstocks. At the higher end of the spectrum, Hifney et al. (2016) recently extracted a fucoidan-rich fraction at a crude yield of 19 % from *Sargassum* sp. using an acidic extraction process, with a very high sulfate content of 48 %. The total sugar and fucose contents of the fraction were not given, but they were quite low for the most similar conditions trialed during method development (11 % and 2.5 %, respectively). Similarly, Yuan and Macquarrie (2015) extracted a fraction from *Ascophyllum nodosum* into acid with a crude yield of 20 % and a sulfate content of 29 % (total sugar and total fucose contents not given), whilst Rodriguez-Jasso et al. (2011) used a microwave-assisted method to extract a fraction from *Fucus vesiculosis* at a

crude yield of 18 %, with sulfate, total sugar, and fucose contents of 21 %, 27 %, and 15 %, respectively. Aside from probable differences in the fucoidan content of the feedstock samples, the techniques used in those studies may have been developed primarily to extract fucoidans, whereas the effects on the alginates were also considered during our method development. It should also be noted that apart from fucoidan yield, different extraction techniques vary in their efficiency, scalability, safety, eco-friendliness, and capital investment requirements. As such, techno-economic and environmental impact analyses are useful in assessing the overall efficacy of potential industrial-scale extraction techniques.

Significant quantities of the remainder of the fucoidans were co-extracted with the alginates (thus reducing the purity of the sodium alginate products) during the sodium carbonate treatments, or remained within the cellulosic final residues. In the case of *S. axillaris*, two-thirds of the bound fucose detected after the sequential process was observed in the cellulosic residue, perhaps indicating close associations between the fucoidans and cellulose in this feedstock sample (Deniaud-Bouët et al., 2014). Furthermore, it was observed that a large proportion (25-48 %) of bound fucose in the starting material was not detected within any of those fractions, appearing instead to be lost during the sequential extraction process. This could have occurred through the hydrolysis of the fucoidans during the heated extractions, followed by the elimination of the liberated monosaccharides during the precipitation and washing steps. This supposition is supported by our previous study, in which we demonstrated that fucoidans are susceptible to rapid hydrolysis in dilute acid (pH 2) under mild heating (60 °C), showing an 87 % reduction in molecular weight over three hours (Lorbeer et al., 2015b). The resistance of fucoidans to comprehensive extraction, coupled with their tendency toward hydrolysis and degradation when exposed to heat and acids, represents a significant challenge for brown algae biorefinery, especially considering that fucoidans are a key fraction of potential value.

Most of the fucoidan-containing extracts contained significant quantities of galactose, along with some xylose, mannose and glucuronic acid, which are all commonly reported components of fucoidans (Ale & Meyer, 2013; Li et al., 2008). The extracts from *M. pyrifera* obtained here differed in monosaccharide composition from the commercial *M. pyrifera* extract, as shown in Table 2. The presence of higher levels of guluronic and mannuronic acids in the *M. pyrifera* extracts obtained using the sequential extraction process can be explained by the co-extraction of

alginates, and indeed, a large proportion of those were removed during acetone fractionation. However, our *M. pyrifera* extracts also contained significant amounts of xylose, which was not detected in the commercial extract. This may have belonged to polysaccharides (perhaps other fucoidans), which were either not extracted (perhaps due to different extraction conditions), or removed after extraction by the manufacturer (Marinova Pty Ltd). It may also conceivably represent differences in the plants used as feedstock, due to anatomy, life stage, or growing conditions (Winberg et al., 2014). Fitton et al. (2015b) from Marinova Pty Ltd reported the composition of another fucoidan extract from *M. pyrifera*, which did contain xylose. These authors also obtained extracts from *E. radiata* and *D. potatorum* which showed various differences in monosaccharide composition and sulfate content compared to the extracts obtained here, demonstrating the variability that can arise between fucoidan extracts, even from the same species.

While fucoidans have also been reported to contain some glucosyl residues, the high levels of glucose detected within some of the extracts are more likely to have indicated the co-extraction of laminarins. It should be noted that for *E. radiata,* the FRPF extracts obtained from the two samples collected in March contained significant proportions of glucose (molar ratio relative to fucose of 25:100 to 149:100), compared with the sample collected in July which had a negligible amount of glucose (molar ratio to fucose of 3:100). This can perhaps be explained by the fact that laminarin is a secondary photosynthate, which has been shown in some species to accumulate during the warmer months of the year, before being expended during maturation processes early during the cooler season (Iwao et al., 2008; Stewart et al., 1961). As such, the harvesting time may affect the composition of polysaccharide extracts from brown algae. The FRPF from the *D. potatorum* sample, which was also collected during March, also contained a high proportion of glucose (2.56 times the amount of fucose), whereas the *S. axillaris* and *M. pyrifera* FRPFs contained very little (molar ratios to fucose of 7:100 and 3:100, respectively). However, more comprehensive comparisons between plants belonging to the same species, but collected at different times of the year, would be required in order to confirm that this was a direct result of seasonal influences.

The pH 1 hydrochloric acid solution was previously shown to effectively prevent the co-extraction of alginates from *E. radiata* (Lorbeer et al., 2015a). However, in this study, undesirable alginate contaminations were observed in the FRPFs extracted from all feedstock samples apart from the two *E. radiata* samples collected in

Beachport. This may have resulted from the insufficient exchange of alginate-associated monovalent cations (e.g. sodium and potassium) with protons during the acidic treatment. Other studies have reported the extraction of fucoidan in calcium chloride solutions in order to prevent the co-extraction of soluble alginate salts through their conversion into insoluble calcium alginate (Chale-Dzul et al., 2015; Imbs et al., 2015; Ponce et al., 2003). However, this is not suitable when alginate is to be sequentially extracted, as calcium alginate is more resistant to conversion back to sodium alginate, compared with alginic acid (Hahn et al., 2011). As the acidic treatment used in the sequential extraction process was optimized with the *E. radiata* sample collected from Beachport in 2014, the treatment conditions may not be as well suited to the other feedstock samples (Lorbeer et al., 2015a). However, the stronger acids, higher temperature, or longer treatment time required to increase the exchange of cations may result in further degradation of the polymer (Hernández-Carmona et al., 1998).

Alginates can be removed from the fucoidan with the post-extraction addition of calcium, whilst laminarins can be separated from fucoidans using a variety of methods based upon the differences in their charge or MW (Hahn et al., 2012). In this study, however, stepwise acetone precipitation was used to fractionate the FRPFs. This separated a small proportion of the glucose and most of the alginates from the main fucoidan-containing fraction in each case. Interestingly, most of the fucoidans did not precipitate, even with the addition of two volumes of acetone. This contrasts with previous studies using a similar fractionation protocol, where most of the fucoidans were precipitated with 0.8, 1.0, or 1.5 volumes of acetone (Castro et al., 2015; Castro et al., 2014; Silva et al., 2005). These differences in acetone solubility might reflect variations in the polarity (due to sulfate content, for instance) or molecular weight of the extracted fucoidans (Miller-Chou & Koenig, 2003). However, in this study, the fucoidan extracts in the AFS fractions showed diverse sulfate contents of 13-28 % (w/w), and yet all remained soluble after the addition of two volumes of acetone.

5.5.2 Alginate extracts produced by the sequential extraction process

In addition to preventing the co-extraction of alginates with the fucoidans, the acidic treatment was also shown to facilitate a higher yield and purity of alginate during the sequential sodium carbonate treatment (Lorbeer et al., 2015a). This results from the conversion of calcium and magnesium alginates into alginic acids, which are then easier to extract as sodium alginates, as well as the partial removal of potential

contaminants and extraction inhibitors including other polysaccharides, polyphenols, and proteins (Lorbeer et al., 2015a). The relatively high alginate yields and purities achieved in this study, despite the use of mild extraction conditions, suggest that the acid pre-treatment performed these functions effectively.

The presence of the multivalent cations (calcium and magnesium ions) in crude alginate extracts also causes problems from a processing perspective. Small amounts can substantially increase the viscosity of the crude extracts, which necessitates more dilution in order to achieve a low enough viscosity to allow for the clarification of the crude extract using floatation and/or filtration processes (McHugh, 2003). Therefore, multivalent cation removal prior to alginate extraction is highly desirable. An indicator of the extent to which multivalent cations are removed is the RV value of the alginate extract. McHugh et al. (2001) stated that commercial alginates are expected to have a RV of lower than 40 %. All of the alginate extracts obtained here had a RV value of less than 15 %, with the exception of those from *D. potatorum* (34 %) and *S. axillaris* (48 %). Perhaps in those species, higher Ca^{2+}/H^{+} exchange could be achieved during the acidic pre-treatment with modified treatment conditions (e.g. a lower pH). It seems that the relatively low pH (pH 1) used for the acidic treatment step in the optimized sequential extraction process was crucial in removing polyvalent cations. To illustrate this point, the alginates obtained previously from *E. radiata* after acidic treatments at a pH of 2-5 (Lorbeer et al., 2015a) had RV values of 71-78 % (Appendix 5.1) compared to the alginates extracted from the same feedstock sample during this study, which had a RV of 9 %.

However, caution must be exercised when treating the algae with acids prior to alginate extraction, in order to avoid excessive polymer degradation. And indeed, the alginates extracted from *D. potatorum* and *S. axillaris* had low viscosities (120 and 118 mPa s, respectively). In those cases, the alginates might have been more prone to degradation, or naturally low in MW/viscosity. The alginates extracted from the other species had medium to high MWs and viscosities, indicating that undesirable degradation was successfully reduced. As a benchmark, the MW of the alginate extracted from *M. pyrifera* was much higher than that reported by Gomez et al. (2009) using a very similar process (297-396 kDa). It should be noted that some further degradation would be expected to occur with post-extraction processing, which is usually necessary to remove the color in crude alginate extracts.

That said, the clarity of the crude alginate extracted from *M. pyrifera* (after the

turbidity was removed), was roughly equal or slightly lower than that reported by Rodríguez-Montesinos' group (1998; 2001), after formalin treatment, post-extraction precipitation, and/or bleach treatment. However, the other alginate extracts had much higher color intensity, suggesting the need for more up- or down-stream processing to remove it, or different potential applications (i.e. lower grades). McHugh (2003) has mentioned that *M. pyrifera* yields a lightly colored alginate, compared with most other species.

Between the *E. radiata* alginate extracts, the one obtained from the O'Sullivan Beach feedstock sample was much darker than those from the Beachport samples (42 % transmittance compared to 74 %). Dark colored extracts from brown algae have been attributed to high phenol content (Deniaud-Bouët et al., 2014; Hifney et al., 2016), and may also signal the presence of other colored pigments, such as fucoxanthin. Stiger et al. (2004) has noted that some brown algae produce more polyphenols when in nutrient- or grazer-rich sites, which may have been the case in O'Sullivan Beach, as indicated by the abundance of epiphytes on the surface of the plants. However, compared to the Beachport samples, the O'Sullivan beach algae differed in numerous ways. Aside from the different geographic location, they were smaller (probably less mature), growing in shallower water, and still attached to their substrate when obtained, so it is not possible to ascertain the reason for the darker color of their extracts. However, it may be useful to investigate the effects of growing conditions on the color of the extracts, as it is an important determinant of value in commercial alginate products.

It was also noted that the brown pigmentation was associated with different fractions for different feedstock samples. With some samples, the FRPFs had a darker color, and for others, the crude alginates did. This was also observed by Deniaud-Bouët et al. (2014), who demonstrated that phenolic compounds were most commonly associated with alginates, but also occasionally linked to fucoidans via connecting proteins. As such, the phenolics were extracted with different conditions depending on the seaweed species.

In terms of the G/M ratios of the alginates, the *S. axillaris* extract had the highest at 1.83, compared with the second highest of 0.94 belonging to the *M. pyrifera* alginate. *D. potatorum* had a very low G/M ratio of 0.40. While all of the other feedstock samples contained whole plants or mixtures of their anatomical parts (with the exception of holdfasts), the *D. potatorum* sample did not contain any stipes

because they could not be milled in the blender after drying. It has been shown that the G/M ratio within *D. potatorum* tissue has an increasing gradient moving from the ends of the blades to the holdfast, only becoming guluronic acid-rich at the base of the stipe, which explains the low G/M ratio observed here (Cheshire & Hallam, 1985). It is common for the stipes of brown algae to have a higher G content compared with the fronds and blades, owing to their strong and rigid morphology (McHugh, 1987). However, McKee et al. (1992) reported *M. pyrifera*, which has slender and flexible stipes, to be an exception.

5.5.3 Antioxidant potential of the extracted fucoidans

In order to assess the antioxidant potential of the fucoidan extracts, we performed two antioxidant assays: FRAP and ORAC. It was noted that all of the FRPFs tested exhibited antioxidant reducing potential in the FRAP assay between 141 (*D. potatorum*) to 217 mmol/g (Fe^{2+} equivalent) (*E. radiata* - O'Sullivan Beach 2014), which was comparable to the commercial *M. pyrifera* extract at 159 mmol/g, which contained fucoidans as well as polyphenols. All tested FRPFs exhibited radical scavenging activity in the ORAC assay between 59-150 µmol/g Trolox equivalent, which was slightly higher than that of the commercial *M. pyrifera* extract (54 µmol/g). This range of ORAC responses is similar to those reported by Ou et al. (2002) for some common vegetables including white onions, red peppers, cauliflowers, beets, and broccolis, but substantially lower than the nineteen commercial green tea dietary supplements tested by Seeram et al. (2006), which had an average response of 5151±3869 µmol/g Trolox equivalent.

It was noted that the two commercial pure fucoidan preparations showed significantly higher activity for the FRAP assay (305-319 mmol/g Fe^{2+} equivalent) but lower activities for the ORAC assay (5-10 µmol/g Trolox equivalent). Ou et al. (2002) also noted discrepancies between the FRAP and ORAC assays, when testing the antioxidant potential of fruits and vegetables. These authors suggested that the ORAC should be considered a more definitive measure of antioxidant potential, as it measures the actual peroxide radical scavenging activity of the sample. The FRAP assay, on the other hand, reflects only the ferric reducing potential, as opposed to the antioxidant preventative effect. However, the ORAC assays fails to detect activity against other relevant reactive oxygen species, and so cannot be considered a total antioxidant activity assay (Ou et al., 2002). In light of these assertions, it seems possible that the pure fucoidan preparations could have displayed activity against a non-peroxyl reactive oxygen species, or were involved in interference

reactions during the FRAP assay not indicative of true antioxidant potential.

There is a strong possibility that polyphenols, as opposed to the fucoidans, were responsible for much of the antioxidant effects exhibited by the FRPFs and the commercial *M. pyrifera* extract, which would explain why their activity profiles differed from the pure fucoidans. Fitton et al. (2015a) reported a relatively high peroxyl scavenging activity for a *Fucus vesiculosis* extract containing 59 % fucoidan and 34 % polyphenols. Furthermore, Charoensiddhi et al. (2014) showed that the antioxidant capacity of fucoidan and polyphenol-containing *E. radiata* extracts was correlated with the total phlorotannin content. The correlation appeared stronger with the FRAP assay (R^2 = 0.83 compared to 0.40 for the ORAC assay), although the opposite would be expected given the assay responses observed here for the commercial pure fucoidan samples.

5.6 Conclusions

Using the process conditions optimized previously with the *E. radiata* sample collected at Beachport in 2014, the sequential extraction process achieved high yields of alginate for all feedstock samples. The alginates extracted from the *E. radiata* and *M. pyrifera* samples had high MWs and viscosities, and low residual calcium, while that from *S. axillaris* was reported for the first time to have a very high G/M ratio, suggesting a high capacity to form strong gels. Meanwhile, the acidic treatment achieved low to medium yields of fucoidans, accompanied by substantial proportions of unwanted materials in most cases. However, some of the acidic extracts exhibited relatively high fucose and sulfate contents (up to 30 % w/w of each) as well as some antioxidant activity, indicating potential applications. The findings suggest that fucoidans and alginates with commercial value could be sequentially extracted from several South Australian seaweed feedstocks, toward a biorefinery strategy for their valorization.

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5.8 References

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5.9 Appendix 5.1. The RV of alginates extracted after different acidic treatments

The acidic treatment conditions used during the previous study, and the viscosities of the alginate extracts (before and after calcium sequestration)

Temperature (C)	pH	Duration (min)	Viscosity (mPa s) Prior to Ca^{2+} removal	After Ca^{2+} removal	RV (%)
35	3.5	60	1455	406	72
35	3.5	60	1918	547	71
35	3.5	60	2130	527	75
25	3.5	10	1573	389	75
25	3.5	110	1555	362	77
45	3.5	10	1490	355	76
45	3.5	110	1323	330	75
35	2.0	10	1318	355	73
35	2.0	110	1800	429	76
35	5.0	10	1953	427	78
35	5.0	110	1690	393	77
25	2.0	60	1388	358	74
25	5.0	60	1323	323	76
45	2.0	60	1395	354	75
45	5.0	60	1115	276	75

The extracts were obtained during the optimization experiment in Chapter 4 (Lorbeer et al., 2015a).
The viscosity measurements and RV calculations were performed as described within this chapter.

6 Comparative techno-economic analysis of valorization strategies for southern Australian brown algae for the production of fucoidans, alginates, and fertilizers

After gaining unprecedented insights into the nature and potential applications of South Australia's seaweed resources, a comparative techno-economic analysis was conducted in order to bring all of the information together.

Author contributions: PS provided helpful information and advice regarding the logistics of industrial-scale processes; SC shared experimental data that contributed to the mass balance used within the simulations; WZ provided advice regarding techno-economic analyses and the specific software used, and helped dictate experimental directions; and he and CMMF assisted with the editing of the article. I designed all of simulations, performed the techno-economic analyses, analyzed all of the data and wrote all of the content.

We intend on submitting this chapter to Bioresource Technology for publication.

Comparative techno-economic analysis of the production of fucoidans, alginates, and fertilizers from the brown algae *Ecklonia radiata*, *Macrocystis pyrifera*, and *Durvillaea potatorum*

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6.1 Abstract

A seaweed processing industry operates in rural South Australia, based on the manufacture of agricultural commodities using beach-cast biomass. It was previously demonstrated that some of the brown seaweed species abundant in the resource could alternatively be used for the production of alginates and fucoidans with commercially attractive qualities. This study simulated the industry-scale production of fertilizer, alginate, and fucoidan from three common South Australian species: *Ecklonia radiata*, *Macrocystis pyrifera*, and *Durvillaea potatorum*, using SuperPro Designer v8.0. Five processes were considered, which manufactured the products alone or in combination using integrated biorefinery strategies, and comparative techno-economic analyses were conducted. The production of fucoidans and fertilizers appeared to be profitable using all of the brown seaweed feedstocks, given gross margins of 65-78 %, as long as more than136 dry metric tons of suitable feedstock could be accessed per year. The production of alginate, however, did not appear to be economically feasible due to the high costs of production, unless a premium price (>US$26.20 /kg) or high processing scale (>3830 dry metric tons of feedstock per year) could be achieved; but neither seemed likely, given the nature of the available beach-cast seaweed resource. The sharing of materials and infrastructure resulted in economic benefits associated with biorefinery process integration. For example, the integrated production of fucoidan and alginate had total capital investment and operating costs that were 20 % and 25 % lower, respectively, than the sum of the two individual processes, but generated

revenues that were only 3 % lower, when using *M. pyrifera*.

Keywords:

Seaweed; biorefinery; sequential extraction; integrated process; techno-economic analysis; simulation.

6.2 Introduction

South Australia is situated within a global hotspot for seaweed diversity and endemism (Kerswell, 2006; Phillips, 2001). However, the state's only commercialized seaweed resource is the beach-cast wrack in its South-East region (PIRSA, 2014). Currently, a single license-holder harvests the seaweed biomass from the region's beaches – mostly at Rivoli Bay, Beachport – and then processes it into agricultural commodities at a facility in nearby Millicent.

Preliminary surveys by our group have found that brown algae account for the bulk of the harvestable biomass at Rivoli Bay – with *Ecklonia radiata* (C.Agardh) J.Agardh, *Macrocystis pyrifera* (Linnaeus) C.Agardh, and *Durvillaea potatorum* (Labillardière) Areschoug (unpublished). Due to their abundance and large, robust morphologies, these species can be isolated from the beach-cast wrack, perhaps at commercially relevant quantities. The latter should be confirmed with a comprehensive, long-term survey of this resource.

The main product currently generated at the facility in Millicent is a liquid fertilizer, produced from *D. potatorum* biomass. Such products are most often manufactured by the alkaline hydrolysis of the whole seaweed tissue, with or without the application of heat (Briceño-Domínguez et al., 2014; Khan et al., 2009). They have been shown to confer a broad range of benefits to crops, including the elicitation of plant growth, and resistance to pests, diseases, and environmental shock, due to the presence of plant hormones, buffering molecules, active polysaccharides, minerals, and trace elements (Arioli et al., 2015).

However, brown algae contain other metabolites with substantially higher economic value than the fertilizers. Alginate – an industrial polysaccharide gum that forms viscous solutions and gels with water – currently dominates the market for non-whole food brown algal products. In 2009, 26,500 metric tonnes (t) were sold at an average price of US$12 per kilogram (Bixler & Porse, 2011). Meanwhile, the complex and heterologous sulfated polysaccharides, the fucoidans, are now the

focus of a large body of research due to their interesting profiles of biological activities, which include anticoagulant, antiviral, antiaging, and immunomodulatory effects (Winberg et al., 2014). As a result, they are increasingly being marketed in health and cosmetic products, with annual global production being estimated at 250 t at a total value of US$125 million (average price of $500 per kilogram), as of 2008 (Lee, 2008). Other products of potential value from brown algae include the storage compounds laminarin and mannitol, the carotenoid fucoxanthin, as well as various polyphenols, terpenoids and halogenated compounds (Lorbeer et al., 2013).

Of the aforementioned species in the beach-cast wrack harvested at Rivoli Bay, *M. pyrifera* and *D. potatorum* have both been used extensively in other regions for the commercial production of alginates (with *D. potatorum* being harvested from beaches in King Island, Tasmania, and exported to Europe for extraction) (Bixler & Porse, 2011).

We have also shown that fucoidans and alginates with commercially attractive attributes can be sequentially extracted from all three species, using an optimized process (Lorbeer et al., 2015a; Lorbeer et al., 2015b). Process integration potentially offers a number of benefits to manufacturers and the environment, including a more efficient utilization of materials, the sharing of infrastructure, a reduction of waste streams, and the diversification of product catalogs. For example, Balboa's group have suggested a number of biorefinery strategies, as well as a comparative environmental assessment, for the valorization of the invasive brown algae *Sargassum muticum* (Balboa et al., 2015; Pérez-López et al., 2014). These have included the application of supercritical CO_2 extraction of fucoxanthin and lipids, the conventional extraction of alginates, the autohydrolysis-assisted extraction of fucoidan and polyphenols, and membrane fractionation.

However, the perceived environmental and logistical benefits associated with integrated processing may not necessarily translate into economic viability. Therefore, industrial process modelling and techno-economic analyses are necessary to compare the various avenues and strategies for seaweed biomass valorization. This approach has been used to assess the feasibility of biogas and bioethanol production from brown algae (Dave et al., 2013; Fasahati et al., 2015), but there have been no reports that have investigated the production of different polysaccharide-containing products. Therefore, the aim of this study is to conduct a comparative techno-economic assessment of the production of fucoidans, alginates,

and fertilizers – individually, and combined in integrated biorefinery processes – from the common southern Australian seaweeds *E. radiata*, *M. pyrifera*, and *D. potatorum*.

6.3 Materials and methods

6.3.1 Simulation description

The simulations were all performed using the proprietary software package SuperPro Designer v8.0 (Intelligen, Inc.). All of the models used batch processes, with processing capacities of 2000 kg of dried (10 % moisture) seaweed per batch. The default maximum annual operating time of 7920 hours per year was employed.

All currencies were converted to US$, using exchange rates as of 4th November 2015, where necessary (US$1.00= AU$1.39).

6.3.2 Models

Processes were simulated for the individual production of sodium alginate, crude fucoidan, and liquid fertilizer. Where the processes were considered compatible for integration, the sequential production of two products was also simulated. The alginate and liquid fertilizer production processes were not integrated, because the extraction of alginates would remove a large proportion of the components required for an efficacious liquid fertilizer. However, it was assumed that the fucoidan extraction process could precede either of the other two processes, without having significantly deleterious effects on their products. Fucoidans have not been directly associated with the elicitation of plant growth or health (Khan et al., 2009), and are a potential contaminant in alginate production.

Fortuitously, an acidic pre-treatment is common practice in the alginate industry, even when no fucoidan is recovered, because it can significantly improve the alginate yield by converting insoluble alginate salts into alginic acids, which are subsequently easier to extract as sodium alginate (Hahn et al., 2011). As such, both the fucoidan and alginate production processes simulated here included the same acidic treatment step, using a previously developed set of conditions that is beneficial for both products (Lorbeer et al., 2015b).

Table 1 shows the batch time (the duration of each batch, from start to finish) and cycle time (the shortest possible time between the commencements of consecutive batches) of the various processes, as well as the number of batches achievable per year, when operating at full-time capacity. The cycle times were shorter than the

batch times for all processes, because different operations within each process occupied different equipment units. Therefore, a new batch could be commenced before the preceding batch was completed, provided the equipment units required for early process operations had been vacated. Therefore, it was not the batch time, but the cycle time that dictated how many batches per year were possible. In fact, it was predicted that the alginate process would have the longest batch time (29.2 hours), but the shortest cycle time (12.7 hours), due to the distribution of the process between many relatively short-duration unit operations. This allowed it to have the highest productivity. However, a greater number of unit operations was associated with higher capital expenditure, and a more labor intensive process, as reflected by the annual labor hours.

Table 1. Scheduling information for the various processes, when operating at full-time capacity (7920 hr/yr)

Process	Batch time (hr)	Cycle time (hr)	Batches per year	Annual labor hours
Fertilizer	14.0	13.0	609	17,610
Alginate	29.2	12.7	623	36,671
Fucoidan	23.2	14.5	545	19,772
Fucoidan + fertilizer	23.2	15.5	510	33,249
Fucoidan + alginate	29.2	14.5	545	43,888

Fucoidan production process

The process flow-chart simulated for the production of crude fucoidan is represented in Figure 1. Briefly, the dried and milled (<1.4mm granules) seaweed was extracted in 0.1 M hydrochloric acid, at a solvent to biomass ratio of 20 (v/w of algal starting material), at 42 °C for 160 minutes in a blending tank. The mixture was then cooled and neutralized with the addition of sodium hydroxide, and transferred through a plate-and-frame filter to remove the residual biomass. The biomass was washed with water, and the wash-fluid and extract were both transferred into a holding tank. From there, they were subjected to ultrafiltration using a 30 kDa molecular weight cut-off (MWCO) membrane to enrich the fucoidan in the concentrate. It was assumed that this process would operate at a filtrate flux rate of 20 $L/m^2/h$. At the maximum simulated scale of operation, it was estimated that three ultrafiltration units, with a combined membrane area of 188 m^2, would be required to complete the process within 11 hours. The concentrate was then dried to form the crude fucoidan product.

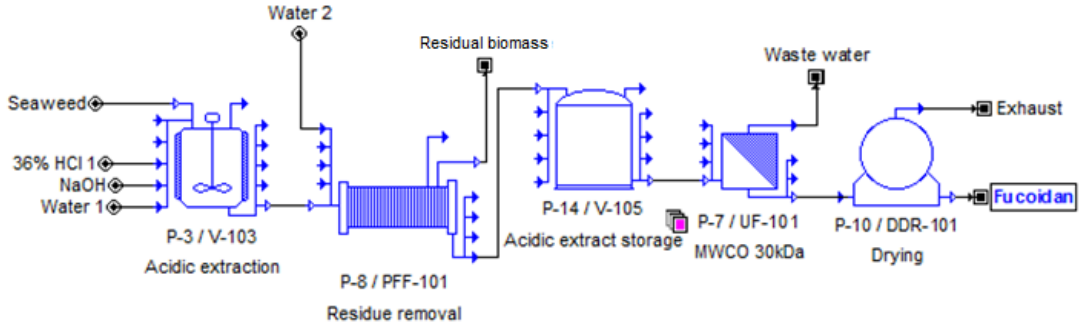

Figure 1. Fucoidan production process flow-chart

Alginate production process

The simulated process for the production of alginate (Figure 2) first involved an acidic pre-treatment conducted using the same conditions as the acidic treatment described above for the extraction of fucoidan. However, after the filtration, the fluid extract was discarded and the acid-treated biomass was transferred to another extraction tank. There, it was treated with aqueous sodium carbonate at a final concentration of 0.15 M and a solvent to biomass ratio of 27 (v/w of algal starting material) for two hours while being heated to a final temperature of 45 °C. At that point, heating was ceased, the pH was neutralized, and the slurry was diluted with water to reach a final solvent to biomass ratio of 67 (v/w of algal starting material). The residue was then removed using an industrial centrifuge.

The viscous sodium alginate extract was then subjected to a refining process similar to that commonly used in commercial alginate production processes (McHugh, 1987). First, it was added to a tank containing a 10% solution of calcium chloride in order to precipitate calcium alginate fibers. The fibers were then recovered using a filter screen, and treated with 0.5 M hydrochloric acid to convert them into alginic acid fibers. In practice, this is usually done sequentially in three separate baths; two containing the acid solutions from previous batches (McHugh, 1987). But, for simplicity, the process is represented here with one bath containing the total volume of acid, with two thirds of the cost of the acid being recovered from each batch as credit. The alginic acid fibers were then recovered using another filter screen, and mixed with sodium carbonate in a mixing vessel to form a sodium alginate paste, which was dried to become the sodium alginate product. Usually, the alginic acid fibers would be dewatered in a screw press, and the sodium alginate paste would be extruded into pellets before drying (McHugh, 1987), but that was not represented here (for simplicity, and due to the absence of suitable functions in the software). It was assumed that 10 % of the sodium alginate extracted from the seaweed was lost

during the refining process.

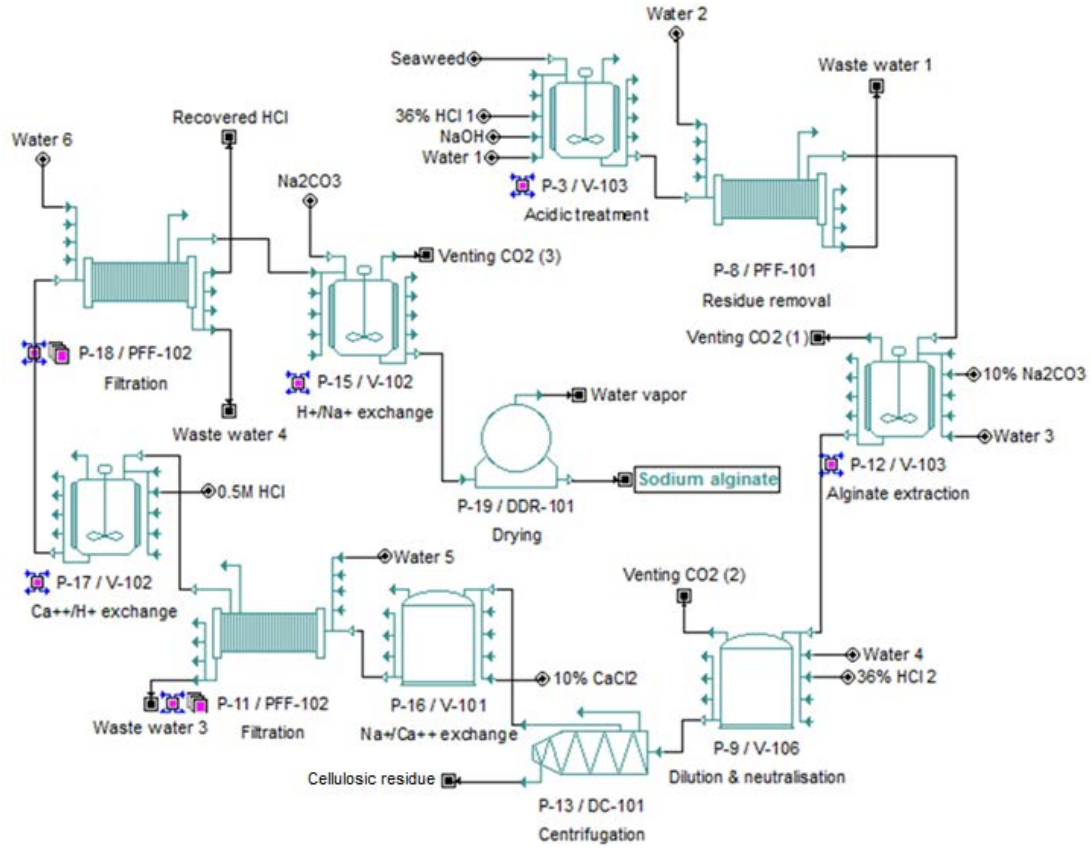

Figure 2. Alginate production process flow-chart

Liquid fertilizer production process

The simulated production of liquid fertilizer from seaweed, represented in Figure 3, was based on the commonly used alkaline hydrolysis process, with heating to increase the rate of reaction (Briceño-Domínguez et al., 2014; Khan et al., 2009). The dried and milled seaweed was placed in a blending tank with a 0.16 M solution of potassium hydroxide, at a solvent to biomass ratio of 10 (v/w dry alga). The mixture was then heated to 80 °C for 8 hours to digest the seaweed and liquefy the slurry, at which point citric acid was added to neutralize the pH. The residual solids were then removed from the liquid fertilizer product using a plate and frame filter.

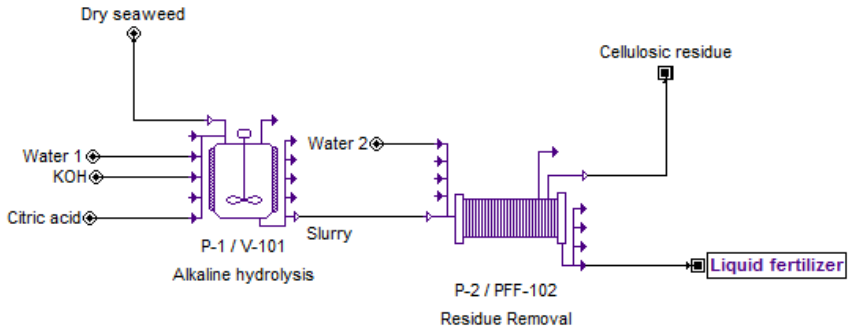

Figure 3. Fertilizer production process flow-chart

Integrated processes

Two integrated production systems were also simulated. They both involved the same fucoidan extraction process used for its individual production, with the acid-treated biomass then being subjected to either the fertilizer (Figure 4) or alginate production process (replacing the identical acid treatment and filtration steps in the latter, as shown in Figure 5). For the fucoidan and fertilizer integrated process, it was assumed that the filtrate coming from the ultrafiltration step, containing laminarins and other solutes, would be recovered for use in the alkaline hydrolysis step of the following batch.

Organic certification is an important characteristic of seaweed-derived liquid fertilizers, and could also be expected to enhance the value of fucoidan extracts. So, in practice, the hydrochloric acid and sodium hydroxide used in the fucoidan production process would be replaced with organic-allowable alternatives. However, it is unlikely that this modification would significantly affect the overall production economics, as the total water and chemical inputs account for a very small proportion of the total operating costs for those processes (3 % for fucoidan production and 6% for fertilizer production), as shown in Figure 7. Therefore, it was not undertaken in the simulations.

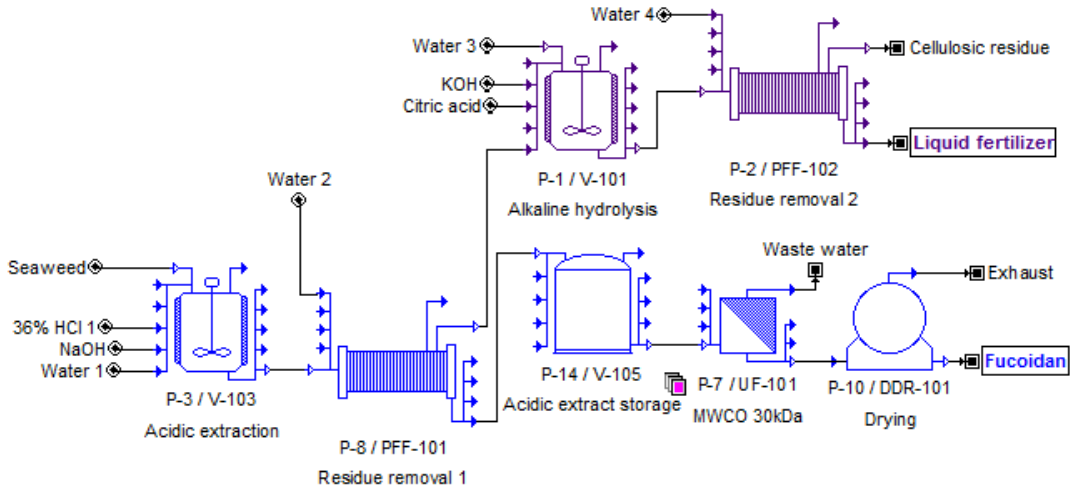

Figure 4. Integrated fucoidan and fertilizer production process flow-chart

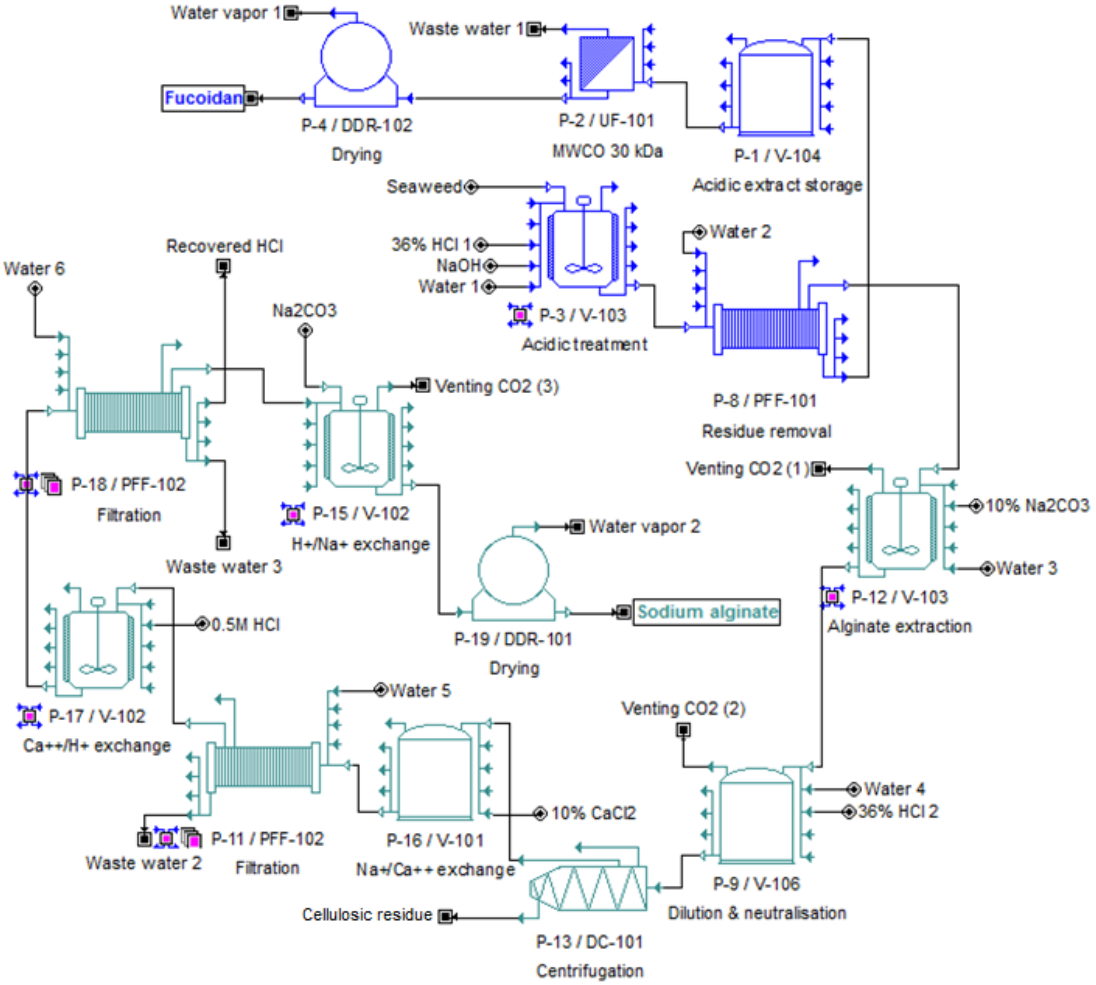

Figure 5. Integrated fucoidan and alginate production process flow-chart

Feedstocks

Four feedstock samples were considered for all processes, including one sample

each of *D. potatorum* and *M. pyrifera*, as well as two samples of *E. radiata* showing high variability in their chemical composition, in order to demonstrate the potential economic effects of intraspecies variability that may occur seasonally, for instance (Lorbeer et al., 2015a).

6.3.3 Capital cost estimation

It was assumed that the processing facility would be built on empty land in Millicent, South Australia. As such, the capital costs included the construction of all required buildings and yard improvements (e.g. added infrastructure), as well as the purchase and installation of all equipment. The default costings in the software were used for capital (which were automatically adjusted according to the year of commencement, which was 2015), with a construction period, startup period and project lifetime of 30 months, 4 months and 15 years, respectively.

The size (and therefore the cost) of the equipment used in each process was scaled according to the capacity required. Therefore, even for the same process, capital costs differed slightly according to the nature of the biomass (e.g. equipment used for alginate refining had to be larger for the feedstocks that yielded larger volumes of alginate, such as *D. potatorum*). However, equal numbers of equipment units were used between different feedstocks. Where possible, equipment was shared between different steps in each process in order to reduce capital expenditure. However, equipment sharing was abandoned in those cases where it led to bottlenecks that counteracted the financial savings by prolonging the batch cycle time. Vessels were priced with the assumption that they were to be made from materials resistant to the dilute acids and bases and the salty water that they would be exposed to during operation.

6.3.4 Operating cost estimation

The software default settings were used to estimate the facility-dependent costs, which included equipment maintenance, depreciation, insurance, property taxes and possibly other overhead costs, depending on the facility set-up.

Utility costs were set at figures reflective of the intended location, with electricity charged at $0.216 per kWh, and water at $2.42 per kiloliter. Meanwhile, a basic labor rate of $30 /hr was used, which equated to a lumped rate of $69 /hr once the specific types of operators, along with the associated supervision, supplies, administration, and overhead costs, had been taken into account. The default value of 15 % was added to the labor costs to cover quality assurance and checking.

Raw materials were costed according to quotes received from local suppliers, including Ixom Operations Pty Ltd and Redox Pty Ltd. Given Millicent's rural location, an additional freight cost of $43 /t was added, based on advice received from suppliers (assuming materials were to be sent by full-truck load of 20 t). Diluted solutions (e.g. 0.5 M HCl) were assumed to be mixed on site, and their costs were therefore calculated according to the sum of the mixed components. The unit value of the various raw materials is shown in Table 2.

Table 2. Estimated values of materials

Stream	Material	Value ($/kg[a])
Raw materials	Dried, milled seaweed	0.86
	Calcium chloride	0.48
	Sodium hydroxide	0.66
	Sodium carbonate	0.40
	36 % hydrochloric acid	0.38
	Potassium hydroxide	1.49
	Citric acid	1.12
Consumables	UF membrane ($/m^2)	400
Products	Liquid fertilizer	0.94
	Fucoidan	700
	Alginate (*M. pyrifera*)	21.6[b]
	Alginate (*E. radiata*)	18.0[b]
	Alginate (*D. potatorum*)	14.4[b]

Costs given in terms of 100% purity, adjusted from industrial-grade, after adding cost of freight.
[a] Unless another unit measure is specified.
[b] Priced according to the quality of the alginates, as observed previously (Lorbeer et al., 2015a).

With regard to the seaweed biomass, no reliable figures could be obtained for the costs associated with harvesting and transporting the seaweeds from Rivoli Bay. So, a generic purchasing price of $864 /t was applied to all feedstocks, which was roughly equal to the price of dried Australian beach-cast *D. potatorum*, based on industry advice.

No waste disposal costs were applied in the simulations. The waste waters were salty, but not hazardous, and could be treated fairly inexpensively. This would allow for a large portion of water to be recycled, which could partly offset the costs of its treatment or disposal (McHugh, 2003). Meanwhile, as per industry advice, it was assumed that the residual biomass from the processes could be removed free-of-charge for use in other applications, such as aquaculture feeds and soil conditioners. It was not practical to include other company costs such as packaging, marketing, and research and development, so the total costs predicted by the models would most likely be underestimated. For a company producing fucoidan, research and development could indeed account for a significant proportion of operating costs, if it chose to pursue comprehensive efficacy testing on its products, for instance (although, some of those costs would likely be offset by the value added to the products).

6.3.5 Revenues

The selling prices of the three revenue streams – the crude fucoidans, sodium alginates, and liquid fertilizers – were all based on advice obtained from commercial-

in-confidence suppliers. The fucoidans were valued at $700 /kg of pure product, with the price of the crude fucoidan products reduced proportionately according to their purity. This was based on the assumption that they would be sold to functional food and cosmetic manufacturers. While the value of fucoidans may vary according to their source species, it was assumed here that all of the fucoidans had equal value.

Meanwhile, it was assumed that the sodium alginates would be sold to the food industry at a rate dependent on their quality (refer Table 2), as assessed in a previous study (Lorbeer et al., 2015a). Finally, the liquid fertilizers were all prescribed an equal value of $936 /t, regardless of the feedstock used.

The yields and purity of fucoidan and alginate that could be extracted from each feedstock were estimated from observations made in previous lab-scale experiments (Lorbeer et al., 2015a). It is difficult to accurately determine the purity of crude fucoidan extracts due to the structural diversity of the polymers. Therefore, the purity was estimated using the fucose content of the extracts as an indicator. Holtkamp et al. stated that fucose tends to account for 34‑44 % of the weight of fucoidans (2009), so here it was assumed that all fucoidans were composed of an average of 39 % fucose (w/w), although in reality this would not be the case. It was also assumed that most of the laminarin and other relatively low molecular weight compounds in the liquid extracts were removed by the ultrafiltration step, thus enriching the fucoidans.

6.3.6 Indicators of process economic performance

Four profitability measures were utilized: the gross margin, payback time, net present value (NPV); and return on investment. The gross margin is the percentage of annual revenues that become gross profit; the payback time is the time it takes once processing operations commence for cumulative annual net profits to balance the total capital investment (TCI); the NPV is the total value of future net cash flows during the lifetime of the project (discounted to reflect the value of money at the present year, done here using the default interest rate of 7%); and the ROI is the annual percentage return on the TCI. When comparing multiple projects, the one with the highest NPV would generally be considered the most financially attractive, assuming the TCI is considered affordable. And of course, a project with a negative NPV should not be undertaken.

6.4 Results and discussion

6.4.1 Differences between the feedstocks

The yield and purity of the fucoidan and alginate products were estimated based on the results previously obtained in laboratory-scale experiments (Lorbeer et al., 2015a), with some modifications according to the extra refining steps included in the simulations. With all of the fucoidans being assigned an equal value (per kg of pure product) and being produced at similar levels of purity (57-65 %), the main determinant of revenue generated from the fucoidans was their yield. Therefore, as shown in Table 3, *M. pyrifera* was predicted to generate the highest revenue from fucoidans ($42,032 per batch), and *D. potatorum* the lowest ($26,151). It should also be noted that there was some variability between the fucoidan revenues generated from the two *E. radiata* feedstocks, resulting mainly from their different levels of purity.

The quality of the alginates, as observed in previous studies (Lorbeer et al., 2015a), was also taken into account when assigning product prices. Of the three species considered here, the crude alginate from *M. pyrifera* had the highest viscosity, the highest G/M ratio (indicating the ability to form strong gels), and the lightest color. Therefore, in the simulations it was assigned the highest unit price, as shown in Table 2. Meanwhile, *E. radiata* produced intermediate quality alginates, and *D. potatorum* performed relatively poorly in all three measures. However, in addition to the quality of the alginate extracts, their yield and purity were also important in determining the value of the products. Therefore, as shown in Table 3, the alginates from *D. potatorum* were estimated to generate the most revenue ($14,647 per batch) despite having the poorest quality, due to their very high yield. Meanwhile, the differences in revenue predicted for the two *E. radiata* feedstocks demonstrate the economic risk that can result from feedstock variability. Such variability has a broad range of contributors, including seasonal and environmental influences, as well as life history, and so could be expected to be higher for wild-harvested seaweeds, compared with those sourced from aquaculture, which is inherently more controllable (Winberg et al., 2014).

Table 3. The nature of the fucoidan and alginate products from the various feedstocks

		M. pyrifera	*E. radiata* (Mar-13)	*E. radiata* (July-14)	*D. potatorum*
Fucoidan product	Yield (kg/batch)	103	85.1	86.2	57.9
	Purity (%)	58.4	56.9	64.1	64.5
	Price (US$/kg)	409	398	449	451
	Revenue (US$/batch)	42,032	33,877	38,680	26,151
Alginate product	Yield (kg/batch)	683	503	768	1053
	Purity (%)	92.8	94.8	97.1	96.6
	Price (US$/kg)	20.1	17.1	17.5	13.9
	Revenue (US$/batch)	13,699	8,588	13,421	14,647

Yield and purity estimations based on previous observations (Lorbeer et al., 2015a).
Price adjusted according to the purity of the products.

6.4.2 Overall economic performance of different processing strategies

Table 4 presents a range of economic indicators for the performance of the different production processes when operating at full-time capacity, using the various feedstocks. Over the lifetime of the project, all of the processing options appear to be profitable, apart from the production of alginate as the only product, which resulted in a total loss of at least $13 million for all feedstocks in the simulations (and more than double that for the low yielding *E. radiata* sample), as a result of negative gross margins and very high total capital investment requirements. The production of liquid fertilizer, which is currently conducted in Millicent, was predicted to require very low total capital investment (approximately $4m) compared to all other processes. Given its high gross operating margin, this resulted in the shortest payback time and the highest ROI out of all the simulations, of 0.81 years and 123 %, respectively. Therefore, it would appear to be an attractive and relatively low risk investment, particularly if high amounts of capital were not initially available. However, fucoidan production from *E. radiata* and *M. pyrifera* was predicted to be significantly more profitable over the lifetime of the project due to annual revenues of approximately double that of the fertilizer production process ($18.5-22.9m compared with $10.8m). When using *D. potatorum* as the feedstock, however, processing fucoidan appeared marginally more profitable than fertilizer production over the lifetime of the project (NPV of $35.1m compared to $30.7m) (PIRSA, 2014). But, with the much higher TCI ($11.1m compared to $4.0m) and payback time (1.7 compared to 0.81 years), and lower ROI (59 % compared to 123 %) associated with the fucoidan project, in that case, fertilizer production seems the more attractive option.

Given the sharing of infrastructure, processes and materials, the production efficiency of fucoidan and alginate were improved through integrated processing

was improved. These are some of the commonly cited advantages of integrated processing systems (Dias et al., 2012). For instance, the integrated process had a TCI and operating costs that were 20 % and 25 % lower, respectively, than the sum of the individual processes, but generated revenues that were only 3 % lower, when using *M. pyrifera*. Also, the cycle time of the integrated process was no longer than that of the fucoidan production alone. As a result, the NPV of the sequential process was very high (approximately equal to that of the fucoidan production process for all feedstocks apart from *E. radiata* '14), despite the cost burdens associated with the production of the alginate. However, the TCI and payback time were approximately double that of the production of fucoidan alone, and the ROI was roughly half, so the integrated process would not be worth pursuing given the more attractive alternative. The sequential production of fucoidan and fertilizer from *M. pyrifera* was predicted to generate the most profits over the lifetime of the projects.

Table 4. The economic performance of the processes, when operating at full-time capacity, using different feedstocks.

Process	Feedstock	TCI ($)	Operating cost ($/yr)	Revenues ($/yr)	Gross Margin (%)	Payback time (yr)	NPV ($mil)	ROI (%)
Fertilizer	Brown algae	4,014,000	3,146,988	10,793,000	70.8	0.81	30.65	123
Alginate	*E. radiata* ('14)	21,528,000	9,052,782	8,361,000	-8.27	17.8	-15.77	5.62
	E. radiata ('13)	20,696,000	8,713,327	5,351,000	-62.8	N/A	-33.23	-7.41
	M. pyrifera	21,312,000	8,928,928	8,533,000	-4.64	14.3	-13.46	6.98
	D. potatorum	23,232,000	9,580,495	9,128,000	-4.95	14.5	-15.49	6.89
Fucoidan	*E. radiata* ('14)	11,100,000	4,976,000	21,080,000	76.4	1.04	64.56	95.9
	E. radiata ('13)	11,084,000	4,983,000	18,463,000	73.0	1.22	53.26	81.8
	M. pyrifera	11,141,000	4,983,000	22,907,000	78.3	0.95	72.31	105
	D. potatorum	11,100,000	4,977,000	14,252,000	65.1	1.70	35.14	59.0
Fucoidan + fertilizer	*E. radiata* ('14)	14,949,000	6,616,929	28,353,000	76.7	1.04	86.82	96.1
	E. radiata ('13)	14,539,000	6,520,059	23,864,000	72.7	1.24	68.04	80.4
	M. pyrifera	14,821,000	6,586,819	29,152,000	77.4	1.00	90,43	100
	D. potatorum	15,067,000	6,511,810	21,925,000	70.3	1.42	59.59	70.2
Fucoidan + alginate	*E. radiata* ('14)	26,075,000	10,536,662	28,394,000	62.9	2.00	64.63	50.0
	E. radiata ('13)	25,245,000	10,222,239	23,144,000	55.8	2.53	44.06	39.6
	M. pyrifera	25,860,000	10,423,572	30,372,000	65.7	1.81	73.88	55.2
	D. potatorum	27,774,000	11,036,141	22,237,000	50.4	3.02	34.97	33.1

TCI: Total capital investment.
NPV: Net present value.

6.4.3 Fixed capital costs

As shown in Figure 6, the integrated fucoidan and fertilizer production process was estimated to require fixed capital investment approximately equal to the sum of both individual processes. This is because, unlike the fucoidan and alginate production

processes, no processing steps or equipment are shared between the two processes. Indeed, the extraction vessel and plate and frame filter used for both fucoidan and fertilizer production could be shared, but this was found to substantially increase the cycle time of the integrated process, thus reducing annual production capacity and negating the savings in capital. Figure 6 also demonstrates how the costs associated with the large amount of equipment required for the alginate production process were substantially amplified by the related building, engineering and construction expenses.

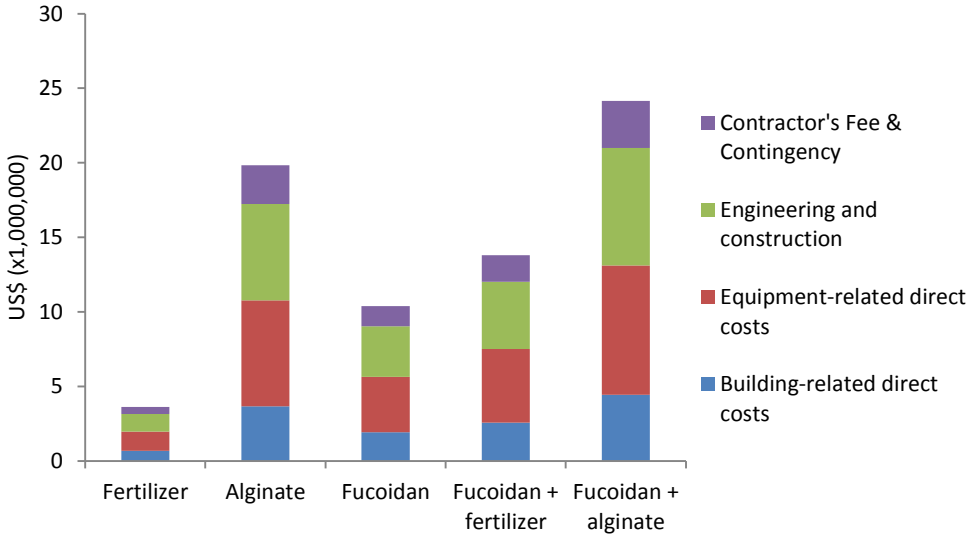

Figure 6. The fixed capital costs of the different processes
M. pyrifera used as the feedstock in each scenario.

6.4.4 Operating costs and revenues

As shown in Figure 7, the facility-dependent operating costs of the alginate production process, which accounted for 41% of its total operating costs, were also significantly higher than the fertilizer and fucoidan processes. This was due mainly to the maintenance of the equipment. The costs of water and chemicals were 8- and 10-fold higher for the production of alginate, compared with fertilizer and fucoidan, respectively, and the labor costs were also twice as high for alginate production.

However, the processes involved in the production of fucoidan include a high consumables cost, mainly accounted for by the membrane used in the ultrafiltration step. The membrane costs of \$400 /m^2 equated to a total annual cost of \$452,000, or 9 % of the total operating costs, in the simulated production of fucoidan from *M. pyrifera*. Ultrafiltration was also estimated to be a time-consuming step (~11 hours), and was responsible for a large proportion of the fucoidan process cycle time. In

fact, it caused the cycle time of the fucoidan process to be longer than that of the much more complicated alginate extraction process. However, compared with alternative methods of recovering the fucoidans from the liquid extract, such as with the addition of organic solvent or surfactants, membrane technology does not require the addition of chemicals, improves the safety and recyclability of waste water, is less likely to affect the structure of the polymers, and can selectively recover molecules based on their size (Balboa et al., 2015).

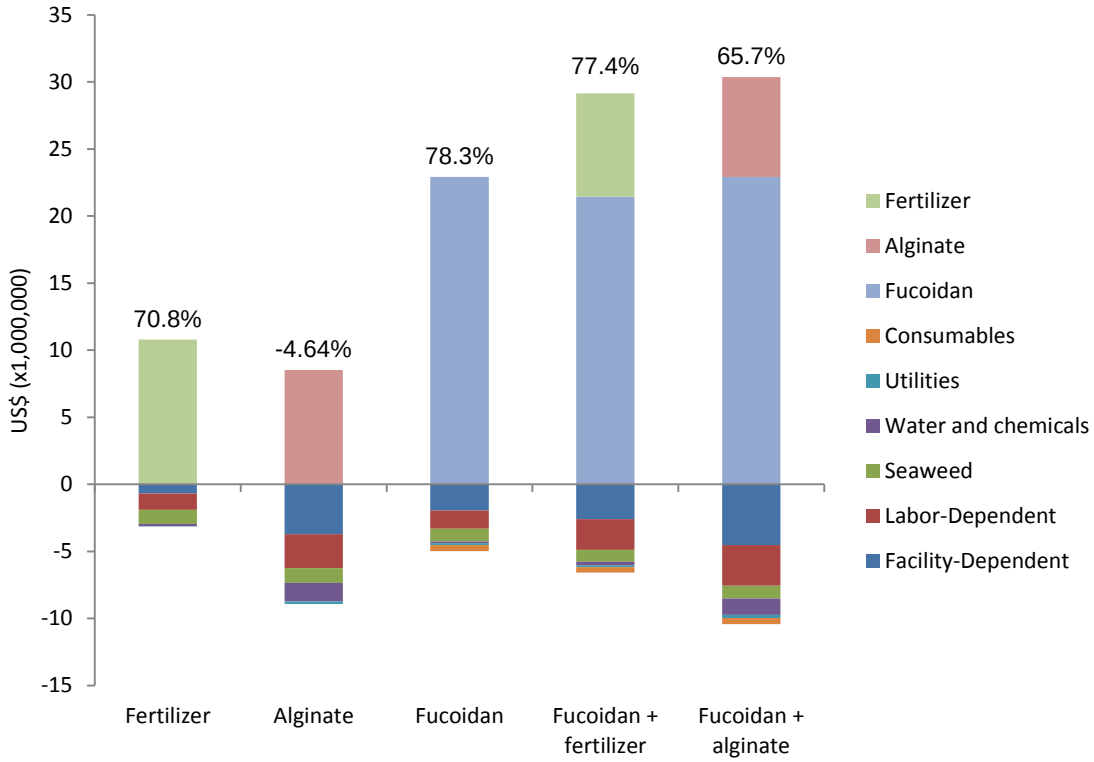

Figure 7. Annual operating costs and revenues of the different processes, when operating at full-time capacity
M. pyrifera used as the feedstock in each scenario.
The numbers above the bars refer to the gross margin of each process.

6.4.5 The economic effects of using different feedstocks

With regard to the economic effects of using different feedstocks within the same processes, as shown in Figure 8, the operating costs varied slightly, but the revenues were altered considerably as a result of the different yields, purities and qualities of the products. In reality, the value of fucoidans depends on a number of additional factors that have not been considered here, some of which relate to the species of origin (e.g. whether clinical studies have been conducted using fucoidans from a particular species), so the differences in revenue could be more pronounced (Lee, 2008). The variations in the operating costs associated with the feedstocks can be explained by the different amounts of chemicals and water required as a

result of the product yields. For example, *D. potatorum* produced the largest quantity of alginate, meaning larger amounts of water and reagents were required during the refining steps. This also affected the capital investment costs estimated by the software (as seen in Table 4), as larger filters and dryers would be required to handle the extra material. If equipment sizing was held constant between the models, the filtering and drying times (and therefore process cycle time) would have been longer for high yielding species, again affecting the process economics.

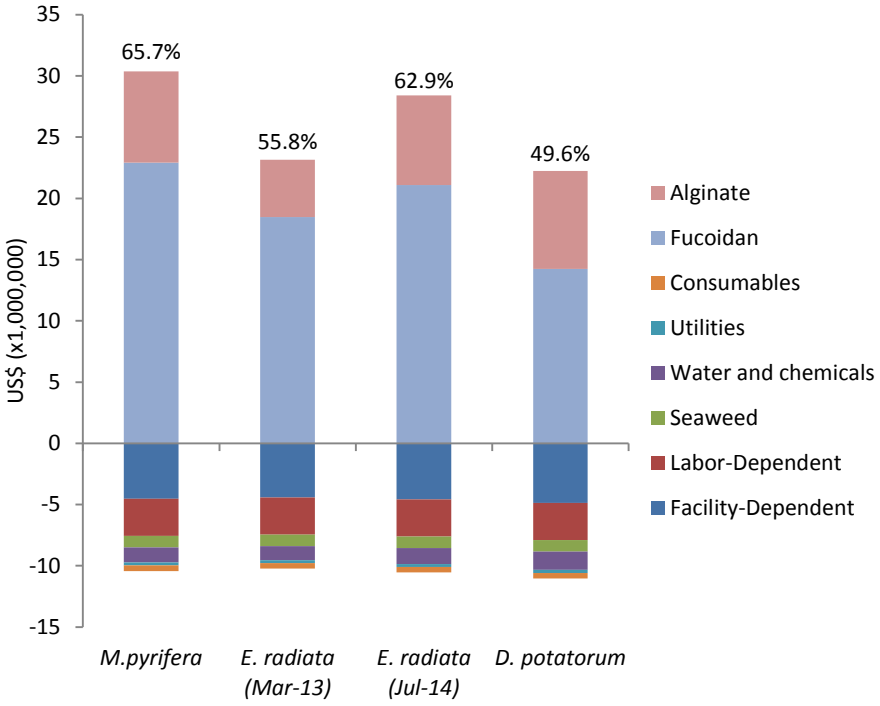

Figure 8. Annual operating costs and revenues of the sequential fucoidan and alginate production process, when operating at full-time capacity, using different feedstocks.
The numbers above the bars refer to the gross margin of each process.

6.4.6 Minimum acceptable price analysis

With regard to the sensitivity of the various processes to change, we have shown that the yield and selling price of the products would have a considerable impact on the overall economic feasibility of the projects. This is particularly true for fucoidan, given its relatively low yield, high unit value, and the fact that the market is still developing and subject to rapid change (from both the supply and demand sides). The seaweed feedstock generally accounted for a fairly low proportion of the operating costs (except in the case of the fertilizer process, where it accounted for 33 % of the total operating costs), so variations in its price would be unlikely to have significant impacts on the economic feasibility of the processes. However, sudden changes in the availability of feedstocks with favorable traits would indeed have important implications. Operations relying on locally harvested biomass would be

particularly sensitive to this, as a result of changes in legislation, water quality, and weather/climate patterns, for instance. However, alternative biomass could probably be imported to supplement feedstocks in such scenarios.

The sensitivity of the various projects to economic change can be assessed by examining the differences between the predicted product prices, and the prices that would achieve a break-even scenario (where NPV = $0). This data is shown in Table 5. The project based around the production of liquid fertilizers was predicted to break-even if the fertilizer was sold at a price of $320 per metric ton, which is 66% lower than its predicted current market value. Therefore, this project would be fairly economically robust in the face of unforeseen economic changes. This, along with the low capital investment requirements, suggests that it would be a relatively low risk venture. Likewise, the projects involving the production of fucoidan from *M. pyrifera*, either by itself or together with fertilizer, would break even if the revenues achieved were one quarter of that predicted. However, when producing multiple products using an integrated process, the minimum acceptable price of one product can be further reduced, if a higher price can be maintained for the other product. For instance, for the integrated fucoidan and fertilizer process, the price of fucoidan could be reduced to as low as 18 % of its current predicted value ($74 /kg), if the price of fertilizer was maintained at 50 % of its current predicted value. Whereas, when fucoidan was the sole product, the lowest acceptable price was $113 /kg. In this way, the integrated process would be less sensitive to market fluctuations for one product or the other, when compared with the individual processes, further demonstrating the benefits of the biorefinery process strategy.

For the integrated production of crude fucoidan and alginate from *M. pyrifera*, the two products could be sold for as low as $175 and $8.61 per kilogram, respectively, which is 57% lower than the predicted market prices for each. However, for the production of alginate as the sole product, it would need to command a premium price of $26.20 per kilogram (when using *M. pyrifera* as the feedstock) in order to break even. The price would need to be higher again if using the other feedstocks, which would be highly unlikely given their lower product quality.

Table 5. The product pricing required to break-even for each process, if operating at full-time scale

Process	Minimum acceptable price ($/kg)			Proportion of predicted price
	Fertilizer	Alginate	Fucoidan	
Fertilizer	0.32	-	-	0.34
Alginate	-	26.2	-	1.31

Fucoidan	-	-	108	0.26
Fucoidan + fertilizer	0.26	-	113	0.28
Fucoidan + alginate	-	8.61	175	0.43

M. pyrifera used as the feedstock in each case.

6.4.7 Feasibility of the projects in a limited biomass scenario

So far, we have looked at the processes when operating at a maximum production capacity of 7920 hours per year, in order to assess their viability in a scenario of plentiful feedstock availability. With each batch processing 2 mt of dry (10 % moisture) seaweed, this resulted in annual feedstock consumption of between 1020 mt for the fucoidan and fertilizer process, to 1246 mt for the alginate process.

This is modest in comparison to the annual harvest of *D. potatorum* at King Island, where approximately 4500 mt of dry seaweed is exported to Europe for alginate extraction each year (Bixler & Porse, 2011), and additional biomass supplies local manufacturers of agricultural commodities (Kirkman & Kendrick, 1997). However, the harvest at the seaweed fishery in South Australia's south-east has traditionally been much smaller, with an average of 79.5 mt and a maximum of 305 mt of fresh seaweed (or approximately 13.3 mt and 50.8 mt of dry algae) being collected each year, from 2000 to 2014. Therefore, in order to fully supply the fucoidan and fertilizer process operating at full-time capacity, the annual harvest at Beachport would need to be twenty times larger than the maximum harvest carried out thus far, and would need to consist of selected species of brown algae, as opposed to the mixed-species wrack.

AKP typically harvested a very small proportion of the available beach-cast wrack from a single 8 km-long beach (Rivoli Bay) within their 102 km license area (PIRSA, 2014). Therefore, in lieu of a comprehensive and long-term survey of the resource at Beachport, it is not currently possible to accurately predict how much seaweed could be harvested sustainably, let alone if targeting a particular species. On this basis, we can determine the lowest amount of feedstock at which the different projects appear to be economically viable, as a yardstick to determine what is feasible as more data regarding the resource becomes available.

For the processes that appeared to be economically viable at full-time production capacity (i.e. all except the production of alginate alone), the scale of production was lowered to the point where the project was predicted to break-even by either reducing the batch size and holding the annual operating time constant, or vice

versa. When the batch size was reduced, the TCI and the facility-dependent costs fell as smaller equipment and fewer equipment units were required. When the operating hours were reduced, the labor-related costs fell. In both cases, the annual expenditure on raw materials was also reduced. For the production of alginate as the sole product, as the operating hours could not be increased beyond full-time capacity, only the batch size was increased. This necessitated increased total capital investment ($55.9 m for 6.1 mt batch process) as more and larger units of equipment were required, but reduced the costs per unit of production (15.72 $/kg of alginate for 6.1 mt batch, compared to 20.98 $/kg of alginate for the 2 mt batch, for *M. pyrifera*) through economies of scale.

The data from this analysis are presented in Table 6, using *M. pyrifera* feedstock in each case. In projects where labor accounted for a large proportion of the total costs, such as the fertilizer process, it was more effective to reduce the annual operating hours, as opposed to the processing capacity, in order to achieve the lowest break-even production level. On the other hand, if the facility-dependent costs accounted for a larger proportion of the operating costs than labor, as was the case for the production of fucoidan, it was more effective to reduce the processing capacity.

The fucoidan production project was shown to make a profit with the lowest annual seaweed consumption of 136 dry mt of *M. pyrifera* per year. However, the profitability of the fucoidan production process was very sensitive to the feedstock used. So, if a species that yields less fucoidan, such as *D. potatorum*, were more easily accessible than *M. pyrifera* in a scenario of limited biomass, then a fertilizer production project would have better economic prospects. The integrated production of fucoidan and fertilizer, and the production of fertilizer alone, were predicted to require 156and 152 mt of dry *M. pyrifera* per year to break-even, which equates to a fresh weight of approximately three-fold higher than AKP's maximum reported harvest at Beachport.

On the other hand, the alginate production project was predicted to break-even only when operating at full-time capacity, with a batch size of 6.1 mt of feedstock, which would equate to 3830 mt of dry seaweed per year, or approximately 4 % of the global harvest for alginate feedstock (Bixler & Porse, 2011). For the projects involving the production of fucoidan or alginate, if the most favorable species – *M. pyrifera* – was not available in sufficient quantities to be used as the sole feedstock,

then the production scale required to break-even would increase, due to the reduced revenues achieved when using the other feedstocks (or higher production costs, when using *D. potatorum* for alginate production).

Table 6. The production scales at which the various processes break-even

Process	Annual operating time (hr)	Batch size (dry[a] mt)	Batches	Annual seaweed Consumption (dry[a] mt)	(wet[b] mt)
Fertilizer	990	2.00	76	152	912
	7920	0.43	609	260	1560
Fucoidan	1160	2.00	79	158	948
	7920	0.25	545	136	814
Fucoidan + fertilizer	1230	2.00	78	156	936
	7920	0.32	510	163	978
Fucoidan + alginate	2225	2.00	152	304	1824
	7920	0.51	545	275	1652
Alginate	7920	6.15	623	3830	22980

Batch size refers to the amount of dry seaweed (10 % moisture) being processed per batched.
M. pyrifera was used as the feedstock in each case.
[a] Moisture content of 10 %
[b] Moisture content of 85 %

6.4.8 Circumstances under which alginate production may be economically viable

According to the simulations, the production of alginate did not appear to be economically feasible in South Australia, at the processing scale of 1246 mt of dry feedstock per year. Alginate producers in Western countries that have remained in operation after the recent decade of heavy consolidation and increased competition from Asia, have done so by focusing on the high-end of the market, and through economies of scale (Bixler & Porse, 2011). During this study, both of these outcomes were demonstrated when it was shown that the alginate production project could break-even in South Australia, if a product price of $26.20 /kg, or a processing scale of 3830 mt/year were achieved. According to the processing model considered here, that scale of production would require a facility with the capacity to process 6.1 mt of dry seaweed every 12.7 hours, accompanied by a gross margin of approximately 21.3 % (Figure 9 A).

However, these criteria might not be necessary in order to achieve economic feasibility, if the project were located in a country with lower labor rates. Figure 9 B shows the economic effects of reducing the basic labor rate below the $30 /hr used in the simulations, while keeping the processing scale and product price the same. A positive gross margin was achieved when the basic labor rate was below $25.30 /hr, and the project break-even was achieved when the basic labor rate was reduced to $2 /hr, which was close to the average labor rate for manufacturing employees in China in 2009 ($1.74 /hr) (U.S._Bureau_of_Labor_Statistics, 2013). However, in these simulations, the facility-dependent costs were not altered, but in reality, the

reduction in the basic labor rate would also reduce the costs of construction, installation, and maintenance. Meanwhile, land and infrastructure also tend to be cheaper in countries with lower labor rates. Therefore, the TCI and non-labor operating costs would also be reduced, and the NPV would be significantly higher than indicated in the graph.

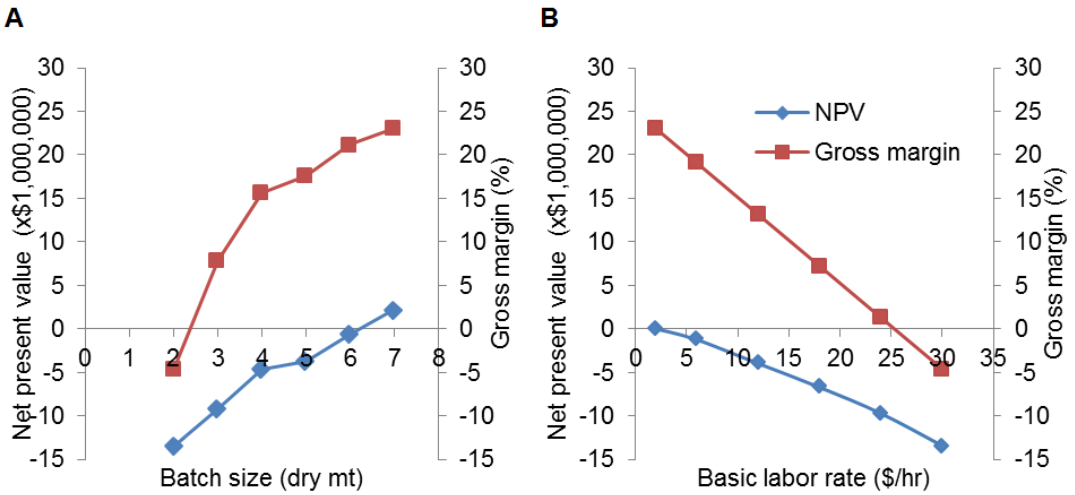

Figure 9. The effect of (A) production scale and (B) basic labor rate on the economic viability of the alginate production process
Batch size refers to the amount of dry seaweed (10 % moisture) being processed per batch.
When the batch size was altered, the basic labor rate was held at $30 /hr.
When the labor rate was altered, the batch size was held at 2 dry metric tons.
Changes in the basic labor rate were only reflected in the labor costs; not in the facility-dependent costs.
All other parameters were held constant.

6.4.9 Other potential valorization strategies

It must be considered that the valorization strategies considered here are not exhaustive. Other compounds of potential value that could be extracted from the brown algae include laminarins, phlorotannins, and fucoxanthin. Building on the integrated fucoidan and fertilizer process considered here, laminarin could also be recovered from the acidic extract (Lorbeer et al., 2015a), perhaps using a second ultrafiltration membrane with a MWCO of ~2 kDa, to filter the permeate from the first 30 kDa MWCO membrane, for instance. Some phlorotannin-rich material could also be recovered from the low MW fractions. Otherwise, organic solvent leaching or supercritical fluid extraction could be used to recover phlorotannins, fucoxanthin and oils from the starting material, prior to the extraction of polysaccharides, as has been demonstrated previously (Balboa et al., 2015; Conde et al., 2014; Pérez-López et al., 2014; Quitain et al., 2013). Meanwhile, the final cellulosic residue remaining after the integrated process could potentially be incorporated into soil conditioners, or treated with autohydrolysis for the production of sugars, antioxidant fractions, or bulk chemicals (Ansari et al., 2015; González-López et al., 2012; Pérez-López et al.,

2014; Yuan & Macquarrie, 2015).

Aside from integrated extraction strategies, brown algae can also be used to produce bioenergy. Dave et al. (2013) have shown that an anaerobic digestion process may be able to produce heat and electricity in Northern Europe at the break-even electricity selling price of $132 /MWh. However, the simulated processing scale was 8.64 dry mt per day – more than twice the full-time capacity of the models considered here.

6.5 Conclusions

Judging from the economic indicators obtained from the simulations, the production of liquid fertilizer from brown algae, as is currently undertaken in Millicent, South Australia, is an attractive investment and relatively low risk venture, due to low capital and operational costs, and high return on investment. Meanwhile, the production of crude fucoidan was predicted to be substantially more profitable over the lifetime of the project, considering the very high current market value of the product and the relatively simple production process required. However, it would be more sensitive to changes within the industry and the availability of favorable biomass. Given the high costs of land, labor, utilities and skilled services in Australia, and the commodity status of alginate, the economic feasibility of its production in a small to medium enterprise (i.e. in lieu of economies of scale) appeared to be low. Its feasibility improved when integrated into a sequential process with fucoidan, but this would not be undertaken, as the production of fucoidan alone, or together with liquid fertilizer, appeared to be much more attractive alternatives. Integrated processing was shown to have economic benefits, particularly in the case of the sequential extraction of fucoidan and alginate, where materials, processes and infrastructure could be shared between processes. Finally, it was found that the *M. pyrifera* sample performed best out of the tested feedstocks in all models involving the production of fucoidan and/or alginate, but comprehensive and long-term surveying of the resource is required to determine whether it could be reliably sourced from South Australia's seaweed fishery at a commercially viable scale. In general, reliance on locally harvested beach-cast wrack poses an economic risk due to its sensitivity to environmental and legislative change, but it could be used for small-scale industry development, and supplemented with imported or, preferably, aquacultured biomass as required.

6.6 Acknowledgements

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7 Conclusions

7.1 Summary of key findings

This project was undertaken with the aim of facilitating the early development of a seaweed processing industry in rural South Australia, involving the production of high-value products. In large measure, these aims were achieved as the harvestable seaweed resources were assessed for their potential utilization; improved processing technologies were developed; and a number of valorization strategies were simulated at industry-scale, and compared for their economic feasibility.

It was also observed that the bulk of the harvestable biomass comprised brown algae; especially *Ecklonia radiata*, *Macrocystis pyrifera* and *Durvillaea potatorum,* but also a number of endemic species belonging to the families Sargassaceae and Seirococcaceae. Sugar analysis of their whole tissues was used to screen for species that might be good sources of commercially relevant polysaccharides. Notably, the biomass samples belonging to the endemic Seirococcaceae species, *Seirococcus axillaris* and *Phyllospora comosa,* were reported for the first time to contain high amounts (36-46%) of guluronate-rich alginate.

Extractive studies were undertaken in order to gain clearer insights into the limitations of conventional processing techniques, and improve upon them accordingly. When applied to *E. radiata*, classical fucoidan extraction conditions (dilute HCl at pH 2, 60 °C, 3 hours) were shown to achieve a low yield of fucoidan (22 % of the total available, with logarithmic extraction kinetics), while causing a significant loss of structural integrity (exponential decay of molecular weight, and gradual cleavage of sulfate groups), and a gradual reduction in purity (due to the increased co-extraction of laminarin). In circumstances where conserving the native structure, or maximizing the purity of the fucoidans is a priority, a short-duration (approximately six minutes) extraction could be recommended. Open-vessel microwave-assisted extraction was identified as an efficient and highly controllable alternative to convective heating, which did not significantly affect the nature of the products.

It was hypothesized that the extraction of fucoidan may be inhibited by associations between it and other components of the cell wall. However, pre-treatment of the algal tissue with commercial protease and carbohydrase preparations, and pre-treatment with deep eutectic solvents formed between urea and choline chloride

both failed to significantly improve the yield of extracted fucoidan.

As the extraction of fucoidans only utilized a small proportion of the brown algal biomass, integrated processing technologies were then investigated. A sequential extraction process was developed, involving the acidic extraction of fucoidans, followed by the sodium carbonate extraction of alginates. It is well known that the acidic treatment could be beneficial both for the extraction of fucoidans, and as a pre-treatment for alginate extraction. Here, we reported for the first time the multiple response optimization of the acidic treatment to simultaneously perform both functions effectively. Contrary to previous reports, a solvent with a pH as low as one could be used during the acid treatment of *E. radiata* without causing excessive degradation to the subsequently extracted alginates, as long as the temperature and duration were tightly controlled at 42 °C and 159 minutes, respectively. This enhanced the recovery of the fucoidan-containing acidic extract (by reducing fluid retention by the algae), maximized sequential alginate yields, and reduced the viscosity of the crude alginate extract (without reducing its viscosifying capacity) by removing most of the polyvalent cations (thereby reducing the need for dilution prior to filtration, thus saving water.

When the optimized sequential extraction method was applied to other brown algae, some of the extracted products showed potential for commercial utilization. The *S. axillaris*-produced alginates were very rich in guluronic acid (G/M ratio of 1.83), as was predicted by the earlier screening of whole tissues. Meanwhile, the alginate extract from *M. pyrifera* had a high viscosity (599 mPa s for a 1 % solution) and very little color. Preliminary studies also indicated that the fucoidan-rich extract obtained from *E. radiata* stimulated the proliferation of human skin fibroblasts.

Finally, a comparative techno-economic analysis indicated that the production of fucoidan or seaweed-based fertilizer, either alone or together in a sequential process, would be economically viable in the rural south-east of South Australia, provided that a minimum of 140 dry metric tons of *M. pyrifera* (or slightly more for *E. radiata* or *D. potatorum*, given lower yields of fucoidan) could be obtained per year. Alginate production would not be feasible, unless a high processing scale (3830 dry metric tons of *M. pyrifera* per year) and premium price were achieved, which were considered unlikely, given the nature of the beach-cast seaweed resource. However, the feasibility of its production could be greatly improved if the sequential fucoidan and alginate production process were utilized, through improved production

efficiency resulting from the sharing of equipment, materials and processes. With the existence of more profitable alternatives, it is unlikely that such a project would be undertaken in South Australia.

7.2 Future directions

The studies detailed within this thesis have made important contributions to the state of knowledge within the field of brown algae processing and utilization. But, as is so often the case, a litany of new questions has also arisen, which will hopefully become the focus of exciting new research.

First and foremost, as was mentioned on numerous occasions throughout the thesis, consistent, long-term, and comprehensive surveying of the harvestable resource within the South Australian beach-cast seaweed fishery should be undertaken. The unknowns currently surrounding the magnitude, composition, and variability of the resource make it difficult to set informed and ecologically responsible limitations on harvesting activities, and leave the small seaweed processing industry based in Beachport highly sensitive to environmental and legislative changes. It is therefore within the best interests of commercial operators (Gather Australian Treasure Shareholding Co. Ltd) and the relevant government agencies (e.g. Primary Industries and Regions SA) to foster the sustainable commercialization of this unique resource through collaborative research.

Perhaps the biggest challenge encountered throughout the project was the low yield of fucoidan obtained using any of the extraction processes trialed. The issue was further complicated by the observed lability of the fucoidan structure during solvent extraction, even when using relatively mild conditions. Therefore, methods of disaggregating the cell wall complex while preserving the structural integrity of the fucoidans should continue to be pursued. Enzyme preparations that cleave structurally-important cell wall components offer one avenue. However, their access to minor components, such as celluloses, may be obstructed by the alginate-dominated mucilage (one hypothesis for the low activity observed during our enzymatic investigations). Furthermore, enzymes may perform poorly when highly specific environmental criteria are not met. For example, Leskinen et al. (2015) demonstrated that cellulase activity on cellulosic substrates was enhanced in the presence of sterols from wood. Therefore, enzymatic preparations specifically suited to the brown algae cell wall environment need to be developed.

It might also be worth further investigating whether any ionic liquids and deep eutectic solvents could aid in the dissolution of the brown algal cell wall, as they have with ligno-cellulosic materials. However, non-toxic, cheap and biodegradable solvents would be advisable, and even then, they might cause problems if they are difficult to remove completely.

Pulsed electric field (PEF) -assisted extraction has also shown high potential as a rapid and scalable method for the extraction of polysaccharides and other metabolites (Loginova et al., 2010; Yongguang et al., 2006; Zhao et al., 2011). This approach might also cause less polymer degradation than currently used alternatives, such as hot acid extraction and autohydrolysis. However, while PEF has been shown to puncture and lyse cells to extract intracellular metabolites, it is unclear whether it could disaggregate tightly associated cell wall components.

In any case, a deeper fundamental understanding of the nature of the brown algal cell wall complex, and the factors influencing the associative behavior of its various components, is required, in order to guide the rational improvement of extractive strategies. One tool that might assist with this would be the labelling and microscopic examination of the cell wall before and after different dissociative treatments, such as those suggested.

During the project, fucoidans were extracted from a number of different species using various extractive conditions. Given the processing focus, the comprehensive elucidation of their structure and activity was considered beyond the scope of the project. However, it would be very interesting to test the activity of the extracted fucoidans – particularly those from endemic and poorly studied species – for a range of potential applications. There would also be value in assessing how their biological activities were affected by the changes in structure that resulted from the different extraction conditions. The amount of evidence available in relation to the biological activity of specific fucoidans is an important determinant of their economic value, so these studies would have significant commercial benefits if the production of fucoidans is eventually pursued in South Australia.

With regard to the multiple response optimization carried out in Chapter 4, fucoidan and alginate were selected as the products of focus due to the sharing of similar processing steps (the acidic treatment), and because alginates are currently our industry partner’s main product. For alginate producers who wish to expand their

operations into fucoidan production as a secondary product, it would be important that the yield and quality of their alginates were not adversely affected, so the optimization approach reported here would have high relevance. However, the results from the techno-economic analysis in Chapter 6 suggest that prospective small- to medium-scale operators in Australia (and other regions with high costs of production) would benefit more from a similar study, which focused instead on the integrated production of fucoidan and liquid fertilizer. Furthermore, given the high relative economic importance of the fucoidan product stream, the fucoidan yield response should be prioritized using scaling factors, when utilizing desirability functions. The integrated process could then be trialed at pilot-scale, and the data obtained could be used to conduct a comprehensive techno-economic analysis, to gain a clearer understanding of its commercial prospects.

7.3 References

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