The viability of commercially relevant New Zealand seaweeds as a novel source of protein: The impact of processing on the extraction efficiency of protein

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Zoe Battershill

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Abstract

The world's population is increasing and with it so too is the demand for food production specifically protein which is essential for human nutrition. However, relying solely on animal-based proteins is increasingly problematic given growing global environmental impacts. Thus, novel protein sources are increasingly investigated, particularly plant-based alternatives due to their smaller carbon footprint. Seaweeds hold potential as a novel protein source due to their relatively high protein content and quality as evidenced by essential amino acid content. However, seaweeds' cell structure, with unique polysaccharides, complicate protein availability in terms of human digestion, necessitating procedures to disrupt the cell if seaweeds are to be a reliable protein source.

This research thesis sought to investigate which of six New Zealand seaweeds (five different species) had the highest raw potential as a protein source, and if this protein's extraction could be improved by food grade standard, industry relevant processing techniques.

Seaweeds assessed included: *Pyropia plicata* wild harvested from the Tauranga, Bay of Plenty (North Island) and Kaikōura, Canterbury (South Island); *Macrocystis pyrifera* and *Undaria pinnatifida* provided by commercial supplier (NZ Kelp); and *Ulva* sp. B and *Ulva ralfsii* harvested from land-based cultivation at Coastal Marine Field Station (CMFS), University of Waikato, Tauranga. All six were analysed for proximate composition (C, H, N, S, and ash), content of protein (as amino acids), total lipids, carbohydrates, fibre, minerals, and heavy metals. Antioxidant capacity was evaluated as new research shows this can influence protein bioavailability. From these results *Ulva* sp. B and *Ulva ralfsii* were chosen as the potential protein sources with their protein content 151 mg/ g and 138 mg/ g, respectively.

The effects of two alternative processing methods, which were applicable to industry for human nutrition, were then applied to these two species to assess their impact on the

extractability of protein. The two chosen processing treatments were enzyme hydrolysis using a commercial mixture of enzymes (Viscozyme®) to target the chemical degradation of cell wall polysaccharides, and homogenisation to target the mechanical degradation of the cell wall structure. These were used alone and combined in a factorial experimental design and found that the combination of both processing methods yielded the greatest extraction efficiency 55.2% and 63.7% extraction efficiency for *U. sp*. B and *U. ralfsii*, respectively.

While this study was limited in scope, it demonstrated the potential for cultivated seaweeds in New Zealand to be successfully processed to enhance their available protein and serve as a novel protein source for human nutrition.

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Additionally, I wish to thank all those who contributed vital components to this research. Many thanks to our commercial supplier of Phaeophycaea, NZ Kelp; David Schiel and his team for the Kaikoura *Pyropia plicata*; and the EU team, in particular Peter Ranrup, for the *Ulva* spp. From collection and provision to follow-up emails regarding information vital to the methods, you were integral in this putting this research together. A special thank you to Ariane Bradenburg for her support as laboratory technician at the Field Station; from laboratory procedures to sample shipment for outsourced analyses, her assistance was invaluable especially with Covid-19 interruptions. I also very much appreciate Rebecca Lawton for her assistance with the genetic identification of the *Pyropia plicata*, without her input in this process it would not have been possible.

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1. Chapter 1: Literature Review

Protein fuels the human body's everyday function, hastens muscle recovery (Phillips & Van Loon, 2011), reduces bone loss (particularly in older adults) (Groenendijk et al., 2019), and supports function during acute and chronic illnesses (Biolo, 2013; Hoffer, 2007). Being such a critical component of nutrition, consumption of protein worldwide rose roughly 30% between 1961 and 2011; however, the plant protein proportion fell 8% while animal-based protein surged (Sans & Combris, 2015). This increase of animal-based protein consumption is expected to continue at 1.6% per annum for the next 10 years (Sans & Combris, 2015). The agriculture industry produces the majority of this protein, contributing to major environmental impacts due to high use of land surface and fresh water (Reguant-Closa et al., 2020) (Figure 1.1). However, those are not the only global impacts; energy, transportation, processing, by-products, biodiversity loss, habitat degradation, nutrient release and contributions to greenhouse gas (GHG) emissions are also significant concerns (Hilborn et al., 2018).

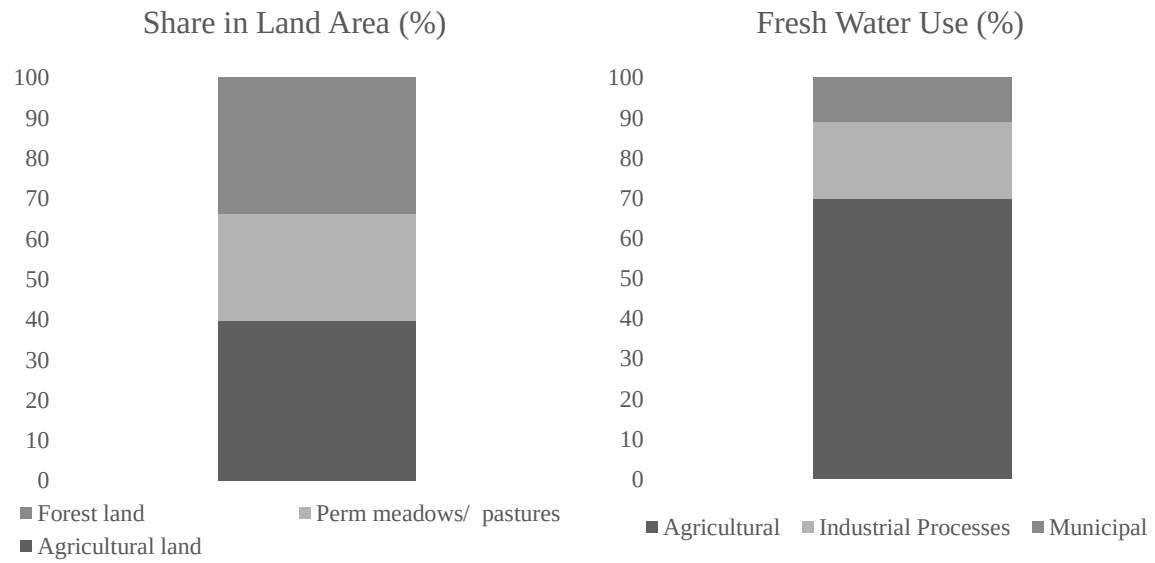

Figure 1.1 Agricultural impact on land (Food and Agriculture Organization of the United Nations [FAO], 2021) and water use (FAO, 2011)

The Intergovernmental Panel on Climate Change (IPCC) estimate that 24% of total emissions worldwide are from the "Agriculture, Forestry and Other Land Use Sector", with agriculture having the predominant output; Food and Agriculture Organization of the United Nations (FAO) supports this with their estimate that the livestock system comprises 14.5% of worldwide GHG emissions (Webster & D'Silva, 2017). Greenhouse gas emissions vary greatly between livestock varieties used for meat; for example, ruminant meat has a greater impact than any other (beef 8-fold higher emission intensity than poultry per gram protein) (FAO, 2021; Swain et al., 2018). Animal products such as eggs and dairy in turn have a smaller footprint than the same animal's meat; and plant-based protein's GHG emissions are even smaller again (Sabate, 2019) (Figure 1.2).

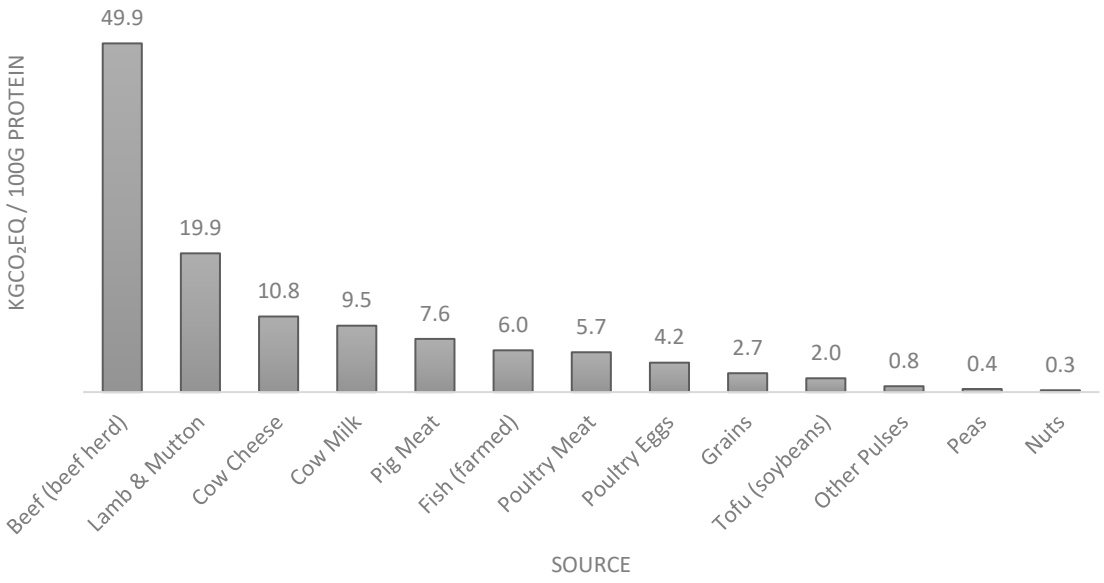

Figure 1.2 2010 Greenhouse gas Emission Intensity per 100g Protein. Adapted from Poore & Nemecek, (2018). $KgCO_2eq$ is a measure of emissions inclusive of other greenhouse gases, calculated to equate to the amount of CO_2 that would have the same global warming potential over time.

While plant-based protein sources have a lower GHG footprint compared to animal-based, their protein quality is lower as they generally do not contain all essential amino acids (EAAs) (Hoffman & Falvo, 2004; Lopez & Mohiuddin, 2021). Essential amino acids are amino acids (AA) that cannot be produced freely by the human body and are required as part of the diet of humans (Hoffer, 2016). Furthermore, low digestibility of plant-based protein is a challenge when seeking a source for sustainable nutrition (Drewnowski et al., 2015;

Millward, 1999; Rogerson, 2017; Wu et al., 2014). For these reasons, the identification of a nutritionally and environmentally viable alternative source of protein is critical. The ideal protein source is a high quality locally produced (to minimise long distance transportation) source of protein that requires minimal processing with little to no waste. Seaweeds are a viable option as they can have a high content of protein, can be subjected to cascading usage leading to low waste production and are cultivated in saltwater. Brown seaweed (Ochrophyta, Phaeophycaea, (Ruggiero et al., 2015)), green seaweed (Chlorophyta), and red seaweed (Rhodophyta) have all been found to have species containing all EAAs (i.e., complete protein) (Fujiwara-Arasaki et al., 1984; Kazir et al., 2019; Ortiz et al., 2009; Wong & Cheung, 2000).

1.1 Nutritional Requirements of Protein and Amino Acids Quality Implication

The protein requirement for humans can be denoted as the minimal amount of dietary protein required to be able to maintain energy balance and body cell mass (Hoffer, 2016; Li et al., 2014; Millward, 1999). However, this is more accurately described as the minimum protein requirement and is not necessarily the optimum protein requirement – particularly for active individuals who require significantly more protein than the minimum required for basic function (Hoffer, 2016; Phillips & Van Loon, 2011). Variability between individuals can also heavily influence the amount of protein required. For sedentary individuals 0.75 – 0.8g of protein/ kg of bodyweight / day is the recommended amount, which is approximately half of that recommended for active individuals (1.2 – 2 g/ kg of bodyweight / day) (active defined as those involved in strength or resistance training) (Phillips & Van Loon, 2011; Poortmans et al., 2012). For athletes in particular, protein, whether supplemented or via whole foods, is used as a muscle recovery supplement to increase muscle mass and performance (Morton et al., 2018; Phillips & Van Loon, 2011; Tagawa et al., 2021). The quality of protein is imperative (i.e., content and proportions of AAs) to facilitate protein synthesis in the human body (Biolo, 2013). There are 20 proteinogenic AAs in the human

body (Figure 1.3) (Lopez & Mohiuddin, 2021), 11 of which are the EAAs that cannot be produced by the body and therefore must be consumed. Within this group there are branched-chain amino acids (BCAAs; Leucine, Isoleucine and Valine) which are taken up directly by the striated muscles before being partially oxidised (Poortmans et al., 2012; Shimomura et al., 2004). BCAAs stimulate muscle protein synthesis (Jackman et al., 2017) through inclusion of EAAs, and are required for maximal protein synthesis (Jackman et al., 2017; Wolfe, 2017).

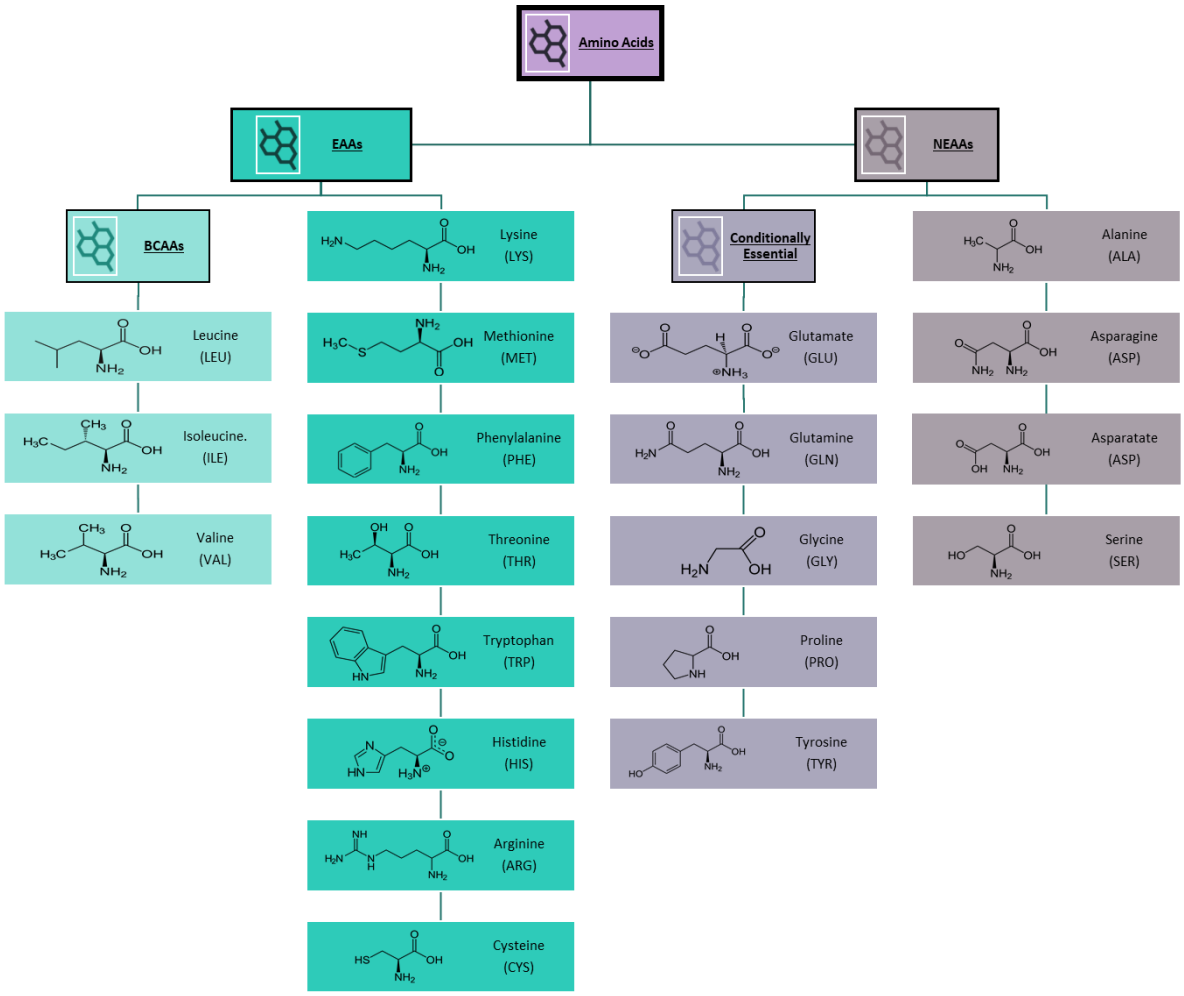

Figure 1.3 AA categories created with category information from Gruca & Dufour, (2018) and chemical structure from (Soult, 2016).

If energy expenditure increases, such as with exercise or when the body is fighting disease, the oxidation of BCAAs increases and an increased intake is required to maintain homeostasis (Shimomura et al., 2004). For this reason, BCAAs are an important consideration in protein sources, as well as the overall protein quality determined by the limiting AA (i.e., the EAA with the lowest quantity or lowest ratio of human nutritional

requirement). Importantly protein quality also depends on the relative levels of EAAs as deficiencies in some (e.g., Leucine) can limit the body's capacity to efficiently utilise other EAAs (Lopez & Mohiuddin, 2021). Thus, a measure of protein quality is essential to assess the nutritional value of different protein sources for human consumption.

1.2 Models for Assessing Protein Quality

Two models are available for assessing the nutritional quality of different protein sources, the Protein Digestibility Corrected Amino Acid Score (PDCAAS) and Digestible Indispensable Amino Acid Score (DIAAS). PDCAAS was the preferred method of quantifying protein quality for human consumption (Hoffman & Falvo, 2004), until recently when FAO changed their recommended measure to the Digestible Indispensable Amino Acid Score (DIAAS) (Figure 1.4). DIAAS more accurately estimates the bioavailability of the protein being digested as it assesses the individual digestibility of EAAs at the end of the small intestine; whereas PDCAAS estimates AAs from the total digestion tract with assumption that all have the same digestibility as crude protein (which is not the case) (Mathai et al., 2017). This differentiation is an essential for evaluating plant-based protein sources with high contents of indigestible fibre known to impede the bioavailability of protein.

$$DIAAS\ \% = 100 \text{ x lowest value } [\frac{(\text{mg of digestible dietary indispensable amino acid in 1g of dietary protein})}{(\text{mg of the same dietary indispensable amino acid in 1g of the reference protein})}]$$

Figure 1.4 DIAAS equation

The reference protein in this equation is the specific "indispensable AA" requirement for the appropriate age group based on FAO scoring; as seen in Table 1.1. "In calculating DIAAS the ratio should be calculated for each dietary indispensable AA and the lowest value designated as the DIAAS" (FAO, 2013). It should be noted that a high DIAAS does not always indicate a good source of protein for human nutrition as it may still have a low quantity of protein per 100g of product – as demonstrated by potatoes in Table 1.2.

Table 1.1 Indispensable AAs Reference for adults (Food and Agriculture Organization of the United Nations, 2013)

Scoring pattern requirement for adults' dietary indispensable AAs (mg/ g protein)								
His	Iso	Leu	Lys	Sulfur AAs	Aromatic AAs	Thr	Try	Val
16	30	61	48	23	41	25	6.6	40

His: Histidine; Iso: Isoleucine; Leu: Leucine; Lys: Lysine; Sulfur AAs: Methionine + Cysteine; Aromatic AAs: Tyrosine + Phenylalanine + Tryptophan; Thr: Threonine; Try: Tryptophan; Val: Valine.

Table 1.2 Calculated Digestible Indispensable Amino Acid Score (DIAAS) vs Protein % of various sources

Protein Sources		DIAAS	% (g of Protein in 100 g serving)
Animal Sourced (Raw)	Whey	85[1]	(Powder) 13 (Concentrate) 57 (Isolate) 90[2]
	Chicken Egg	100[1]	13[3]
	Chicken (Average of breast and thigh)		20[3]
	Tuna		24[3]
	Salmon		20[3]
	Halibut		19[3]
	Beef (Average of beef cuts)		21[3]
	Pork (Average of loin & ham)		19[3]
Tubers	Potato	100[1]	2[4]
Pulse & legumes	Soy	91[1]	(Flour) 50 (Concentrate) 70 (Isolate) 90[2]
	Peas	70[1]	5[3]
Cereal	Rice	47[1]	8[4]
	Wheat	48[1]	12[4]
	Oats	57[1]	13[4]

[1] adapted from Herreman et al., (2020); [2] adapted from Hoffman & Falvo (2004); [3] adapted from Bohrer (2017);[4] adapted from Hayes (2018)

The quality and quantity of protein is influenced by its bioavailability in a particular food substance. Bioavailability consists of two components of the body's absorption processes of that substance: bioaccessibility and bioactivity. 'Bioaccessibility' is the quantity of the food, or in this case the protein, that is accessible for absorption in the intestine. 'Bioactivity' is the biological activity this component has with regards to a physiological response, such as protein synthesis (Shannon et al., 2021). The DIAAS is used in this context to estimate the degree of bioactivity, as the limiting AA will in turn limit the physiological response. To assess the bioaccessibility of the proteins from novel food sources (e.g., seaweed), *in vitro* or simulated digestion can be employed to simulate the level of protein available for absorption in the intestine (Shannon et al., 2021).

1.2 Seaweed as protein

Consumption of seaweed is not a new practice and is a part of traditional diets in many countries (Cian et al., 2014; J. Fleurence et al., 2018; Fleurence, 1999a; Kazir et al., 2019; Nelson & Conroy, 1989; O'Connell-Milne & Hepburn, 2015), including in New Zealand where Māori consider some seaweed species taonga or treasure (O'Connell-Milne & Hepburn, 2015). More recent however, is the evaluation of the nutrient content of different species of macroalgae in a broader international context with the search for sustainably produced nutrition sources (Fleurence, 1999a; Galland-Irmouli et al., 1999; Hou et al., 2015). Phaeophycaea, Chlorophyta, and Rhodophyta, respectively were previously thought to have an ascending amount of protein, with Phaeophycaea thought to have typically less than 15% (of dry weight) (Fleurence, 1999a). However, generalisations across phyla are not appropriate, as protein content also varies broadly with species within phyla (Angell et al. 2016), lifecycle stage, and season/ cultivation conditions (Fleurence, 1999a; Galland-Irmouli et al., 1999; Hou et al., 2015). Furthermore, species and cultivation conditions (environment) also influence the levels of contaminants, such as toxic heavy metals and metalloids that influence the suitability of seaweed for human consumption (Kalia & Khambholja, 2015).

Therefore, careful consideration of species selection, cultivation conditions, and post-harvest processing can lead to considerable improvements in seaweed protein content and quality.

The bioavailability of protein from seaweeds is limited due to their typically high fibre content (Fleurence et al., 2018), which bind and lock up the protein preventing absorption in the human gut (Pliego-Cortés et al., 2020). Both chemical and mechanical techniques have been recommended as part of processing to break down the cell wall and increase protein bioavailability (Amano & Noda, 1990; Mæhre et al., 2016). Viscozyme® is a food grade mixture of carbohydrases (arabanase, cellulase, hemicellulase and xylanase) that are able to hydrolyse polysaccharides present in plant cell walls (eg xylanes and cellulose), and thereby release intracellular components such as protein (Rosset et al., 2014). Homogenisation is also suggested as a mechanical option to break down tertiary structures of the cell wall and increase protein availability in seaweeds (Barbarino & Lourenço, 2005). However, for seaweed protein to be a viable option for NZ, the selection of a high-potential seaweed species and the interaction of scalable processing techniques is needed.

1.3 Thesis Outline

This thesis consists of two research chapters that aimed to evaluate the selected seaweeds grown in New Zealand for their suitability as a protein source for human nutrition. Chapter 2 investigated the nutritional profile of six seaweeds – two cultivated Chlorophyta, two wild harvested Rhodophyta and two commercially harvested Phaeophycaea. The nutritional profile from Chapter 2 was used to select two species based on their content of protein and suitability for human consumption for further investigation in Chapter 3. Chapter 3 investigated the effect of homogenisation and enzymatic hydrolysis processing treatments on the extractability of protein from these two species. Through this research, this thesis aims to answer the question is seaweed available in New Zealand is a viable novel protein for human nutrition.

2. Chapter 2: Nutritional Profile of Six Seaweeds Present in New Zealand

2.1 Introduction

Quantitative analytical investigation into seaweeds for human nutrition began in the 1960s (Iwasaki, 1967) and has since demonstrated that the nutritional value varies amongst species, with effects associated with lifecycle stage, location, season and whether cultivated or wild harvested (Angell et al., 2016; Fleurence, 1999a; Galland-Irmouli et al., 1999; Hou et al., 2015). Sweeping statements concerning differences in protein content between the phyla are misleading (Angell et al., 2016). From a meta-analysis conducted by Angell et al., (2015) across 236 published articles, the difference among phyla's median protein content (% d/w) was minimal; Rhodophyta 9.83% (±8.33); Chlorophyta 9.97% (±8.56); Phaeophycaea (Phaeophycaea) 9.20% (±4.87) (Angell et al., 2015).

All phyla of seaweed typically meet the requirement for a 'complete protein' source as they contain all the essential amino acids (EAA) (Fleurence et al., 2018; Mæhre, 2016; O' Connor et al., 2020). This is part of the reason they have the potential to be more nutritionally valuable than most terrestrial plant-based proteins (Bleakley & Hayes, 2017). It is worth noting, however, that different macroalgae have inherent differences regarding the identity of which AA is most commonly the limiting AA (O' Connor et al., 2020). Differences in compositional components relevant to human nutrition other than amino acids have also been identified across algal species; e.g., *Ecklonia radiata* have prebiotic potential for gut health (Charoensiddhi et al., 2016); Rhodophyta extracts of R-phycoerythrin as a potential food colourant (Fleurence et al., 2012); and many species contain high mineral and iodine content (Fleurence et al., 2018; Mæhre et al., 2014).

The current chapter investigates the nutritional value and suitability for human consumption of seaweeds of commercial interest in New Zealand, and includes species from Rhodophyta

(*Pyropia plicata n.sp.*, Bangiales Nelson, 2017,); Phaeophycaea (*Macrocystis pyrifera*, Laminariales (Linnaeus) C.Agardh, 1820 and *Undaria pinnatifida*, Laminariales (Harvey) Suringar, 1873); and Chlorophyta (*Ulva* sp. *B* and *Ulva ralfsii*, Ulvales, (Harvey) Le Jolis, 1863*)* (World Register of Marine Species (WoRMS), 2022). *Macrocystis pyrifera* is distributed predominantly along the east coast of New Zealand, in southern and central open coastal waters (Brown et al., 1997) and is harvested (White & White, 2020) under the Quota Management System (Ministry for Primary Industries [MPI], 2022.). It is currently sold for agricultural and horticultural use as "Zelp", as well as human nutrition as "Valére Kelp" (NZ Kelp, 2022). *Undaria pinnatifida* is an introduced species that is widespread in New Zealand, predominantly found along the east coast from as far north as the Hauraki Gulf/ Tīkapa Moana to Stuart Island/ Rakiura (White & White, 2020). This versatile seaweed, initially the target of eradication, is one of the 'big five' aquaculture species globally, and currently harvested as by-catch in New Zealand (Nadeeshani et al., 2021; Smith et al., 2010; White & White, 2020), for the production of nutraceuticals (Nadeeshani et al., 2021) and biostimulants (Salcedo et al., 2020). There are 24 genetically distinct species of *Ulva* found in New Zealand (Heesch et al., 2007). Some species are known for creating large algal blooms, or "green tides", such as in Tauranga Harbour in the North Island (White & White, 2020). Despite its somewhat negative connotation as a fouling agent and bloom forming organism, species of the genus *Ulva* are a valuable commercial resource for food (Ohno, 2006; Ohno & Critchley, 1993), chemical products, and for use in bioremediation technologies (Bolton et al., 2008; Bolton et al., 2016; Glasson et al., 2017, 2019; Kidgell et al., 2019; Lamare & Wing, 2001; Lawton et al., 2021). *Ulva* species have also been included in traditional Māori diets, often eaten whole (Smith et al., 2010; White & White, 2020). Karengo, a taonga to Māori, was once thought to only reference *Porphyra* spp., is now understood to reference many edible algae including *Porphyra*, *Pyropia* and *Clymene* genera (O'Connell-Milne & Hepburn, 2015). *Porphyra* is the foundation of the prominent nori industry in Japan and China, and

together with the republic of Korea being the largest consumers of seaweed for human nutrition (Bocanegra et al., 2009). The genus *Porphyra* has also been updated to better define genetic differences between species, so that many of these commercially relevant species are now classified under the genus *Pyropia* (Meynard et al., 2019; Nelson, 2013; Ramirez et al., 2014; Sutherland, 2011). One species of *Pyropia* found in North and South Islands is *P. plicata*, which is endemic and abundant in New Zealand (Diehl et al., 2019; Nelson, 2013).

This chapter investigates the nutritional profile of six New Zealand seaweeds in terms of content of protein (as amino acids), total lipids, carbohydrates, fibre, and mineral profiles. Antioxidant capacity, and heavy metals are also considered as these components influence the nutritional value and safe dietary inclusion levels (Fleurence et al., 2018; Mæhre, 2016). The results from this chapter inform the selection of two species for inclusion in Chapter 3 where processing methods to improve the protein extraction will be investigated.

2.2 Method

2.2.1 Sample Collection and Preparation

Six seaweeds (five different species) were analysed in this study, with each having three biological replicates of ≥60g per sample. Samples were either collected from natural populations, cultivated stock cultures, or sourced from commercial suppliers depending on availability. Karengo species (*Ulva* and *Pyropia*) are noncommercially harvestable under the 1998 Ngāi Tahu Settlement Act unless under a permit which is often only given for scientific purposes in collaboration with relevant Iwi (O'Connell-Milne & Hepburn, 2015). *Macrocystis pyrifera* and *U. pinnatifida* were provided by commercial suppliers. Both species of *Ulva* were cultivated from stock sourced in Tauranga, Bay of Plenty, with original brood stock collected under Ministry of Primary Industries – Special Permit Number (MPI-SP#) 560, following consultation with Tauranga Moana mana whenua (Ngāi Te Rangi, Ngāti Ranginui, and Ngāti Pūkenga). *Pyropia plicata* (T) were collected wild by hand from Mt Maunganui, Tauranga, Bay of Plenty, New Zealand (37º38'1"S, 176^{0}10'1"E) in November

2020 under MPI-SP#742. *Pyropia plicata (*K) was collected at the same time from Kaikōura, Canterbury, New Zealand by Professor David Schiel and his team from University of Canterbury (MPI Special Permit # 728 (2020-2025), following consultation with Ngāi Tahu. *U*lva sp B (GenBank accession number MW250819.1) and *U. ralfsii* (GenBank accession number MW250805.1) were harvested from mixed gametophyte/sporophyte stock cultures cultivated at the Coastal Marine Field Station (CMFS), University of Waikato, Tauranga. These were cultivated indoors under artificial lighting (2 x 4 Seasons LUMA 800 LED Grow Lights at 600mm height with >600 PAR at the surface) at 12/12h light/dark cycle in round tanks (2 x 550 L; 900 diameter x 950mm height) filled to a depth of 0.8m with filtered seawater (5µm, UV processed) collected from Tauranga harbour and adjusted to 35 ppt with tap water as needed to correct for evaporation, and with 50% weekly water change for all tanks. The temperature was controlled at 18^{o}C with a heater / chiller (300W Eheim Thermocontrol Heater / Hailea 1HP Chiller). The tanks were stocked at 0.8 g/ L fresh weight algal biomass and dosed with F/2 nutrients (Cell-Hi F2P, Varicon Aqua Solutions UK, 0.1g L^{-1}, 12.3 mg nitrate-N L^{-1} and 1.1 mg P L^{-1} purchased from Fresh by Design Ltd) as per the manufacturer's directions. Harvesting occurred weekly through October 2020 for both *Ulva* spp.

*Pyropia plicata (*T) was collected by hand at low tide when seaweeds were exposed on rock surfaces. Three separate collections were made all during November 2020. *Pyropia plicata (*K) was also collected in this manner with three separate collections made during November and December 2020.

Three individual *U. pinnatifida* specimens were harvested in December and provided in fully intact as three individual replicates. *M. pyrifera* was provided as three separate samples in dried, milled form (to approximately 1mm), and time of harvest is unknown. All other biomass was frozen fresh (-20˚C) and freeze-dried (BUCHI LyovaporTM L-200 Freeze Dryer) prior to milling (FRITCH cutting mill PULVERISETTE 15 with a 0.5mm sieve). All

seaweed powder biomass was sealed in plastic bags at room temperature with silica gel sachets *in situ* to prevent absorption of moisture.

2.2.2 Species Identification

Macrocystis pyrifera and *U. pinnatifida* were identified based on morphological characteristics (by the supplier for *Macrocystis*). *Ulva* were identified as per Lawton et al., (2021) and have been maintained in culture since the original collection. Due to morphological ambiguity in the genus (Abe et al., 2013; Meynard et al., 2019; Nelson, 2013), *Pyropia* samples were analysed via genetic barcoding. DNA was extracted from 50 mg dried tissue of each sample using the using the Chelex method of Goff & Moon (1993). Approximately 1300bp of the rbcL locus was amplified using the primers F57/RbcS-S (Bartolo et al., 2020). All samples were successfully amplified and were sequenced in both directions by Macrogen Inc. Resulting DNA sequences were trimmed and assembled using Geneious Prime 2021.1.1., and the consensus sequences were compared with sequences in GenBank using BLAST https://blast.ncbi.nlm.nih.gov/). Samples collected from the same location had identical DNA sequences, and sequences only differed between sites by 2 bp. DNA sequences were submitted to Genbank under the following accession numbers: *P. plicata* (T) samples – OK073982, and *P. plicata* (K) samples – OK073983.

2.2.3 Compositional Analyses

Elemental analysis of C, H, N, S, iodine, and ash content was outsourced to OEA labs (http://www.oealabs.com, Callington, UK), where samples were combusted in pure oxygen before being separated and quantified using as chromatography-thermal conductivity detection (GC-TCD).

C, H, N, S, and ash were expressed as a percentage of the total mass of biomass (n= 3). Samples were analysed for total lipid content with a modified protocol (Folch et al., 1957) as described in (Gosch et al., 2012). Briefly, 5 mL of DCM:MeOH solution (2 : 1, v/v; both HPLC-grade) was added to dried milled biomass (approximately 200 mg ± 1 mg) in a 7 mL

Teflon capped glass vial (Supelco 27161) followed by heating at 60 °C in a block heater for 1 hr. The extract was allowed to cool and then vacuum filtered through a Whatman glass microfiber filter [GF/C 47mm diameter; WHA3822-047] to remove particulate matter. A further 5 mL of DCM:MeOH was used to rinse the filter. The total filtered volume was then measured and transferred into a 15 mL centrifuge tube followed by the addition of 0.2 volumes of NaCl-solution (0.9% w/v). The sample was then mixed and the organic phase transferred to a pre-weighed 7mL glass vial using a Pasteur pipette. The solvent was then evaporated under a gentle stream of nitrogen and the total lipid content determined as % of dry weight.

Total carbohydrates were calculated by difference using the equation (Ortiz et al., 2009):

Carbohydrates = 100 - Protein % - Lipids % - Ash %

2.2.4 Fibre Analysis

Total dietary fibre analysis was performed using Megazyme Total Dietary Fibre Assay Kit (K-TDFR-100A), as per the manufacturer's protocol for Method 1 except centrifugation was used for separation of solid and liquid phases. Solids were washed as per the manufacturers protocol and dried at 50^{o}C to remove excess acetone prior to freeze-drying. The solids were milled using a mortar and pestle prior analysis of N and ash by OEA laboratories Limited. A nitrogen (N) to protein conversion factor of 5 was used to calculate the protein content of the solid. The total fibre contents, including the soluble and insoluble contents, were corrected for the content of protein and ash as per equation:

$$\text{Fibre (\%)} = \frac{Residue-p-ash}{Sample}\ x\ 100$$

2.2.5 Antioxidant Analysis

Oxygen Radical Absorbance Capacity (ORAC) was chosen as the measure of antioxidant capacity most relevant to human physiology due to its hydrogen atom transfer reaction (Prior et al., 2005). The ORAC assay used was based on methods by Huang et al., (2002) and sample preparation methods by Wu et al., (2004) without use of methylated β-cyclodextrin as

a solubility enhancer. Butylated hydroxytoluene (BHT) (Supelco PHR1117; pharmaceutical secondary standard) was used as a positive control (Kindleysides et al., 2012) to allow comparison between studies. The protocol targets both lipophilic and hydrophilic antioxidants using a two-step extraction process. Briefly, 5 mL of hexane/dichloromethane (1:1, Hex:DCM) was added to an accurately weighed biomass sample (500 mg ± 1 mg) in a 7 mL Teflon capped glass vial (Supelco 27161) with a magnetic stirrer bar and heated at 30ºC for 2 hours. The liquid and solid states were separated by centrifugation (2561 rcf for 10 minutes) and the supernatant dried under a stream of N_2 gas to afford the lipophilic extract. This was then reconstituted in 1 mL of acetone and further diluted 100 μL in 900 μL of phosphate buffer (75mM, pH7.4) for fluorometric quantification of lipophilic ORAC (BMG Labtech Omega series spectrophotometer). The residual biomass (the pellet) was extracted a second time using 1 mL of acetone/water/acetic acid (70:29.5:0.5, A: W: A) in a at 30ºC stirring water bath for 2 hours followed by centrifugation (2561 rcf for 10 minutes). The supernatant was transferred to a clean 1.5 mL microcentrifuge tube (EP022363212) and further centrifuged as above. The supernatant was then further diluted by adding 100μL of supernatant into 900μL of phosphate buffer (75mM, pH 7.4) before fluorometric quantification of the hydrophilic ORAC (BMG Labtech Omegaseries spectrophotometer). Fluorescence measurements (Ex. 485 nm, Em. 520 nm) for both lipophilic and hydrophilic extracts were measured every 90 seconds for 3 cycles before AAPH was added manually by pipette. Samples were then remeasured at 90 second intervals over 80 minutes (where drop off and plateau were seen).

2.2.6 Metals, Metalloids and Minerals Analysis

The metals and metalloids (25 elements) were analysed by Inductively Coupled Plasma – Mass Spectrometer (ICP-MS) at the University of Waikato Laboratory (The University of Waikato, Te Whare Wananga o Waikato, Gate 1, Knighton Road, Hamilton, 3240, New Zealand) using an Agilent 8900 ICP-MS (Agilent Technologies, Santa Clara, California,

USA) controlled by MassHunter Workstation (version 4.5). Samples (200 mg) were digested with 0.65 mL of double-distilled 65% HNO_3 and 0.6 mL of 30% H_2O_2 before analysis. Samples were then introduced via an SPS4 autosampler (Agilent Technologies, Santa Clara, California, USA) and PVC tubing (Pulse Instrumentation, Mequon, Wisconsin, USA); a 0.05-0.1 mL/min micromist U-Series nebuliser (Glass Expansion, Melbourne, Victoria, Australia) was attached to a quartz Scott Type spray chamber followed a quartz torch with 2.5mm injector (Agilent Technologies, Santa Clara, California, USA). Following this, samples were introduced to the rest of the instrument via a nickel sampler and skimmer cone, followed by an extraction omega lens (Agilent Technologies, Santa Clara, California, USA). The ICP-MS was run in pulse counting mode (with a dwell time of 0.1 s- 0.3 s), with radio frequency (RF) power set to 1550 W and gas flow rates at 15L/min (plasma gas), 1.05L/ min (nebuliser carrier gas), 0.1L/ min (dilution gas). A five-point calibration curve, consisting of concentrations between 0.1 and 500ppb was prepared for all trace elements using stock standard IV71-A (Inorganic Ventures, Christiansburg, VA, USA). A separate calibration curve, consisting of concentrations between 100 and 10,000ppb was prepared for major elements (Ca, Si, P, S, K, Fe) using single-element standards (Inorganic Ventures, Christiansburg, VA, USA). Check standards were analysed every 20 samples and re-calibration was performed every 100 samples. Blank samples were analysed every 10 samples to ensure minimal carryover between samples. An online internal standard containing ^{45}Sc, ^{72}Ge, ^{103}Rh, ^{193}Ir, and ^{205}Tl was used to monitor and correct for instrumental drift and matrix effects.

2.2.7 Amino Acid Analysis

Amino acids were analysed at the Australian Proteome Analysis Facility, Macquarie University, Sydney (Kulshreshtha et al., 2014) using a standard amino acid assay procedure, accredited under ISO 17025 accreditation (Accreditation number 20344). Briefly, biomass was hydrolysed in 6M HCl for 24 hours at 110˚C using Waters AccQTag Ultra chemistry and

quantified on a Waters Acquity Ultra-Performance Liquid Chromatography (UPLC) (Waters Corporation. Milford, Massachusetts). This procedure identifies the proteinogenic amino acids, though it converts glutamine and asparagine to their acid form and breaks down cysteine and tryptophan (Macquarie University, 2021). Cysteine is typically a minor component of the amino acids in seaweeds (0.36% of amino acids) (Kulshreshtha et al., 2014) and though tryptophan is an EAA it is often the least abundant amino acids (Macquarie University, 2021). This combined with their difficulty in quantification means the two are commonly excluded from amino acid analyses (Angell et al., 2016). The protein content was calculated as the sum of individual amino acids to give Total Amino Acids (TAA) and used with nitrogen percentage for calculation of species-specific Nitrogen-to-Protein conversion factor as per equation below.

TAA (species x) / %N (species x) = Species x specific Nitrogen-to-Protein conversion factor

2.2.8 Data Analyses

Difference among the species were analysed using a one factor permutational analyses of variance (PERMANOVA) conducted in Primer v7 (Primer-E Ltd., UK) using Euclidean distances resemblance matrices, 9,999 unrestricted permutations of raw data, Type III sum of squares, and inclusion of Monte Carlo P value. Post-hoc PERMANOVA tests (pairwise) were carried out for all analyses which were significant according to the PERMANOVA test. Raw, unadjusted p(MC) values are reported for all analyses. The patterns of mineral content from ICP analysis and Amino acid were visualised using non-metric multidimensional scaling (nMDS; Primer 7).

2.3 Results

2.3.1 Compositional Analyses

Iodine ranged from non-detectable in *U. ralfsii* to the highest levels in the species *M. pyrifera* 212.46 ± 39.70 μg/g (Table 2.1); both Phaeophycaea species were significantly higher than all other species with *M. pyrifera* also being significantly higher than *U. pinnatifida* (ANOVA, $F_{5,12} = 79.1$, p(MC) < 0.0001). Content of ash also varied significantly between species (ANOVA, $F_{5,12} = 23.7$, p(MC) < 0.0001) with *Ulva* sp. B and *P. plicata* (K) having the lowest ash content and the remaining species all above 30%.

Table 2.1 Content (% of dry weight) of carbon (C), hydrogen (H), nitrogen (N), sulfur (S), iodine, and ash in selected New Zealand seaweed (means ± S.D, n=3). Common letters within columns represent non-significant groupings (pairwise PERMANOVA, p(MC) < 0.05).

	C (%)	H (%)	N (%)	S (%)	Iodine (μg/g)	Ash (%)
U. sp. B	33.6 ± 0.8 [a]	5.9 ± 0.1 [a]	3.4 ± 0.0 [a]	4.5 ± 0.1	3.41 ± 3.12 [a]	18.7 ± 1.1 [a]
U. ralfsii	23.6 ± 0.7 [b]	4.4 ± 0.1 [bcd]	3.5 ± 0.1 [a]	3.2 ± 0.1	ND ± 0 [a]	37.9 ± 1.6 [b]
M. pyrifera	25.4 ± 1.5 [b]	4.0 ± 0.3 [b]	1.4 ± 0.3 [bc]	0.9 ± 0.1	212.46 ± 39.70	34.4 ± 5.5 [bc]
U. pinnatifida	29.4 ± 1.5	4.7 ± 0.3 [d]	1.5 ± 0.1 [b]	1.4 ± 0.2[a]	17.41 ± 5.57	31.5 ± 1.4 [c]
P. plicata (K)	33.6 ± 1.4 [a]	5.7 ± 0.2 [a]	1.9 ± 0.2 [cd]	2.2 ± 0.1	1.45 ± 2.51 [a]	21.9 ± 2.6 [a]
P. plicata (T)	25.5 ± 1.0 [b]	4.3 ± 0.1 [bd]	1.7 ± 0.2 [bd]	1.7 ± 0.1[a]	2.46 ± 4.27 [a]	38.3 ± 3.1 [b]

Protein as by sum of TAA ranged from 6.4 ± 1.3% (*M. pyrifera*) to 15.1 ± 1.1% (*U. sp. B*) (Table 2.2), with both *Ulva* species being significantly different from all species except each other (ANOVA, $F_{5,12} = 40.9$, p(MC) < 0.0001). All species had relatively low lipid levels, ranging from 0.5 ± 0.1 % (*P. plicata* (T)) to 5.3 ± 0.5 % (*U. ralfsii*). *Ulva ralfsii*, with the highest lipid levels was significantly different from all species except *U. pinnatifida* (ANOVA, $F_{5,12} = 54.8$, p(MC) < 0.0001). Total carbohydrate content was above 50% in all seaweed except *U. ralfsii* (35.1 ± 1.1%) which was significantly lower than all other species (ANOVA, $F_{5,12} = 27.3$, p(MC) < 0.0001). Insoluble fibre was highest in *P. plicata* (K) 42.6 ± 20.6% and lowest in *U. ralfsii* ranging from 22.1 ± 2.8%, however there was no statistical difference found between groups (ANOVA, $F_{5,12} = 1.0$, p(MC) 0.4583). There was statistical

difference found between soluble fibre results (ANOVA, $F_{5,8}$ =7.7, p(MC) 0.007), however this data must be reviewed with care due to the low amount of soluble fibre from the *P. plicata* samples, replicates had to be combined to get a result. It is possible that the method used was not applicable to Rhodophyta. Similarly, results for total fibre must also be viewed with care when reviewing the statistical differences between groups (ANOVA, $F_{5,8}$ =4.6, p(MC) 0.028).

Table 2.2 Proximate composition (% of dry weight) of total lipids, carbohydrates, fibre, and protein for selected species of New Zealand seaweeds (means ± S.D, n=3).

Common letters within columns represent non-significant groupings (pairwise PERMANOVA, p(MC) < 0.05).

	Protein (%) By TAA	Lipids (%)	Total Carbohydrates (%)	Total Fibre (%)	Insoluble Fibre (%)	Soluble Fibre (%)
U. sp. B	15.1 ± 1.1 [a]	1.0 ± 0.3 [a]	63.2 ± 1.2 [a]	62.4 ± 7.2 [a]	34.7 ± 4.0 [ab]	27.7 ± 4.6 [a]
U. ralfsii	13.8 ± 0.9 [a]	5.3 ± 0.5 [b]	39.4 ± 1.2	40.1 ± 7.1 [b]	22.1 ± 2.8 [c]	18.1 ± 6.8 [abcd]
M. pyrifera	6.4 ± 1.3 [b]	1.3 ± 0.3 [a]	57.0 ± 7.1 [abcd]	45.4 ± 2.5 [b]	29.2 ± 2.5 [a]	16.3 ± 1.0 [b]
U. pinnatifida	6.8 ± 0.7 [bc]	4.8 ± 1.0 [b]	56.3 ± 1.7 [b]	48.2 ± 2.4 [ab]	38.2 ± 3.5 [b]	10.1 ± 1.4 c
P. plicata (K)	8.9 ± 1.0 [d]	0.7 ± 0.4 [ac]	52.5 ± 1.6 [c]	43.3 ± 20.9 [ab]	42.6 ± 20.6 [abc]	0.7* [ae]
P. plicata (T)	7.8 ± 1.0 [cd]	0.5 ± 0.1 [c]	67.9 ± 2.2 [d]	36.9 ± 12.8 [ab]	36.9 ± 23.8 [abc]	0.0* [de]

*Low values for soluble fibre necessitated consolidation of replicates in *P. plicata* samples.

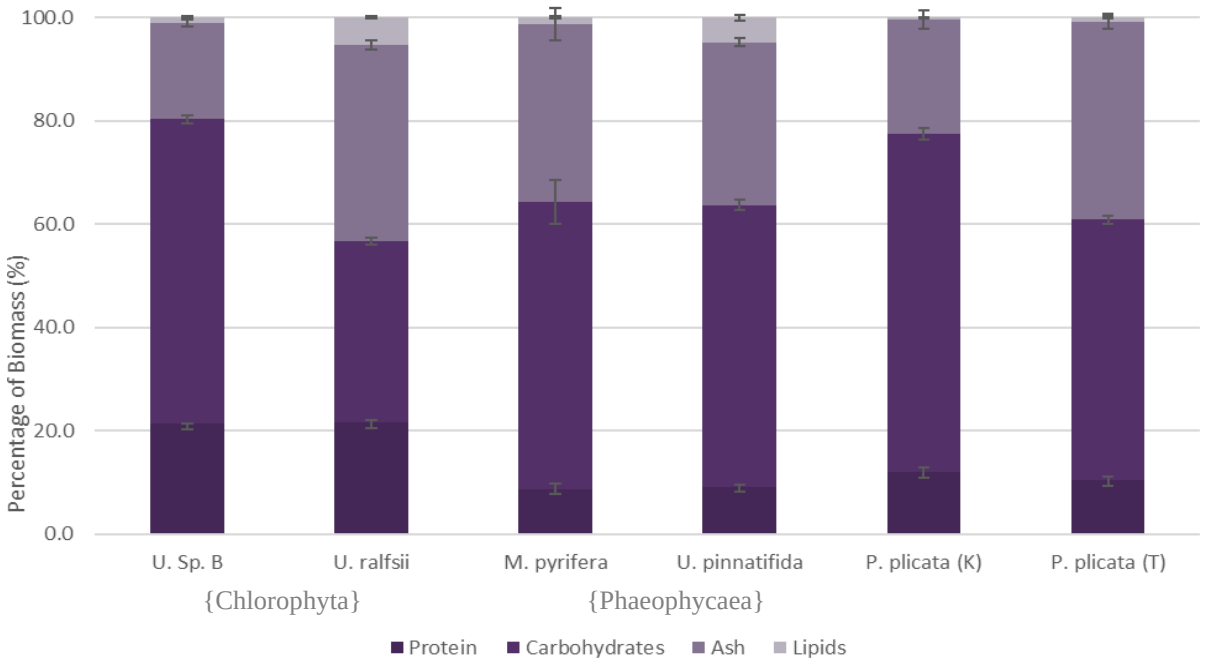

Figure 2.1 Content (% of dry weight) of protein, lipids, carbohydrates, and ash.

Bars represent mean and error bars represent standard error for each component of that species, n = 3.

2.3.2 Antioxidants

Total antioxidant capacity (Table 2.3) was significantly different between species (PERMANOVA, $F_{5,12}$ =8.6, p(MC) 0.0009), with the Phaeophycaea having greater amounts than the Chlorophyta, which in turn had more than the Rhodophyta. This was driven completely by activity in the hydrophilic antioxidant content (ANOVA, $F_{5,12}$ =11.3, p(MC) 0.0003), as lipophilic antioxidants were absent in all species.

Table 2.3 Antioxidant Trolox Equivalents (TE) (average ± S.D, n=3) in selected New Zealand seaweeds.

Common letters within columns represent non-significant groupings (pairwise PERMANOVA, p(MC) < 0.05).

	Lipophilic (TE)	Hydrophilic (TE)	Total (TE)
U. sp. B	0 ± 0	51.3 ± 19.0 ^{a}	68.9 ± 47.9 ^{abc}
U. ralfsii	0 ± 0	66.5 ± 9.6 ^{a}	66.5 ± 9.6 ^{a}
M. pyrifera	0 ± 0	114.6 ± 49.8 ^{ab}	114.6 ± 49.8 ^{ad}
U. pinnatifida	0 ± 0	167.5 ± 60.6 ^{b}	167.5 ± 60.6^{bd}
P. plicata (K)	0 ± 0	4.1 ± 3.6 ^{c}	4.1 ± 3.6 ^{c}
P. plicata (T)	0 ± 0	1.6 ± 2.8 ^{c}	1.6 ± 2.8^{c}

2.3.3 Metals & Metalloids Analysis

Content of metals/metalloids differed significantly between and within species (Figure 2.2, and Tables 2.4 and 2.5). Mineral composition of the six seaweeds were generally separated by phyla, with the two Phaeophycaea (or kelps) grouping together, and the two *Pyropia* grouping together (Figure 2.2). The two species of *Ulva* were surprisingly different considering they were cultivated under identical controlled conditions, and this separation was likely driven by morphology. *Ulva ralfsii* had the highest content of Na which was significantly different from all other species reflecting the high ash content of this species (Table 2.4 and 2.5). As seen in the MDS plot, *Ulva* spp. were not grouped, with Co and Na the key drivers of this separation (Figure 2.2). While *Ulva ralfsii* had a significantly higher level of Pb than *Ulva* sp. B (2.1 mg/ kg compared to 0.5 mg/ kg respectively) (Tables 2.4 and 2.5), this was not a significant driver in separation between species at the R value > 0.85. *Ulva* sp. B, cultivated in the same conditions, had a much lower range of 0.4-0.7 mg/kg of Pb. For separation of the Phaeophycaea, there were several components driving the separation of *M. pyrifera*: namely Ba, Sr, As and K. This is unsurprising as the As levels in *M. pyrifera* were significantly higher at 100 mg/ kg (Table 2.4 and 2.5). This also correlates with *M. pyrifera*'s lower Na:K ratio at 0.25, as compared to *U. pinnatifida*'s 0.75 (Table 2.4 and 2.5).

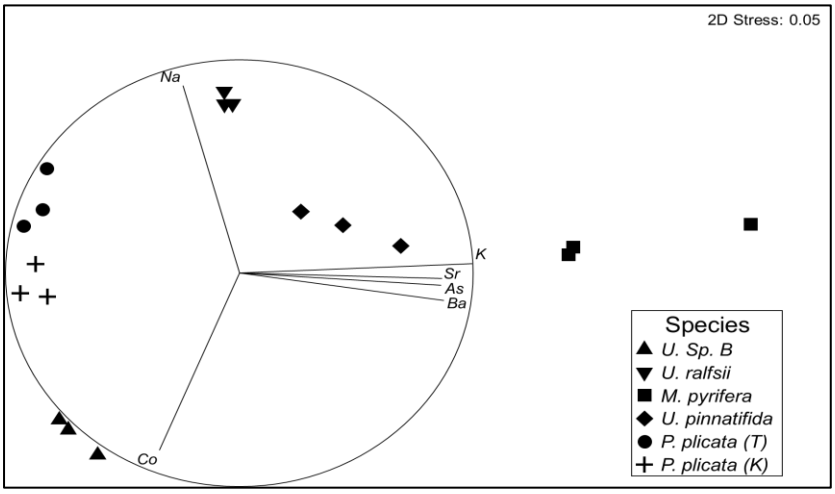

Figure 2.2 The nMDS scaling ordination of metals, metalloids and minerals of each sample (n = 3 biological replicates). Lines represent vector loadings of the specific minerals (Pearson's correlation, R > 0.85), with the size and direction of each vector representing the relative abundance of that element in that region of the plot.

Table 2.4 Results of the main PERMANOVAs testing single factor analysis of ICP analysis of content of metals and metalloids. Pseudo F (F) and P (MC) P values presented; df for all was 5,12.

	F	P		F	P		F	P
Na	59.0	0.0001	Fe	3.6	0.03	Cd	76.0	0.0001
Mg	217.6	0.0001	Co	87.3	0.0001	Hg	4.2	0.02
P	61.3	0.0001	Ni	6.5	0.0035	Pb	25.5	0.0001
S	141.3	0.0001	Cu	571.7	0.0001	K	51.8	0.0001
Ca	17.1	0.0001	Zn	9.6	0.001	Na:K	43.4	0.0001
Cr	3.4	0.0001	As	1123.6	0.0001	Macro	28.5	0.0001
Mn	34.0	0.0001	Se	34.7	0.0001	Trace	0.4	0.9

Table 2.5 Content (mg/ kg dry weight) of nutritionally relevant metals, metalloids, and phosphorus detected in order of atomic number (means ± S.D, n=3). Common letters within rows represent non-significant groupings (pairwise PERMANOVA, p(MC) < 0.05).

	U. sp. B	*U. ralfsii*	*M. pyrifera*	*U. pinnatifida*	*P. plicata (K)*	*P. plicata (T)*	RDI for Adults Aged 20 < 60 years (mg/d)	UL for Adults Aged 20 < 60 years (mg/ d) unless stated otherwise
			(mg/ kg)					
Na	17599 ± 998 [a]	49589 ± 1162	17958 ± 0989 [a]	33344 ± 1535 [b]	25296 ± 2976 [c]	34412 ± 5501 [bc]	1500 [1]	2300 [1]
Mg	16072 ± 1218 [a]	14981 ± 417 [a]	3902 ± 2816 [b]	588 ± 233 [c]	4272 ± 426 [bd]	5361 ± 769 [cd]	55-65[2]	350[2]
P	1792 ± 171 [ab]	2224 ± 221 [a]	1466 ± 138 [bc]	3601 ± 170	1801 ± 265 [ab]	1285 ± 91 [c]	700[3]	4000[3]
S	37930.06 ± 2507.45	23019.90 ± 1654.91 [a]	8096.91 ± 1193.57	12091.50 ± 1403.73	20362.84 ± 978.13 [a]	16447.47 ± 746.31	(SAA) 14 / kg bw [4]	No limit [4]
Ca	3947 ± 27	5464 ± 116	26924 ± 6940 [a]	15556 ± 1163 [a]	8854 ± 1341 [b]	13887 ± 4856 [ab]	1000[1]	3000[1]
Cr	0.4 ± 0.1 [a]	0.2 ± 0.1 [b]	0.4 ± 0.1 [ab]	0.9 ± 0.0 [c]	6.1 ± 5.3 [abc]	3.8 ± 1.9 [c]	100[15]	No limit[15]
Mn	29.1 ± 1.4	19.4 ± 1.5 [a]	6.0 ± 2.1 [b]	9.1 ± 0.3 [bc]	15.4 ± 4.9 [ac]	16.1 ± 1.6 [a]	2-5[67]	11[67]
Fe	316.0 ± 17.8 [ab]	350.1 ± 77.2 [ab]	169.8 ± 116.0 [ab]	317.4 ± 31.5 [ab]	391.6 ± 359.8 [bc]	690.8 ± 14.2 [c]	8(M)/ 18(F)[56]	45[56]
Co	0.84 ± 0.06	0.19 ± 0.07 [ab]	0.26 ± 0.06 [ab]	0.22 ± 0.01 [a]	0.49 ± 0.04	0.17 ± 0.01 [b]	(B_{12}) 0.002[1]	No limit[1]
Ni	1.9 ± 0.1 [a]	0.7 ± 0.3 [bcd]	0.4 ± 0.1 [b]	0.8 ± 0.0 [ce]	3.0 ± 1.5 [ade]	1.6 ± 0.6 [ade]	No RDI[6]	1[6]
Cu	10.7 ± 0.3	5.6 ± 0.5	0.7 ± 0.3 [b]	0.7 ± 0.1 [b]	2.2 ± 0.1 [c]	1.9 ± 0.2 [c]	0.9[5]	10[56]
Zn	10.0 ± 1.8 [ab]	13.4 ± 0.3 [c]	11.9 ± 2.6 [ac]	7.2 ± 1.1 [b]	7.1 ± 0.9 [b]	10.3 ± 0.7 [a]	9-1[5]	45[2]
As	0.3 ± 0.0	0.8 ± 0.1	100.5 ± 2.5	44.0 ± 3.8	21.5 ± 0.8 [a]	20.6 ± 1.1 [a]	No RDI[7]	1 /kg bw[7]
Se	0.02 ± 0.00	0.09 ± 0.02 [a]	0.15 ± 0.04 [b]	0.18 ± 0.02 [b]	0.09 ± 0.03 [a]	0.13 ± 0.00 [a]	0.034 (M) 0.026 (F)[2]	0.4[2]
Cd	0.02 ± 0.00 [a]	0.00 ± 0.00 [a]	1.56 ± 0.26	0.43 ± 0.01 [b]	0.85 ± 0.08	0.47 ± 0.07 [b]	No RDI[7]	0.025 /kg bw[7]
Hg	ND	ND	1.52 ± 1.49	2.61 ± 1.78	ND	ND	No RDI[7]	0[7]
Pb	0.5 ± 0.2 [a]	2.1 ± 0.5	0.2 ± 0.1 [a]	0.4 ± 0.1 [a]	0.4 ± 0.2 [a]	0.5 ± 0.1 [a]	No RDI[7]	12.5 µg/ day[8]
K	19386 ± 2761	32823 ± 1277	72813 ± 12038	44705 ± 6827	10205 ± 1237 [a]	10810 ± 68 [a]	4700[1,5]	No limit[1,5]
Na:K	0.92 ± 0.13	1.51 ± 0.09 [a]	0.25 ± 0.05	0.75 ± 0.09 [a]	2.51 ± 0.47 [b]	3.18 ± 0.51 [b]	0.32[9]	0.49[9]
Macro	96727.1 ± 6889.1	128101.0 ± 2512.5 [a]	131160.5 ± 16646.1 [ab]	115183.0 ± 6731.6 [ab]	70791.0 ± 2821.0	82203.4 ± 1973.2		
Trace	366.5 ± 20.0	388.9 ± 78.7	189.0 ± 121.1	334.9 ± 31.0	416.8 ± 364.0	719.5 ± 15.0		

(SAA)= Sulfur Amino Acids; bw= body weight; Macrominerals: Na+ Mg+ Ca+ P+ S+ K (no Cl analysed); Trace Minerals: Iodine + Mn+ Fe+ Cu+ Zn + Co + Se (no F analysed).
[1] adapted from Cogswell et al., (2012); [2] adapted from World Health Organization,(2003); [3] adapted from Borgi, (2019); [4] Hewlings & Kalman, (2019); [5] adapted from United States National Institutes of Health: Office of Dietary Supplements (2021); [6] adapted from Trumbo et al., (2001); [7] adapted from Nordberg et al., (2014); [8] adapted from United States Food and Drug Administration, (2020); [9] adapted from Drewnowski et al., (2012).

2.3.4 Amino Acids

Protein as sum of TAA (Table 2.7) was highest in the two species of *Ulva*, which differed from all other species (Table 2.6). Both species of *Ulva* had approximately two-fold higher protein content than all other species. Both *Ulva* species were significantly different from all other species in their BCAAs; isoleucine, leucine, valine. All others (*M. pyrifera*, *U. pinnatifida*, *P. plicata* (K), & *P. plicata* (T)) were not significantly different from one another for isoleucine and leucine content. Valine was the only one of the three BCAAs where there were statistical differences between each phylum, but not between species within phyla. This was also the case for NAA serine, CEAA, glycine, and EAA threonine. Almost all amino acids (excluding alanine, tyrosine, and methionine) had significant differences between the two *Ulva* spp. and the others. Given the difference in quantity, the amino acid profiles of the *Ulva* spp., clearly separated them from Phaeophycaea and Rhodophyta species (Figure 2.3a). When analysing the quality via mol % (Figure 2.3b) rather than absolute content (Figure 2.3a), closer similarity between the Phaeophycaea and Chlorophyta is evident together with a clear separation from the Rhodophyta. This correlates with the lack of significant differences seen in EAA: Methionine (Table 2.7) and its role as a driver of separation in the MDS (Figure 2.3b). It is also noted that the key drivers in both separations (Figure 2.3 a & b) are a mix of NEAAs and EAAs.

In Table 2.8 the pseudo-DIAAS scores were calculated without digestion to indicate the limiting amino acid for each species. In the case of all seaweeds selected, the sulfur amino acids (SAA) (methionine + cysteine) were the limiting amino acids. *Ulva* sp. B had the highest DIAAS score (without digestion) with a score of 11.

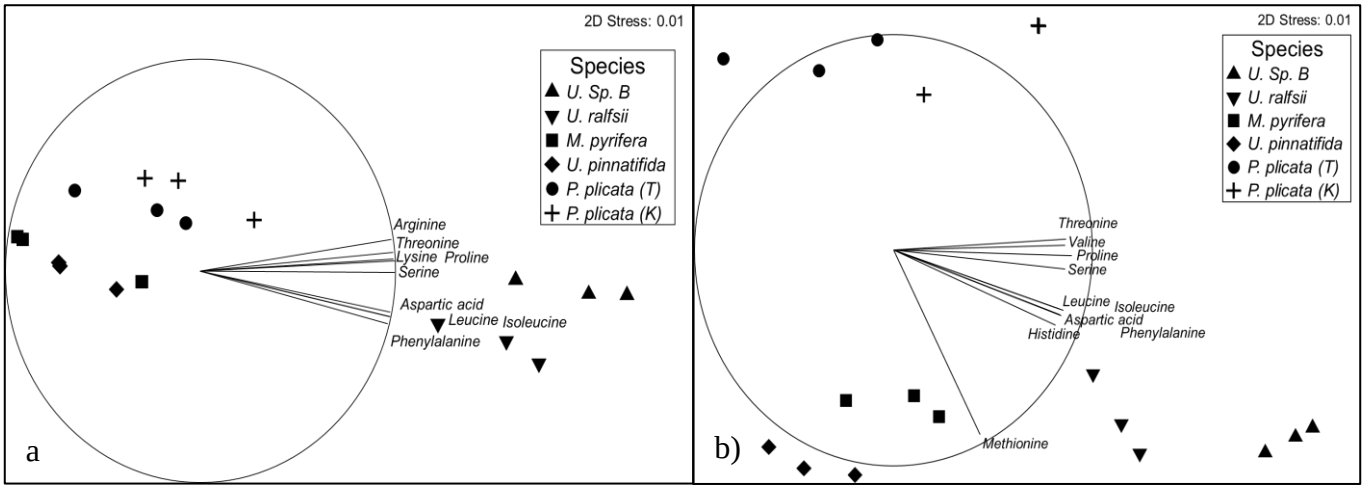

Figure 2.3 The nMDS scaling ordinations of the a) quantity of amino acids as -H2O; mg/ g dw, and b) quality of amino acids as mole %. Lines represent vector loadings of the specific amino acids (Pearson's correlation, a) R > 0.99, b) R > 0.85), with the size and direction of each vector representing the relative abundance of that amino acid in that region of the plot

Table 2.6 Results of main PERMANOVAs testing the effect of species on content of amino acids.

Pseudo F (F) and P (MC) *P* values presented; df for all was 5,12.

N/CEAA	F	P	EAA	F	P	BCAA	F	P
Alanine	7.9	0.0018	Arginine	48.4	0.0001	Isoleucine	69.0	0.0001
Aspartic acid	61.5	0.0001	Histidine	47.6	0.0001	Leucine	60.7	0.0001
Glutamic acid	16.8	0.0001	Lysine	28.1	0.0001	Valine	61.2	0.0001
Serine	50.2	0.0001	Methionine	43.0	0.0001	TAA	40.9	0.0001
Glycine	52.5	0.0001	Phenylalanine	21.7	0.0001	BCAA	62.0	0.0001
Proline	72.0	0.0001	Threonine	43.1	0.0001	EAA	53.7	0.0001
Tyrosine	36.6	0.0001						

Table 2.7 Content of amino acids ((average ± std) -H2O; mg/g in biomass) in selected species of New Zealand seaweed. Common letters within rows represent non-significant groupings (pairwise PERMANOVA, p(MC) < 0.05).

		U. sp. B	*U. ralfsii*	*M. pyrifera*	*U. pinnatifida*	*P. plicata (K)*	*P. plicata (T)*
NEAA	Alanine	11.6 ± 0.7 [a]	12.6 ± 0.7 [a]	6.8 ± 1.7 [b]	7.2 ± 1.1 [b]	9.7 ± 1.7 [ab]	10.1 ± 1.9 [ab]
	Aspartic acid	22.9 ± 1.5 [a]	19.9 ± 2.1 [a]	8.0 ± 1.7 [b]	7.9 ± 0.7 [b]	9.6 ± 1.4 [b]	7.9 ± 1.2 [b]
	Glutamic acid	15.9 ± 0.5 [a]	17.5 ± 1.1 [a]	9.2 ± 2.2 [b]	11.2 ± 1.1 [b]	10.1 ± 1.6 [b]	10.1 ± 1.7 [b]
	Serine	9.0 ± 0.8 [a]	7.4 ± 0.5 [a]	3.2 ± 0.6 [b]	3.3 ± 0.3 [b]	4.9 ± 0.5 [c]	4.5 ± 0.5 [c]
CEAA	Glycine	9.0 ± 0.7 [a]	8.5 ± 0.4 [a]	3.6 ± 0.7 [b]	3.9 ± 0.3 [b]	6.9 ± 0.4 [c]	5.7 ± 0.7 [c]
	Proline	8.1 ± 0.7	6.8 ± 0.3	2.9 ± 0.5 [ab]	2.9 ± 0.2 [a]	5.0 ± 0.3	3.9 ± 0.4 [b]
	Tyrosine	5.1 ± 0.7	2.8 ± 0.2 [a]	1.6 ± 0.4 [bc]	1.6 ± 0.2 [b]	2.5 ± 0.2 [a]	2.3 ± 0.2 [ac]
EAA	Arginine	8.8 ± 0.6	7.5 ± 0.3	3.2 ± 0.6 [a]	3.6 ± 0.3 [a]	6.0 ± 0.7 [b]	5.3 ± 0.6 [b]
	Histidine	3.1 ± 0.3 [a]	2.6 ± 0.1 [a]	1.4 ± 0.2 [b]	1.6 ± 0.1 [b]	1.6 ± 0.2 [b]	1.4 ± 0.1 [b]
	Lysine	8.1 ± 0.5	7.0 ± 0.4	3.9 ± 0.8 [a]	3.9 ± 0.3 [a]	5.7 ± 0.7 [b]	4.8 ± 0.5 [ab]
	Methionine	2.6 ± 0.3 [a]	2.0 ± 0.3 [ab]	1.7 ± 0.3 [b]	1.8 ± 0.2 [b]	0.5 ± 0.1 [c]	0.5 ± 0.1 [c]
	Phenylalanine	9.4 ± 0.7 [a]	9.0 ± 0.5 [a]	3.4 ± 0.5 [b]	3.6 ± 0.4 [b]	4.3 ± 0.3 [b]	3.5 ± 0.4 [b]
	Threonine	8.4 ± 0.8 [a]	7.4 ± 0.6 [a]	3.3 ± 0.6 [b]	3.4 ± 0.3 [b]	5.8 ± 0.5 [c]	4.7 ± 0.5 [c]
BCAA	Isoleucine	7.1 ± 0.5 [a]	6.2 ± 0.3 [a]	2.9 ± 0.5 [b]	3.0 ± 0.3 [b]	3.6 ± 0.3 [b]	2.9 ± 0.4 [b]
	Leucine	12.4 ± 1.0 [a]	11.5 ± 0.5 [a]	5.0 ± 0.8 [b]	5.2 ± 0.6 [b]	6.2 ± 0.9 [b]	5.3 ± 0.6 [b]
	Valine	9.8 ± 0.7 [a]	9.2 ± 0.5 [a]	4.0 ± 0.7 [b]	3.9 ± 0.4 [b]	6.7 ± 0.6 [c]	5.5 ± 0.6 [c]
TAA		151.2 ± 10.8 [a]	137.8 ± 8.5 [a]	64.2 ± 12.8 [b]	68.0 ± 6.6 [bc]	89.3 ± 10.2 [d]	78.4 ± 10.4 [cd]
EAAs (total)		69.6 ± 5.3	62.4 ± 5.3	28.8 ± 5.0 [a]	30.0 ± 2.8 [a]	40.5 ± 4.1 [b]	33.9 ± 3.8 [ab]
BCAAs (total)		29.2 ± 2.1	26.9 ± 1.3	11.9 ± 2.0 [a]	12.1 ± 1.3 [a]	16.6 ± 1.7 [b]	13.7 ±1.6 [ab]
SNP		4.4	4.0	4.6	4.6	4.6	4.7

NEAA= Non-essential amino acids; CEAA= Conditionally essential amino acids; EAA= essential amino acids; BCAA= branched chain amino acids; TAA= Total amino acids;

SNP= Species specific Nitrogen-to-Protein conversion factor.

Table 2.8 Calculated Digestible Indispensable Amino Acid Score (DIAAS) for undigested samples to indicate limiting amino acid which is the essential amino acid with the lowest quantity below human requirement.

DIAAS Scores	His	Lys	SAA, sulfur amino acids (methionine + cysteine [not measured])	AAA, aromatic amino acids (phenylalanine + tyrosine)	Thr	Iso	Leu	Val
U. sp. B	19.4	16.9	**11.3**	35.4	33.6	23.7	20.3	24.5
U. ralfsii	16.3	14.6	**8.7**	28.8	29.6	20.7	18.9	23.0
M. pyrifera	8.8	8.1	**7.4**	12.2	13.2	9.7	8.2	10.0
U. pinnatifida	10.0	8.1	**7.8**	12.7	13.6	10.0	8.5	9.8
P. plicata (K)	10.0	11.9	**2.2**	16.6	23.2	12.0	10.2	16.8
P. plicata (T)	8.8	10.0	**2.2**	14.1	18.8	9.7	8.7	13.8

2.4 Discussion

There were large differences in overall composition among species tested, with the *Ulva* species having approximately two-fold higher content of protein than other species tested. In addition to protein content, proximate composition, metals, minerals, and antioxidants varied among species. This data provides an overview of the nutritional benefit seaweeds can provide as well as possible interactions with protein in seaweed. Therefore, this broad analysis of nutritional components is important for consideration of seaweed as a source of protein for human consumption.

2.4.2 Proximate Composition

Seaweed ash content is high in comparison to terrestrial plants which usually only contain 10% ash (O' Connor et al., 2020). The ash in seaweeds however is removed with relative ease when isolating proteins by simple freshwater rinsing and soaking (Magnusson et al., 2016; Neveux et al., 2015). This results in a simple way to increase the protein content of the selected seaweeds, particularly those with high ash content. In Magnusson, et al., (2016) 24

hours of washing in 40˚C resulted in 30.5% salt removal from filamentous *U. tepida*, and 29% removal from the blade-like *U. ohnoi*. Considering *U. ralfsii* is filamentous and *U.* sp. B is blade-like (Lawton, et al., 2021), expectation of high ash removal from both species to result in increased protein content is reasonable.

Iodine is a necessary mineral needed for thyroid function in humans; the recommended total dose for healthy adults is 150 μg/ day (Rogerson, 2017; World Health Organization [WHO], 2003); however, too much can be detrimental to thyroid function. The WHO has a recommended upper limit of 1100 μg / day to reduce risk of thyroid cancer, metabolic dysfunction and central nervous system impacts (Mæhre et al., 2014; WHO, 2003). Iodine content can vary greatly between species and locations (Roleda et al., 2021). In the current study, the Phaeophycaea contained the highest levels of iodine with *M. pyrifera* containing 212 μg/g and *U. pinnatifida* containing 17 μg/g. All other species had <5μg/g. While iodine has a recommended intake of 2 μg/kg/day for adults it also has an upper limit of 30 μg/kg/day (WHO, 2003). A 60kg adult would have to eat less than 8.5 g of *M. pyrifera* a day to stay under this limit; this would not be an issue if consuming as a garnish, but if consuming for protein, this would limit the amount to be consumed. Although some sources report reduction of iodine levels with processing (Chung et al., 2013; Food Standards Australia New Zealand, 2013), a more recent study reports that these effects are minimal in the context of consumable products (Nitschke & Stengel, 2016). This implies that the Phaeophycaea analysed might be less suitable as protein source in its raw form whereas the other species could be both a source of protein and safe supplementation of iodine.

Seaweeds contain a high content of unique fibre, both soluble and insoluble, inclusive of complex carbohydrates appropriate for human consumption and consumed for their health benefits (Dawczynski et al., 2007; Jiménez-Escrig & Sánchez-Muniz, 2000; Mendis & Kim, 2011; Syad et al., 2013). However, fibre in the cell wall can be problematic for protein availability as the complex matrix of polysaccharides and cell wall bound proteins is not

easily digested in the human gut. To increase bioavailability of seaweed protein, physical and chemical processing methods have been developed to break down the fibres and release the protein for uptake (MacArtain et al., 2007). *Ulva* has been successfully treated using enzymatic processing, with the content of nitrogen increased by a further 12.3–19.8% in salt and ulvan extracted, enzyme-digested biomass compared with salt and ulvan extraction alone (Magnusson et al., 2019). Because of the inherent differences between polysaccharide and cell wall structures in different seaweed, enzymatic processing needs to be optimised for each species, even within the same phyla (Fleurence, 1999b). As seen in this study, seaweeds analysed had 37 - 62% fibre, which is typical for marine algae (Jiménez-Escrig & Sánchez-Muniz, 2000). Seaweeds typically have low lipid levels and high fibre content (Admassu et al., 2018; Ortiz et al., 2009; Taboada et al., 2013). Results found in this study reinforced previous findings of high fibre, and no significant differences were found between phyla (Table 2.2)

2.4.2 Amino Protein Content and Quality

The primary focus of this study was to assess which of the selected New Zealand seaweed species had the greatest protein content as a potential human protein source. The two aquacultured Chlorophyta, *U. sp.* B and *U. ralfsii*, had the highest protein content (15.1 ± 1.0 and 13.8 ± 0.9 % protein in w biomass, respectively) (Table 2.7). These were more than two-fold higher than the kelps, both ~6%, and the two *P. plicata*, with ~9% and ~8% for K and T samples, respectively (Table 2.7). These results are unsurprising due to the controlled nutrient conditions (12 mg N/L) of the *Ulva* spp. compared with natural in-ocean concentrations of N which usually fall well below the fertilised cultivation water and fluctuates with season (being lower in summer and autumn) (Zeldis & Swaney, 2018). The nitrogen loading for these cultivated species is aimed at surpassing the “luxury point” at which the uptake of nitrogen is a luxury and is able to assimilate into free amino acids (Angell et al., 2014).

This is the first study to quantify the content of protein in the abundant and broadly distributed New Zealand species of Ulva (*U.* sp. B/ *U. ralfisii*) however, content of protein was comparable to previously reported for aquacultured *Ulva*. Across other studies similar protein content has been found in Australian cultivated *U. ohnoi* (18.5 ± 1.5% dw, Glasson et al., 2017) and New Zealand wild collected *Ulva stenophylla* (20.4 ± 4.9% dw with Nitrogen-Protein-conversion-Factor (NPF) 6.25, Smith et al., 2010). *Undaria pinnatifida* and *M. pyrifera* on the other hand not only have species-specific research literature available for comparison but also New Zealand commercial, species-specific information (Smith, et al., 2010). Protein content of *U. pinnatifida* has previously been reported as 14.2% dw (with NPF 6.25) (Smith, et al., 2010), more than double the content found in this study. *M. pyrifera* similarly was reported as 11.0% dw (with NPF 6.25) (Smith, et al., 2010). Zhou et al., (2015) reported that the level of nitrogen and amino acid profile in *U. pinnatifida* in New Zealand varies across seasons, with highest quality and quantity in winter. Total nitrogen peaked in July (ranging 22.0-26.2 mg/ g dw) before falling off by spring in September (14.9-23.3 mg/g dw). Westermeier et al., (2012) supports this with *M. pyrifera* in Chile ranging from 12% protein DW in summer months of January - February to 15% dw (both calculated by NPF 6.25) in winter months of March – June. As such it is not unlikely the values could greatly depend on when the commercial species were harvested.

As above, this is the first study to quantify the content of protein for *P. plicata* protein content, though previous studies have determined higher levels for other *Pyropia* spp.; 14.1 ± 0.6% in *Pyropia acanthophora* var. *robusta*, determined using Lowrey's method (Kavale et al., 2017) and 32.7 ± 4.1% dw with NPF 6.25 in *Porphyra* spp. collected in Nelson, New Zealand (Smith et al. 2010). Considering there was no significant difference between the wild *P. plicata* collected in Tauranga compared to Kaikoura in this study, it is unlikely the location of Nelson for the species collected by Smith et al., (2010) had an impact on these differences. It is more likely that the season of collection was the driving factor. In summer months

nitrogen in the water column is depleted (Zeldis & Swaney, 2018) and this was when the *P. plicata* in the current study was collected. Conversely, the *Porphyra* spp. from the study of Smith et al., (2010) was collected across the winter period (May-October 2004). As such it is expected for this study's *Pyropia* spp. to have a lower nitrogen and protein content.

Another reason that results between this study and previous literature may differ is due to the calculation methods used. Lowrey's method with a NPF of 6.25 is not as accurate as TAA and often overestimates the true protein (Angell et al., 2016). N and TAA were used in this study to calculate the species-specific NPF. A low conversion factor indicates more nitrogen is stored as non-protein (not as amino acids). This factor was lowest in *Ulva* sp. B and *ralfsii* (4.4 and 4.0 respectively) compared with the other four seaweeds which were all 4.6 except for *Pyropia plicata* (T) which was 4.7. The NPF determined for *U. pinnatifida* (5.6) was lower than found in Angell et al., (2016); whereas *M. pyrifera* was higher than previously calculated (4.1). *Ulva* sp. B results were comparable to *Ulva spp.* protein data in the literature, but *U. ralfsii* was lower than any *Ulva* species surveyed. *P. plicata* were not included in Angell et al., (2016) but were most comparable to *Porphyra crispata* (4.8). Despite the lower conversion factor, the quality of protein was high in *Ulva* species demonstrated by their essential amino acid (EAA) content. EAA and BCAA, were highest in *U.* sp. B and *Ulva ralfsii* (EAA total: 69.6 and 62.4 mg/g dw; BCAA: 29.2 and 26.9 mg/g dw, respectively). This indicates the luxury uptake employed in their cultivation was beneficial in terms of producing a higher quality of protein in these seaweeds.

This study found that all six seaweeds analysed had all EAAs, with the exception of cysteine and tryptophan, which were not analysed. Note, it is common for these to be excluded as they each require separate extraction and derivatisation for analysis and therefore the cost-benefit for their acquisition is poor (Angell et al., 2016). The EAAs comprised approximately 45% of TAA for the majority of species tested, though *Ulva* had significantly more, in line with their higher nitrogen environment (Table 2.7). This is further highlighted in the pseudo-DIAAS

score calculated in Table 2.8, where SAA were the limiting amino acids for all but *U.* sp. B and *U. ralfsii* had the highest of the six species (11.3 and 8.7 respectively). While this is a relatively low score compared to soy or peas (91 and 70 respectively, Herreman et al., 2020), it is worth noting that the limiting amino acid was the SAA which is methionine + cysteine, and cysteine was not analysed, so their true score may be higher. Whereas the cost-benefit of quantifying cysteine is usually negligible due to the low content found in seaweed (Angell et al., 2016), in this case it may be worth pursuing. As seen in Table 2.9 there have been varied amounts of cysteine found in *Ulva* species (Shuuluka et al., 2013; Tanna et al., 2019), with the highest amount in the farmed species *Ulva lactuca*, forming 20% of the SAA content. Further, processing aimed at breaking down the cell wall has potential to free up amino acids for digestion and make them more bioavailable which is the key in DIAAS.

Table 2.9 Amino acids (mg/g dw) in *Ulva* spp. with percentage of cysteine in total Sulfur Amino Acids (SAA) calculated.

	Shuululka et al., 2012	Tanna et al., 2019	
	U lactuca (farm)	*U fasciata*	*U lactuca*
His	1.8	0.03	0.14
Lys	4.2	0.04	0.08
SAA, sulfur amino acids (methionine + [cysteine])	2	0.12	0.67
AAA, aromatic amino acids (phenylalanine + tyrosine)	6.1	0.24	0.17
Thr	4.7	0.01	0.02
Iso	3.7	0.04	0.08
Leu	6.7	0.05	0.03
Val	6.2	0.11	0.80
Cysteine alone	0.4	0.07	0.21
Cysteine of % SAA	20.0	0.5	0.3

It is interesting that SAA was the limiting amino for *U* sp. B and *U. ralfsii* considering the *Ulva* species contain significant amounts of sulfated polysaccharides (ulvan) (Glasson et al., 2017; Kidgell et al., 2019). This was demonstrated in their high sulfur content (4.5 ± 0.1% and 3.2 ± 0.1% respectively). The metabolic pathways for ulvan and SAA are separate, and it is likely that ulvan takes priority considering the high amount in *Ulva* (up to 45% of dw

mass) (Kidgell et al., 2019). Future studies could investigate if a luxury point could be achieved with sulfur (as exists with nitrogen) to increase these particular amino acids in seaweeds and thereby raise their DIAAS.

2.4.3 Minerals and Heavy Metals

Minerals

The content of minerals is higher in seaweeds than in terrestrial plants (Rupérez, 2002) and stands to be an added benefit when consuming seaweed. *P. plicata* K and T had the highest trace mineral content (iodine, Mn, Fe, Cu, Zn, Co, and Se) (sum total 417 and 720 mg/ kg dw respectively) but macro minerals (Na, Mg, Ca, P, S, and K) were highest in *M. pyrifera* (128,101 mg/ kg dw) and *U. sp*. B (131,160 mg/ kg dw) showing diversity in mineral distribution across species without necessarily being restricted to phyla.

Toxicity concern

Not only are algae natural accumulators of minerals in their environment, but they also have the capacity to accumulate heavy metals (Chekroun & Baghour, 2013; Hwang et al., 2013; Kalia & Khambholja, 2015; Kisten et al., 2016; Pinto et al., 2003; Smith et al., 2010; Yadav et al., 2019), so much so that they are used as bioindicators or for phytoremediation (Chekroun & Baghour, 2013; Hwang et al., 2013; Kalia & Khambholja, 2015; Yadav et al., 2019). Phaeophycaea are often used for these purposes as they typically bioaccumulate higher concentrations of toxic metals such as arsenic than other phyla (Chekroun & Baghour, 2013; Kalia & Khambholja, 2015; Smith et al., 2010; Yadav et al., 2019). Therefore, as well as for review of mineral contents for nutrition, metal analysis is necessary for food safety when considering algae for protein.

Smith et al., (2010) collected and compared New Zealand seaweeds for heavy metals and arsenic. From this analysis, they found that inorganic arsenic in the seaweeds collected fell below 1 mg/kg, though total arsenic levels were up to 97mg/ kg dw in *M. pyrifera* (Smith et

al., 2010). This is comparable with the content of total arsenic found in this study (100 mg/kg). By comparison, the content of arsenic in both species of *Ulva* was very low, below 1 mg/kg each. It is worth noting that this study only analysed the total arsenic and currently inorganic arsenic (<1mg/kg) is the only regulatory measure in New Zealand for seaweed to be used for human consumption. Organic arsenic forms are not specifically assessed due to the lower toxicity when consumed (Smith et al., 2010). A dose of 1-3 mg/ kg body weight (bw)/ day of inorganic arsenic can be lethal (Fowler, Chou, et al., 2014), however it is unlikely that even with the higher amount in *M.* pyrifera found in this study, a toxic dose would be reached in a meal due to the quantity that would need to be consumed (eg. for a 60kg person they would need to eat 600g dw biomass in a day).

Mercury was only detectable in the Phaeophycaea in this study; *M. pyrifera* content averaged 1.5 mg/kg dw and *U. pinnatifida,* 2.6mg/ kg dw. These were both orders of magnitude higher than the mercury levels found in these species (also commercial) by Smith et al., (2010) which were both only 0.05 mg/kg. Most toxic in its methylmercury form, the tolerable weekly intake was set to 1.3 μg/ kg bw (Fowler, Alexander, & Oskarsson, 2014). Assuming a body weight of 60kg, one would need to eat 50g and 30g of unprocessed *M. pyrifera* or *U. pinnatifida* (respectively) in a week to reach this limit (assuming all the mercury found is in the methylmercury speciation which is unlikely but would need to be quantified separately (Jinadasa et al., 2021)). This would not be a concern with *Ulva* or *Pyropia* species as none was detected.

Unlike the other heavy metals, lead was highest in *U. ralfsii* (2.1 mg/kg dw) where all other species in this study fell below 1mg/ kg dw. *Ulva ralfsii*'s lead level is similar to the wild collected *Ulva* in Smith et al., (*U. stenophylla* 1.8mg/ kg dw) (2010). The lead levels are interesting however as the *U.* sp. B grown in the same conditions was a quarter of *U. ralfsii* lead levels, indicating inherent differences in species' bioabsorption pathways for this metal. The previous tolerable daily intake was 3.6 μg/ kg bw, however this threshold was withdrawn

as even this amount was associated with negative health consequences (Fowler, Alexander, & Oskarsson, 2014). The United States Food and Drug Administration (FDA) has calculated an interim reference level of 12.5 μg / day total for adults (FDA, 2020). This would be reached with 6 g dw/ day of unprocessed *U. ralfsii.*

As with many nutritional components addressed thus far, heavy metal contents vary with species, environment and season (Pérez et al., 2007). Nutrient availability such as high nitrogen is also believed to increase the ability of algae to accumulate heavy metals (Pinto et al., 2003), which is of particular note as this is the nutrient linked with protein levels. Given that the *Ulva* spp. were cultivated in elevated nitrogen levels, and *U. ralfsii's* lead levels, further analysis post processing would be recommended if these were to be considered for protein for human nutrition.

2.4.4 Antioxidant Activity

Dietary antioxidants may influence how protein is used in the human body and was therefore analysed in this study to assess New Zealand seaweed's potential as a protein source. Specific mechanisms of antioxidants within the human body are still not well known (Yeum et al., 2004). Pan et al., (2018) found that only "synergistic" groups that contain some lipophilic antioxidants, but had a majority of hydrophilic antioxidants, were absorbed effectively within human diets. When food contained hydrophilic antioxidants without any lipophilic antioxidants, it was found to have no effect on antioxidant capacity within human diets. Considering this, no species analysed in this study would have an antioxidant effect within the scope of human nutrition as none had any detectable lipophilic activity (Table 2.3). This was different to Kindleysides et al., (2012) which found overall higher Trolox equivalent values (150 – 550 TE) using ORAC on lipid extracts of its New Zealand seaweeds studied (*M. pyrifera* and *E. radiata*; and *Champia sp*. and *Porphyra sp.*). However, if the protein from these species is consumed for muscle recovery, rather than general nutrition their low antioxidant levels may be beneficial, as long-term supplementation of antioxidants may

impair exercise adaptions (Margaritelis et al., 2020). Even acute antioxidant supplementation post-exercise may decrease muscle recovery (Merry & Ristow, 2016). Therefore, it is not expected that the antioxidant levels in the species here would have an impact on protein use within the human body.

2.5 Conclusion

Pyropia plicata from the North and South Island, *Macrocystis pyrifera* and *Undaria pinnatifida* from commercial supplier (NZ Kelp, 2022), and *Ulva* sp. B and *Ulva ralfsii* from land-based cultivation, were all analysed for their overall (untreated) nutritional content. This study found that while proximate composition, minerals, and metals varied between species no pattern was detected among phyla. The two cultivated Chlorophyta, *U.* sp. B and *U. ralfsii,* had the greatest quality and quantity of protein of the six seaweeds analysed. The higher EAA and TAA places *Ulva* as a prime candidate for use as a protein source. In addition to this, its high fibre content indicates the potential to further increase protein concentration with processing techniques. While *U. ralfsii* had higher than desirable levels of lead, other contaminants in both *Ulva* species were lower than the Rhodophyta or Phaeophycaea. Processing also has potential to increase the proportion of protein to reduce the amount needed to be consumed and thus mitigate any toxicity concerns. Total amino acids (TAA), indicating protein content, was highest in the two cultivated *Ulva* (151mg/g for *U.* sp. B and 138mg/g for *U. ralfsii*). Using their nitrogen percentage to calculate their species-specific nitrogen-to-protein factor (SNP) found that their SNP was the lowest of six species (4.4 for *U.* sp. B and 4.0 for *U. ralfsii*), indicating more nitrogen is stored as non-protein material (such as chlorophyll, nucleic acid and/ or inorganic nitrogen (Angell et al., 2016) in these species. Thus, their cultivation in a nitrogen rich environment is likely to have successfully surpassed the luxury point to increase nitrogen assimilation into free amino acids (Angell et al., 2014) and facilitated their higher protein content. Protein content for the other four species was lower than previously reported; this may be related to the universal SNP

(6.25) which while often used overestimates the true protein content in seaweeds (Samarathunga et al., 2022). It is also likely that the season of harvest negatively affected the protein values of non-cultivated species as season and temperature is known to impact protein content (Bleakley & Hayes, 2017; Fleurence, 1999a). The controlled temperature and nitrogen in the *Ulva* spp. environment positively benefited their protein content and secured them as the best choice of the six species analysed for raw potential prior to processing.

3. Chapter 3: The effect of processing on the extract efficiency of protein from *Ulva* sp. B and *Ulva ralfsii*

3.1 Introduction

Seaweed is a sustainably produced source of protein, however, the extractability/bioaccessibility of proteins in seaweeds due to cell wall components, including cellulose and pectic polysaccharides (e.g., carrageenan, alginate, and ulvan) is limited (Raja et al., 2022). Processing to disrupt the cell wall has been demonstrated to improve the extractability of seaweed protein. There are a number of processes that have been applied to improve extractability/bioaccessibility of protein from marine and terrestrial plants, including disruption of the cell wall by mechanical (e.g., high pressure homogenisation, ultrasonication, milling, and spray-drying), thermal (microwave, autoclaving, freezing), chemical (e.g., organic solvents, osmotic shock, acid-alkali reactions) and biological (e.g., microbial degradation and enzymatic reactions) treatments (Batista et al., 2020; Fabian & Ju, 2011; Preece et al., 2017). However, cost of treatment needs to be considered to facilitate implementation to industry scale processing (Wang et al., 2019). Enzymatic and homogenisation treatments are broadly used in industry and are known cost-effective approaches that when used in combination have been demonstrated to improve the extractability/bioaccessibility of protein from seaweed (Batista et al., 2020). Their efficiency does vary with species (Batista et al., 2020), and they have not to date been tested with *Ulva* sp. B or *Ulva ralfsii*.

Various enzyme classes have been used for the enzymatic hydrolysis of seaweeds including proteases and carbohydrate active enzymes (CAZymes). However, CAZymes are most commonly employed to chemically degrade the polysaccharide rich cell wall of seaweeds. Previously used CAZyme mixtures include Cellulast® (which has cellulase), Shearzyme® (which has xylanase) and Viscozyme® (which has a mix of: arabinase, cellulase, β-

glucanase, hemicellulase, and xylanase) yielding mixed results (Nadar et al., 2018; Naseri, Jacobsen, et al., 2020; Naseri, Marinho, et al., 2020). As the species *U.* sp. B and *U. ralfsii* have not yet been tested, Viscozyme® with its wide range of CAZymes was considered the best option for this study.

Homogenisation as a processing method is useful for its mechanical cell fragmentation and increased surface area within the treated biomass for greater effect in further processing steps or digestive enzymes. It has often been used in conjunction with other processes for protein extracts. For example, homogenisation prior to pH-shift led to an increase in protein extraction yields in microalgae *Tetraselmis suecica* from 50%-80% (Harrysson et al., 2018). It has also been shown to have value as a stand-alone treatment. For example, the protein concentration from multiple macroalgae extractions (*Porphyra acanthophora* var. *acanthophora, Sargassum vulgare,* and *Ulva fasciata*) was improved by approximately 50% where homogenization used, regardless of aqueous treatment (water or trichloroacetic acid) (Barbarino & Lourenço, 2005). In non-seaweed applications, homogenisation has been found to have to improve protein extraction yield in soy by 90% (Fayaz et al., 2019). While once aimed at increasing stability of emulsions (i.e. for milk) research is increasing for the use of homogenisation as a process to improve bioavailability, but once again the results are dependent on the biomass being processed (Mesa et al., 2020).

This study aims to break down the cell wall of green seaweeds, *Ulva* sp. B and *U. ralfsii*, to increase the extractability of protein. A factorial experimental design was used to analyse the effect of enzyme hydrolysis of cell wall polysaccharides and/ or mechanical disruption of cell walls on the extraction efficiency of protein from *U.* sp. B and *U. ralfsii*. To analyse the impact of these processing methods on protein extractability, samples were subjected to an alkaline extraction process and the subsequent extract analysed for content of protein by using spectrophotometry.

3.2 Methods

3.2.1 Seaweed Biomass

Ulva sp B (GenBank accession number MW250819.1) and *U. ralfsii* (GenBank accession number MW250805.1) were harvested from mixed gametophyte/sporophyte stock cultures cultivated at the Facility for Aquaculture Research of Macroalgae (FARM) at the Coastal Marine Field Station (CMFS) of the University of Waikato, Tauranga. These were cultivated indoors under artificial lighting (2 x 4Seasons LUMA 800 LED Grow Lights at 600mm height with >600 PAR at the surface) at 12/12h light/dark cycle in round tanks (2 x 550 L; 900 diameter x 950mm height) filled to a depth of 0.8m with UV treated, 5 µm filtered seawater (35ppt) collected from Tauranga harbour. The temperature was controlled at 18ºC with a heater / chiller (300W Eheim Thermocontrol Heater / Hailea 1HP Chiller). The tanks were stocked at 0.8 g/ L fresh weight algal biomass and dosed with F/2 nutrients (Cell-Hi F2P, Varicon Aqua Solutions UK, 0.1g L^{-1}, 12.3 mg nitrate-N L^{-1} and 1.1 mg P L^{-1} purchased from Fresh by Design Ltd) as per the manufacturer's directions. Both species were harvested weekly through October 2020 and frozen fresh (-20˚C). They were then freeze-dried (BUCHI LyovaporTM L-200 Freeze Dryer) prior to milling (FRITCH cutting mill PULVERISETTE 15 with a 0.5mm sieve). All seaweed powder biomass was sealed in plastic bags at room temperature with silica gel sachets *in situ* to prevent absorption of moisture.

3.2.2 Processing

The effects of enzyme hydrolysis and homogenisation on the extractability of protein were quantified using a factorial experimental design (Figure 3.1; hydrolysis and homogenisation (EH); hydrolysis only (EN); homogenisation only (NH); neither treatment (NN)). Enzyme hydrolysis was conducted using a commercial mixture of enzymes (Viscozyme®) to target the chemical degradation of cell wall polysaccharides, while homogenisation targeted the mechanical degradation of the cell wall structure. Treatments were replicated on three

biomass samples from separate harvests of either *Ulva* sp. B or *U. ralfsii* producing a total of 24 samples for protein extraction and analysis.

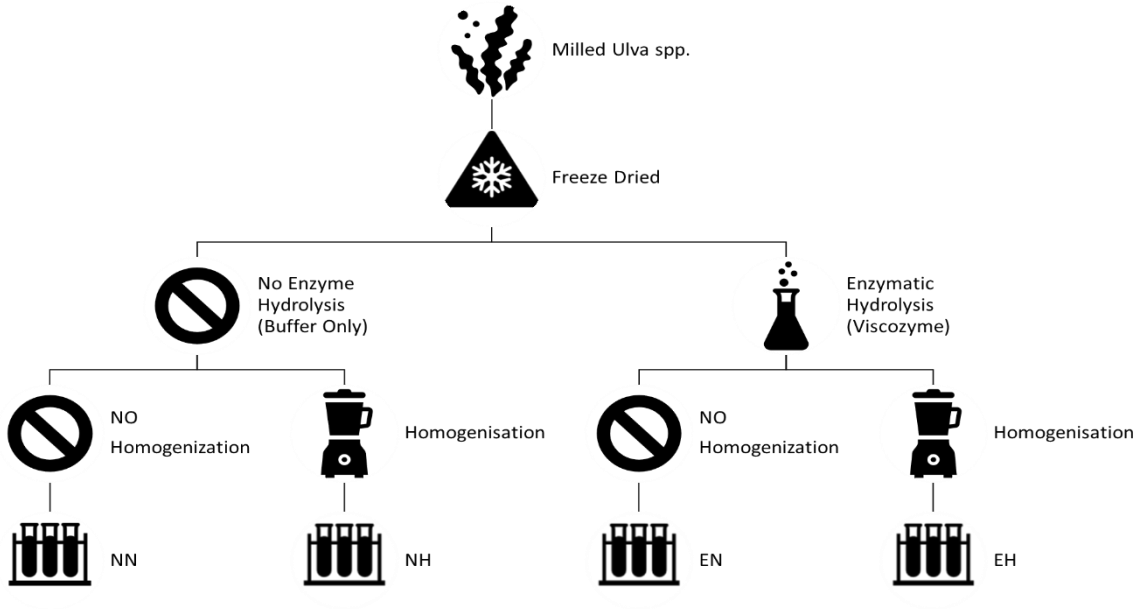

Figure 3.1 Processing experimental design flow chart

3.2.2.1 Enzyme treatment

Dried milled biomass (5 g) was enzymatically hydrolysed with Viscozyme® (5 mL) in 0.05M citrate buffer (45 mL) at pH 5 with stirring at 50 ºC for 24 hours. For control treatment biomass was subjected to the same conditions except 5 mL of citrate buffer at 5 pH was added in place of the Viscozyme®™. At the end of the treatments all samples were heated at 100ºC for 10 minutes to deactivate the enzymes. NN and EN (see figure 3.1) were freeze dried and milled in preparation for protein extraction, while NH and EH were subjected to homogenisation.

3.2.2.2 Homogenisation treatment

Biomass samples were homogenised using an Omni General Laboratory Homogeniser (GLH 850) with a 10 x 115mm saw tooth probe at 25,000rpm for 5 minutes. Homogenised samples were freeze dried and milled in preparation for protein extraction.

3.2.3 Protein extraction

Protein was extracted from dried milled treated biomass samples using an alkaline protein extraction method (Angell et al., 2017). Freeze dried biomass from processing (see appendix Table 6.8) was hydrolysed in 5 mL of 0.01M NaOH (pH 12) at 30˚C with stirring for 2 hours. The extract and residual biomass were then separated by centrifugation at 2561 rcf for 10 minutes and the liquid fraction decanted into a 50 mL Falcon tube. Residual biomass samples were freeze dried and milled to prepare for nitrogen and ash analysis.

3.2.4 Compositional Analyses

Analysis of the contents of nitrogen and ash was outsourced to OEA labs (http://www.oealabs.com, Callington, UK), where samples were combusted in pure oxygen before being separated and quantified by chromatography-thermal conductivity detection (GC-TCD). Protein yield was then calculated from nitrogen percentage using the species-specific nitrogen to protein conversion factors (SNP) established in Chapter 2 (i.e., *Ulva* sp. B: N% * 4.4 = P%; *Ulva ralfsii*: N% * 4.0 = P%).

3.2.5 Extraction efficiency

Extraction efficiency for each treatment was calculated by difference, comparing the protein in protein extracted residual biomasses with the protein in the equivalent weight of raw (unprocessed) biomass.

$$Extraction\ Efficiency = \frac{100\ x\ (Protein\ (mg)\ in\ raw\ biomass\ -\ Protein\ (mg)\ in\ extracted\ biomass)}{Protein\ (mg)\ in\ Raw\ biomass}$$

3.2.6 Data Analyses

Variances on solubilisation of biomass of different treatments were analysed using two factor (species and treatment) permutational analyses of variance (PERMANOVA) conducted in Primer v7 (Primer-E Ltd., UK) using Euclidean distances resemblance matrices, 9,999 unrestricted permutations of raw data, Type III sum of squares, and inclusion of Monte Carlo P value. Post-hoc PERMANOVA tests (pairwise) carried out for all analyses which were

significant according to the main PERMANOVA test. Replicate samples for analysis of protein and ash content in residual biomass from each treatment, post-extraction, and extraction efficiency required pooling due to small sample volumes. Due to the resulting lack of replication for some treatments, these were excluded from any statistical analyses.

3.3 Results

3.3.1 Processing Impact on Solubilisation of Biomass into Liquid

Solubilisation of biomass differed among treatments for each species with the same trend for both species (Table 3.1 and 3.2). Processing treatments that included enzymatic hydrolysis (EN and EH) showed a higher degree of solubilisation than those without (Table 3.2 and Figure 3.2). The high degree of solubilisation in these treatments unfortunately resulted in the replicates of extracted residual biomasses needing to be pooled for subsequent analyses. For these they were therefore excluded from full statistical compositional analysis and extraction efficiency analysis due to the lack of replicates. There was also difference between species (Table 3.1), however this was only the case for treatments without enzymatic hydrolysis (NN and NH; Table 3.3 and Figure 3.2).

Table 3.1 Results of two factor PERMANOVA testing treatment and species factors in percentage of biomass solubilised into liquid during processing treatments. Pseudo F (F) and P (MC) P values presented.

	df	F	P
Tr	3, 16	62.9	0.0001
Sp	1, 16	26.9	0.0001
TrxSp	3, 16	2.2	0.1228

Table 3.2 Results of two factor PERMANOVA (pairwise) for interaction of treatment and species for pairs of levels of factor 'Treatment' in percentage of biomass solubilised into liquid during processing treatments. Presented are t- statistic and Pseudo P (MC) P values; df 1,3.

Species	Treatment	t	P
U. sp. B	NN, NH	0.84746	0.4457
	NN, EN	7.0227	0.0025
	NN, EH	7.9297	0.0016
	NH, EN	6.6267	0.0030
	NH, EH	7.6121	0.0012
	EN, EH	0.45752	0.6747
U. ralfsii	NN, NH	1.0244	0.3639
	NN, EN	4.9977	0.0066
	NN, EH	6.0272	0.0039
	NH, EN	7.4597	0.0016
	NH, EH	12.397	0.0003
	EN, EH	1.2868	0.2662

Table 3.3 Results of two factor PERMANOVA (pairwise) for interaction of treatment and species for factor "Species" (comparing *U.* sp. B and *U. ralfsii)* in percentage of biomass solubilised into liquid during processing treatments. Presented are t- statistic and Pseudo P (MC) P values; df 1,1.

Treatment	t	P
NN	2.7799	0.0497
NH	4.6366	0.0114
EN	1.3054	0.2635
EH	1.9209	0.1278

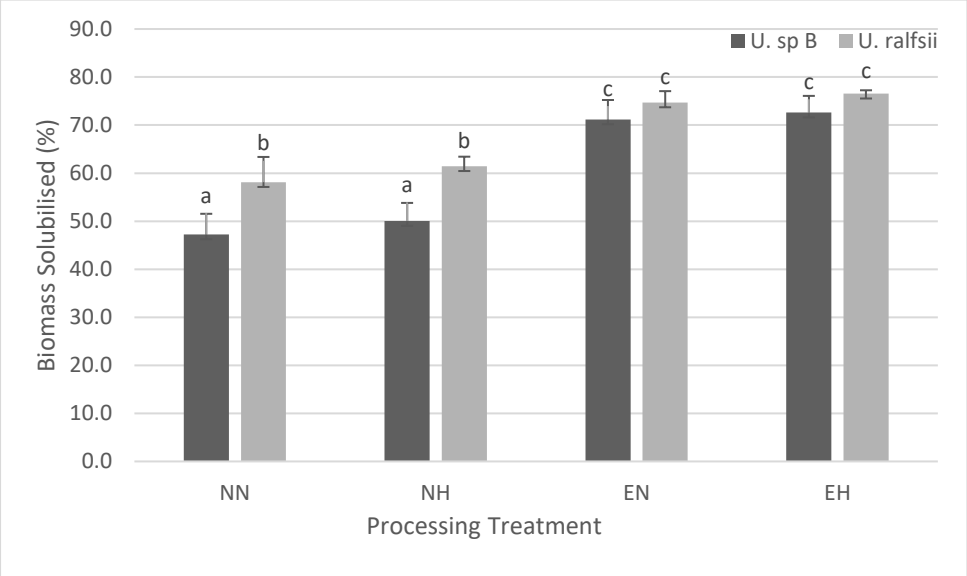

Figure 3.2 Percentage of biomass solubilised into liquid during processing treatments, representing loss of biomass due to solubilisation into liquid with processing. Bars represent means and error bars represent standard error (n = 3), for NN (no treatment), NH (homogenised), EN (enzymatically hydrolysed) and EH (enzyme plus homogenisation). Common letters within columns represent non-significant groupings (pairwise PERMANOVA, Pseudo p(MC) < 0.05).

3.3.2 Processing Impact on Composition of Biomass

The composition (ash and protein content) of residual biomass from each treatment, post-extraction, was impacted by processing compared to raw biomass (Table 3.4 and 3.5). Enzymatic treatments were not statistically analysed (Section 3.2.6). Homogenisation (NH) and simple "washing" of the control treatment (NN) both had an impact on composition compared to raw biomass, but only ash in *U.* sp. B varied when comparing these treatments (NN/ NH) (Table 3.5). The content of protein and ash ranged 15.1-24.5% and 8.5-18.7% respectively for *U.* sp. B and 13.8-25.7% and 9.1-37.9% respectively for *U. ralfsii* (Figure 3.3 and 3.4). For both species ash content was reduced with treatment and the content of protein in protein extracted residual biomasses increased. Notably, the residual biomass from enzymatically hydrolysed extracted residual biomasses had the highest protein for both *U.* sp. B (24.5%) and *U. ralfsii* (25.7%).

Table 3.4 Results of two factor PERMANOVA testing treatment and species factors in percentage of protein and ash in biomass for respective samples. Pseudo F (F) and P (MC) P values presented.

		df	F	P
Protein (%)	Tr	2, 12	100.2	0.0001
	Sp	1, 12	13.6	0.0034
	TrxSp	2, 12	11.4	0.0013
Ash (%)	Tr	2, 12	863.8	0.0001
	Sp	1, 12	239.5	0.0001
	TrxSp	2, 12	216.9	0.0001

Table 3.5 Results of two factor PERMANOVA (pairwise) for interaction of treatment and species for pairs of levels of factor 'Treatment' in percentage of protein and ash in biomass for respective samples. Presented are t-statistic and Pseudo P (MC) P values; df 1,2.

Composition	Species	Treatment	t	P
Protein (%)	*U.* sp. B	Raw, NN	17.911	0.0001
		Raw, NH	14.984	0.0002
		NN, NH	1.1013	0.2278
	U. ralfsii	Raw, NN	9.5742	0.0010
		Raw, NH	9.5506	0.0007
		NN, NH	0.3993	0.3245
Ash (%)	*U.* sp. B	Raw, NN	12.3	0.0006
		Raw, NH	14.133	0.0001
		NN, NH	3.3971	0.0238
	U. ralfsii	Raw, NN	29.648	0.0001
		Raw, NH	28.338	0.0001
		NN, NH	2.495	0.0673

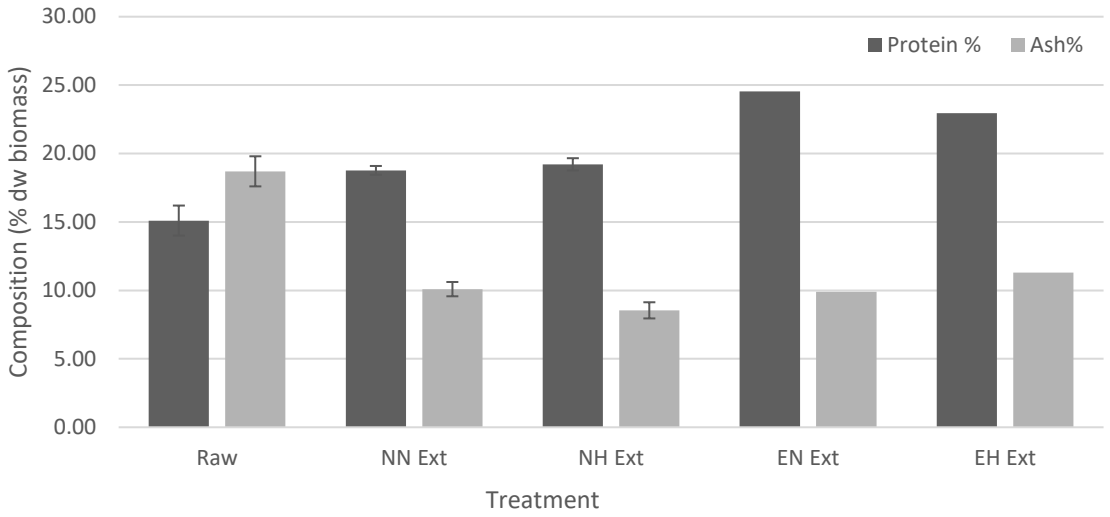

Figure 3.3 Percentage of protein and ash in biomass and protein extracted residual biomasses of *U*lva sp. B. Bars represent mean and error bars represent standard error (n = 3), for raw, NN (no treatment), and NH (homogenised), except for EN (enzymatically hydrolysed) and EH (enzyme plus homogenisation) for which the analysis was conducted on a composite sample (n = 1).

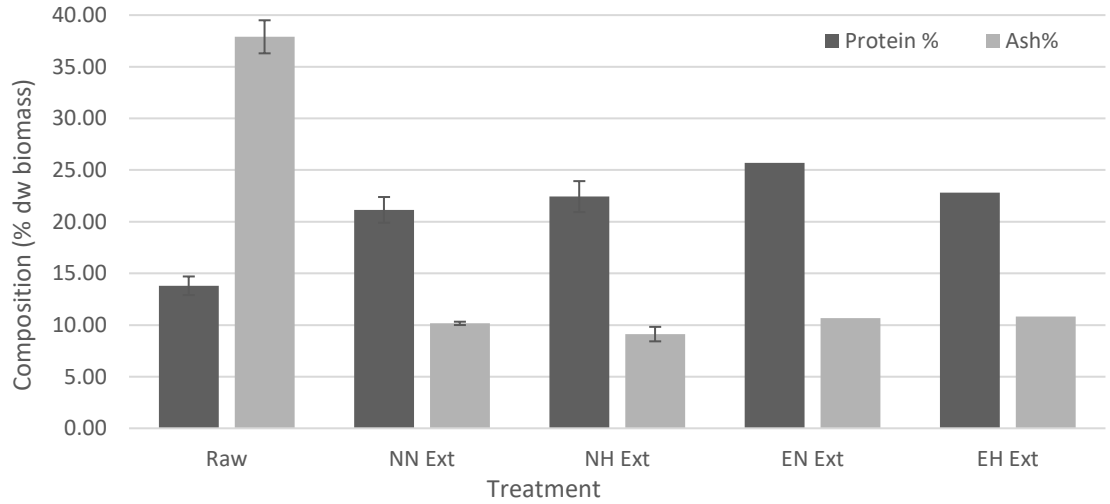

Figure 3.4 Percentage of protein and ash in biomass and protein extracted residual biomasses of *U*lva *ralfsii*. Bars represent mean and error bars represent standard error (n = 3), for raw, NN (no treatment), and NH (homogenised), except for EN (enzymatically hydrolysed) and EH (enzyme plus homogenisation) for which the analysis was conducted on a composite sample (n = 1).

3.3.3 Processing Impact on Extraction Efficiency

The extraction efficiency of protein was not significantly different between treatments without enzymatic hydrolysis (NN and NH) (Table 3.6). Due to the solubilisation for enzymatic treatments (EN and EH), their reduced biomass resulted in the triplicates being combined for nitrogen and ash quantification; hence the lack of standard errors (Figure 3.5)

and lack of inclusion in statistical analysis. While enzymatic treatments (EN and EH) were not included in statistical analysis, the data is likely robust given the small standard error across treatments (Figure 3.5) despite only three replicates in NN and NH. Graphical interpolation shows a clear increase in efficiency of protein extraction; 15.6-24.5% and 16.0-25.7% over untreated (NN) biomass in *U.* sp. B and *U. ralfsii*, respectively.

Table 3.6 Results of two factor PERMANOVA testing treatment (control washing and homogenisation) and species (*U.* sp. B and *U. ralfsii*) factors in extraction efficiency of protein across processing treatments. Pseudo F (F) and P (MC) P values presented.

	df	F	P
Tr	1, 8	0.2	0.6643
Sp	1, 8	0.3	0.6625
TrxSp	1, 8	0.1	0.8026

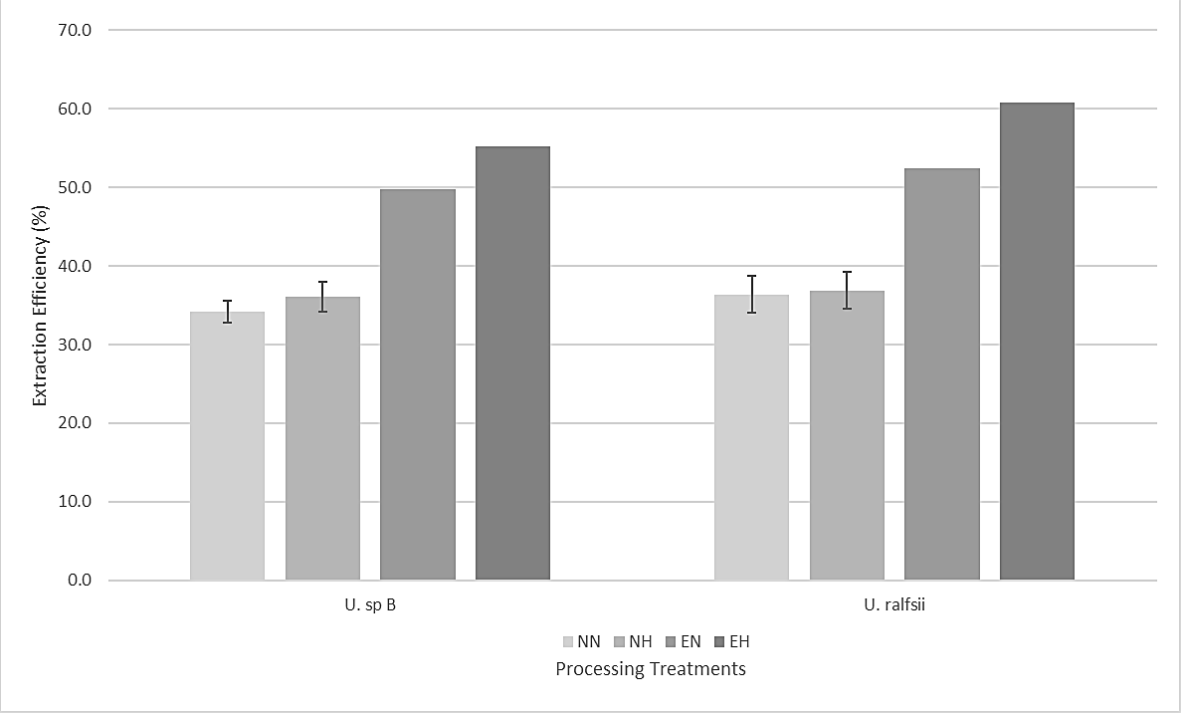

Figure 3.5 Extraction efficiency of protein across processing treatments applied to *Ulva* sp. B and *Ulva ralfsii*. Bars represent mean and error bars represent standard error (n = 3), for raw, NN (no treatment), and NH (homogenised), except for EN (enzymatically hydrolysed) and EH (enzyme plus homogenisation) for which the analysis was conducted on a composite sample (n = 1).

3.4 Discussion

Cell wall polysaccharides inhibit the extractability and bioaccessibility of protein in seaweeds (Naseri, Marinho et al., 2020; Samarathunga et al., 2022). This study investigated two methods (enzymatic hydrolysis and homogenisation) that targeted the disruption of the cell walls of *U. sp. B* and *U. ralfsii* and its effect on protein extractability. The methods were chosen to compare and assess the influence of chemical, mechanical and combined treatments on the extraction efficiency of proteins. The combined treatment (hydrolysed and homogenised) had the highest extraction efficiency of protein; 55.2% for *U. sp*. B and 60.8% for *U. ralfsii* (Figure 3.5). Enzymatic hydrolysis had a larger extraction efficiency of proteins than homogenisation giving increases of 15.6% and 16.0% compared to 2.0% and 0.5% over untreated (NN) biomass for *U*. sp. B and *U. ralfsii*, respectively. Enzyme hydrolysis also indicated greater enrichment of the extracted biomass protein (Figure 3.3 and 3.4). Protein content in the enzymatic hydrolysis residual biomasses post-extraction were increased by 9.4% and 11.6% compared to untreated biomass (raw) for *U*. sp. B and *U. ralfsii*, respectively. Whereas homogenisation was increased by 4.1% and 8.6% compared to untreated biomass (raw) for *U*. sp. B and *U. ralfsii*, respectively.

3.4.1 Enzyme Hydrolysis

In the current study treatment with Viscozyme® led to an increase in extraction efficiency of protein of 15.6-24.5% and 16.0-25.7% over untreated (NN) biomass for both *U.* sp. B and *U. ralfsii*, respectively. The extraction efficiency of Viscozyme® treated samples were 49.8% (EN) and 55.2% (EH) for *U*. sp. B and 54.2% (EN) and 63.73% (EH) for *U. ralfsii*. Similar improvements in extraction efficiency have been recorded for other species of seaweed (Mendez & Kwon, 2021; Naseri, Jacobsen, et al., 2020; Naseri, Marinho, et al., 2020). For example, Naseri, Jacobsen, et al., (2020) calculated extraction efficiency by difference (between pre and post biomass protein) and found that Viscozyme® treatments of *Eucheuma denticulatum* biomass allowed a 48% extraction efficiency of protein; a 33% increase over

untreated biomass. Other studies demonstrated similar improvements with treatments of Viscozyme® ((Mendez & Kwon, 2021; Naseri, Marinho, et al., 2020). It is worth noting that the addition of Viscozyme® can contribute as much as 12 mg/ ml of protein to the extract (Anthon & Barrett, 2008) and may contribute to overestimation of the extraction efficiency of protein from seaweeds if not calculated by difference. In the current study the degree of solubilisation led to only small quantities of protein being extracted from residual biomasses for treatments with Viscozyme® (EN and EH). The extent of this was so great, that resulting biomass triplicates needed to be combined to achieve the minimum quantities required for nitrogen and ash analysis. This limited the ability of the statistical analyses to detect differences between treatments that included Viscozyme® treatments. Though there was no replication for EN and EH treatments, disallowing statistical comparison, graphical interpolation (Figure 3.3, 3.4, and 3.5) shows the data is likely robust given the small standard error across treatments despite only three replicates.

3.4.2 Homogenisation

Homogenisation led to only minor increases in extraction efficiency (however this was non-significant, $p>0.05$; $F_{1,8}$) over untreated biomass (NN), with an apparent greater influence on the bladed *U.* sp. B than the filamentous *U. ralfsii*, if only in ash content. This result was surprising as previous studies investigating the effect of homogenisation on extraction of protein, found homogenisation had improvement in extraction efficiency compared to that from untreated biomass (Barbarino & Lourenço, 2005; Fayaz et al., 2019). However, in Barbarino & Lourenço, (2005), *Sargassum vulgare* with hard and leathery thalli showed greater extraction improvement with homogenisation than flattened soft thalli *Ulva fasciata* or *Porphyra acanthophora* var. *acanthophora*. Thus, the morphology of species may impact the effectiveness of homogenisation. It is also likely that the homogeniser used in this study limited the effectiveness of the homogenisation treatment. For example, Fayaz et al., (2019) demonstrated that increases of pressure (i.e., shear) using a high pressure homogenisation

(HGH) progressively increased the protein extraction yield from soybean okra (11.5 g/ 100 g protein untreated; 37.1 g/ 100 g protein at 50MPA; 65.9 g /100 g protein at 150 MPa). The Favaz et al., (2019) study applied much higher shear forces than the homogeniser used in the current study, allowing the disruption of the cell wall structure and the subsequent increased extraction efficiency of protein.

Homogenisation following enzymatic hydrolysis (EH) did lead to an increase in the extraction efficiency of protein by 5.4% and 8.4% compared to enzymatically treated (EN) *U.* sp. B and *U. ralfsii*, respectively. This indicates that the enzymatic treatment weakened the cell wall structure allowing the lower shear forces to further disrupt the cell wall structure and lead to higher extraction efficiencies. This is an interesting finding that might have implications on cost of processing / equipment required for the production of protein from seaweed biomasses.

3.5 Conclusion

Chapter 3 investigated industrially relevant processing methods (i.e., scalable and food safe) for improving the extractability of protein from *U.* sp B and *U. ralfsii*. Treatments included chemical hydrolysis with a commercially available food grade enzyme mixture (Viscozyme®) and mechanical cell wall disruption using homogenisation. The combined treatment had the greatest effect on the extractability of protein from both Ulva species with the enzymatic hydrolysis treatment having a greater effect that homogenisation. Homogenisation treatments were not solubilised to the same degree but showed no statistical difference from unprocessed biomass (NN), with only 2.0% and 0.5% extraction efficiency difference from untreated (NN) biomass for *U.* sp. B and *U. ralfsii* respectively. While this did not correlate with prior literature regarding homogenisation, it is assumed this is likely owing to the different equipment used across studies and the discrepancy in shear forces applied (Mesa et al., 2020)

4. Chapter 4: General Discussion

With global population expected to increase, so too is the increase in food production and the need for novel protein sources (Bleakley & Hayes, 2017). Seaweeds are increasingly being investigated as a potential source, but protein content is largely dependent on species, location, and season (Samarathunga et al., 2022). This thesis sought to first investigate which of six seaweeds (five different species) of New Zealand (NZ) had the highest yield of raw protein, and if the extractability/bioaccessibility could be improved through processing.

Of the six seaweeds (five different species) examined, this study found that cultivated *U.* sp. B and *U. ralfsii* had the best raw protein content, and that processing by enzymatic hydrolysis (Viscozyme®), resulted in the greatest extraction efficiency. Extraction efficiencies achieved in this study approached that of processed soy extraction (de Figueiredo et al., 2018). Therefore, seaweed should indeed be regarded as a novel protein source for New Zealand (and globally), however there remains a significant amount of research to select the best species, optimal growing conditions, and efficient processing methods to enhance yields and improve bioaccessibility in the human gut. Economic industrial-scale production can then be assessed.

4.1 Limitations and Future Research

This study was limited in selection of seaweed species for comparison, with those chosen by accessibility over spring/summer (October-December 2020). A consequence of this, is that available nitrogen is low in marine systems at this time of year (Zeldis & Swaney, 2018), and seaweeds are known to have lower protein content compared to winter months (Fleurence, 1999a; Galland-Irmouli et al., 1999; Zhou et al., 2015). This means that the comparison biased the *Ulva* spp. with their cultivated environment against other wild harvested species. While it could be said that this is an advantage for land-based cultivated species, in that they are suitable sources of protein year-round, future studies would benefit from a selection of

seaweeds across a range of geographical, temporal and life cycle stages for comprehensive comparison. Investigation could also encompass algae grown in nitrogen polluted waters (Spicer et al., 2021) to both benefit the algal protein production as well as reduce the nitrogen load in these systems.

Processing treatments were limited as they were not optimised for species but applied at a single concentration (Viscozyme®), time (hydrolysis period and homogenisation period), and speed (homogenisation). This was to compare treatments' potential for improvement in extraction efficiency, with optimisation of the most efficient protein extraction methodologies possible in future studies and at industrial scales. However, despite homogenisation having lower extraction efficiency than enzyme hydrolysis, it's use should not be ruled out as comparison to prior studies indicate that this may be due to the lack of shearing force in the homogeniser used in this study. Therefore, future research should investigate optimisation of enzyme hydrolysis for extraction of protein from cultivated species, in addition to high-pressure homogenisation (increasingly used for novel proteins (Mesa et al., 2020)).

Lastly, it can be assumed that the processing treatment applied in this study would improve the bioaccessibility of protein from these species, due to the increase of extraction efficiency; however, this cannot be directly correlated to human digestion. A valuable future study would be to directly investigate the bioaccessibility of enzymatically processed *U.* sp. B and *U. ralfsii* with simulated digestion such as INFOGEST 2.0 (Brodkorb et al., 2019). This would facilitate an accurate Digestible Indispensable Amino Acid Score for the two *Ulva* spp. and allow direct comparison to already available protein sources.

4.2 Potential Applications and Value of Research

The New Zealand seaweed industry is changing in line with macroalgal resources available, farming/cultivation potential, and the scope of new products possible (White & White, 2020). The findings of this research highlight the potential for New Zealand seaweed as a protein

source, with the best path to success in this industry from land-based cultivation. This enables controlled conditions to ensure high nitrogen loading to improve protein content yea round, but also to minimise contaminants such as heavy metals or fouling agents, an essential component for reliable production of a human protein source. The University of Waikato Marine Facility for Aquaculture Research of Macroalgae, (FARM), demonstrates the capacity of cultivated *Ulva* production: 35-70 tonnes / hectare/ annum (unpublished data). The extraction efficiency of protein from *U.* sp. B and *U. ralfsii* with enzyme hydrolysis and homogenisation processing treatments was 55.2 – 63.73%, respectively. With optimisation of processing, this could see a return of up to 7 tonnes/ hectare/ annum of protein – surpassing total soybean yields even prior to protein processing (Cornelius & Goldsmith, 2020; Wang et al., 2015). While future research would be required to analyse if the DIAAS of processed seaweed is comparable to soy, its range of essential amino acids is promising. Furthermore, as it is a marine plant, seaweed would not need the large extent of freshwater soy requires in cultivation, and they can be grown in three dimensions. This yield of protein return, assuming this study's processing methods were scaled to commercial capacity, would require 9,000L of water and 1000L of Viscozyme® per 1 tonne of raw biomass. A scaled production would require an industrial homogeniser large enough for high throughput; a scaled decanter/centrifuge to remove the residual biomass from the extract; and ultrafiltration and drying of the extract by either freeze-drying or by spray drying; all to produce 150 kg of protein powder. Note, many of these manufacturing technologies are already integrated in soy protein production (Ai et al., 2017) so the comparative environmental and economic impacts may indeed be in favour of Seaweed, though this would need further investigation. Finally, while seaweed protein would be well suitable for general nutrition, with the higher quality protein, seaweed protein powder could be marketed as a high-value sports supplement, a multi-billion-dollar industry (Grand View Research, 2022), to support higher costs of production.

4.3 Final Conclusion

This study validated the potential for seaweed as a novel protein source in New Zealand. Further investigation into this field could open up a sustainable, high production protein resource to feed the growing population with numerous advantages on current sources. While still new, this is an exciting avenue as the potential for a novel human protein source can be explored with different species, cultivation and/ or farming options, optimisation of processing methods, additionally advancing bioremediation possibilities. All these factors allow the possibility for even greater benefit to New Zealand as a producer of seaweed protein for human nutrition.

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6. Appendix

Table 6.1 CHNS, Ash & Iodine Results (triplicates)

Sample:	%C	%H	%N	%S	%I
U. sp. B	32.80	5.83	3.41	4.65	0.00612
U. sp. B	33.70	5.94	3.38	4.50	0.00411
U. sp. B	34.42	5.94	3.46	4.38	0.00000
U. ralfsii	23.42	4.29	3.40	3.20	0.00000
U. ralfsii	24.43	4.55	3.59	3.15	0.00000
U. ralfsii	23.06	4.30	3.39	3.25	0.00000
M. pyrifera	26.01	4.12	1.22	0.99	0.19369
M. pyrifera	23.72	3.63	1.73	0.79	0.25807
M. pyrifera	26.60	4.13	1.26	0.90	0.18563
U. pinnatifida	30.79	4.97	1.52	1.68	0.01256
U. pinnatifida	29.73	4.61	1.38	1.43	0.02349
U. pinnatifida	27.81	4.45	1.53	1.21	0.01619
P. plicata (T)	24.65	4.16	1.68	1.71	0.00000
P. plicata (T)	26.61	4.45	1.89	1.81	0.00000
P. plicata (T)	25.32	4.24	1.44	1.65	0.00739
P. plicata (K)	35.07	5.93	2.19	2.26	0.00435
P. plicata (K)	32.24	5.49	1.75	2.16	0.00000
P. plicata (K)	33.46	5.62	1.83	2.31	0.00000

Table 6.2 Carbohydrates, Lipids, Protein & Ash Results (triplicates)

Species	Protein	Ash	Lipids	Carbohydrates
U. sp. B	21.3	19.97	1.22	57.51
U. sp. B	21.1	18.12	1.12	59.66
U. sp. B	21.6	18.03	0.75	59.62
U. ralfsii	21.3	39.63	5.25	33.82
U. ralfsii	22.4	36.41	5.92	35.27
U. ralfsii	21.2	37.79	4.88	36.13
M. pyrifera	7.6	31.57	0.92	59.91
M. pyrifera	10.8	40.68	1.55	46.97
M. pyrifera	7.9	30.85	1.41	59.84
U. pinnatifida	9.5	31.63	5.8	53.07
U. pinnatifida	8.6	30.02	4.86	56.52
U. pinnatifida	9.6	32.8	3.84	53.76
P. plicata (K)	13.7	19.17	0.52	66.61
P. plicata (K)	10.9	25.36	0.53	63.21
P. plicata (K)	11.4	21.46	0.37	66.77
P. plicata (T)	10.5	37.76	1.2	50.54
P. plicata (T)	11.8	36.13	0.41	51.66
P. plicata (T)	9	41.25	0.74	49.01

Table 6.3 ORAC Antioxidant Results (triplicates)

	Lipophilic	Hydrophilic
Sample	TE	TE
U. sp. B	70.48	52.64
U. sp. B	51.24	0
U. sp. B	32.40	0
U. ralfsii	58.11	0
U. ralfsii	64.43	0
U. ralfsii	76.96	0
M. pyrifera	165.03	0
M. pyrifera	65.42	0
M. pyrifera	113.47	0
U. pinnatifida	130.27	0
U. pinnatifida	134.78	0
U. pinnatifida	237.34	0
P. plicata (K)	0	0
P. plicata (K)	6.50	0
P. plicata (K)	5.88	0
P. plicata (T)	4.91	0
P. plicata (T)	0	0
P. plicata (T)	0	0

Table 6.4 ICP Results (triplicates)

Sample:	Na	Mg	Al	K	Ca	P	S	V	Cr	Mn	Fe	Co	Ni
U. sp. B	18303.35	17195.17	44.45	22319.68	3941.89	1986.27	40824.60	0.2636	0.3399	28.07	330.06	0.9040	1.8029
U. sp. B	16456.88	16242.18	25.86	19000.41	3976.97	1732.49	36542.56	0.1510	0.4236	30.73	321.87	0.7807	1.9258
U. sp. B	18036.79	14777.21	17.99	16838.95	3922.96	1659.82	36423.03	0.1109	0.3066	28.47	295.91	0.8412	1.8626
U. ralfsii	49373.95	15050.39	7.02	32179.51	5520.82	2003.54	21119.71	0.1042	0.1581	18.72	266.14	0.1188	1.0298
U. ralfsii	48549.22	15358.18	6.81	34294.42	5541.14	2445.52	23794.85	0.1298	0.1921	18.38	366.13	0.2561	0.4500
U. ralfsii	50842.47	14533.47	13.65	31996.03	5330.20	2224.29	24145.14	0.1695	0.2609	21.10	417.97	0.2079	0.6410
M. pyrifera	18149.53	4033.72	32.38	65648.34	21531.68	1312.11	8937.70	1.7251	0.2767	4.59	94.59	0.2108	0.3206
M. pyrifera	16887.97	3579.96	133.31	86711.02	34753.10	1578.76	6730.78	1.9046	0.4900	8.41	303.39	0.3226	0.4630
M. pyrifera	18837.76	4093.78	48.97	66079.67	24486.07	1507.42	8622.26	1.4841	0.3491	4.87	111.27	0.2341	0.3586
U. pinnatifida	32160.59	5750.16	323.80	43918.62	15905.93	3720.22	13638.53	0.9400	0.9396	9.51	348.63	0.2332	0.8146
U. pinnatifida	32792.30	5751.51	291.84	38304.68	16504.57	3406.42	11736.87	0.8427	0.9713	9.03	317.94	0.2075	0.7505
U. pinnatifida	35078.74	6155.11	251.92	51890.29	14257.95	3677.53	10899.09	0.9003	0.9115	8.98	285.58	0.2100	0.8145
P. plicata (T)	34950.55	5583.00	457.57	10732.40	11708.59	1326.34	17265.02	4.1388	2.0656	16.33	704.45	0.1664	1.1779
P. plicata (T)	28661.41	4505.48	414.36	10859.12	19451.21	1347.95	15802.72	4.2366	5.7399	17.54	692.03	0.1854	2.3007
P. plicata (T)	39624.69	5995.47	414.10	10839.87	10501.98	1179.87	16274.67	4.2271	3.4703	14.46	676.16	0.1667	1.1899
P. plicata (K)	23200.23	4012.87	31.34	11592.82	7305.91	2107.05	21407.50	3.4735	1.2114	12.27	98.26	0.5078	1.3954
P. plicata (K)	28701.85	4763.41	315.18	9806.54	9622.51	1644.78	19468.70	2.9715	11.7670	20.97	793.05	0.5286	4.2137
P. plicata (K)	23986.08	4038.35	127.31	9216.42	9634.02	1651.52	20212.31	2.2661	5.4125	12.82	283.46	0.4476	3.4365

Sample	Cu	Zn	As	Se	Sr	Se	Ag	Cd	Ba	Hg	Pb	U
U. sp. B	10.6332	11.4513	0.3646	0.0144	53.04	0.0494	0.7348	0.0233	2.2891	ND	0.7530	0.0546
U. sp. B	10.9909	10.3011	0.3509	0.0177	54.12	0.0488	0.2706	0.0206	2.4624	ND	0.4388	0.0307
U. sp. B	10.4196	8.0992	0.3094	0.0157	52.07	0.0526	0.4084	0.0166	2.3484	ND	0.3536	0.0228
U. ralfsii	5.0841	13.1896	0.7177	0.0741	81.46	0.0905	0.1350	0.0042	1.2875	ND	2.3805	0.0513
U. ralfsii	6.0077	13.7216	0.7327	0.1025	80.34	0.1229	0.2962	0.0037	1.4581	ND	1.5480	0.0695
U. ralfsii	5.7003	13.2995	0.8070	0.1021	79.67	0.1441	0.2061	0.0038	1.4698	ND	2.4407	0.0660
M. pyrifera	0.5099	10.7536	103.0595	0.1209	726.27	0.1592	0.0376	1.4945	13.7579	ND	0.1207	0.1321
M. pyrifera	1.1032	14.8581	100.3352	0.1956	730.97	0.1800	0.0485	1.8464	9.7925	0.0016	0.3421	0.2053
M. pyrifera	0.6149	10.1094	98.1251	0.1379	724.43	0.1702	0.0291	1.3326	7.9447	0.0030	0.1692	0.1751
U. pinnatifida	0.7870	6.7603	42.2146	0.1970	621.69	0.1825	1.0840	0.4240	7.5049	0.0019	0.4749	0.3617
U. pinnatifida	0.6701	6.3072	41.3063	0.1634	588.28	0.1477	0.5507	0.4207	7.2328	0.0013	0.3037	0.3557
U. pinnatifida	0.7617	8.3728	48.3613	0.1910	657.05	0.1899	0.5640	0.4349	8.2256	0.0046	0.4125	0.4044
P. plicata (T)	2.1685	10.2583	20.8338	0.1274	124.49	0.0846	0.0573	0.4839	2.3712	ND	0.3859	0.0836
P. plicata (T)	1.8149	9.5904	21.4485	0.1317	192.59	0.0740	0.0580	0.5233	2.5030	ND	0.4131	0.1069
P. plicata (T)	1.7329	10.9302	19.3700	0.1218	102.14	0.0867	0.0553	0.3951	2.0335	ND	0.5683	0.0720
P. plicata (K)	2.2674	8.0940	22.4023	0.0590	147.75	0.0641	0.1268	0.9442	1.3538	ND	0.3052	0.0724
P. plicata (K)	2.1320	6.6896	21.1759	0.1244	180.83	0.0816	0.0778	0.7936	1.5461	ND	0.6031	0.1334
P. plicata (K)	2.0940	6.4686	20.8998	0.0892	199.30	0.0801	0.0729	0.8176	1.2996	ND	0.2514	0.1061

Table 6.5 ICP Results (Average & std d n=3)

	Ulva Sp. B (mg/kg dry wt.)	*U. ralfsii* (mg/kg dry wt.)	*M pyrifera* (mg/kg dry wt.)	*U pinnatifida* (mg/kg dry wt.)	*P. plicata (K)* (mg/kg dry wt.)	*P. plicata (T)* (mg/kg dry wt.)
Na	17599.01 ± 998.05	49588.55 ± 1161.59	17958.42 ± 988.84	33343.88 ± 1535.28	25296.05 ± 2975.56	34412.22 ± 5501.43
Mg	16071.52 ± 1217.98	14980.68 ± 416.75	3902.49 ± 280.93	5885.59 ± 233.41	4271.55 ± 426.16	5361.32 ± 769.33
Al	29.43 ± 13.59	9.16 ± 3.89	71.55 ± 54.12	289.19 ± 36.01	157.94 ± 144.38	428.67 ± 25.02
K	19386.34 ± 2760.67	32823.32 ± 1277.31	72813.01 ± 12037.96	44704.53 ± 6826.82	10205.26 ± 1237.36	10810.46 ± 68.29
Ca	3947.27 ± 27.40	5464.05 ± 116.36	26923.62 ± 6939.58	15556.15 ± 1163.44	8854.15 ± 1340.82	13887.26 ± 4856.14
P	1792.86 ± 171.39	2224.45 ± 220.99	1466.10 ± 138.04	3601.39 ± 170.19	1801.12 ± 264.97	1284.72 ± 91.44
S	37930.06 ± 2507.45	23019.90 ± 1654.91	8096.91 ± 1193.57	12091.50 ± 1403.73	20362.84 ± 978.13	16447.47 ± 746.31
V	0.17 ± 0.08	0.13 ± 0.03	1.70 ± 0.21	0.89 ± 0.05	2.90 ± 0.61	4.20 ± 0.05
Cr	0.36 ± 0.06	0.20 ± 0.05	0.37 ± 0.11	0.94 ± 0.03	6.13 ± 5.31	3.76 ± 1.85
Mn	29.09 ± 1.43	19.40 ± 1.48	5.96 ± 2.13	9.17 ± 0.29	15.35 ± 4.87	16.11 ± 1.55
Fe	315.95 ± 17.83	350.08 ± 77.18	169.75 ± 116.04	317.38 ± 31.53	391.59 ± 359.79	690.8 ± 14.18
Co	0.84 ± 0.06	0.19 ± 0.07	0.26 ± 0.06	0.22 ± 0.01	0.49 ± 0.04	0.17 ± 0.01
Ni	1.86 ± 0.06	0.71 ± 0.30	0.38 ± 0.07	0.79 ± 0.04	3.02 ± 1.46	1.56 ± 0.64
Cu	10.68 ± 0.29	5.60 ± 0.47	0.74 ± 0.32	0.74 ± 0.06	2.16 ± 0.09	1.91 ± 0.23
Zn	9.95 ± 1.70	13.40 ± 0.28	11.91 ± 2.58	7.15 ± 1.09	7.08 ± 0.88	10.26 ± 0.67
As	0.34 ± 0.03	0.75 ± 0.05	100.51 ± 2.47	43.96 ± 3.84	21.49 ± 0.80	20.55 ± 1.07
Se	0.02 ± 0.00	0.09 ± 0.02	0.15 ± 0.04	0.18 ± 0.02	0.09 ± 0.03	0.13 ± 0.00
Sr	53.08 ± 1.03	80.49 ± 0.90	727.22 ± 3.37	622.34 ± 34.39	175.96 ± 26.12	139.74 ± 47.11
Se	0.05 ± 0.00	0.12 ± 0.03	0.17 ± 0.01	0.17 ± 0.02	0.08 ± 0.01	0.08 ± 0.01
Ag	0.47 ± 0.24	0.21 ± 0.08	0.04 ± 0.01	0.73 ± 0.30	0.09 ± 0.03	0.06 ± 0.00
Cd	0.02 ± 0.00	0.00 ± 0.00	1.56 ± 0.26	0.43 ± 0.01	0.85 ± 0.08	0.47 ± 0.07
Ba	2.37 ± 0.09	1.41 ± 0.10	10.50 ± 2.97	7.65 ± 0.51	1.40 ± 0.13	2.30 ± 0.24
Hg	ND	ND	0.0023 ± 0.00	0.0026 ± 0.00	ND	ND
Pb	0.52 ± 0.21	2.12 ± 0.50	0.21 ± 0.12	0.40 ± 0.09	0.39 ± 0.19	0.46 ± 0.10
U	0.04 ± 0.02	0.06 ± 0.01	0.17 ± 0.04	0.37 ± 0.03	0.11 ± 0.03	0.09 ± 0.02

Table 6.6 Amino Acid Results (mg/g) (triplicates)

		U. sp. B	*U. sp. B*	*U. sp. B*	*U. ralfsii*	*U. ralfsii*	*U. ralfsii*	*M. pyrifera*	*M. pyrifera*	*M. pyrifera*	*U. pinnatifida*	*U. pinnatifida*	*U. pinnatifida*	*P. plicata (T)*	*P. plicata (T)*	*P. plicata (T)*	*P. plicata (K)*	*P. plicata (K)*	*P. plicata (K)*
Amino Acids mg/g Protein	Histidine	2.76	3.11	3.29	2.58	2.76	2.56	1.28	1.62	1.29	1.60	1.54	1.74	1.44	1.51	1.23	1.85	1.49	1.58
	Serine	8.04	9.15	9.66	7.44	7.90	6.81	2.88	3.92	2.94	3.20	3.14	3.62	4.70	4.86	3.91	5.46	4.49	4.77
	Arginine	8.18	8.84	9.42	7.59	7.78	7.15	2.80	3.89	2.85	3.47	3.34	3.93	5.59	5.73	4.58	6.78	5.52	5.83
	Glycine	8.25	9.14	9.55	8.46	9.01	8.16	3.21	4.40	3.26	3.71	3.75	4.22	5.97	6.19	4.94	7.25	6.44	6.95
	Aspartic acid	21.30	23.24	24.19	21.04	21.20	17.40	6.98	9.99	7.03	7.50	7.44	8.76	8.43	8.83	6.56	11.17	8.56	9.21
	Glutamic acid	15.33	15.95	16.39	17.55	18.55	16.28	7.83	11.66	8.00	10.39	10.71	12.37	10.60	11.52	8.15	11.90	8.88	9.54
	Threonine	7.56	8.57	9.09	7.44	8.02	6.88	2.97	3.99	3.04	3.30	3.21	3.70	4.98	5.08	4.17	6.37	5.38	5.70
	Alanine	10.89	11.87	12.18	12.44	13.31	11.95	5.68	8.86	5.99	6.39	6.77	8.51	10.39	11.80	7.97	11.72	8.45	9.04
	Proline	7.38	8.23	8.73	6.68	7.19	6.65	2.59	3.50	2.56	2.73	2.73	3.14	4.08	4.13	3.44	5.31	4.62	4.97
	Lysine	7.57	8.28	8.54	6.90	7.35	6.64	3.40	4.86	3.54	3.79	3.71	4.29	5.12	5.17	4.21	6.41	5.14	5.51
	Tyrosine	4.33	5.27	5.72	2.68	3.00	2.64	1.38	2.06	1.41	1.69	1.44	1.75	2.38	2.47	2.01	2.75	2.35	2.49
	Methionine	2.23	2.61	2.82	2.24	1.96	1.66	1.52	2.00	1.50	1.75	1.68	2.00	0.55	0.53	0.42	0.56	0.44	0.43
	Valine	9.03	9.93	10.31	9.02	9.71	8.84	3.56	4.73	3.63	3.70	3.71	4.35	5.75	5.82	4.78	7.33	6.23	6.61
	Isoleucine	6.56	7.19	7.47	6.17	6.61	5.93	2.67	3.52	2.64	2.78	2.79	3.36	3.08	3.19	2.53	3.91	3.33	3.54
	Leucine	11.34	12.62	13.26	11.36	12.04	10.98	4.49	5.96	4.50	4.86	4.86	5.81	5.54	5.75	4.56	7.22	5.54	5.97
	Phenylalanine	8.62	9.55	10.02	8.88	9.51	8.51	3.06	4.04	3.16	3.36	3.41	4.06	3.65	3.72	3.07	4.60	4.01	4.26
	Total	139.36	153.56	160.63	138.46	145.90	129.03	56.30	78.99	57.33	64.20	64.24	75.62	82.24	86.30	66.52	100.59	80.88	86.41
Amino Acids mol %	Histidine	1.51	1.55	1.57	1.41	1.43	1.50	1.73	1.56	1.71	1.91	1.83	1.75	1.29	1.28	1.37	1.36	1.36	1.35
	Serine	6.95	7.18	7.25	6.42	6.46	6.28	6.12	5.91	6.12	6.00	5.86	5.73	6.62	6.49	6.81	6.32	6.45	6.41
	Arginine	3.94	3.87	3.94	3.65	3.55	3.68	3.32	3.27	3.31	3.63	3.47	3.46	4.39	4.27	4.45	4.37	4.42	4.36
	Glycine	10.89	10.94	10.94	11.14	11.24	11.49	10.42	10.13	10.36	10.62	10.68	10.19	12.83	12.63	13.15	12.80	14.11	14.24
	Aspartic acid	13.94	13.80	13.74	13.74	13.11	12.15	11.22	11.41	11.08	10.63	10.49	10.48	8.98	8.93	8.65	9.77	9.30	9.36
	Glutamic acid	8.94	8.44	8.30	10.22	10.22	10.13	11.22	11.87	11.24	13.13	13.46	13.19	10.07	10.39	9.58	9.28	8.60	8.64
	Threonine	5.63	5.79	5.88	5.53	5.64	5.47	5.43	5.19	5.46	5.33	5.16	5.04	6.04	5.85	6.27	6.34	6.65	6.59
	Alanine	11.54	11.42	11.20	13.15	13.33	13.51	14.79	16.38	15.30	14.67	15.47	16.50	17.92	19.32	17.03	16.60	14.86	14.88
	Proline	5.72	5.79	5.88	5.17	5.27	5.50	4.93	4.73	4.77	4.58	4.56	4.46	5.15	4.95	5.37	5.51	5.95	5.98
	Lysine	4.45	4.41	4.36	4.05	4.08	4.16	4.91	4.98	5.01	4.83	4.69	4.61	4.90	4.70	4.99	5.03	5.01	5.02
	Tyrosine	2.00	2.21	2.29	1.24	1.31	1.30	1.56	1.66	1.57	1.69	1.44	1.47	1.79	1.76	1.87	1.70	1.80	1.79
	Methionine	1.28	1.36	1.40	1.28	1.06	1.02	2.15	2.01	2.08	2.18	2.08	2.10	0.51	0.47	0.48	0.43	0.42	0.38
	Valine	6.86	6.85	6.80	6.84	6.97	7.17	6.65	6.27	6.64	6.09	6.08	6.04	7.12	6.83	7.33	7.45	7.85	7.79
	Isoleucine	4.37	4.34	4.32	4.10	4.16	4.21	4.36	4.09	4.24	4.00	4.01	4.10	3.34	3.28	3.39	3.48	3.68	3.66
	Leucine	7.55	7.62	7.66	7.54	7.57	7.80	7.34	6.93	7.21	7.01	6.97	7.08	6.00	5.91	6.11	6.42	6.12	6.17
	Phenylalanine	4.41	4.43	4.45	4.54	4.60	4.65	3.85	3.61	3.90	3.73	3.76	3.80	3.04	2.94	3.16	3.15	3.40	3.39

Table 6.7 Processed Treatments mass, nitrogen and ash (triplicate except where had to be combined (EN and EH nitrogen and ash)

Ulva Sp B				*Ulva ralfsii*			
Biomass	Biomass (mg)	%N	%Ash	Biomass	Biomass (mg)	%N	%Ash
Raw (NN)	71.49	3.41	19.97	Raw (NN)	84.53	3.40	39.63
Raw (NN)	71.91	3.38	18.12	Raw (NN)	81.43	3.59	36.41
Raw (NN)	65.13	3.46	18.03	Raw (NN)	89.10	3.39	37.79
NNEx	35.80	4.25	10.54	NNEx	36.70	4.95	10.23
NNEx	36.30	4.20	10.22	NNEx	37.60	5.34	10.27
NNEx	37.60	4.34	9.52	NNEx	32.10	5.57	9.98
Raw (NH)	85.78	3.41	19.97	Raw (NH)	95.69	3.41	39.63
Raw (NH)	74.20	3.38	18.12	Raw (NH)	95.23	3.38	36.41
Raw (NH)	77.10	3.46	18.03	Raw (NH)	105.26	3.46	37.79
NHEx	39.50	4.25	9.22	NHEx	38.70	5.20	9.76
NHEx	37.30	4.44	8.30	NHEx	36.90	5.70	9.24
NHEx	41.30	4.41	8.11	NHEx	38.40	5.93	8.37
Raw (EN)	89.63	3.41	19.97	Raw (EN)	110.14	3.41	39.63
Raw (EN)	112.36	3.38	18.12	Raw (EN)	98.43	3.38	36.41
Raw (EN)	87.35	3.46	18.03	Raw (EN)	108.42	3.46	37.79
ENEx	24.30	5.58	9.89	ENEx	30.80	6.42	10.66
ENEx	29.10			ENEx	24.00		
ENEx	29.20			ENEx	25.50		
Raw (EH)	83.23	3.41	19.97	Raw (EH)	115.04	3.41	39.63
Raw (EH)	107.39	3.38	18.12	Raw (EH)	84.61	3.38	36.41
Raw (EH)	79.25	3.46	18.03	Raw (EH)	118.79	3.46	37.79
EHEx	25.90	5.22	11.30	EHEx	26.30	5.71	10.81
EHEx	26.00			EHEx	19.70		
EHEx	21.30			EHEx	28.80		

Table 6.8 Extracted Treatments Solubilisation (%) (triplicate)

Solubilisation (%)		
	Sp B	ralfsii
NNEx	49.92	56.58
NNEx	49.52	53.83
NNEx	42.27	63.97
NHEx	53.95	59.56
NHEx	49.73	61.25
NHEx	46.43	63.52
ENEx	72.89	72.04
ENEx	74.10	75.62
ENEx	66.57	76.48
EHEx	68.88	77.14
EHEx	75.79	76.72
EHEx	73.12	75.76

Table 6.9 Processed Treatments Extraction Efficiency (%) (triplicate except where had to be combined (EN and EH)

Extraction efficiency (%)		
Biomass	Sp B	ralfsii
NNEx	37.59	36.79
NNEx	37.27	31.23
NNEx	27.59	40.92
NHEx	42.61	38.36
NHEx	33.97	34.63
NHEx	31.73	37.51
ENEx	49.77	54.22
EHEx	55.16	63.73