Seaweeds as an alternative crop for the production of protein

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Abstract

The total global demand for protein from both humans and livestock will rise substantially into the future due to the combined increase in population and per capita consumption of animal protein. Currently, net protein is primarily produced by agricultural crops. However, the future production of agricultural crops is limited by a finite supply of arable land, fresh water and synthetic fertilisers. Alternative crops such as seaweeds have the potential to help meet the protein demand without applying additional stress on traditional agricultural resources. This thesis investigates the potential of seaweeds as an alternative crop for the production of protein.

Chapter 1 provides a general introduction to the thesis. The chapter begins by introducing the current supply and future demand of protein globally, with a specific focus on the demands of mono-gastric livestock (poultry, swine and fish). This is followed by a summary of potential alternative protein sources that are currently being explored. Finally, seaweeds are introduced in the context of their potential as a biomass crop for the production of protein.

Many seaweed species have considerable plasticity in nitrogen content, yet the relationship between nitrogen content, protein concentration, protein quality and growth rate are poorly understood. Therefore, in Chapter 2, the plasticity in protein content in the green seaweed *Ulva ohnoi* was investigated. This was done by assessing the quantitative and qualitative changes in protein in *Ulva ohnoi* and relating these to changes in internal nitrogen content and growth rate. To do this water nitrogen concentrations and water renewal rates were varied simultaneously to manipulating the supply of nitrogen to outdoor cultures of *U. ohnoi*. Both internal nitrogen content and growth rate varied substantially, and the quantitative and qualitative changes in total amino acids were described in the context of three internal nitrogen states; nitrogen-limited, metabolic, and luxury. The nitrogen-limited state was defined by increases in all amino acids with increasing nitrogen content and growth rates up until 1.2 % internal nitrogen. The metabolic nitrogen state was defined by increases in all amino acids with increasing internal nitrogen content up to 2.6 % with no increases in growth rate. Luxury state was defined by internal nitrogen contents above 2.6 % which occurred only when nitrogen availability was high but growth rates were reduced. In this luxury

circumstance, excess nitrogen was accumulated as free amino acids, in two phases. The first phase is distinguished by a small increase in the majority of amino acids up to ≈ 3.3 % internal nitrogen, and the second by a large increase in glutamic acid/glutamine and arginine up to 4.2 % internal nitrogen. This chapter demonstrates that the relationship between internal nitrogen content and amino acid quality is dynamic but predictable, and could be used for holding seaweeds in a desired nitrogen state during culture.

In Chapter 3, I assessed the relative importance of direct and indirect effects of salinity on protein in seaweed. Indirect effects, through altering growth rates, and direct effects, through altering the synthesis of specific amino acids and osmolytes, were examined in the context of the concentration and quality of protein in *Ulva ohnoi*. To do this, *U. ohnoi* was cultured under a range of salinities without nutrient limitation. Both the concentration and quality of protein varied across the salinity treatments. Protein concentration was strongly related to the growth rate of the seaweed and was highest in the slowest growing seaweed. In contrast, the quality of protein (individual amino acids as a proportion of total amino acid content) was strongly related to salinity for all amino acids, although this varied substantially amongst individual amino acids. Increases in salinity were positively correlated with the proportion of proline (46 % increase), tyrosine (36 % increase) and histidine (26 % increase), whereas there was a negative correlation with alanine (29 % decrease). The proportion of methionine, with strong links to the synthesis of the osmolyte dimethylsulphoniopropionate (DMSP), did not correlate linearly with salinity and instead was moderately higher at the optimal salinities for growth. This chapter demonstrates that salinity simultaneously affects the concentration and quality of protein in seaweed through both indirect and direct mechanisms, with growth rates playing the overarching role in determining the concentration of protein.

During my investigations into the protein physiology and nutrition of seaweeds, it became evident that there were many inconsistencies and potential inaccuracies with the way protein concentrations are reported. Therefore, in Chapter 4, I assessed these issues on a broad scale by systematically analysing the literature to assess the way that people measure and report protein in seaweeds with the aim to provide an evidence-based conversion factor for nitrogen to protein that is specific to seaweeds. Almost 95 % of studies on seaweeds determined protein either by direct extraction procedures (42 % of all studies) or by applying an indirect nitrogen-to-protein conversion factor of 6.25 (52

% of all studies), with the latter the most widely used method in the last 6 years. Meta-analysis of the true protein content, defined as the sum of the proteomic amino acids, demonstrated that direct extraction procedures under-estimated protein content by 33 %, while the most commonly used indirect nitrogen-to-protein conversion factor of 6.25 overestimated protein content by 43 %. I then questioned whether a single nitrogen-to-protein conversion factor could be used for seaweeds and evaluated how robust this would be by analysing the variation in N-to-protein conversion factors for 103 species across 44 studies that span three taxonomic groups, multiple geographic regions and a range of nitrogen contents. This resulted in an overall median nitrogen-to-protein conversion factor of 4.97 and a mean nitrogen-to-protein conversion factor of 4.76. Based on these results I proposed that the value of 5 be adopted as the universal seaweed nitrogen-to-protein (SNP) conversion factor. This chapter highlighted that most of the quantitative data on the protein contents of seaweeds have been under- or overestimated and was in need of review in regards to the potential applications of seaweed protein.

Therefore, in Chapter 5, seaweeds were quantitatively assessed as a protein source in livestock feeds using the dataset established in Chapter 4 as a platform to compare the quality and concentration of protein to traditional protein sources (soybean meal and fishmeal) and then benchmarking the seaweeds against the amino acid requirements of mono-gastric livestock (chicken, swine and fish). The quality of seaweed protein (% of essential amino acids in total amino acids) is similar to, if not better than, traditional protein sources. However, seaweeds without exception have substantially lower concentrations of essential amino acids, including methionine and lysine, than traditional protein sources (on a whole biomass basis, % dw). Correspondingly, seaweeds in their whole form contain insufficient protein, and specifically insufficient essential amino acids, to meet the requirements of most mono-gastric livestock. This chapter highlights that the protein from seaweeds must be concentrated or extracted, and these techniques are the most important goals for developing seaweeds as alternative source of protein for mono-gastric livestock.

Therefore, in Chapter 6, I examined multiple techniques to isolate and concentrate protein in a seaweed, returning to the model organism the green seaweed *Ulva ohnoi*. The aim of this chapter was to compare the protein isolation and concentration efficiency of a mechanical-based method (as applied to leaves) to the solvent based

method (as applied to seed crops). Protein isolate yields ranged from 12.28 ± 1.32 % to 21.57 ± 0.57 % and were higher using the methods established for leaves compared to those for seeds. Protein isolates from all treatment combinations were ~ 250 – 400 % higher in the concentration of protein and essential amino acids compared to the original whole biomass, reaching a maximum concentration of 56.04 ± 2.35 % and 27.56 ± 1.16 % for protein and total essential amino acids, respectively. In contrast, protein and essential amino acid concentrations were only ~ 30 – 50 % higher in protein concentrates compared to the original whole seaweed, reaching a maximum of 19.65 ± 0.21 % and 9.52 ± 0.11 % for protein and total essential amino acids, respectively. This chapter demonstrated that the methodologies used for the isolation of protein in leaves are more suited to seaweeds than those that are based on seed crops, which have traditionally been applied to seaweeds. This chapter also demonstrated that protein isolation methods are more suited to seaweeds with low concentrations of protein, such as *Ulva ohnoi*, compared to protein concentration methods.

In summary, the research presented throughout this thesis establishes that seaweeds, irrespective of cultivation conditions and species, are not viable as a protein source for mono-gastric livestock in a whole form and will need to be processed post-harvest to concentrate their protein. Therefore, it is proposed that the most important strategy for developing seaweeds as a protein crop is the development of protein isolates and concentrates from seaweeds produced under intensive cultivation.

Abbreviations

AA = amino acid
ACTFR = Australian Centre for Tropical Freshwater Research
Ala = alanine
ANCOVA = analysis of co-variance
ANOVA = analysis of variance
Arg = arginine
Asn = asparagine
Asp = aspartic acid
BCA = Bicinchoninic acid assay
Cys = cysteine
d = day
DI = de-ionised
DM = dry and milled
DMSP = dimethylsulphoniopropionate
dw = dry weight
EAA = essential amino acid
Eq = equation
FAAP = free amino acid pool
FCR = feed conversion ratio
FP = fresh and pulped
fw = fresh weight
Gln = glutamine
Glu = glutamic acid
Gly = glycine
His = histidine
HN = high nitrogen
HSD = honest significance test
Ile = isoleucine
Ip = isoelectric point
Leu = leucine
LM = leaf method
LN = low nitrogen
Lys = lysine
MARFU = Marine and Aquaculture Research Facilities Unit
MC = Monte-Carlo
Met = methionine
MN = medium nitrogen
MS = mean sum of squares
NA = not applicable
N*6.25 = nitrogen-to-protein conversion by 6.25 factor
nMDS = non-metric multidimensional scaling plot

NPN = non-protein nitrogen
NPU = net protein utilisation
N*X = nitrogen content by a unique factor determined specifically for seaweeds
OEA = Organic Elemental Analyser
PAR = photosynthetically active radiation
PER = protein efficiency ratio
PERMANOVA = permutation analysis of variance
Phe = phenylalanine
PI = protein isolate
PRIMER = Plymouth Routines In Multivariate Ecological Research
PRISMA = Preferred Reporting Items for Systematic Reviews and Meta-Analyses
Pro = proline
SA = surface area
SD = standard error
Ser = serine
SGR = specific growth rate
SM = seed method
SNP = Seaweed Nitrogen-to-Protein factor
TAA = total amino acid
TEAA = total essential amino acid
Thr = threonine
Trp = tryptophan
Tyr = tyrosine
UPLC = Ultra Performance Liquid Chromatography
UV = ultraviolet
Val = valine
W_f = final weight
W_i = initial weight

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Chapter 1: General introduction

1.1. Protein – current supply and future demand

Protein provides the foundation of human and livestock nutrition, however, the global production of protein is having a significant effect on the environment. Agriculture, which delivers the majority of protein from crop and animal production, uses approximately 30 % of all ice-free land, 70 % of available freshwater and 20 % of energy (Aiking et al. 2006; Aiking 2011). Furthermore, agriculture has negative impacts on biodiversity loss, habitat loss and climate change (Verweij et al. 2009; Godfray et al. 2010). All of these impacts are compounded by the prediction that global protein supply will need to double by 2050 to support the demand from an increase in 2.3 billion people and higher per capita incomes in developing countries (Tilman et al. 2011). Meeting this demand, while simultaneously reducing the environmental impact of food production, is critical to securing the sustainable supply of protein.

Agricultural improvements in yield per hectare from improved irrigation and fertiliser use has meant that the supply of protein from agriculture has kept pace with growing demand (Aiking 2011). However, the resources on which these practices depend – fresh water, arable land and fertilisers – are finite and have reached their limits (Godfray et al. 2010; Lott et al. 2011). Many strategies have since been suggested to reduce the environmental impact of protein production while increasing supply. These include a focus on increasing the productivity of low yielding agricultural crops in developing countries and feeding down the food chain through the increased per capita consumption of plant protein (Godfray et al. 2010; Boland et al. 2013). However, while these strategies are integral in meeting future protein demand, they are only part of the solution as the demand for livestock will inevitably increase due to increases in per capita wealth in large developing countries (Godfray et al. 2010). This will consequently increase the demand of protein for livestock, which consume approximately 80 million tonnes of protein per year that is potentially suitable for human consumption (Steinfeld et al. 2012).

1.2. Livestock – nutrition and protein demand

Livestock are domesticated animals that are raised for the production of food, fibre or labour. Livestock can be broadly divided based on their digestive physiology into ruminant or mono-gastric livestock. Ruminants, which include cattle, sheep and goats, possess a complex four-chambered stomach that includes a rumen where plant material is fermented by symbiotic microflora prior to digestion. In contrast, mono-gastric livestock, which include poultry, swine and fish, possess a simple single chambered stomach that primarily digests food directly by means of stomach acid and enzymes. The difference in gut physiology between ruminant and mono-gastric livestock distinguishes their nutritional requirements for protein. While all livestock require protein, and more specifically its amino acids, ruminants do not require amino acids in their feed as they can be obtained indirectly from their symbiotic microflora. Symbiotic microflora in the rumen, which include bacteria, protozoa and yeast, synthesise complex plant material into their own biomass as protein, which is subsequently digested by the ruminant (Van Soest 1994).

Mono-gastric livestock, in contrast to ruminants, must obtain amino acids directly from their diet in the form of proteins, peptides or free amino acids. During digestion, mono-gastric livestock break down dietary proteins and polypeptides into smaller polypeptides and ultimately amino acids through acid hydrolysis and protease enzymatic activity. These amino acids are then either used to synthesise other amino acids or to build the proteins that are required by the livestock. For mono-gastric livestock, amino acids are classified as non-essential (those that can be synthesised from other amino acids), essential (those that cannot be synthesised) and conditionally essential (those that are essential under specific pathological or physiological conditions). Each essential amino acid is required at a specific concentration in the diet depending on the species and age of the livestock. If an essential amino acid is not provided at that concentration, then the amino acid is limiting because it restricts the utilisation of protein by the livestock. It is, therefore, not the concentration of protein in a feed source that is critical in mono-gastric livestock nutrition *per se*, but the concentrations of these limiting essential amino acids. If we consider livestock fed a legume and cereal based diet, then the concentration of the essential amino acids methionine and lysine are most critical as one of these amino acids is usually the "first" limiting amino acid (NRC 1994, 1998; McDonald et al. 2002; NRC 2011; Boland et al. 2013). Therefore, in addition to the concentration of protein, the proportion of protein as these essential amino acids

(protein "quality") is important when evaluating a potential protein source for mono-gastric livestock.

Currently, livestock protein demands are primarily met by agricultural crops and fishmeal, with soybean meal providing the vast majority of this (Boland et al. 2013). However, these protein sources cannot continue to sustainably meet the demand of protein by livestock. The production of soybean requires large amounts of arable land, fertiliser and fresh water and is responsible for considerable levels of deforestation and habitat loss (Verweij et al. 2009). Likewise, fishmeal, which is primarily used for aquaculture livestock, relies on the harvest of wild fish from fisheries and therefore represents a finite resource that is currently at a sustainable production limit. Furthermore, the use of soybean meal and fishmeal protein as a livestock feed competes directly with its use for human consumption, which is highlighted by the feed conversion ratios (kg of feed used to produce 1 kg of meat) of poultry meat, pork and grain fed beef at 2.3, 4.0 and 8.8, respectively (Wilkinson 2011). Therefore, finding alternative sources of protein that can substitute for traditional agricultural crops will play a critical role in securing the future supply and sustainability of protein for livestock and ultimately for human nutrition as well.

1.3. Alternative protein crops for mono-gastric livestock

1.3.1. Terrestrial leaf protein

Leaves are the photosynthetic organs of higher plants and represent an under-utilised protein resource. The leaves of traditional crops can be utilised as a co-product along with the production of seeds or other agricultural products that are the focus of crop production (Dale et al. 2009; Bals and Dale 2011; Chiesa and Gnansounou 2011). In this sense, the production of leaf protein is a sustainable addition to crops such as soybean meal, as leaf production does require additional agricultural resources. The concentration of protein in leaves is relatively consistent (10 – 20 % dw) and is of a quality, determined by limiting essential amino acids, comparable to seed crops (Fiorentini and Galoppini 1983). However, the digestible energy in leaves (proportion of potential energy in a food that is available upon digestion) and their digestible protein concentration (proportion of protein in a food that is available upon digestion) are too low for mono-gastric livestock due to high concentrations of cellulosic material.

Therefore, proteins from leaves must be extracted into a more concentrated form ("leaf protein concentrates" – LPCs) if they are to be fed to mono-gastric livestock. The extraction of protein from leaves has been investigated for a number of decades (Pirie 1969; Betschart and Kinsella 1973; Fernández et al. 1999; Sinclair 2009) with yields of up to 70 % (Pirie 1969; Huang et al. 1971; Betschart and Kinsella 1973; Antonov and Tolstoguzov 1990; Bals and Dale 2011) and a high quality of protein that is comparable to soybean meal (Dale et al. 2009; Chiesa and Gnansounou 2011). Despite this, LPCs are not used as a commercial protein source for livestock because the economics are not comparable to existing protein sources (Dale et al. 2009; Bals and Dale 2011). However, the extraction of leaf protein has recently been examined in the context of the biorefinery concept, where proteins are initially extracted from biofuel crops prior to the generation of energy. Multiple products increase the overall value of the crop and may alleviate, in part, the competition between food and fuels (Dale et al. 2009; Chiesa and Gnansounou 2011).

1.3.2. Fungal and bacterial protein

Fungal and bacterial protein sources include single-cell fungi (yeast), filamentous fungi and bacteria. Unlike traditional, or even alternative, crops which are autotrophic, fungal and bacterial protein crops are mostly heterotrophic and can use a wide range of substrates as a source of carbon and nitrogen for growth. These substrates include lignocellulosic waste streams that are inexpensive and abundant (Kuhad et al. 1997; Ravindra 2000). In this sense, fungal and bacterial crops are capable of producing protein without additional resources as they can be integrated into existing agricultural production systems using waste. This includes, for example, rice husk, which is the waste product that is separated from the rice grain during processing and represents 20 – 25 % of the harvested rice grain on a dry weight basis (Sims 2004).

Both fungi and bacteria can have high biomass productivities and both have a high concentration (30 – 80 % dw) and quality of protein (Kuhad et al. 1997; Ravindra 2000). However, there are a number of issues that restrict the use of fungal and bacterial protein in the diets of humans and livestock. These issues include their potential toxicity (especially in the case of bacteria) due to toxic and carcinogenic compounds originating from the substrate or biosynthesised by the organism. Another issue is their high nucleic acid content, which has been linked to a number of health issues in humans such as gout

and kidney stones due to the accumulation of uric acid from nucleic acid degradation (Sinskey and Tannenbaum 1975). Furthermore, the small size of bacteria and fungi incurs a high recovery cost (Ravindra 2000). Consequently, the most prominent issue preventing the further development of fungal and bacterial protein is the cost of production, which is simply not competitive with currently used sources such as soybean meal. The best example of where these issues have been overcome to provide a protein source is with the development of the filamentous fungi product Quorn, which has had great success as a vegetarian meat substitute for human consumption (Wiebe 2004). The success of Quorn as a commercial fungi protein product has been attributed to species selection for protein content, non-toxicity and suitable growth and morphology, development of economic production systems, development of post-harvest processing to reduce RNA content and effective marketing highlighting their high fibre and low cholesterol content (Wiebe 2004).

1.3.3. Microalgae

Microalgae are a diverse group of single-celled eukaryotes that are predominantly photosynthetic. Microalgae are being considered as alternative crops because their production does not require arable land or freshwater (for marine species) and generally have a high concentration (28 – 71 % dw) and quality of protein (Becker 2007). The use of whole microalgae as a source of whole nutrition, protein and lipids is well developed in the aquaculture hatchery industry where they are used to feed fish, crustacean and mollusc larvae and live feeds such as rotifers (Borowitzka 1997). However, the use of whole microalgae as a protein source in compound mono-gastric animal diets is limited. This is predominantly because their cost of production is high relative to other protein sources (Becker 2007). Consequently, microalgae are only utilised in the animal feed industry for high value products such as the fatty acid docosahexaenoic acid (DHA, DHA-SCO^{TM}) (Ratledge 2013) and the pigment astaxanthin (Higuera-Ciapara et al. 2006). Similar to leaf protein, the high production costs of microalgae have highlighted the need for an integrated biorefinery approach when targeting lower value products such as biofuels and protein. In this scenario, efficient and economical protein isolation techniques that can be easily be integrated with the co-production of biofuels will need to be developed if the microalgal resource is to be developed at scale (Wijffels and Barbosa 2010; Williams and Laurens 2010; Suganya et al. 2016).

1.3.4. Freshwater macroalgae

Freshwater macroalgae are multicellular, macroscopic, eukaryotic algae that inhabit freshwater environments. Despite requiring freshwater for their production, freshwater macroalgae can be grown as an alternative protein crop because they do not require arable land and can use wastewater rather than potable water. Freshwater macroalgae have variable concentration of protein, depending on species and environmental conditions (4 – 44 % dw), but a consistent high quality of protein and high biomass productivities (Wilkie and Mulbry 2002; Cole et al. 2015a; Cole et al. 2015b; Neveux et al. 2015). Selection for reliable and robust species with high biomass productivities in culture has resulted in a research and development focus on the genus *Oedogonium* (Lawton et al. 2013a), which has a concentration of protein in the range of 18 – 27 % under non-N limiting conditions (Cole et al. 2015a; Cole et al. 2015b). However, unlike microalgae and marine macroalgae (seaweeds) (see below), there are no high-value products identified from freshwater macroalgae. As a consequence there is no commercial production of freshwater macroalgae and their development as biomass crop remains nascent. Research and development is focused on utilising freshwater macroalgae as a bioremediation tool for waste water streams from agriculture (Wilkie and Mulbry 2002), aquaculture (Cole et al. 2014) and municipal wastewater treatment (Neveux et al. 2016). In this sense, the primary product of freshwater macroalgae culture is reusable freshwater, with biomass as a co-product. However, like terrestrial leaf protein, it is likely that freshwater macroalgae may be unsuitable for mono-gastric livestock in a raw form due to low concentrations of protein and high concentrations of fibre, and instead may need to be processed into a concentrated form.

1.4. Seaweeds as an alternative protein crop

1.4.1. Overview of seaweed

Seaweeds are multicellular, or macroscopic, eukaryotic algae (macroalgae) that inhabit the marine environment. Seaweeds are broadly grouped based on their phylogenetic origin and pigmentation into brown (Phaeophyceae), red (Rhodophyta) and green (Chlorophyta) seaweeds (Lobban and Harrison 1997). These three distinct lineages represent over 8,000 species of seaweed (Lüning et al. 1990) that are distributed throughout tropical, temperate and artic regions (Bolton 1994). Seaweeds are generally

ephemeral and have a much simpler structure than terrestrial crops and terrestrial plants, characterised by limited cell differentiation (no differentiated root system for nutrient absorption) and a high surface area to volume ratio (Lobban and Harrison 1997). This essentially means that most, if not all, cells are able to photosynthesise and assimilate nutrients. These characteristics make seaweeds a highly productive biomass crop (Bolton et al. 2009; Mata et al. 2010; Nielsen et al. 2012; Mata et al. 2016). Seaweeds also have diverse life-history strategies, and as a consequence a large range of morphologies and environmental tolerances (Lobban and Harrison 1997; Cohen and Fong 2004; Larsen and Sand-Jensen 2006). The highly productive nature of seaweeds and their diversity offers the potential to select suitable species for the primary production of protein. However, it is the ability of seaweed to be cultivated in seawater without using traditional agricultural resources that has attracted attention to developing them as an alternative protein crop.

1.4.2. Current use and production of seaweed

In contrast to other alternative protein crops, seaweeds are commercially produced in abundance at a global scale. In 2012, there was an estimated 20 million tonnes of seaweed harvested globally (FAO 2014). Approximately half of this seaweed was used directly as a food source for humans and half as a source of phycocolloids for use in the gelling agent industry, with a minor proportion of the global harvest being used in the fertiliser industry and as feed supplements for animals (FAO 2014). Despite the dominance of seaweed use as a food source for humans, seaweeds are seldom utilised as a protein source. As a human food source, seaweeds are a high value, low volume product that provide little protein nutrition at even the highest rates of consumption. For example, the average per capita consumption of seaweed in Japan is estimated to be 5.3 g per day (Matsumura 2001) which, at a maximum protein concentration of approximately 47 % (Fleurence 1999b) provides only 2.5 g of protein in comparison to a recommended daily consumption of 46 and 56 g for an average adult female and male, respectively (Food & Nutrition Board 2004). Further, the small amount of seaweed, approximately 100,000 tonnes, that is used annually for the production of feed supplements for livestock are provided in diets at low inclusion levels (< 5 %) and also contributes a negligible amount of protein (Dierick et al. 2009; Katayama et al. 2011; Abudabos et al. 2013; Evans and Critchley 2014). Instead, seaweeds are used as a source of fibre and minerals in human food and as a functional ingredient (a food that

has proven health benefits for the prevention, management or treatment of chronic disease) in animal feeds (Evans and Critchley 2014). The only case where seaweeds are produced commercially and used as a protein source is for those animals that feed naturally on seaweeds. For example, the green seaweed *Ulva* is produced commercially using the effluent from abalone aquaculture and then used as a feed for the abalone (Bolton et al. 2009), although additional sources of protein are also required.

The vast majority of the global seaweed harvest comes from cultured seaweed (93 %), with a small and decreasing amount from wild harvested stocks (Paul et al. 2012). Seaweeds are predominantly cultured off-shore using low-technology systems, predominantly net and long-line culture. While net culture is favoured for small to medium sized red seaweeds with non-motile spores (*Pyropia* species (formally *Porphyra*)), long-line culture is used to culture large brown seaweeds (*Saccharina* and *Undaria* species) predominantly in China, and red seaweeds from fragments (*Eucheuma, Kappaphycus* and *Gracilaria*) predominantly in South-East Asia (Paul et al. 2012). However, long-line culture is most common and is responsible for over 90 % of cultured seaweed production (Paul et al. 2012). Long-line culture involves hanging seaweed from suspended synthetic ropes (10 – 60 m) that are attached to anchored buoys or directly to the substrate. These off-shore cultivation methods have high biomass productivities (120 -150 t wet weight ha^{-1} $year^{-1}$) (Gao and McKinley 1994b; Lüning and Pang 2003; Titlyanov and Titlyanova 2010), however, they are labour intensive and are only economically viable in countries with low labour costs. Despite the mass culture of seaweed traditionally taking place extensively off-shore, recent efforts in the culture of seaweed has focused on intensive inland culture systems (Bolton et al. 2009; Magnusson et al. 2014; Mata et al. 2016). These systems enable the culture of seaweeds with morphologies unsuitable for off-shore production and provide a high degree of control over the culture environment that can be geared to maximise biomass productivities while minimising land and labour requirements. However, the future success of intensive land based systems in cultivating seaweeds at scale will depend on their ability to utilise waste streams (Bolton et al. 2009; Nielsen et al. 2012; Neveux et al. 2016). This integrated approach not only provides water and nutrients at a low cost, but also provides value adding bioremediation benefits. Further, intensive land based systems also allow for the manipulation of biochemical compositions that could be directed towards the production of protein.

1.4.3. The nutritional value of seaweeds

1.4.3.1. Digestible energy

Seaweeds contain very little digestible energy, which is the proportion of potential energy in a food that is available upon digestion. The low digestible energy of seaweeds is primarily due to the high concentrations of polysaccharides and ash in seaweeds. Polysaccharides are the largest component of seaweed biomass and can represent up to 75 % of the dry weight (Kraan 2012). Most of the polysaccharide content in seaweeds is associated with cell wall material that is needed to maintain the multicellular structure of seaweeds. While the concentration of cell wall material is not as high as in terrestrial plants, the concentration of structural polysaccharides in seaweeds range from 35 to 62 % of dry weight (Kraan 2012) and represents a considerable proportion of the biomass that cannot be utilised by mono-gastric livestock for energy. The other major component in seaweeds that contributes to their low digestible energy content is ash. Ash contents are comprised of external and internal salts and minerals and usually constitute between 20 – 50 % of dry weight (McDermid and Stuercke 2003; McDermid et al. 2007). While the high concentration of ash in seaweeds make them a good source of minerals (MacArtain et al. 2007), it lowers the digestible energy content.

On the other hand, seaweeds contain low concentrations of lipids including high-energy oils. The total lipid content of most seaweeds range from 3 – 5 % of dry weight (Montgomery and Gerking 1980; McDermid and Stuercke 2003; McDermid et al. 2007), which is much less than most microalgae (~ 10 – 50 % dw) (Becker 2007; Griffiths and Harrison 2009; Huerlimann et al. 2010), terrestrial seed crops (~ 20 – 50 % dw) (NRC 2011; Issariyakul and Dalai 2014) and fishmeal (~ 10 % dw) (NRC 2011). However, there are exceptions to this as a small number of seaweed species have lipid contents higher than 10 % of dry weight (Gosch et al. 2012). Overall, the low concentration of lipids and high concentrations of polysaccharides and ash in seaweeds define them as a high fibre, high mineral content food for humans (Holdt and Kraan 2011) and a functional feed ingredient for mono-gastric livestock (Evans and Critchley 2014).

1.4.3.2. Protein and amino acids

After polysaccharides and ash, protein is the next largest fraction of seaweed biomass. Biologically, the proteins in seaweed are composed of enzymes, metabolic proteins

(such as ion pumps) and structural proteins (Lobban and Harrison 1997; Naldi and Wheeler 1999). Nutritionally, however, the 'protein' fraction in seaweed is interchangeable with the total amino acid content (TAA), which also includes free amino acids. Free amino acids are amino acids that are not bound to any other amino acids and act as the major nitrogen storage pool in seaweeds (Naldi and Wheeler 1999). The concentration of protein or TAA in seaweeds varies substantially between species and has been reported to range from as low as 3 % dry weight to as high as 47 % dry weight (Fleurence 1999b; Fleurence et al. 2012). Red seaweeds generally have the highest concentration of protein, followed by green and then brown seaweeds (Fleurence 1999b), however, the variation within these taxonomic divisions is high. Similarly, the quality of protein in seaweeds (proportion of protein as essential amino acids) is variable, but generally considered high compared to traditional agricultural crops. Seaweed proteins contain particularly high concentrations of aspartic acid and glutamic acid, but also relatively high concentrations of essential amino acids compared to traditional agricultural crops (Fleurence 1999b; McDermid and Stuercke 2003; McDermid et al. 2007; Nielsen et al. 2012), in particular the essential amino acid methionine (Boland et al. 2013).

In addition to the high variation between species of seaweed, the concentration of protein is also highly variable within species of seaweed. This within-species variation is directly linked to variation in the concentration of internal nitrogen (N), which in turn is heavily dependent on environmental conditions, particularly in relation to the external supply of N in the environment (Hanisak 1977, 1979, 1983; Lignell and Pedersen 1987; Hanisak 1990; Pedersen and Borum 1996; Harrison and Hurd 2001). For example, when the supply of external N is increased for *Ulva lactuca* grown under N-limiting conditions, the internal concentration of N in this seaweed increases from approximately 1 to 5.5 % dry weight and this increases the specific growth rate asymptotically from less than 5 % d^{-1} to over 40 % d^{-1} (Pedersen and Borum 1996).

Despite many empirical studies focused on profiling the protein and amino acid nutrition of seaweeds, few have taken into account the high within-species variability in internal N and its relationship with the concentration and quality of protein, and the critical factors of growth rate and environmental parameters, for developing seaweeds as a protein crop.

1.5. Aims and chapter summary

The overarching aim of this thesis is to investigate the potential of seaweeds as a new biomass crop for the production of protein. The first two chapters of this thesis (Chapters 2 and 3) investigate the effects that growth and biomass production rates have on the protein nutrition of seaweeds. This was done by examining the relationship between culture conditions, growth rate and the considerable variability of the concentration and quality of protein within species of seaweeds using the green seaweed *Ulva ohnoi* as a model. *Ulva ohnoi* is from a cosmopolitan genus that can be easily cultured in intensive land-based systems.

Chapter 2 investigates the within-species variation in the concentration of protein and amino acids in seaweeds by examining their relationship with N supply and growth rate for *Ulva ohnoi*. The first aim was to quantify the relationship between the internal N content and growth rate of *U. ohnoi* under a range of external N supplies that encompassed both N limiting conditions and non-N limiting conditions. The supply of N and non-N resources were manipulated in a unique two-way assessment by manipulating water N concentration and water renewal rates. This resulted in a variety of N supply conditions for cultures of *U. ohnoi* that were, in turn, growing at variable rates and generated a large range of internal N contents. The second aim was to quantify the relationship between the internal N content and both the concentration and quality of protein in *U. ohnoi*, with a specific focus on the concentration of two essential amino acids that are often limiting in livestock diets, methionine and lysine. A conceptual relationship between growth rate, internal N and the concentration of protein and amino acids was developed to explain within-species variation in protein content that is important when also considering biomass productivity as critical factor.

The concentration and quality of protein in *U. ohnoi* was found to be strongly related to growth rate under non-N limiting conditions. This suggested that environmental variables could have the primary influence on within-species variation in the concentration and quality of protein under non-N limiting conditions, doing so indirectly by dictating the growth rate of the seaweed.

Therefore, in **Chapter 3** the within-species variation in the concentration and quality of protein in *U. ohnoi* was further investigated by examining the indirect and direct effects

of salinity. The first aim of this chapter was to investigate whether salinity, an important example of a "non-N" environmental parameter, can also indirectly influence the concentration and quality of protein through growth rate. The second aim of this chapter was to investigate the relative importance of the direct effects of salinity on the concentration and quality of protein in *U. ohnoi*, especially in regards to the essential amino acid methionine because of its links to the osmolyte dimethylsulfoniopropionate (DMSP). *U. ohnoi* was grown under a range of salinities (from 10 to 60 ‰) under non-nutrient limiting conditions. The salinity treatments selected represent the broad range associated with the tropical environment under which the species grows. Additionally, biomass productivities were measured at each salinity in conjunction with the concentration and quality of protein to estimate the areal productivities of protein and essential amino acids for *U. ohnoi* and to evaluate the implications of within-species changes in the concentration and quality of protein for developing this seaweed as a protein crop.

The high within-species variability in the concentration and quality of protein, and its dynamic relationship with the internal N content of seaweeds, highlighted the importance of accurate methods for determination of protein in seaweeds, especially when nitrogen-to-protein conversion factors are used.

Therefore, **Chapter 4** assessed the accuracy of different methods for measuring protein with the goal of recommending the most appropriate method to determine protein in seaweeds. The first aim of this chapter was to review the literature to quantitatively describe the methods that are currently used and their suitability. The second aim of this chapter was to provide improvements to (and insights into) the commonly used method of determining protein through the N*6.25 conversion factor by developing and proofing a universal N-protein conversion factor that is specific to seaweeds. This was done by consolidating available nitrogen and total amino acid data and calculating N-protein conversion factors for 103 seaweed species. These species-specific N-protein conversion factors were then analysed for associations between the critical variables of taxonomic groups, geographic regions, cultivated and wild harvested seaweeds, and internal N content.

In **Chapter 4** it was demonstrated that most of the quantitative measures of protein in the literature have been overestimated through the use of the generic N-protein

conversion factor of 6.25. Further, it became evident that the concentration of essential amino acids on a whole biomass basis was rarely used to assess the nutritional value of seaweeds.

Therefore, in **Chapter 5** the data on the essential amino acid extracted from the literature compiled in **Chapter 4** were used to re-assess the potential of seaweeds more broadly as a protein source for mono-gastric livestock. The first aim of this chapter was to present the quantitative amino acid data for > 100 seaweeds, both as a proportion of protein and whole biomass, and compare these to the traditional protein sources of soybean meal and fishmeal. The second aim was to then compare the concentration of essential amino acids in seaweeds on a whole biomass basis to the requirements of mono-gastric livestock. This quantitative data, in conjunction with a review of published feeding trials using seaweed, was used to assess the positive and negative aspects of using seaweeds in a whole form in the compound diets of mono-gastric livestock. Finally, it was proposed that the isolation and concentration of protein will make seaweeds more accessible as an ingredient in compound diets, although there was a distinct knowledge gap for such an application in this field.

Therefore, in **Chapter 6** multiple techniques were examined to isolate and concentrate protein in a seaweed, returning to the model organism the green seaweed *Ulva ohnoi*. The aim of this chapter was to compare the protein isolation and concentration efficiency of a mechanical-based method (as applied to leaves) to the solvent based method (as applied to seed crops). The procedural variables that optimised protein isolation and concentration in these methods were assessed in a factorial design. The three factors examined were (1) the starting material as fresh and pulped biomass or dry and milled biomass, (2) the biomass to solvent ratio in the aqueous extraction and (3) the extraction time of the aqueous extraction.

In **Chapter 7** the implications of the major findings of the previous chapters were synthesised and discussed in the context of three key factors that, in my opinion, are the key to success for developing seaweeds as a protein crop. These factors are (1) identifying suitable species for protein production, (2) optimising culture protocols for protein production, and (3) processing seaweed biomass post-harvest for protein production.

Chapter 2: Variation in amino acid content and its relationship to nitrogen content and growth rate in *Ulva ohnoi* (Chlorophyta)[1]

2.1. Introduction

Proteins, or the amino acids from which they are made, are the critical constituent in animal feeds, specifically the essential amino acids methionine and lysine as these are the "first" limiting amino acids in plant-based feed formulations (McDonald et al. 2002; Boland et al. 2013). Amino acids are also targeted as a feedstock for biorefinery in the bio-based chemical industry (Scott et al. 2007; Jung et al. 2013). In this scenario, it is the non-essential amino acids that are the preferred primary substrates for bio-based chemicals, specifically glutamic acid which resembles many industrial intermediates (Lammens et al. 2012). The extraction and concentration of nitrogenous biochemicals is now proposed as a common value-added component of most biofuel conversion and modelling (Ragauskas et al. 2006). Together these applications promote the use of high productivity biological feedstocks for feed and bio-based chemicals before the remaining biomass is converted to a biofuel, for which algae have received much attention (Ragauskas et al. 2006; Rowbotham et al. 2012). However, relatively little is known about the relationship between internal nitrogen content, growth rate and the quantitative and qualitative changes in amino acids for seaweeds compared to terrestrial plants (see Steinlein et al. 1993; Heilmeier et al. 1994; Lipson et al. 1996), and, correspondingly, whether internal nitrogen content can be manipulated to maximise the yields of specific amino acids.

Many plants have considerable compositional plasticity in nitrogen content with strong links to growth and nitrogen availability (Greenwood et al. 1991; Gastal and Lemaire 2002). This plasticity is related to nitrogen limitation (Greenwood et al. 1990) or the luxury uptake of nitrogen when it is available in excess to that required for immediate growth (Chapin et al. 1990; Lipson et al. 1996). These two nitrogen states revolve around the critical N content, which is defined as the minimum nitrogen content that allows for maximum growth rate (Ulrich 1952). Nitrogen contents above this value therefore represent nitrogen stores. The idea of storing nitrogen for use at a later date is

[1] **Chapter 2** is adapted from Angell, A. R., Mata, L., de Nys, R. & Paul, N. A. 2014. Variation in amino acid content and its relationship to nitrogen content and growth rate in *Ulva ohnoi* (Chlorophyta). *Journal of Phycology* **50**:216-26

a well-founded concept for long-lived terrestrial plants, but some marine macroalgae (seaweeds) also have considerable nitrogen content plasticity (Hanisak 1983). Seaweeds are typically ephemeral and have a much simpler structure than terrestrial plants, characterised by limited cell differentiation and a high surface area to volume ratio (Lobban and Harrison 1997). This essentially means that all cells are able to both photosynthesise and assimilate nutrients. Seaweeds can also be cultured intensively in tumble culture to create a homogenous environment in which the entire biomass has equal access to all resources, including light and nutrients. Such a cultivation system allows for the delivery of nitrogen to be manipulated by either varying both water nitrogen concentration and renewal rates simultaneously. Water nitrogen concentration (Hanisak 1979; Bjornsater and Wheeler 1990; Pedersen and Borum 1996) and water renewal rates (Mata et al. 2010) influence both internal nitrogen content and growth rate, but have not been examined simultaneously for their effects on nitrogen storage and partitioning in the production of amino acids.

Green seaweeds (Chlorophyta) belonging to the genus *Ulva* are strong candidates for the production of protein due to their high growth rates in excess of 20 g dry weight m^{-2} d^{-1} (Bolton et al. 2009; Nielsen et al. 2012; Mata et al. 2016) and wide environmental tolerances (Cohen and Fong 2004; Larsen and Sand-Jensen 2006; Mata et al. 2016). *Ulva* spp. are also particularly plastic in nitrogen content, ranging from 0.51 % (Renaud and Luong-Van 2006) to over 5 % of dry weight (Mata et al. 2010; Nielsen et al. 2012). This large range likely encompasses nitrogen limitation, where internal nitrogen content limits growth (Hanisak 1983; Harrison and Hurd 2001) through to luxury uptake, where additional nitrogen beyond requirements for growth is accumulated (Harrison and Hurd 2001; Naldi and Viaroli 2002). The controlled cultivation of *Ulva* for protein production is complicated because nitrogen assimilation can promote the synthesis of metabolic, structural or storage compounds including nitrate (Duke et al. 1986; Naldi and Wheeler 1999), free amino acids (Bird et al. 1982; Jones et al. 1996; Naldi and Wheeler 1999), proteins (Bird et al. 1982; Smit et al. 1996; Naldi and Wheeler 1999), enzymes (Duke et al. 1987) and pigments (Bird et al. 1982; Smit et al. 1996; Naldi and Wheeler 1999). In addition, the common target amino acids, methionine, lysine, glutamic acid and glutamine have different functions in the cells, and may therefore respond differentially to culture manipulations (Taylor et al. 2006). Notably, the relationship between internal

nitrogen content and the concentration and quality of protein has not been elucidated or related to the targeted production of amino acids in *Ulva* spp.

Therefore, the aim of this chapter was to manipulate the internal nitrogen content in outdoor cultures by manipulating the supply of nitrogen to examine the interactions between protein concentration, quality and productivity in the green seaweed *Ulva ohnoi* M. Hiraoka & S. Shimada. The overall goal was to characterise, for the first time, the nitrogen states of *U. ohnoi* in intensive cultivation. Nitrogen was supplied in a unique two-way assessment by manipulating water nitrogen concentration and water renewals to assess the quantitative changes in the concentration and quality of protein with internal nitrogen content and growth rate. This data was then used to create a conceptual relationship between internal nitrogen content, growth rates and the concentration and quality of protein.

2.2. Materials and methods

2.2.1. Study organism

The green seaweed *Ulva ohnoi* (Lawton et al. 2013b; Genbank accession number KF195501, KF195536) was collected from an aquaculture facility in Guthalungra, Queensland, Australia (19° 55' 27'' S, 147° 50' 37'' E) and domesticated at the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University for > 12 months prior to experiments. The culture experiment was run in an outdoor greenhouse in the austral winter (photoperiod; 12.5h light:11.5 h dark). *U. ohnoi* was cultured in individual 4 L cylindrical opaque containers (surface area = 0.035 m^2, height = 170 mm), with a constant supply of air provided at the base to tumble the biomass, which was situated inside a water bath to maintain temperature control.

2.2.2. Experimental design and procedure

The interactive effects of water nitrogen concentration and water renewal rate on internal N content, protein concentration (total amino acid concentration) and protein quality (individual amino acids as a proportion of protein of TAA) were investigated. A stocking density of 4 g L^{-1} (fw) was used as this density has previously been identified as providing a higher N content and slightly higher areal biomass productivities than 1 g L^{-1} (Angell et al. 2014). *U. ohnoi* was cultured at three water nitrogen concentrations (low nitrogen LN = 20.65 μM L^{-1}, medium nitrogen MN = 86.41 μM L^{-1} and high

nitrogen HN = 183.15 μM L^{-1}) and ten water renewal rates ranging from ~ 14 % h^{-1} to ~ 333 % h^{-1}. The combinations of these water nitrogen concentrations and flow rates resulted in N flux ranges of 3.10 – 68.74 μM L^{-1} h^{-1} for LN, 7.89 – 96.25 μM L^{-1} h^{-1} for MN, and 14.89 – 163.71 μM L^{-1} h^{-1} for HN. The combinations of flow rates provided overlapping N flux for each water nitrogen concentration enabled direct comparisons of nitrogen fluxes from 7.89 to 96.25 μM L^{-1} h^{-1} (Fig. 2.1).

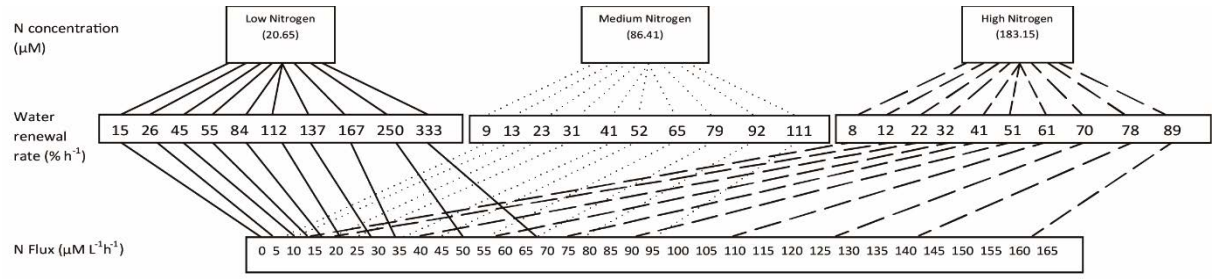

Figure 2.1. Experimental design. Three nitrogen concentration treatments (low = 20.65 μM, medium = 86.41 μM and high = 183.15 μM) were provided at ten water renewal rates. The water renewal rates were selected so that multiple nitrogen fluxes were provided by either two or three nitrogen concentration treatments. This experimental design allowed for the delivery of nitrogen flux to be manipulated over a large range of nitrogen fluxes which was aimed at varying growth rates under varying nitrogen supplies (nitrogen flux).

Cultures were maintained in a flow-through, single pass system to provide the water N concentration treatments dosed with sodium nitrate ($NaNO_3$). Total N (16.31 ± 0.61 μM) and P (0.92 ± 0.11 μM) were measured in seawater using OI Analytical Flow IV Segmented Flow Analysers (APHA 4500-NO_3^- F and APHA 4500-P F) after alkaline persulfate digestion prior to the addition of $NaNO_3$. Inorganic N (NO_3^-, NO_2^- and NH_4^+) was measured in each treatment header tank (APHA 4500-NO_3^- F, APHA 4500-NO_2^- F and APHA 4500-NH_3 G) throughout the experimental period and a mean calculated for each treatment (see above). Water quality analysis for this experiment was carried out by the Australian Centre for Tropical Freshwater Research (ACTFR), James Cook University, Townsville (APHA 2005). Water renewal rates were measured and adjusted daily throughout the entire culture period. Cultures underwent an initial acclimation period of 25 days before a final six day experimental period. At the end of each week of acclimation, all cultures were harvested and weighed before being stocked back to their respective stocking densities. The initial (fw_i) and final weights (fw_f) of each culture in the final experimental period (final six days) were used to calculate the specific growth rate (Equation 2.1):

$$Specific\ growth\ rate\ (SGR) = {ln\left(\frac{fw_f}{fw_i}\right)}/{days} \quad \textbf{Eq. 2.1}$$

After the final experimental period, all biomass from each culture was freeze-dried for internal N and C content and amino acid analysis (see section 2.2.3.). The fresh weight (fw) to dry weight (dw) ratio was calculated for each culture (60˚C oven dried until constant weight) to convert fresh biomass growth rates to dry weight biomass productivity (g dw m^{-2} d^{-1}) using the surface area of each culture (0.035 m^2). Biomass productivity was then converted to protein productivity using the total amino acid content (% dw) for each culture (see section 2.2.3.).

Temperature and pH were measured hourly during the experimental growth period from 07:00 to 18:00 in all cultures at the beginning (day 1) and end (day 5) using a portable probe (YSI model 63, USA). Maximum pH occurred at 14:00 and ranged from 8.03 ± 0.01 – 8.66 ± 0.02. Temperature ranged from a minimum of 20.19 ± 0.10 ˚C to a maximum of 26.02 ± 0.07 ˚C. Salinity was monitored throughout the experiment using a portable probe (YSI model 63, USA) and was constant at 33.5 ‰. The photosynthetically active radiation (PAR) at the surface of the cultures inside the greenhouse was logged every five minutes using a Li-190SA Quantum Sensor connected to a Li-1400 Data Logger (Li-Cor, Lincoln, NE, USA) close to the experimental setup. Total irradiance during the six day experimental period was 126.24 mol photons m^{-2} at the surface of the cultures, with intensity reaching a maximum of 1163 µmol m^{-2} s^{-1}.

2.2.3. Compositional analysis

Nitrogen and Carbon content were quantified using an elemental analyser (OEA laboratory Ltd., UK). The internal N and C (see Table S2.1) content is reported as grams per 100 g dry weight (% dw).

To quantify changes in the concentration and quality of protein with varying internal N content, all cultures were analysed for amino acids. All cultures were analysed for aspartic acid, asparagine, glutamic acid, glutamine, serine, histidine, glycine, threonine, alanine, arginine, tyrosine, valine, methionine, phenylalanine, isoleucine, leucine, lysine

and proline (Table S2.1). As asparagine is hydrolysed to aspartic acid and glutamine to glutamic acid during analysis, the sum of these amino acids are reported as asparagine/aspartic acid or glutamic acid/glutamine. The two remaining proteome amino acids, cysteine and tryptophan, were not analysed as they are minor constituents in *Ulva* spp. (Angell et al. 2012; Angell et al. 2014). Amino acids were analysed after 24 hour liquid hydrolysis in 6M HCl at 110˚C using a Waters ACQUITY UPLC at the Australian Proteome Analysis Facility, Macquarie University, Sydney using procedures based on the Waters AccQTag amino acid methodology (Cohen 2000; Bosch et al. 2006).

2.2.4. Data Analysis

Internal N content (% dw) and SGR (% d^{-1}) were plotted against N flux. Curves of best fit were applied for both relationships using SigmaPlot 10.0 (R^2 values reported). The quality of protein in biomass was analysed using non-metric multidimensional scaling (nMDS) using the statistical software PRIMER (PRIMER-E Ltd., Lutton, UK). A similarity matrix was calculated from the 4^{th} root transformed with individual amino acids contents (as a percentage of total amino acid content), N % and SGR as variables in the MDS cluster diagram and vector plot. Protein (as total amino acids), methionine, lysine and glutamine/glutamic acid contents (g $100g^{-1}$ dw) were plotted against internal N content for each water N concentration treatment. Linear correlations were made for internal nitrogen content versus total amino acids, methionine, lysine and glutamic acid/glutamine contents (SigmaPlot 10.0, r values reported). A linear correlation was also made for specific growth rate versus glutamic acid/glutamine contents.

2.3. Results

2.3.1. Growth rate and nitrogen content

Both internal N content and SGR varied substantially across the range of N fluxes supplied through the three water N concentrations and varying water renewal rates. Overall, internal N contents varied from 0.6 % to 4.2 % and SGR from 2.0 % d^{-1} to 11.7 % d^{-1} (Fig. 2.2A & B). The internal N content can be allocated to one of three nitrogen states based on the relationship with growth rate.

The first N state is defined by the critical nitrogen (hereafter referred to as critical N) content as the upper limit, 1.2 %, which corresponded with the maximal growth rate 11.7 % d^{-1}. This nitrogen-limited state (0.6 – 1.2 %) occurred in algae cultivated with N flux < ≈ 17 μM L^{-1} h^{-1}, supplied by the low nitrogen concentration (LN - 20.65 μM) treatment. Increases in internal N content in this state were coupled with an asymptotic increase in SGR, which reached a maximum at ≈ 11.7 % d^{-1} at a N flux of ≈ 17.2 μM L^{-1} h^{-1}.

The second nitrogen state is immediately above the critical N content (1.2 %), where additional N was assimilated beyond the requirements for growth. However, this additional N assimilation only occurred up until a threshold of 2.6 % N when *U. ohnoi* was growing at maximal rates. Internal N contents within this range occurred in seaweed cultivated with N fluxes of 17 - 69 μM L^{-1} h^{-1} supplied by the low nitrogen concentration at higher water renewal rates. Cultures within this internal N content range had SGRs that were the highest of all cultures (11.7 % d^{-1}).

The third N state is where internal N content increased beyond 2.6 % until the maximum of 4.2 % and growth rates were below maximum (11.7 % d^{-1}). This only occurred in the medium (86.41 μM) and high (183.15 μM) water N concentration treatments. In these cultures SGR increased linearly with N flux to maxima of 10.0 and 8.6 % d^{-1} at N fluxes of 95.6 μM L^{-1} h^{-1} and 163.7 μM L^{-1} h^{-1}, respectively for MN and HN cultures.

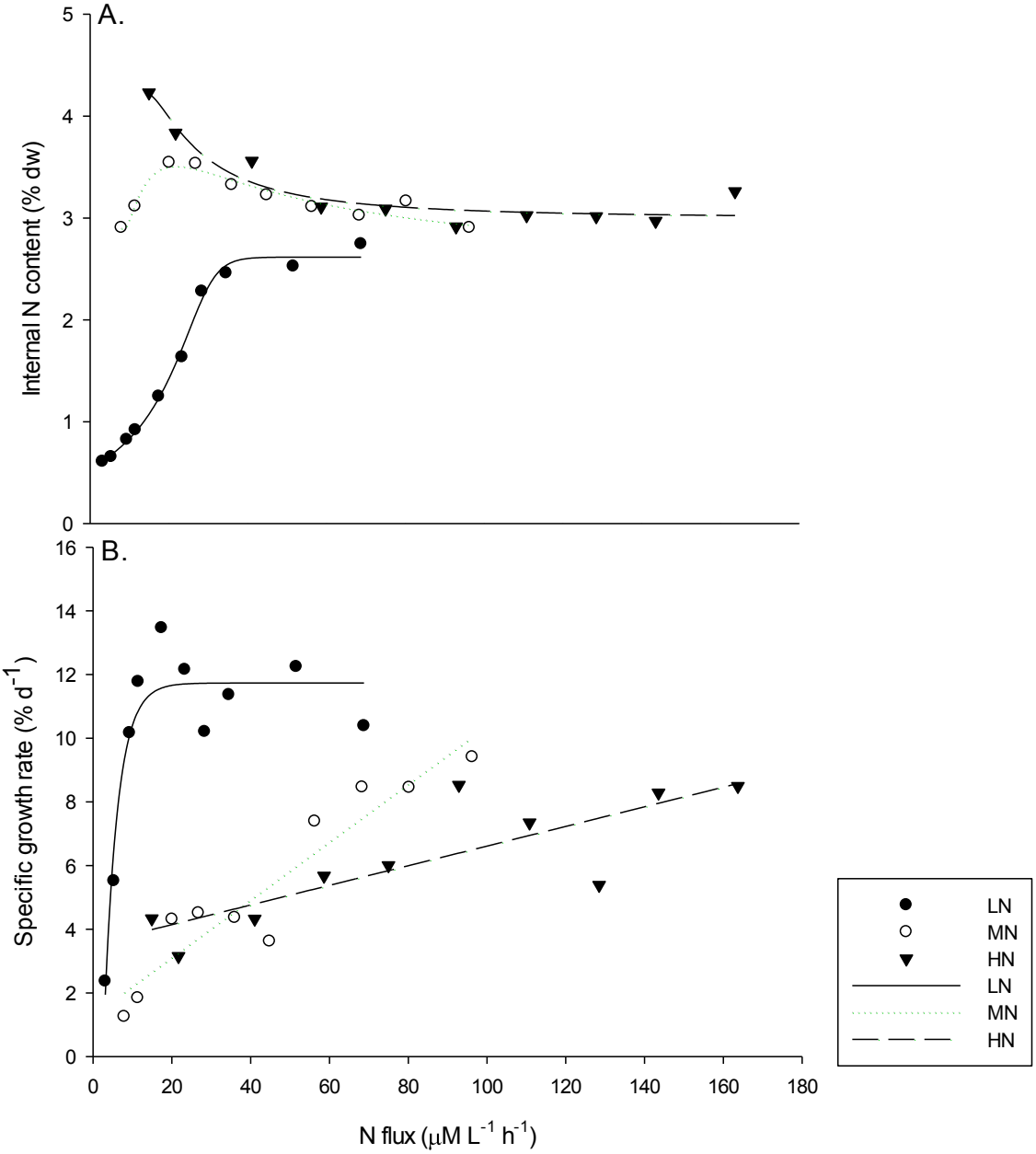

Figure 2.2. (A) Internal N content and (B) specific growth rate against N flux for low (LN – 20.65 μM), medium (MN – 86.41 μM) and high (HN – 183.15 μM) water nitrogen concentrations. (A) R^2 = 0.993, 0.911 and 0.916 for LN, MN and HN respectively. (B) R^2 = 0.901, 0.890 and 0.677 for LN, MN and HN respectively.

2.3.2. Amino acid quality

The substantial variation in internal N content was coupled with quantitative and qualitative variation in amino acids. The nMDS plot and vector loadings (Fig. 2.3A & B) illustrate the major qualitative changes in amino acid profile (each amino acid as a proportion of TAA) as internal N content shifts from 0.6 – 4.2 %. Low nitrogen content

U. ohnoi (Fig. 2.3A, "1: 0.6 – 1.2 % N") had higher proportions of valine, alanine, threonine, serine, glycine and phenylalanine relative to the other N states (Fig. 2.3B). As internal N content increased (Fig. 2.3A, "2: 1.2 – 2.6 % N"), there was a shift in the proportion of specific amino acids. Histidine, tyrosine, methionine, isoleucine, leucine aspartic acid/asparagine and proline were all present at relatively higher proportions in *U. ohnoi* (Fig. 2.3B) where nitrogen was not limiting and growth rate was high (1.2 – 2.6 % N). When internal N content increased beyond 2.6 % there was a major increase in the proportion of the amino acids glutamic acid/glutamine and arginine (Fig. 2.3A, "3: 2.6 – 4.2 % N"), which negatively correlated with growth rate ($r = -0.809$, $F_{1,18} = 33.99$, $p < 0.0001$). This qualitative variation was related to the substantial increases in the concentration of these amino acids rather than any decrease in the concentration of other amino acids (see below).

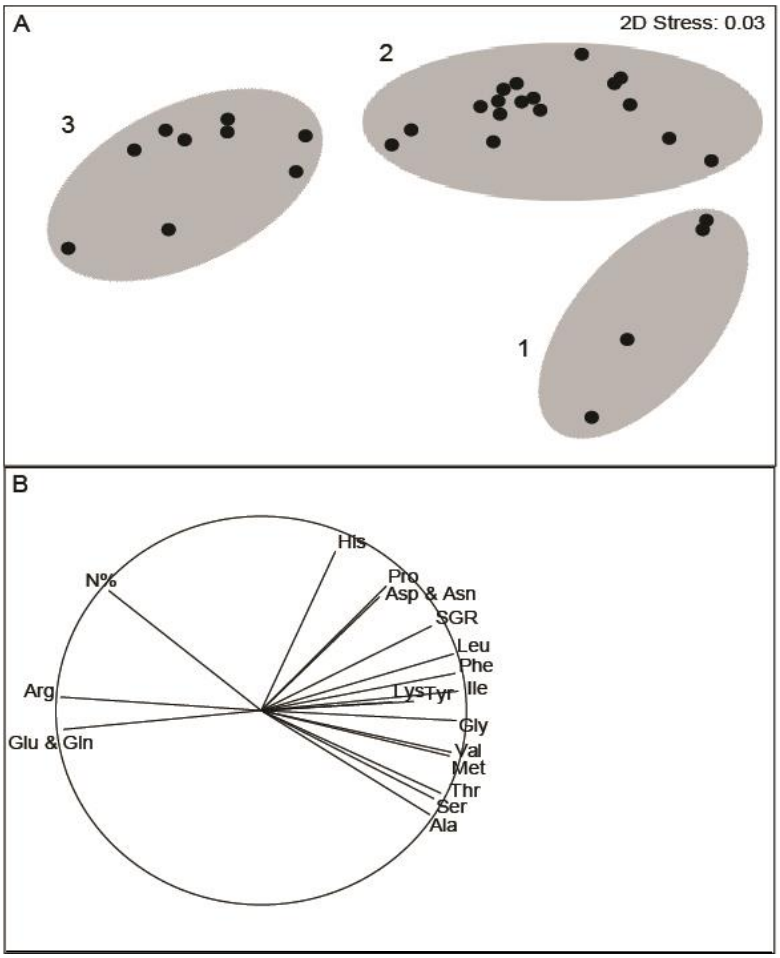

Figure 2.3. Multidimensional scaling plot showing similarity between *U. ohnoi* cultured under different N-fluxes using low (LN – 20.65 µM), medium (MN – 86.41 µM) and high (HN – 183.15 µM) water nitrogen concentrations. (A) MDS plot (Stress = 0.03) with groups superimposed (1 = 0.6 – 1.2 % N, 2 = 1.2 – 2.6 % N, 3 = 2.6 – 4.2 % N). (B) Associated vector plot of the MDS. The length and direction of which indicates the strength of the correlation and direction of change between the two MDS axes.

2.3.3. Amino acid concentration

The protein concentration (TAA concentration) varied from 2.98 g $100g^{-1}$ dw to 18.72 g $100g^{-1}$ dw and increased linearly with internal N content ($r = 0.987$, $F_{1,28} = 1044.47$, $p < 0.0001$; Fig. 2.5A). However, there was also variation in specific amino acids relative to internal N content and these trends could be divided into three groups of amino acids best represented by methionine, lysine and glutamic acid/glutamine (Fig 2.4B - D). Methionine (trend 1) increased from a low of 0.05 $100g^{-1}$ dw to a maximum threshold of 0.22 g $100g^{-1}$ dw with an increase in internal N content up to 2.6 % (Fig. 2.5B; $r = 0.971$, $F_{1,8} = 131.95$, $p < 0.0001$ for linear increase up to 2.6 %). Concentrations of proline, tyrosine and leucine also followed this trend (Table S2.1). Secondly, lysine (trend 2) increased in a similar fashion to methionine up to the internal N content of 2.6 % from a low of 0.16 g $100g^{-1}$ dw in the most N limiting cultures to 0.69 g $100g^{-1}$ dw at an internal N content of 2.6 % (Fig. 2.4C). However, the lysine concentration continued to rise linearly with internal N content, until a threshold of ~ 0.95 g $100g^{-1}$ dw at an internal N content of ~ 3.3 % N ($r = 0.983$, $F_{1,18} = 528.91$, $p < 0.0001$). This trend was similar for aspartic acid/asparagine, alanine, phenyalanine, isoleucine, glysine, histidine, serine, threonine and valine. Thirdly, glutamic acid/glutamine (trend 3) increased linearly with increasing internal N content up to 2.6 % ($r = 0.992$, $F_{1,8} = 475.98$, $p < 0.0001$). However, glutamic acid/glutamine continued to increase in concentration until the maximum N content (4.2 %), almost tripling from 1.3 g 100 g^{-1} (at 2.6 % N) to 3.7 g 100 g^{-1} (Fig. 2.4D). This corresponded to almost a doubling in the proportion of total amino acids to 20 %, with 38 % of free amino acids represented by glutamic acid/glutamine. Arginine was the only other amino acid that also followed this trend, increasing from 0.8 to 2.4 g $100g^{-1}$ (up to 13 % of total and 26 % of free amino acids) when internal N content increased from 2.6 to 4.2 %.

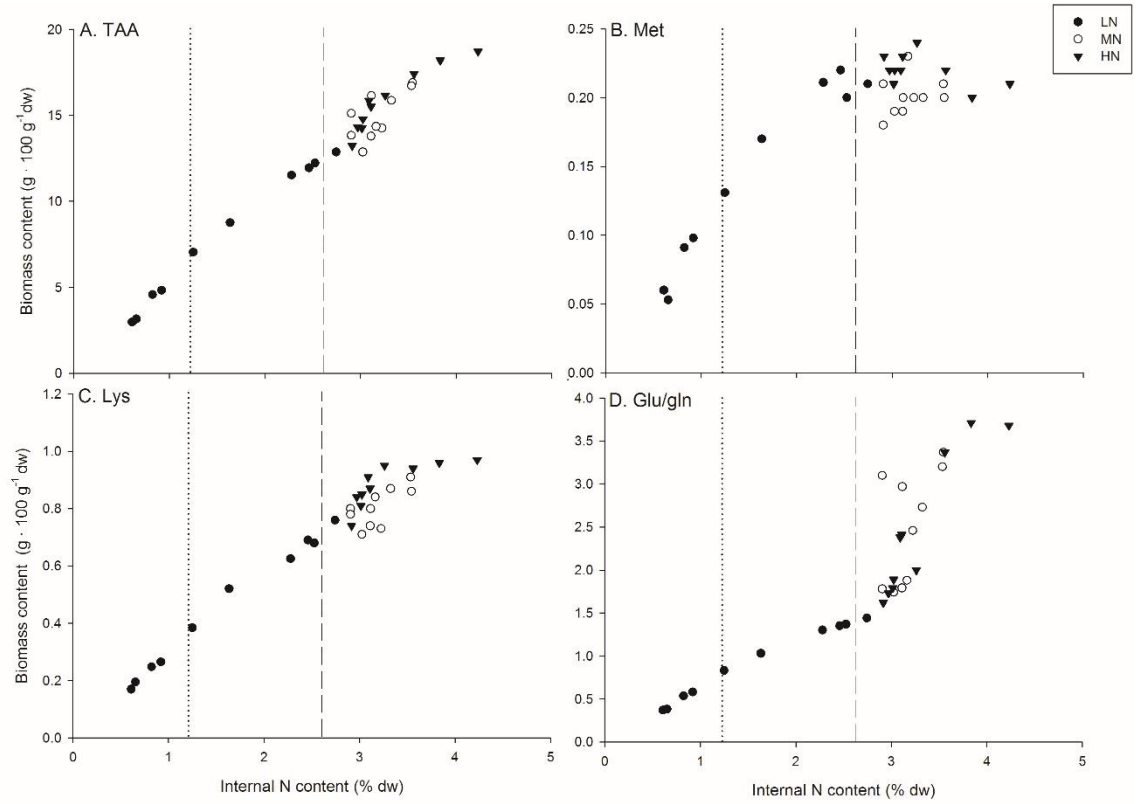

Figure 2.4. Content (g) of (A) total amino acids, (B) methionine, (C) lysine and (D) glutamic acid/glutamine per 100 g dry biomass in relation to internal N content in *U. ohnoi* cultured under varying nitrogen fluxes and water nitrogen concentrations (LN = 20.65, MN = 86.41 and HN = 183.15 µM). Dotted lines indicate the critical N content measured in this study (1.2 %) and dashed lines indicate a N content of 2.6 % where amino acids such as methionine reached a threshold. Correlations: (A) $r = 0.987$, $p < 0.0001$ over all water nitrogen concentration treatments; (B) $r = 0.971$, $p < 0.0001$ LN treatment only; (C) $r = 0.983$, $p < 0.0001$ LN and MN treatments; (D) $r = 0.992$, $p < 0.0001$ LN treatment only.

The protein productivities (as a function of biomass productivity and % total amino acid, dw) varied between each of the N concentration treatments (Fig. S2.1). The highest protein productivity of 2 g m^{-2} d^{-1} occurred in the low N treatment at a N flux of 68.74 µM h^{-1} (331.6 % d^{-1}) in seaweed with an internal N content of 2.6 %. The highest protein productivities for the medium (1.83 g m^{-2} d^{-1}) and high (1.60 g m^{-2} d^{-1}) N concentration treatments occurred at a N flux of 96.25 µM L^{-1} h^{-1} (111.4 % d^{-1}) and 163.71 µM L^{-1} h^{-1} (89.4 % d^{-1}), respectively in seaweed with an internal N content of ~ 3 %.

2.3.4. Amino acids and the N status in *Ulva ohnoi*

The interaction between amino acids and the internal N content in the three nitrogen states of *Ulva ohnoi* is best summarised in a conceptual schematic of internal nitrogen content versus growth rate (Fig. 2.5). Below the critical internal N content (dotted line)

the seaweed is in a nitrogen limited state and nitrogenous compounds are structural and metabolic in nature. Increases in internal N content up to the critical N content are represented by increases in all amino acids, which correlate strongly with growth rate (see Fig 2.2B). Above the critical internal N content, changes in internal N do not influence growth rate yet N is taken up and assimilated into all amino acids. Because methionine is the start codon for protein synthesis, the increases in all amino acids immediately above the critical N content and up until 2.6 % internal N suggest that these amino acids have metabolic function. This nitrogen state is therefore referred to as the metabolic nitrogen state. However, beyond an internal N content of 2.6 %, methionine no longer increases in concentration (Fig. 2.4B). Further increases in internal N content are therefore referred to as the luxury nitrogen state and the point at which this occurs (2.6 % internal N) is nominally the luxury point (dashed line). The luxury state only occurred when growth rate was limited but nitrogen was not. Amino acid synthesis in this luxury state is in the form of free amino acid pools (FAAP) and is divided into two stages. The primary stage (1˚ luxury uptake from 2.6 – 3.3 % internal N) is defined by increases in all amino acids other than methionine, proline, tyrosine and leucine (e.g. lysine, Fig. 2.4C). However, the majority of the increases in the FAAP (2˚ luxury uptake: ≈ 3.3 – 4.2 % internal N) related only to glutamic acid/glutamine (Fig. 2.4D) and arginine.

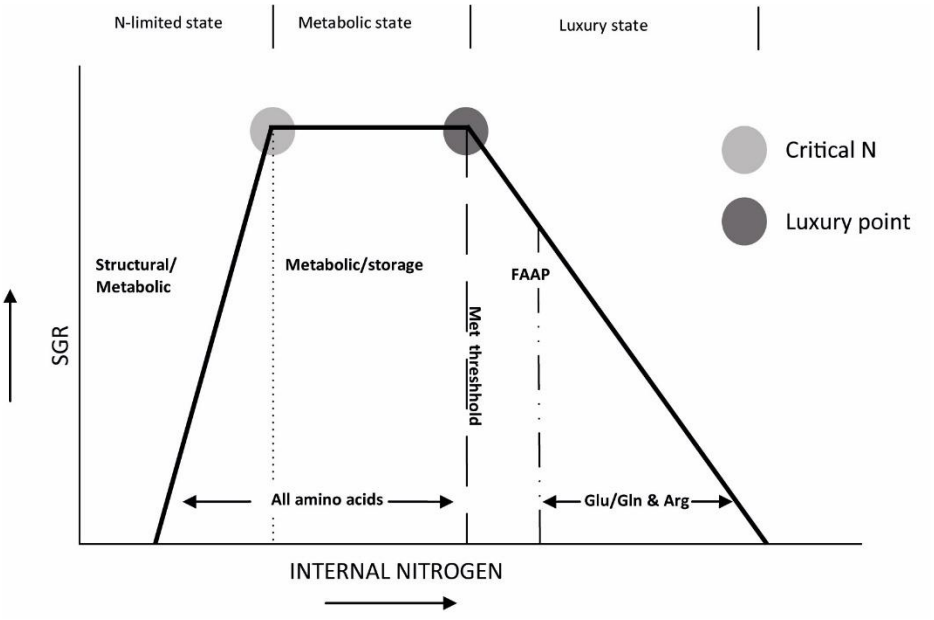

Figure 2.5. Conceptual diagram highlighting the correlative relationships between internal nitrogen content, specific growth rate (SGR) and qualitative variation in amino acids observed over the three N states for *U. ohnoi*. Key points highlighted: critical N (dotted), luxury point (dashed) and 2˚ shift of luxury (dot-dash). Met = methionine, Glu/Gln = glutamic acid/glutamine and Arg = arginine.

2.4. Discussion

Opportunistic green seaweeds such as *Ulva* have many attributes that make them attractive for the commercial production of amino acids, including high biomass productivities (Mata et al. 2016) and wide environmental tolerances (Cohen and Fong 2004; Larsen and Sand-Jensen 2006). In this study both the internal N content and growth rate of *Ulva ohnoi* were manipulated in culture by altering the way in which nitrogen flux is achieved, by either changing water nitrogen concentration or renewal rates. These results demonstrate that variation in internal nitrogen content is hinged at two points: a critical N content (1.2 % N), below which growth was limited, and what is defined here as the "luxury point" (2.6 % N), above which there is luxury uptake of N and assimilation into free amino acids. The three nitrogen states of *U. ohnoi* (Fig. 2.5) – N-limited (0.6 – 1.2 %), metabolic (1.2 – 2.6 %), and luxury (2.6 – 4.2 %) – were defined by the quantitative and qualitative differences in amino acids and importantly represent steady state biomass that can be maintained in culture with a stable supply of water nitrogen concentration and water renewals. This enabled, for the first time, the qualitative changes in free amino acids in the luxury state to be differentiated into two phases, the first, a small increase in the majority of amino acids (including lysine) followed by a second large increase in only three amino acids (glutamic acid/glutamine and arginine). Together these empirical results for *U. ohnoi* contribute to the fundamental understanding of the nitrogen physiology of seaweeds (Hanisak 1979, 1983; Lignell and Pedersen 1987; Hanisak 1990; Pedersen and Borum 1996; Harrison and Hurd 2001) but also provide new insights on manipulating N states in the emerging biomass applications of seaweeds to target amino acids for nutrition or bio-based chemicals.

Nitrogen limitation in seaweeds hinges on a variable known as the critical N content, which is the internal N content that just limits growth (Ulrich 1952). Internal N contents above or below this critical value indicate nitrogen reserves or nitrogen limitation, respectively. In this study, the growth rate of *U. ohnoi* peaked at the relatively low internal N content of 1.2 %, which is therefore the critical N content in the outdoor tank-based cultivation system used in this study. The critical N content of *U. ohnoi* is lower than those reported for other *Ulva* species, for example, 2.5 and 3.2 % for *U.*

intestinalis and *U. fenestrata* respectively (Bjornsater and Wheeler 1990) and also lower than other seaweed genera, for example 1.9 % for the green seaweed *Codium fragile* (Hanisak 1979) and 2 % for the red seaweed *Gracilaria tikvahiae* (Hanisak 1987). The low critical N for *U. ohnoi* in this study highlights that this species is able to maintain growth rates with a low internal N content, which is a positive trait for biomass crops that aim to maximise productivity with minimal nutrient inputs.

The qualitative changes in amino acid up to the critical N content represent structural and metabolic proteins required for growth rather than free amino acid pools (Hanisak 1983). Given that *Ulva* can grow at considerably higher growth rates than observed in the nitrogen flux experiment (~ 40 % d^{-1} (Pedersen and Borum 1996)), it is proposed that the proteins synthesised immediately above the critical N are also metabolic in nature and, if other resources were not limited at high stocking densities, would enable the seaweed to grow at similar rates. These increases in metabolic proteins are beyond what is required for immediate growth, but are available for growth as a rapid response to changes in resource availability. In this sense, the critical N for a seaweed is defined by the system in which it is grown, and may increase or decrease depending on the maximum growth rate allowed by the system (see Pedersen and Borum (1996)). The maximum growth rate in this study was 11.7 % d^{-1}. The growth rate plateaued with increasing water renewals, which suggests that the biomass in high-density tumble cultures will be light limited at this point. An SGR of 11.7% d^{-1} is lower than other studies using individual thalli for which up to ~ 40 % d^{-1} can be attained (e.g. Pedersen and Borum (1996)). Correspondingly, the present study has a lower critical N (1.2 %) compared to 2.17 % N in Pedersen and Borum (1996). Therefore, the theoretical critical N content of *U. ohnoi* growing with unlimited resources, limited only by its innate physiology, should be equal to the luxury point. However, in any growth-limiting system, the difference between the critical N content and luxury point will be defined by the luxury uptake of excess nitrogen with no change in growth rate. This represents an interpretation of luxury uptake that differs from most terrestrial plants that react on longer timeframes, and better reflects the plastic ability of seaweeds to respond to variation in resources.

Unlike the initial metabolic uptake state that leads to increased protein synthesis, the luxury uptake state did not yield any increase in methionine – the start codon for

proteins (Garrett and Grisham 2013). This supports the idea that the increases in amino acid content in the luxury state were from free amino acids, not proteins. The luxury uptake of nitrogen and assimilation into free amino acids was characterised by two phases. The first phase includes essential and non-essential amino acids (including lysine), while the second is dominated by glutamic acid/glutamine and arginine. Free amino acids are the major contributors to total internal N storage in both green and red seaweeds (Lignell and Pedersen 1987; McGlathery et al. 1996; Naldi and Wheeler 1999). However, much of the physiological data on luxury uptake relates to "surge uptake" studies. For example, in *U. intestinalis* there is a short-term increase in the free amino acids glutamine and asparagine following the addition of high concentrations of ammonium and nitrate (Taylor et al. 2006). Similar surge increases in amino acids occur in *Gracilaria* spp. (Jones et al. 1996) and *U. fenestrata* (Naldi and Wheeler 1999). These phenomena allow seaweeds to opportunistically compete for nitrogen which can be patchy in nature (Lobban and Harrison 1997), however, the longer term and steady state system demonstrates that glutamic acid/glutamine and arginine are the dominant luxury uptake free amino acids.

Glutamic acid and glutamine specifically are the substrate for all organic nitrogen based compounds and therefore represent the most energy efficient way to store excess nitrogen (Garrett and Grisham 2013). These free amino acids equated to almost 40 % of the free amino acid pool and 20 % of the total amino acid content for seaweed in the luxury state, almost twice that of the metabolic state. Arginine represented over 25 % of the free amino acid pool and almost 13 % of total amino acids in the luxury uptake state. In terrestrial plants, arginine synthesis eliminates excess nitrogen (Nasholm 1994) as well as storage to support future growth (Lipson et al. 1996). High concentrations of arginine have also been reported for long term studies in *Gracilaria secundata* (Lignell and Pedersen 1987). Notably, the synthesis of arginine uses glutamine and asparagine for the amide group (Lobban and Harrison 1997; Garrett and Grisham 2013), and the synthesis of high levels of arginine is proposed as the rationale for the minimal free asparagine quantified in this study.

Although the internal N content and the total amino acid content was highest for seaweed in the luxury state, there was a clear trade-off with growth rates, and therefore with potential biomass production. Internal N contents did not increase beyond the

luxury point (2.6 % internal N) unless growth rate was limited by a resource other than nitrogen. The main non-N limiting resources in intensive seaweed cultivation includes light, which was limiting for the majority of the low N treatment cultures, but in contrast it was the dissolved resources (other macro-nutrients (P), trace elements or carbon (Lobban and Harrison 1997)) that are delivered by increasing water renewals which limited growth in the luxury N state. Although it is difficult to identify exactly what the limiting resource was for these luxury state seaweeds, there is opportunity to enhance luxury N production by removing the next limiting resource and potentially maintaining the luxury N state at higher total amino acid productivities (> 1.6 g m^{-2} d^{-1}) with lower water renewal rates.

In conclusion, the current study quantified the variation in internal N content and amino acid concentration and quality in the green seaweed *U. ohnoi* using an innovative provision of nitrogen flux by simultaneously manipulating nitrogen concentration and water renewals. This study demonstrated that amino acid concentration and quality varied substantially based on the nitrogen state of the seaweed, which was determined by N flux and growth rate. Amino acid synthesis above the luxury point had limited (lysine) or no (methionine) further gains in amino acids essential to nutrition and any targeted production of these compounds should focus solely on maximising biomass productivity through high growth rates to ensure that the biomass is maintained in the metabolic state. Alternatively, the luxury uptake abilities of seaweeds, such as *U. ohnoi*, may be best utilised in bioremediation applications (Neori et al. 2003) as low flow, nutrient-rich waste streams could be most efficient for the production of amino acids. This concept of managing amino acid production of seaweeds using the luxury point as a fulcrum emphasises the inextricable link between understanding the fundamental physiology of seaweeds and innovative strategies for their production.

Chapter 3: Indirect and direct effects of salinity on the concentration and quality of protein in *Ulva ohnoi* (Chlorophyta)[1]

3.1. Introduction

Marine macroalgae (seaweeds) are a promising source of sustainable protein with many advantages over conventional terrestrial crops by avoiding the use of arable land, fresh water and – despite a few exceptions – human consumption. As with any terrestrial plant-based protein source, it is both the concentration (percent dry weight biomass) and quality (percent of individual amino acids) of protein (or TAA) that often limits the nutritional applications of seaweed. However, the concentration and quality of protein are highly plastic in seaweeds (Chapter 2) (Nielsen et al. 2012). Much of this plasticity is related to within-species variation in total nitrogen (N) content which in turn is primarily related to the availability of N under N-limited conditions or the indirect effect of the dilution of internal N content with increasing growth rate under non-N limiting conditions (Chapter 2) (Greenwood and Barnes 1978; Hanisak 1983; Harrison and Hurd 2001). The latter relationship is a result of the indirect dilution effect from the synthesis of non-amino acid biomass (carbohydrates and lipids), which decreases as growth rate is reduced – a well-established concept for terrestrial plants (Greenwood and Barnes 1978; Wong 1990; Kuehny et al. 1991; Gifford et al. 2000; Gastal and Lemaire 2002; Taub and Wang 2008) yet only recently highlighted for seaweeds (Chapter 2). It is therefore difficult to attribute any direct effect of an environmental variable on the concentration and quality of protein in seaweed without considering its indirect effect on growth rate.

The land-based aquaculture of seaweeds provides conditions under which growth is not limited by nutrients but rather by environmental factors, in particular salinity (de Paula Silva et al. 2008; de Paula Silva et al. 2012). Under these conditions, the concentration and quality of protein may not only be directly affected by salinity but also indirectly through its effect on growth rate (Chapter 2). Salinity is affected most strongly by climatic conditions and can vary substantially, especially in the tropics, with high rainfall during the monsoon season and high evaporation rates during the dry season.

[1] **Chapter 3** is adapted from Angell, A. R., Mata, L., de Nys, R. & Paul, N. A. 2015. Indirect and direct effects of salinity on the quantity and quality of total amino acids in *Ulva ohnoi* (Chlorophyta). *Journal of Phycology* **51**:536-45.

These contrasting effects result in large changes in the salinity of coastal waters, from 5 to 45 ‰, and potentially even larger changes in more isolated systems such as rockpools, lagoons, and aquaculture systems (Lobban and Harrison 1997; de Paula Silva et al. 2008). The acclimation to hypo- or hyper-tonic conditions, i.e. the longer term response that takes place on a time scale of days to weeks in macroalgae, is relatively rare in nature, but may be more important in smaller water bodies such as land-based aquaculture in the tropics (Kirst 1990). This acclimation may affect growth rate in a number of ways including a reduction in the availability of ions such as K^+ (low salinities) which are required to maintain metabolic activity (Ritchie and Larkum 1985), the negative effects of high concentrations of ions (high salinities) that reduce water potential and have an adverse effect on metabolism (Kirst 1990), a decrease or increase in the vacuolar component of cells (Edwards et al. 1988), and the synthesis or degradation of osmolytes which draws on metabolites and energy needed for the synthesis of amino acids and for growth (Edwards et al. 1988; Kirst 1990). Together, these negative effects may act to reduce the rate of carbon accumulation (as carbohydrates and lipids) and thereby concentrate protein and amino acids in the biomass.

Long term acclimation to hypo- or hyper-saline conditions involves the synthesis or degradation of organic osmolytes which can directly affect the concentration and quality of protein as many are N-based or derived from N metabolism. For the green intertidal seaweed *Ulva,* which has a high tolerance to a wide range of salinities (Cohen and Fong 2004; Larsen and Sand-Jensen 2006), these osmolytes include the amino acid proline (Edwards et al. 1988; Liu et al. 2000; Kakinuma et al. 2006) as well as dimethylsulphoniopropionate (DMSP) (Van Alstyne 2008) which is derived from the essential amino acid methionine (Gage et al. 1997). The concentration of DMSP increases with salinity in a number of marine macroalgae (Stefels 2000) including species of *Ulva* (Edwards et al. 1987; Edwards et al. 1988; Karsten et al. 1992), however, concentrations of methionine have not been examined in response to long term osmotic stress. As methionine is often the first amino acid to limit the nutritional value of plant-based diets (Boland et al. 2013), understanding how methionine changes with osmotic stress is of interest both for understanding the physiological responses of *Ulva* to salinity gradients and for applications in protein production, especially

considering the high rates of areal productivity for *Ulva* in land-based cultivation (Bolton et al. 2009; Mata et al. 2010; Nielsen et al. 2012; Mata et al. 2016).

Therefore, this chapter examined whether quantitative and qualitative changes in the protein content in *Ulva ohnoi* are indirectly related to the effect of salinity on growth rate, or directly related to the osmotic stress. Specifically, the proportion of the essential amino acid methionine is examined in relation to osmotic stress. The salinity treatments selected represent the broad range associated with the tropical environment in Australia under which the species grows. Additionally, biomass productivities at each salinity were measured, in conjunction with the concentration and quality of protein, to calculate the potential areal productivities of protein and methionine for *Ulva ohnoi* and evaluate whether physiological changes have important implications for the production of protein using seaweed.

3.2. Materials and methods

3.2.1. Study organism and culture techniques

The green seaweed *Ulva ohnoi* M. Hiroka and S. Shimada (Lawton et al. 2013; Genbank accession numbers KF195501, KF195536) was sourced from the Marine and Aquaculture Research Facility Unit (MARFU) at James Cook University. The strain used was not sterile, however, no reproductive events were observed over the course of the experiment.

To quantify the effect of salinity on growth rate, the concentration and quality of protein and the proportion of methionine of acclimated *U. ohnoi*, the seaweed was grown in salinities ranging from 10 to 60 ‰ in increments of 5 ‰ for 21 days (n = 4). The experiment was run in an outdoor greenhouse in the early tropical austral dry season (May, Photoperiod 12:12 light:dark) using 4 L opaque containers (surface area (SA) = 0.035 m^2, height = 170 mm) with bottom aeration to tumble the biomass. The containers were situated inside a water bath, which fluctuated in temperature over the day from a minimum of 22.4 – 23.3 ˚C (05:30 – 7:00) to a maximum of 26.0 – 27.6 ˚C (13:00 – 14:00) over the final three day experimental period (see below). All the biomass used for the experiment was collected from one stock tank in which it had been cultured at a salinity of 35 ‰ for seven days. The experimental buckets (n=44) were all stocked with

4 g fresh weight (fw) L^{-1} as this has previously been identified as providing the highest protein productivities (Angell et al. 2014). The biomass was acclimated to the experimental system condition (at a salinity of 35 ‰) for three days prior to the commencement of the experiment. Seawater (salinity range: 33-35 ‰) was used as the basal culture water with lower salinities created by diluting with de-chlorinated tap water and higher salinities with the addition of sea salt (Cheetham Salt Flossy). The cultures were run as batch cultures with 100 % water and f/2 nutrient enriched media exchanged every three days. De-chlorinated freshwater was added twice daily to adjust for evaporation. The pH of all cultures was measured throughout the day (0700 to 1800 h) using a Hach HQ40d hand held probe. Maximum pH occurred at 1200 h and ranged from 9.82 ± 0.01 for 60 ‰ to 10.16 ± 0.01 for 20 ‰. The photosynthetic radiation at the surface of the cultures was logged every 5 minutes throughout the experiment using a Li-1400 Data Logger (Li-Cor, Lincoln, NE, USA). The total irradiance of the final three day experimental period was 104.1 mol photons m^{-2}, with intensity reaching a maximum of 1,969 µmol photons m^{-2} s^{-1}.

3.2.2. Growth rate

All cultures were harvested when the water was exchanged (every three days) and the biomass weighed and restocked to original stocking density. The specific growth rates were calculated according to equation 3.1, where fw_f and fw_i are the final and initial fresh weights, respectively.

$$Specific\ growth\ rate\ (SGR) = {ln\left(\frac{fw_f}{fw_i}\right)}\Big/{days} \qquad \textbf{Eq. 3.1}$$

The biomass from the final harvest (final three day experimental period) was selected for the remaining analyses as by this time the biomass was acclimated to its respective salinity treatment with stable growth rates and composition (Fig. S3.1). The 21 d biomass was analysed for: SGR (Eq. 3.1), fresh weight (fw) to dry weight (dw) ratio, ash content, N content, C content and amino acid content (see below).

3.2.3. Compositional analysis

The fw:dw for each culture was determined by drying freshly harvested biomass in a food dehydrator (Ultra FD1000; Ezidri) at 50 ˚C for 48 hours. The ash content was calculated in these samples by incinerating at 550 ˚C for six hours. For all other

biochemical analyses, freshly harvested biomass was freeze-dried and milled prior to analyses.

N and C content were quantified using an elemental analyser to provide the percentage as dry weight (OEA Laboratory Ltd., Callington, UK). Internal N and C content are reported as grams per 100 g dry weight (% dw).

3.2.4. Concentration and quality of protein

The concentration of protein was calculated based on the sum of the following amino acids (TAA): aspartic acid, asparagine, glutamic acid, glutamine, serine, histidine, glysine, threonine, alanine, arginine, tyrosine, valine, methionine, phenylalanine, isoleucine, leucine, lysine, and proline. As asparagine is hydrolysed to aspartic acid and glutamine to glutamic acid during analysis, the sum of these amino acids were reported as asparagine/aspartic acid or glutamine/glutamic acid. The two remaining proteome amino acids, cysteine and tryptophan, were not analysed as they are minor constituents in *Ulva* spp. (Angell et al. 2012).

Protein quality was calculated as the proportion of each amino acid with respect to TAA. Amino acids were analysed after 24 h liquid hydrolysis in 6 M HCl at 110 ˚C using a Waters ACQUITY UPLC at the Australian Proteome Analysis Facility, Macquarie University, Sydney using procedures based on the Waters AccQTag amino acid methodology (Cohen 2000; Bosch et al. 2006).

3.2.5. Productivity

Biomass, protein and methionine productivities were calculated from 21 d biomass (Eq. 3.2, 3.3 and 3.4, respectively). Areal productivity was calculated as a means to standardise the effects of different fresh biomass composition (fw:dw) between salinities. Protein and methionine productivities were calculated to standardise for the effects of protein concentration (TAA concentration) and protein quality, respectively. Methionine was selected because it is often the first limiting essential amino acid for mono-gastric livestock (Boland et al. 2013) and also because of its potential primary relationship with salinity through DMSP production.

$$Biomass\ productivity = \left(\frac{fw_f - fw_i}{fw{:}dw}\right) / SA \Big/ days \qquad \textbf{Eq. 3.2}$$

$$Protein\ productivity = (Eq.\ 3.2) \times TAA/100 \quad \textbf{Eq. 3.3}$$

$$Methionine\ productivity = (Eq.\ 3.3) \times MET/100 \quad \textbf{Eq. 3.4}$$

Where fw_f and dw_i are the final and initial fresh weights, respectively, fw:dw is the fresh to dry weight ratio of harvested biomass, SA is the surface area of the cultures in m^2, TAA is the total amino acid concentration as % dw biomass, and MET is the proportion of methionine as a percentage of the protein content (% TAA).

3.2.6. Data analysis

The effect of salinity on SGR (% d^{-1}), on the compositional analyses of fw:dw, ash, C and N and on the biomass, protein and methionine productivity were each analysed using one-way analysis of variance (ANOVA – Statistica 12; StatSoft Inc.). Salinity treatments were compared using Tukey's post hoc comparisons, as appropriate. ANOVA assumptions of homogeneity of variance and normality were assessed using Levene's test and histograms of the residuals, respectively.

Correlations were made for the concentration of protein versus salinity and SGR using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA). R^2 values are reported as the measure of goodness of fit and p values ($p < 0.05$) for the significance of relationship.

Protein quality of the biomass was analysed using non-metric multidimensional scaling (nMDS) using the statistical software PRIMER (PRIMER-E Ltd., Lutton, UK). A similarity matrix was calculated from the 4^{th} root transformed data with individual amino acid contents (as a percentage of TAA content) as variables in the MDS cluster diagram and vector plot. Pairwise correlations were subsequently run for each individual amino acid (% TAA) versus salinity and SGR, R^2 values reported and compared to assess the relative influence of each.

3.3. Results

3.3.1. Growth rate and compositional analysis

Salinity had a strong effect on specific growth rate (SGR). *U. ohnoi* could not tolerate the most extreme of the low salinities (10 and 15 ‰), with rapidly declining growth rates after the first three days (Fig. S3.1). All other salinity treatments survived for the

entire 21 day culture period and could be divided into three groups based on the SGRs from the final three day culture period (low (20 ‰), optimal (25 – 40 ‰), and high (45 – 60 ‰)) (Fig. 3.1). The SGR was 9.60 ± 0.50 % d^{-1} for the low salinity group (20 ‰), 13.02 ± 0.32 to 15.04 ± 0.32 % d^{-1} for the optimal salinity group (25 – 40 ‰), and 7.89 ±0.49 to 11.55 ± 0.67 % d^{-1} for the high salinity group (45 – 60 ‰) (See Fig. 3.1 for Tukey's HSD comparisons, ANOVA: $F_{1,8} = 28.30$, $p < 0.001$). Acclimation – defined by stabilisation in SGR, fw:dw and ash content – occurred by day 9 in the high salinities (Fig. S3.1).

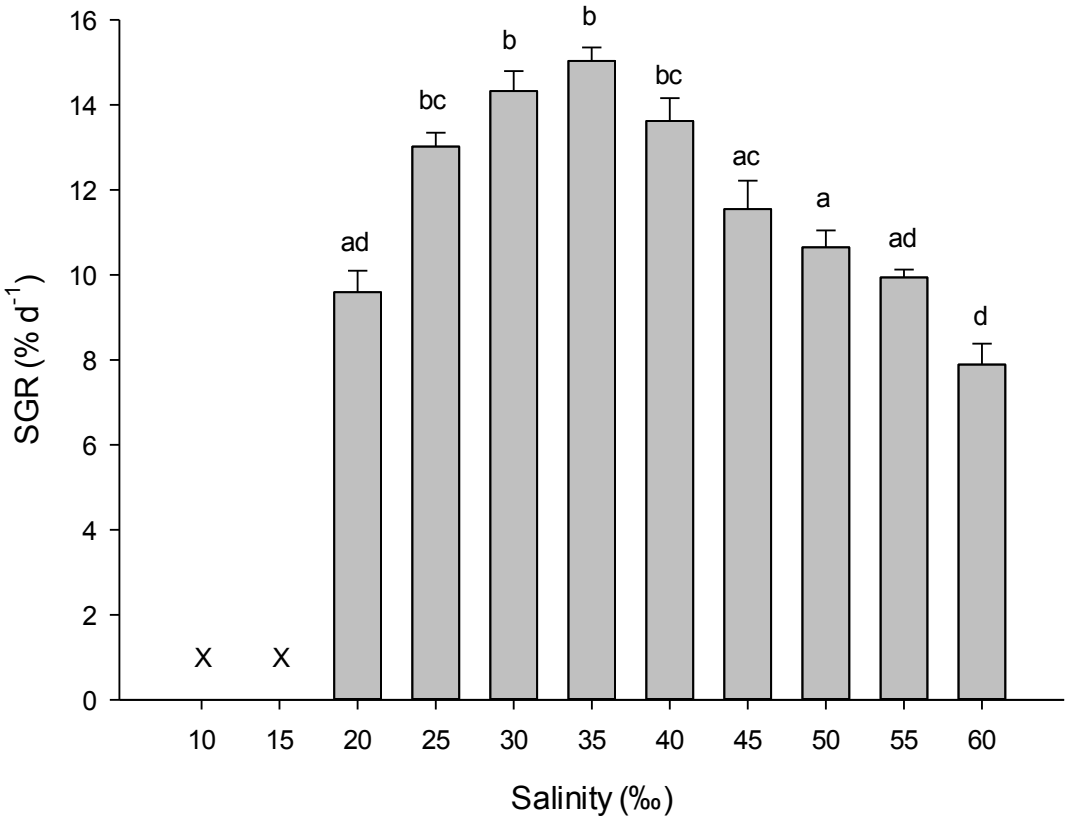

Figure 3.1. Fresh weight specific growth rate (SGR: mean + SE) of *U. ohnoi* cultured at various salinities (n = 4). SGR was calculated for the final 3 day period of 21 days of culture. An 'X' indicates the crash of salinity treatment 10 on day 3 and of salinity treatment 15 on day 9. Common letters above columns indicate no significant difference (Tukey's HSD, $p < 0.05$).

Salinity had a significant effect on the fw:dw ratio, ash content, N content and C content (Table 3.1). The fw:dw was highest for low and optimal salinities (4.66 ± 0.17 – 5.14 ± 0.13) and lowest for high salinities (3.09 ± 0.08 – 4.24 ± 0.07) (ANOVA: $F_{8,27} = 87.03$, $p < 0.001$). Ash content was lowest for 20, 25 and 30 ‰ treatments (23.93 ± 0.25, 26.16

±0.35 and 27.80 ± 0.50 % dw, respectively) and similarly high for salinity treatments above 30 ‰ (32.67 ± 0.94 – 34.65 ± 0.47 % dw) (ANOVA: $F_{8,27} = 30.72$, $p < 0.001$). C and N content showed similar changes with salinity: highest for the extreme salinities 20, 25, 55 and 60 ‰ and lowest for 35 and 40 ‰ (see Table 3.1). C content was highest at 20 ‰ (29.23 ± 0.15 % dw) and lowest at 40 ‰ (24.31 ±0.33 % dw) (ANOVA: $F_{8,27} = 36.50$, $p < 0.001$), and N content was highest at 60 ‰ (3.78 ± 0.09 % dw) and lowest at 35 ‰ (3.00 ± 0.05 % dw) (ANOVA: $F_{8,27} = 10.95$, $p < 0.001$).

Table 3.1. Fresh to dry weight ratio (fw:dw), ash content, carbon content (C) and nitrogen content (N) of Ulva ohnoi cultured at various salinities. Analyses were done on biomass harvested from final 3 day period of 21 day culture (mean ± SE, n = 4). Concentration values are % dw and common letters in superscripts indicate no significant difference (Tukey's HSD, $p < 0.05$).

Salinity (‰)	fw:dw	Ash	C	N
20	4.66 ± 0.17^{a}	23.93 ± 0.25^{a}	29.23 ± 0.15^{a}	3.58 ± 0.05^{ad}
25	4.88 ± 0.10^{b}	26.16 ± 0.35^{ab}	27.32 ± 0.27^{b}	3.34 ± 0.15^{abc}
30	5.14 ± 0.13^{b}	27.80 ± 0.50^{b}	25.86 ± 0.13^{cd}	3.18 ± 0.09^{bc}
35	4.84 ± 0.07^{ab}	32.67 ± 0.94^{c}	24.61 ± 0.25^{e}	3.00 ± 0.05^{b}
40	4.71 ± 0.06^{a}	32.93 ± 0.66^{c}	24.31 ± 0.33^{e}	3.09 ± 0.06^{b}
45	4.24 ± 0.07^{c}	34.65 ± 0.47^{c}	25.18 ± 0.18^{de}	3.27 ± 0.07^{abc}
50	3.94 ± 0.06^{cd}	32.97 ± 0.77^{c}	26.09 ± 0.22^{cd}	3.51 ± 0.04^{cd}
55	3.56 ± 0.05^{de}	34.25 ± 1.36^{c}	26.49 ± 0.26^{bc}	3.55 ± 0.05^{cd}
60	3.09 ± 0.08^{e}	34.28 ± 0.54^{c}	27.42 ± 0.40^{b}	3.78 ± 0.09^{d}

3.3.2. Concentration of protein

The concentration of protein (TAA content) had a strong positive quadratic relationship with salinity (Fig. 3.2A: $R^2 = 0.684$, $p < 0.05$) as it was highest at the most extreme salinities and lowest at the optimal salinities. However, protein concentration had a stronger relationship with SGR than salinity (Fig. 3.2B: c.f. R^2 values, $R^2 = 0.842$, $p < 0.05$). Protein concentration had a negative linear correlation with SGR and was highest in the slowest growing cultures (19.26 % dw) and lowest in the fastest growing cultures (14.45 % dw). This relationship was independent of changes in ash content as no correlation existed between protein concentration and ash content.

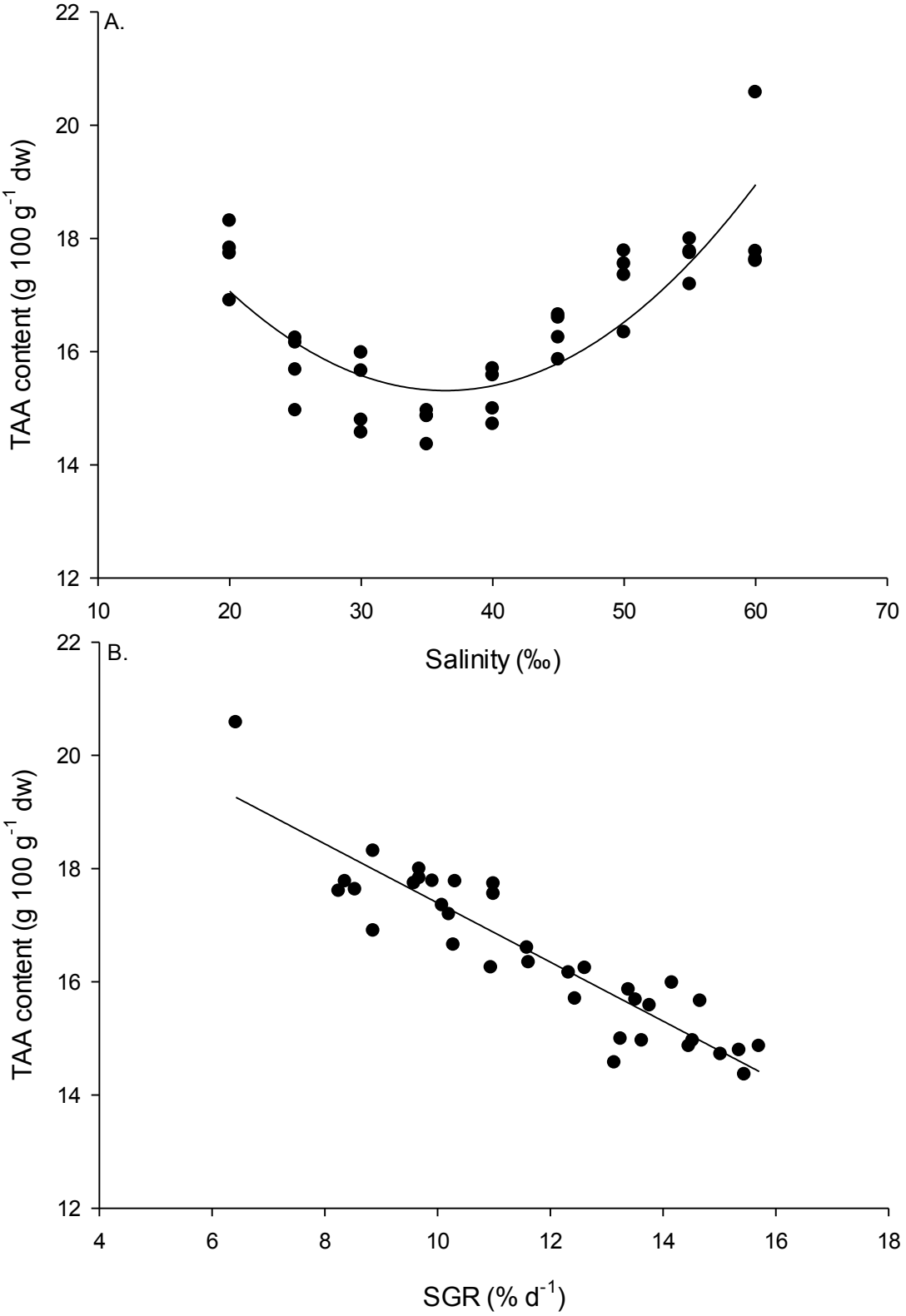

Figure 3.2. Protein concentration (TAA % dw) correlated against (A) salinity and (B) SGR for *U. ohnoi* cultured at various salinities (n = 36). Results represent the final 3 day period of 21 days of culture.

3.3.3. Quality of protein

The nMDS and vector loadings (Fig. 3.3A and B) illustrate qualitative differences in the composition of amino acids in protein between the low and high salinity groups (Fig. 3.3A). Protein in seaweeds from the low salinity group (Fig. 3.3A, group 1: 20 ‰) had higher proportions of serine, glysine, threonine, alanine, valine, and isoleucine relative to the other salinity groups (Fig. 3.3B). Alternatively, protein in seaweeds from the high salinity group (Fig. 3.3A, group 3: 45 – 60 ‰) had higher proportions of histidine, arginine, proline, and tyrosine (Fig. 3.3B). Protein in seaweeds from the optimal salinity group (Fig. 3.3A, group 2: 25 – 40 ‰) had no strong correlation with any particular amino acid relative to low and high salinity groups.

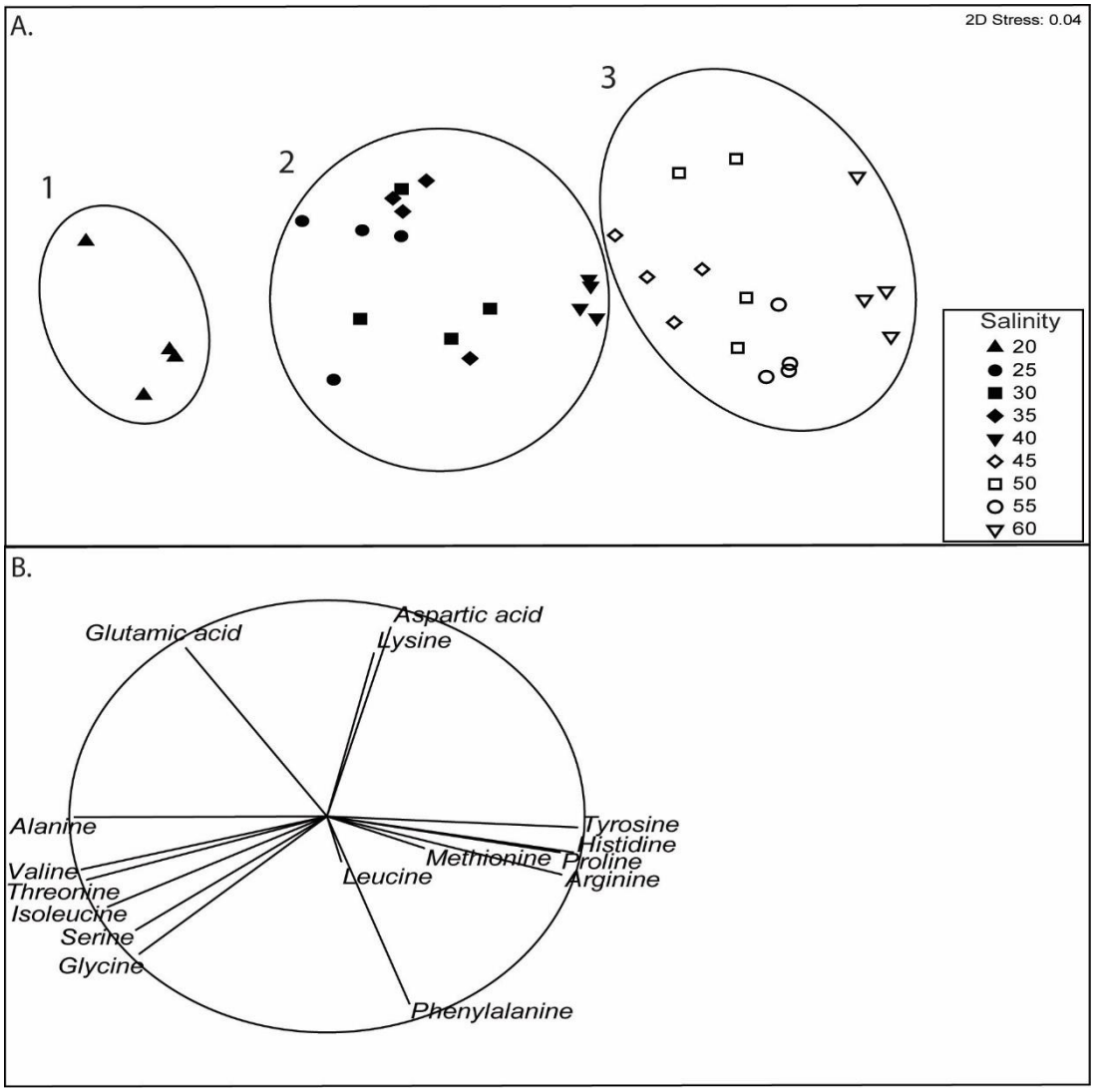

Figure 3.3. Non-metric multidimensional scaling (nMDS) plot showing similarity in the proportion of protein as individual amino acids between *U. ohnoi* cultured at different salinities. (A) nMDS plot (stress = 0.04) with groups superimposed (1 = low salinity 20 ‰; 2 = optimal salinities 25-40 ‰; 3 = high salinities 45-60 ‰). (B) Associated vector plot of the nMDS. The length and direction of which indicates the strength of the correlation and the direction of change between the two nMDS axes.

In contrast to the changes in the concentration of protein, the qualitative changes in protein for all of the amino acids (Table 3.2) were more strongly related to salinity than SGR (c.f. R^2 values in Table 3.3). However, the magnitude of these changes varied for different amino acids, with the greatest changes occurring for the amino acids proline, tyrosine, histidine and alanine (Table 3.3). Proline (Fig. 3.4A) had the largest change with salinity, increasing exponentially from 4.53 % (in 20 ‰) to 6.61 % (in 60 ‰). Similarly, tyrosine and histidine were also positively related to salinity, increasing from 2.62 and 1.67 % in 20 ‰ to 3.56 and 2.11 % in 60 ‰, respectively. Alanine (Fig. 3.4B), however, was negatively correlated (exponentially) with salinity, increasing from 7.38 % in 60 ‰ to 9.51 % in 20 ‰. Methionine had a quadratic relationship with salinity, peaking 19.02 % higher at 40 ‰ (Fig. 3.4C).

Table 3.2. The quality (proportion of individual amino acids - % TAA) and concentration (TAA, % dw) of protein in *Ulva ohnoi* cultured at various salinities. Analyses were done on biomass harvested from final 3 day period of 21 day culture (mean ± SE, n=4). TAA = total amino acids, His = histidine, Ser = serine, Arg = arginine, Gly = Glycine, Asp = aspartic acid, Asn = asparagine, Glu = glutamic acid, Gln = glutamine, Thr = threonine, Ala = alanine, Pro = proline, Lys = lysine, Tyr = tyrosine, Met = methionine, Val = valine, Ile = isoleucine, Leu = leucine and Phe = phenylalanine.

	Salinity (‰)								
AA	20	25	30	35	40	45	50	55	60
His	1.60 ± 0.01	1.78 ± 0.02	1.84 ± 0.03	1.79 ± 0.03	1.90 ± 0.01	1.93 ± 0.02	1.97 ± 0.04	2.09 ± 0.01	2.09 ± 0.03
Ser	5.98 ± 0.05	5.56 ± 0.08	5.51 ± 0.06	5.45 ± 0.03	5.38 ± 0.03	5.48 ± 0.04	5.32 ± 0.06	5.47 ± 0.03	5.31 ± 0.03
Arg	5.75 ± 0.09	5.74 ± 0.08	5.95 ± 0.06	5.86 ± 0.08	6.21 ± 0.03	6.29 ± 0.06	6.33 ± 0.07	6.49 ± 0.01	6.86 ± 0.09
Gly	6.19 ± 0.05	5.98 ± 0.08	5.96 ± 0.06	5.84 ± 0.06	5.80 ± 0.04	5.80 ± 0.03	5.68 ± 0.08	5.86 ± 0.04	5.68 ± 0.04
Asp/Asn	12.91 ± 0.19	13.47 ± 0.22	13.50 ± 0.19	13.74 ± 0.20	13.69 ± 0.07	13.82 ± 0.07	13.79 ± 0.28	13.04 ± 0.20	13.63 ± 0.17
Glu/Gln	12.14 ± 0.16	12.49 ± 0.20	12.30 ± 0.14	12.42 ± 0.22	11.95 ± 0.03	11.89 ± 0.11	12.08 ± 0.19	11.54 ± 0.02	11.89 ± 0.16
Thr	5.68 ± 0.02	5.44 ± 0.04	5.39 ± 0.03	5.39 ± 0.01	5.28 ± 0.01	5.26 ± 0.02	5.16 ± 0.04	5.22 ± 0.01	5.15 ± 0.01
Ala	9.53 ± 0.05	8.93 ± 0.04	8.67 ± 0.11	8.60 ± 0.04	7.94 ± 0.04	7.68 ± 0.07	7.60 ± 0.06	7.63 ± 0.02	7.39 ± 0.04
Pro	4.63 ± 0.03	4.69 ± 0.04	4.70 ± 0.02	4.72 ± 0.05	5.10 ± 0.06	5.42 ± 0.00	5.73 ± 0.08	6.30 ± 0.08	6.48 ± 0.04
Lys	5.77 ± 0.03	5.81 ± 0.07	5.81 ± 0.08	6.07 ± 0.08	5.94 ± 0.05	5.92 ± 0.05	6.04 ± 0.07	5.88 ± 0.03	5.82 ± 0.05
Tyr	2.60 ± 0.05	2.89 ± 0.04	3.03 ± 0.06	2.96 ± 0.04	3.28 ± 0.02	3.40 ± 0.04	3.49 ± 0.03	3.54 ± 0.01	3.49 ± 0.04
Met	1.62 ± 0.05	1.75 ± 0.03	1.88 ± 0.04	1.85 ± 0.04	2.00 ± 0.02	1.91 ± 0.07	1.87 ± 0.02	1.83 ± 0.01	1.73 ± 0.04
Val	7.04 ± 0.02	6.79 ± 0.04	6.72 ± 0.05	6.72 ± 0.04	6.61 ± 0.01	6.49 ± 0.03	6.46 ± 0.03	6.49 ± 0.02	6.29 ± 0.01
Ile	4.86 ± 0.02	4.74 ± 0.04	4.73 ± 0.05	4.71 ± 0.04	4.71 ± 0.01	4.60 ± 0.02	4.56 ± 0.03	4.58 ± 0.02	4.46 ± 0.02
Leu	7.67 ± 0.03	7.95 ± 0.05	7.95 ± 0.09	7.89 ± 0.05	8.05 ± 0.03	8.00 ± 0.03	7.92 ± 0.05	7.88 ± 0.04	7.62 ± 0.04
Phe	6.04 ± 0.07	5.99 ± 0.08	6.05 ± 0.07	5.98 ± 0.08	6.17 ± 0.02	6.12 ± 0.04	6.01 ± 0.10	6.15 ± 0.03	6.11 ± 0.07
Protein (% dw)	17.69 ± 0.29	15.76 ± 0.29	15.25 ± 0.34	14.76 ± 0.14	15.25 ± 0.23	16.34 ± 0.18	17.26 ± 0.32	17.67 ± 0.17	18.40 ± 0.73

Table 3.3. Relationships of individual amino acid as a proportion of protein (% TAA) with salinity and specific growth rate (SGR) based on R^2 values ($p < 0.05$). Direction and type of relationship shown along with range and relative change in each amino acid as a proportion of protein concentration. AA = amino acid and TAA = total amino acid.

	R^2 value (direction and type)			
Amino acid	Salinity	SGR	Range (% TAA)	Change (%)
Histidine	0.856 (+ve, linear)	0.130 (-ve, linear)	1.67 – 2.11	26.34
Serine	0.750 (-ve, exponential)	-	5.39 – 5.97	10.76
Arginine	0.827 (+ve, linear)	0.344 (-ve, linear)	5.64 – 6.69	18.62
Glycine	0.614 (-ve, exponential)	-	5.74 – 6.17	7.49
Aspartic acid/asparagine	0.251 (-ve, quadratic)	-	13.04 – 13.75	5.45
Glutamic acid/glutamine	0.292 (-ve, linear)	0.202 (+ve, linear)	11.76 – 12.40	5.44
Threonine	0.876 (-ve, exponential)	-	5.10 – 5.65	10.78
Alanine	0.946 (-ve, exponential)	0.116 (+ve, linear)	7.38 – 9.51	28.86
Proline	0.956 (+ve, exponential)	0.463 (-ve, linear)	4.53 – 6.61	45.92
Lysine	0.288 (-ve, quadratic)	-	5.73 – 5.98	4.36
Tyrosine	0.905 (+ve, exponential)	-	2.62 – 3.56	35.88
Methionine	0.629 (-ve, quadratic)	0.184 (+ve, linear)	1.63 – 1.94	19.02
Valine	0.853 (-ve, linear)	0.116 (+ve, linear)	6.32 – 6.93	9.65
Isoleucine	0.749 (-ve, linear)	0.177 (+ve, linear)	4.49 – 4.83	7.57
Leucine	0.581 (-ve, quadratic)	0.353 (+ve, linear)	7.67 – 8.02	4.56
Phenylalanine	-	-	-	-

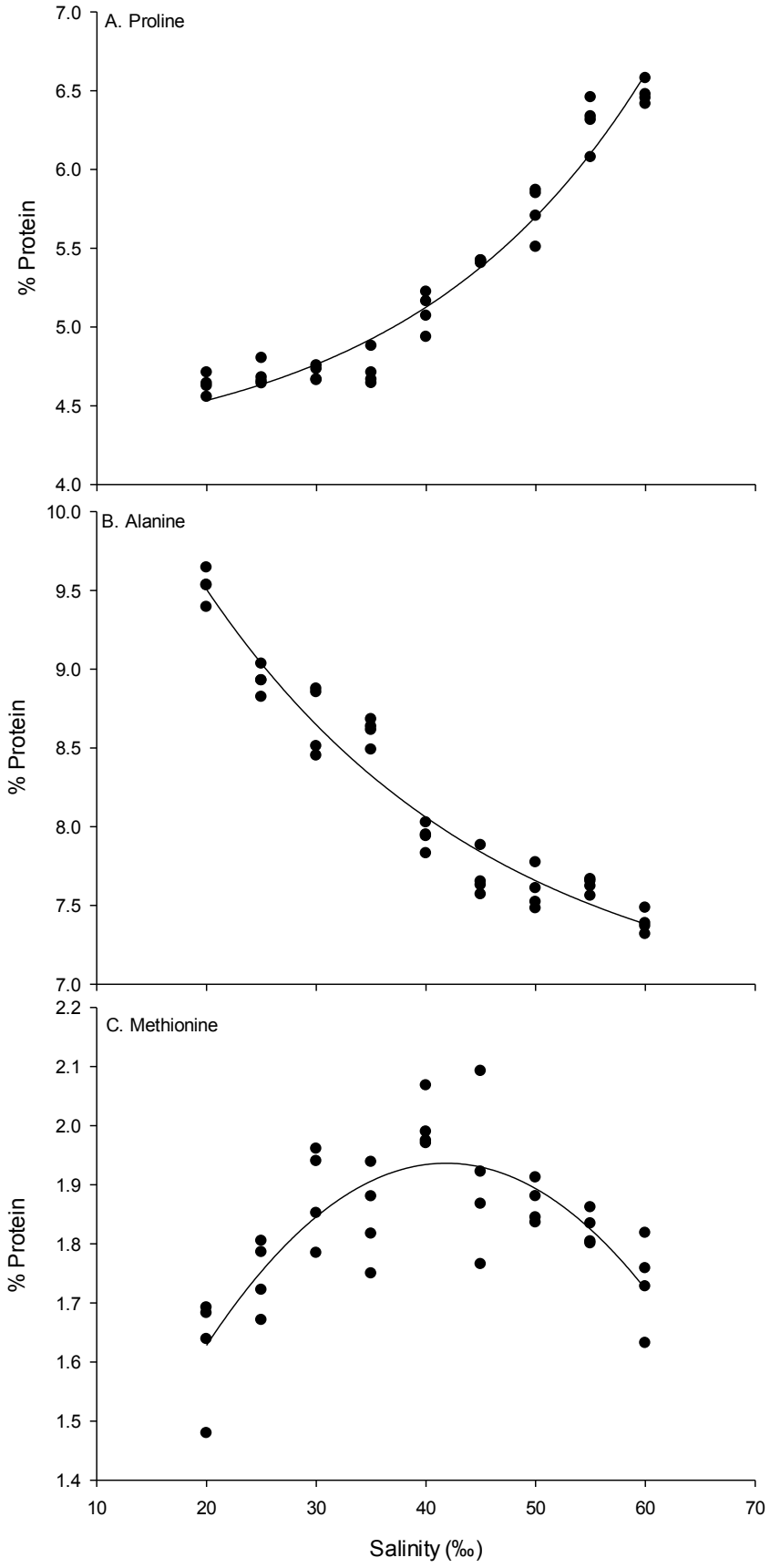

Figure 3.4. Varying responses of individual amino acids as a proportion of protein concentration (% TAA) to salinity: (A) proline, (B) alanine, and the essential amino acid (C) methionine. See Table 3.3 for R^2 values.

3.3.4. Productivity

Biomass productivity (dw) was lowest for the most extreme salinities 20 and 60 ‰ (10.96 ± 0.67 and 13.53 ± 0.93 g dw m^{-2} d^{-1}, respectively), followed by 25 ‰ (14.09 ± 0.42 g dw m^{-2} d^{-1}) and highest for the other salinity treatments (15.64 ± 0.62 – 17.63 ± 0.46 g dw m^{-2} d^{-1}) (see Fig. 3.5A for Tukey's HSD comparisons, ANOVA: $F_{8,27} = 8.04$, $p < 0.001$). Protein productivity mirrored these results and was low in 20 and 25 ‰ (1.94 ± 0.13 and 2.22 ± 0.04 g TAA m^{-2} d^{-1}, respectively) and high in all other salinities (2.39 ± 0.11 – 2.80 ± 0.05 g TAA m^{-2} d^{-1}), although 25 ‰ was similar to all treatments except 50 and 55 ‰ (see Fig. 3.5B for Tukey's HSD comparisons, ANOVA: $F_{8,27} = 7.72$, $p < 0.001$). Methionine (MET) productivities were lowest when *U. ohnoi* was cultured at 20 ‰ (31.64 ± 2.83 mg MET m^{-2} d^{-1}) and highest when cultured at salinities equal to or greater than 30 ‰ (45.06 ± 2.90 – 51.74 ± 1.79 mg MET m^{-2} d^{-1}), with the exception of 60 ‰ (42.38 ± 2.14 mg MET m^{-2} d^{-1}) (see Fig. 3.5C for Tukey's HSD comparisons, ANOVA: $F_{8,27} = 12.41$, $p < 0.001$).

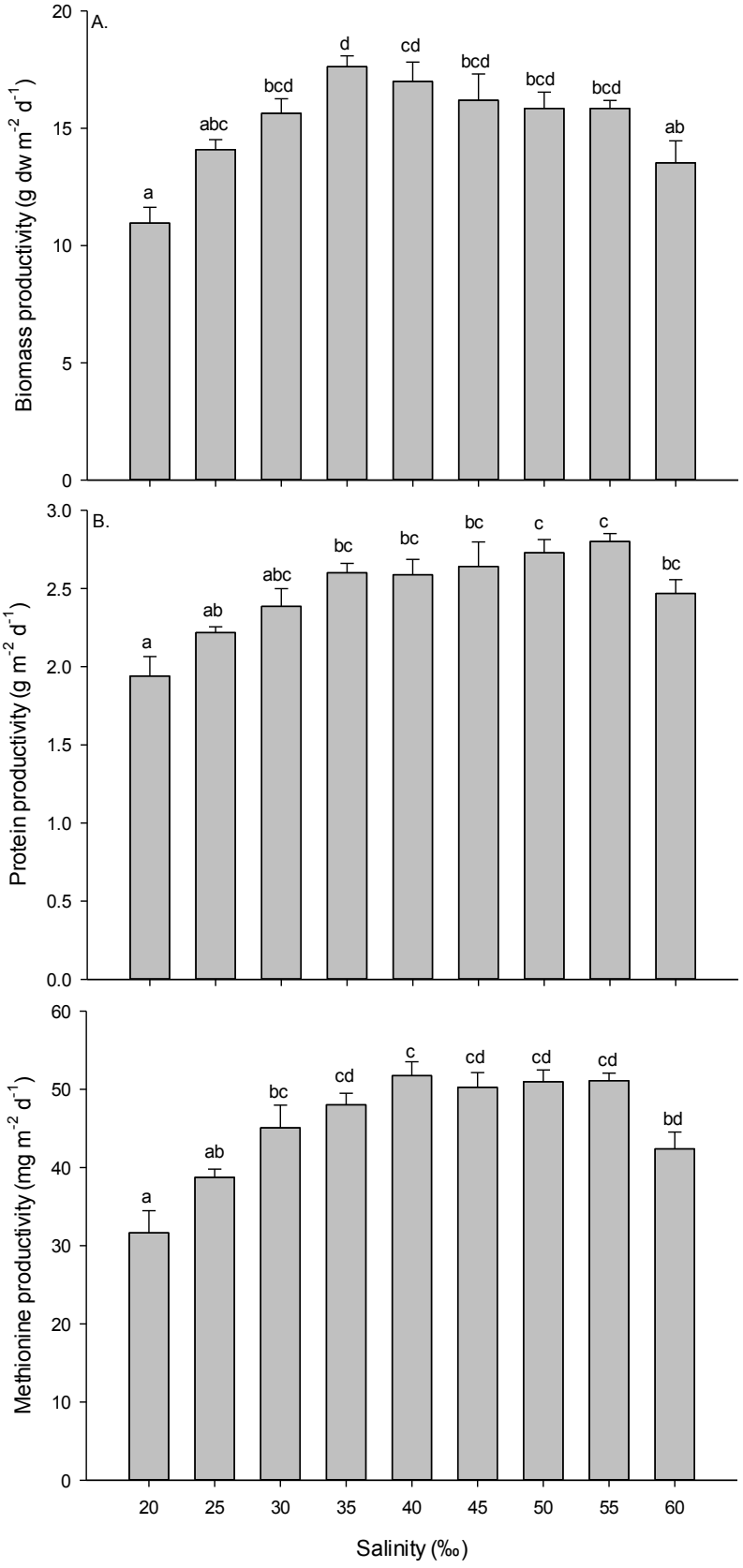

Figure 3.5. Dry weight biomass (A), protein (B) and methionine (C) areal productivities of *U. ohnoi* cultured at various salinities (mean ± SE, n = 4). Productivities were calculated for the final 3 day period of 21 days of culture. Common letters above columns indicate no significant difference (Tukey's HSD, $p < 0.05$).

3.4. Discussion

In this study, salinity strongly affected the growth rate of *Ulva ohnoi* to the extent that this indirect effect explained more of the variation in the concentration of protein than its direct effect on the metabolism and accumulation of amino acids. In contrast, the quality of protein was directly affected by salinity, with large increases in relative amounts of proline, tyrosine and histidine, and a large decrease in alanine, with increasing salinity from 20 to 60 ‰. However, there was only a moderate and non-linear relationship between salinity and the proportion of the essential amino acid methionine suggesting that increases in the osmolyte DMSP, which can increase with increasing salinity in *Ulva* (Edwards et al. 1988; Karsten et al. 1992; Stefels 2000), do not influence the concentration of methionine. These results demonstrate that growth rate, rather than salinity directly, is the primary driver of changes in the concentration of protein, whereas the quality of protein can be related directly to salinity.

Growth rates have frequently been used to assess salinity tolerance in algae as it represents the sum of all physiological processes (Kirst 1990). In this study, *U. ohnoi* had optimal growth under normal seawater conditions, a common trait for many intertidal seaweeds (Jacob et al. 1991; Karsten et al. 1994). However, when cultured at salinities below 20 ‰, the growth rate of *U. ohnoi* declined after three days and could not tolerate these low salinities over a longer period. This is somewhat different to other studies on species of *Ulva* that report moderate to high growth in salinities in the range of 10 – 20 ‰ (Taylor et al. 2001; de Paula Silva et al. 2008; Choi et al. 2010), which may be attributed to species-specific tolerances or the shorter time scale of such studies. Indeed, *U. ohnoi* cultured at 10 and 15 ‰ in this chapter actually had high growth rates for the first three days of the experiment, suggesting that this species can tolerate lower salinities in the short term. However, this tolerance may be an adaptation to the frequent short-term changes that occur in nature (Kirst 1990) but cannot be sustained for the prolonged exposure to very low salinities that are common for land-based aquaculture in the tropics (Lobban and Harrison 1997; de Paula Silva et al. 2008). Over the longer term (21 days), *U. ohnoi* was able to tolerate all the remaining salinity treatments (20 – 60 ‰), although acclimation to higher salinities appeared to take more than three days. This delay could relate to the time needed to synthesise organic osmolytes that serve to

restore turgor pressure and cell volume while having a minimal effect on metabolism (Kirst 1990).

The concentration of protein had a negative linear relationship with growth rate and a weaker non-linear relationship with salinity. This demonstrates that a change in the growth rate – as affected by salinity – was the most likely cause of the variability in the concentration of protein. The dilution of protein concentration through increased growth, or concentration through reduced growth, is a result of unbalanced growth where the processes of nutrient acquisition are uncoupled from those of carbon fixation (Berman-Frank and Dubinsky 1999). Similarly, N dilution is predicted in terrestrial plants that have been exposed to elevated CO_2 levels through the increased synthesis of carbohydrates (Wong 1990; Kuehny et al. 1991; Gifford et al. 2000), whereas N concentration occurs in *U. ohnoi* when growth rates are low under non-N limiting environments (Chapter 2). This suggests that hypo- or hyper-saline conditions limited carbon fixation in *U. ohnoi* more than these conditions limited N uptake and assimilation into amino acids and protein. Hypo- and hypersaline conditions can reduce the net rates of photosynthesis and carbon fixation in macroalgae (Dawes et al. 1978; Reed et al. 1980; Satoh et al. 1983; Macler 1988; Kirst 1990) while having a limited effect on N-uptake (Rueter and Robinson 1986; Lartigue et al. 2003; Choi et al. 2010), although data in regards to N-uptake is scarce. In addition, Macler (1988) suggested that there was no inhibition of inorganic N assimilation when the red seaweed *Gelidium coulteri* was cultured under varying salinities. To our knowledge, no study has directly examined the influence of salinity on the assimilation rates of amino acids and the presence of a strong negative correlation between the concentration of protein and growth rate in this study indicates that this metabolic response is relatively less important than the changes to carbon fixation.

Although salinity indirectly influenced the concentration of protein through growth rate, there was a direct effect of salinity on the quality of protein. While proline, tyrosine and histidine increased substantially, alanine decreased substantially as a proportion of protein with increasing salinity from 20 to 60 ‰. Proline is an osmolyte in seaweeds with increased concentrations under high salinities (Edwards et al. 1987; Edwards et al. 1988; Kirst 1990; Lee and Liu 1999). To my knowledge, tyrosine, histidine and alanine have not previously been reported to respond to changes in salinity, however, the increase in tyrosine and histidine with increased salinity suggests that these amino acids

may also act as osmolytes in *U. ohnoi*. These amino acids also changed in concentration on a whole biomass basis (% dw) in a similar manner, without a proportional change in response to changes in other amino acids or protein concentration. Changes in these amino acids are therefore likely a result of an increase in biosynthesis through the up-regulation of enzymes, a decrease in catabolic enzyme activity, an increase in their substrates or an increase in proteins that are rich in these amino acids. Indeed for proline, increases have been shown to be partly due to a decrease in the catabolic enzyme proline dehydrogenase (Madan et al. 1995). In contrast, the relationship between the proportion of methionine and salinity was not linear but was instead lowest at the extreme salinities and highest at 40 ‰. This suggests that the synthesis of DMSP, which increases with salinity (Edwards et al. 1988; Karsten et al. 1992; Stefels 2000), has no net effect on the proportion of methionine.

Although growth rates were substantially higher in the optimal salinity treatments, the biomass productivities were less structured because of the lower fw:dw ratios in the high salinity treatments, with clear differences only at the extremes of 20 and 60 ‰. When the concentration of protein was taken into account by calculating protein productivities, the productivity gap between the low, optimal and high salinity groups was further reduced as all treatments other than 20 and 25 ‰ had similar productivities of protein. This overall similarity in productivities of protein further illustrates the limited effect salinity has on the assimilation of amino acids in *U. ohnoi* relative to growth rate. Similarly, when considering an individual amino acid such as methionine, the relative productivities of salinity treatments remained essentially unchanged compared to protein productivities. This demonstrates that the qualitative influence of salinity has a negligible overall effect on the productivity of methionine compared to the quantitative influence of SGR on protein concentration. This trade-off between growth and protein concentration was only unbalanced for low (20 and 25 ‰) and extremely high salinities (60 ‰), with moderately high salinities (45 – 55 ‰) compensating for lower growth rates with higher protein concentrations and lower ratios of fresh to dry weight. This suggests that higher salinities will likely not impact on the commercial productivity of protein or methionine, although lower and extremely high salinities will.

In conclusion, this study examined the effects of long-term acclimation responses to salinity on the growth rate and concentration and quality of protein for the green seaweed *Ulva ohnoi* under non-N limiting conditions. The results demonstrated that

salinity has both an indirect and direct effect on the concentration and quality of protein, and that the concentration of protein was more related to the indirect effects of salinity on growth rate than the direct effects of salinity on amino acid metabolism. In contrast, there was a direct effect of salinity on the quality of protein, however, this was limited in the case of the essential amino acid methionine. These findings highlight that growth rate rather than salinity *per se* is more important to the concentration of protein in seaweeds and, along with N supply, is a critical consideration for the commercial production of seaweeds for protein.

Chapter 4: The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five[1]

4.1. Introduction

The fundamental role of nitrogen and protein in the nutrition, physiology and ecology of seaweeds has been a key research topic for decades (Dawes et al. 1974; Rosell and Srivastava 1985; Hurd et al. 1996; McGlathery et al. 1996; Harrison and Hurd 2001; Nelson et al. 2008; Angell et al. 2014). However, the nitrogen and protein content of seaweeds has more recently become a focus of applied research (Harnedy and FitzGerald 2011; Boland et al. 2013), in particular for applications where the biochemical composition of species must be well characterised. These applications range from human and animal nutrition and health (Fleurence 1999b), and fertilisers and plant growth stimulants (Craigie 2011; Sharma et al. 2014) to bioenergy (Neveux et al. 2015). Together these studies have generated a significant database on the protein biochemistry of seaweeds across diverse disciplines. However, there are inconsistencies and potential inaccuracies in the methods used to determine protein content arising from the use of direct extraction procedures for the measurement of soluble protein and the indirect (proxy) method of protein determination using a nitrogen-to-protein (N-protein) conversion factor of 6.25 (N*6.25 – crude protein).

Protein determination using direct extraction procedures employs multiple options for the extraction component and for the subsequent quantification of soluble protein (mainly the alkaline copper assay (Lowry et al. 1951) and the Coomassie Brilliant Blue assay (Bradford 1976)). Both the extraction of the protein and the quantification of the extracted soluble protein are susceptible to inaccuracies. First, protein extraction yields are generally low for seaweeds due to the presence of cell wall mucilages and phenolic compounds (Fleurence et al. 1995; Wong and Cheung 2001b). Second, the initial method of protein extraction is not a standardised process and consequently varies between studies. For example, protein extraction procedures vary with the pre-treatment of the sample (raw, milled, freeze/thawed, enzymatic digestion etc.), the volume of water and exposure time used for the extraction of water-soluble proteins, the type and

[1] **Chapter 4** is adapted from Angell, A. R., Mata, L., de Nys, R. & Paul, N. A. 2016. The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five. *Journal of Applied Phycology* **28**:511-524.

exposure time of buffer used, whether or not the protein is precipitated, the method of precipitation (e.g. trichloroacetic acid to supernatant ratio), centrifuge time and force, and the type of standard used (Berges et al. 1993; Fleurence et al. 1995; Fleurence 1999a; Wong and Cheung 2001b; Barbarino and Lourenco 2005; Wong et al. 2006). These direct extraction procedures differ in efficiency in their own right and there is also an influence of the chemical and morphological features of the seaweed themselves (Barbarino and Lourenco 2005). For example, tough leathery brown seaweeds may be more resistant to certain extraction procedures compared to seaweeds with soft thalli. Finally, irrespective of the extraction procedure, the main methods for quantifying protein in the extract are colorimetric assays (Bradford and Lowry assays) and these methods are also subject to interference from a number of factors depending on the biochemistry of the seaweed (Lowry et al. 1951; Compton and Jones 1985; Crossman et al. 2000). For example, the Bradford assay can underestimate protein in plant tissues rich in phenols and phenolases (Mattoo et al. 1987), which includes many brown seaweeds. Taken together, the number of unique combinations of extraction procedure, colorimetric assays, and type of seaweed substrate leads to considerable variation in the quantitative determination of protein.

In contrast to the technical issues related to the direct extraction of protein, the determination of the total nitrogen content does not require any extraction of material and is simple, inexpensive and easily reproducible. Total nitrogen in tissue is determined mainly using either the Kjeldahl method (or a variation thereof) or through combustion using CHN analysers. While the methods for quantifying total tissue nitrogen content are less variable than the direct extraction procedures, the fallibility of this approach is the conversion factor then used to calculate the total protein. The traditional conversion factor of 6.25, which is used as the standard factor for seaweeds and many other materials (Mariotti et al. 2008), assumes that the total protein constitutes 16 % (100/6.25) nitrogen and, more erroneously, also assumes that all nitrogen is in the form of protein. In reality, all plant material including algae have significant sources of non-protein nitrogenous material such as chlorophyll, nucleic acids, free amino acids and inorganic nitrogen (e.g. nitrate, nitrite and ammonia) (Naldi and Wheeler 1999; Lourenço et al. 2002; Lourenço et al. 2004). This can therefore lead to an over-estimate of protein contents in seaweeds when the 6.25 conversion factor is applied (Lourenço et al. 2002; Diniz et al. 2011; Shuuluka et al. 2013). As a result,

many studies have determined specific N-protein factors for commercially important terrestrial plants (Mossé et al. 1985; Mossé 1990; Sosulski and Imafidon 1990; Yeoh and Wee 1994; Yeoh and Truong 1996), fungal material (Danell and Eaker 1992; Fujihara et al. 1995), microalgae (Lourenço et al. 2004) and seaweed (Aitken et al. 1991; Lourenço et al. 2002; Diniz et al. 2011; Shuuluka et al. 2013). However, these published factors are seldom used for seaweeds with most authors reverting to the traditional conversion factor of 6.25. Failing to implement a specific factor has the potential to cause economic losses, as it has threatened to do with established industries such as dairy (Mariotti et al. 2008). Therefore, recalibrating with a universal seaweed-specific factor when the seaweed industry is relatively in its infancy, could avoid economic losses in the future.

N-protein conversion factors are assumed to be based on the quantification of total amino acids which is considered to be the most accurate way of determining protein (Heidelbaugh et al. 1975). Conversion factors have been calculated using two different methods. The first, which is referred to as k_A, uses the known molecular proportion of nitrogen of each individual amino acid, determined by quantitative amino acid analysis, to quantify the overall proportion of nitrogen in the total amino acid pool (Mossé et al. 1985; Mossé 1990). Although this method takes into account the specific amino acid profile of the material, it will overestimate the conversion factor if it is applied to total nitrogen content as it does not take into account the non-protein nitrogen. For this reason, conversion factors have also been calculated using another method, referred to as k_P, which is based on the ratio of the total amino acids to total nitrogen determined using independent methods (Mossé et al. 1985; Mossé 1990). Although k_P takes into account non-protein nitrogen, it relies on the assumption that the total amino acid analysis is a true determination of protein. However, amino acid analyses may underestimate protein contents due to the partial or full destruction of some amino acids during hydrolysis (in particular cysteine, tryptophan, methionine and serine) as well as the use of a single hydrolysis time that cannot guarantee the complete hydrolysis of certain amino acids without the destruction of others (Darragh and Moughan 2005). As a result of these inaccuracies, it has been suggested that k_P will underestimate the true conversion factor (Mossé 1990), although some authors argue, for algae at least, that free amino acids also analysed in the process compensate for the amino acids lost during hydrolysis (Lourenço et al. 2002). Therefore, because seaweeds typically contain

high concentrations of non-protein nitrogen, the most accurate way for estimating the nitrogen-to-protein conversion factor is the determination of protein by total amino acid analysis and the independent determination of total N (k_P) (Lourenço et al. 2002; Diniz et al. 2011).

Despite the established science for the calculation of N-protein conversion factors, and much evidence to suggest that high concentrations of non-protein nitrogen are common for seaweeds, only a few empirical studies have calculated seaweed-specific nitrogen to protein conversion factors – all of which were lower than 6.25 (Aitken et al. 1991; Lourenço et al. 2002; Diniz et al. 2011; Shuuluka et al. 2013). Aside from these studies, the use of the N-protein factor of 6.25 remains the default factor for seaweeds. Similarly, authors continue to report direct extraction procedures and quantification of soluble protein in the literature despite these being highly variable and generally perceived to underestimate the content of protein in seaweeds (Crossman et al. 2000; Barbarino and Lourenco 2005; Shuuluka et al. 2013).

There is, therefore, a strong rationale for synthesising the body of data in the literature on the protein content of seaweeds to provide the simplest and most accurate standardised method for determining the content (proportion of dry weight) of protein in seaweed biomass. The aim of this chapter is to quantitatively list the methods used in the literature, assess their suitability in quantifying protein and recommend the most appropriate method to determine protein in seaweeds. To do this, I consolidated available nitrogen and total amino acid data to calculate seaweed-specific N-protein conversion factors and analyse associations between these and the critical variables of taxonomic groups, geographic regions, cultivated and wild harvested seaweeds, and internal N content. The overarching goal of this meta-analysis is to determine if there is an acceptable universal seaweed-specific conversion factor, and if so provide a justifiable value.

4.2. Materials and methods

4.2.1. Literature search

To retrieve a large number of original research articles that reported the protein or amino acid content in seaweeds, the Web of Science core collection (1945-present) was

searched on the 4th November 2014 using the following search string for terms in the title, key words or abstract: ((protein* OR amino*) AND (nutrition* OR nitrogen* OR lipid* OR carbohydrate* OR nutrient* OR biochemical* OR aquaculture*) AND (macroalga* OR seaweed*) NOT (enzyme* OR mycosporine*)). This search string was determined to be the most efficient at reducing the number of irrelevant articles while maintaining a large number of relevant articles. No constraints on the year of publication or the language of publication were imposed on the database search. In addition, 17 articles that were not found by this search string were also included in the meta-analysis (see Annex to Chapter 4 for all articles included in meta-analysis).

To ensure I only included articles that met my aim, I screened the results by reading the title, abstract and materials and methods to exclude those articles that did not contain a measure of protein or amino acids of unprocessed seaweed. I did not have access to 23 of the 259 articles that remained after the first screening. These were not examined further. I recorded the number of articles included and excluded according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement (see Fig. 4.1) (Moher et al. 2009).

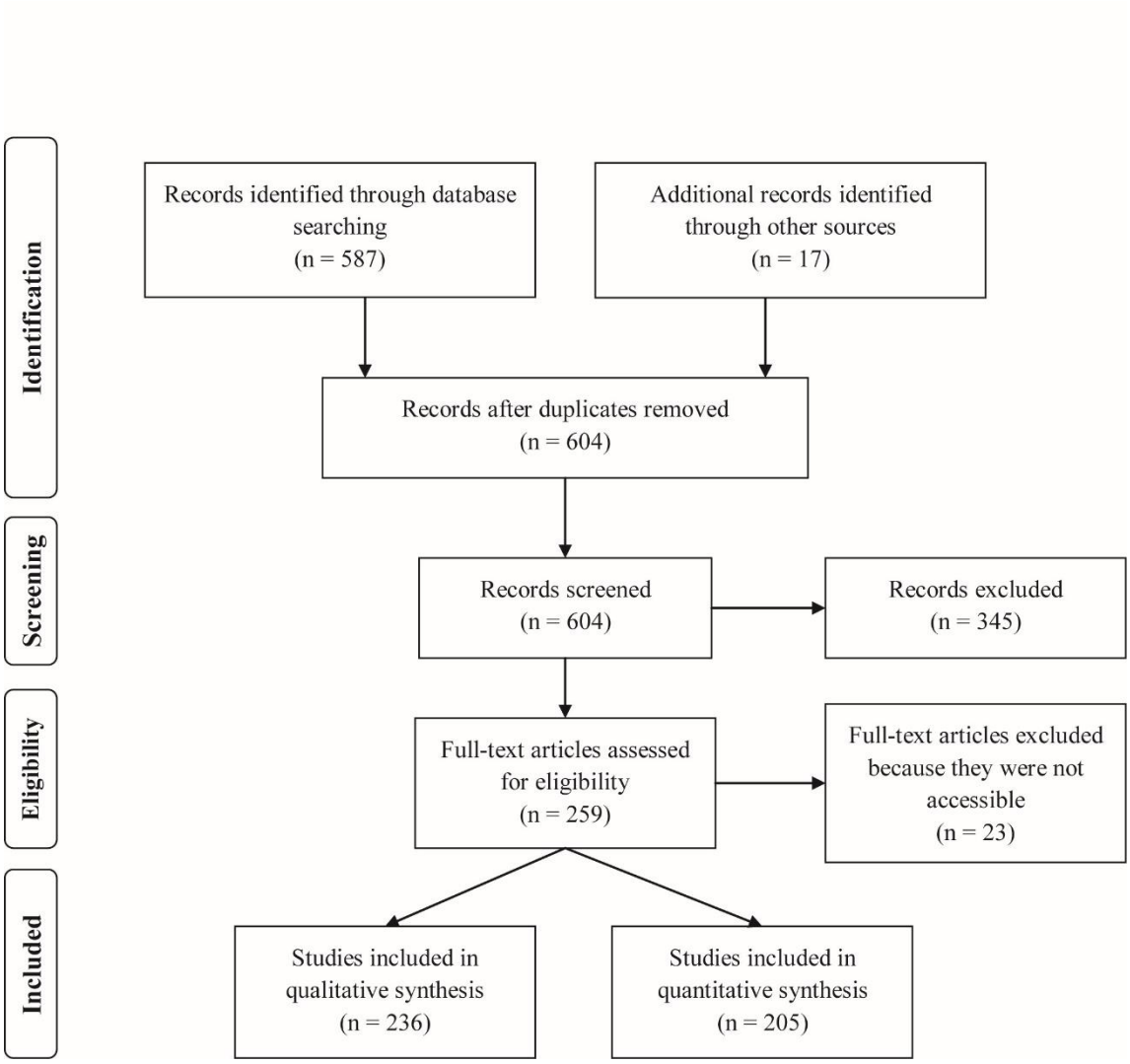

Figure 4.1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) literature search flow diagram (Moher et al., 2009).

4.2.2. Extraction of qualitative data

For each article included in the meta-analysis (236 articles), the following qualitative information was recorded: (1) the year of the study, (2) the journal of publication, (3) the discipline of the article, (4) the phylum, genus and species of each seaweed analysed, (5) the geographic region of the seaweed (tropical, temperate or polar), (6) whether the seaweed was wild harvested or from a cultivation system, (7) the method used to determine the protein content in each sample, (8) the method used to determine the total tissue nitrogen content in each sample (if measured), (9) whether the total amino acids were measured and (10) the units in which the amino acids were reported.

Articles were divided into eight scientific disciplines, as defined in Table 4.1. Tropical regions were defined by the Tropic of Cancer (23˚26'16''N) and Tropic of Capricorn (23˚26'16''S), Polar Regions by the Arctic Circle (66˚33'44''N) and the Antarctic Circle (66˚33'44''S), and temperate regions between these latitudes. Wild harvested seaweeds were defined as any seaweed that was harvested from natural seawater (including sea-ranched seaweed) and cultivated seaweeds as any seaweed that were cultivated in an artificial land-based system. The protein determination methods that involved the extraction of protein were grouped together as 'extraction' methods and the method of protein quantification for each was recorded. The total amino acid (TAA) content as a protein determination method was defined by: (1) the article reported the sum of amino acids as a proportion of dry weight and (2) there was no other method of protein determination. Only those articles that met these criteria were considered to have used TAA as a protein measurement. However, all amino acid data was used in the quantitative section of this meta-analysis, irrespective of whether it was used as the primary method for determining protein in an article (36 articles also reported TAA data in addition to the 14 which used TAA content as a protein determination method).

4.2.3. Extraction of quantitative data

The following quantitative information was recorded: (1) the protein content in % dry weight (dw), (2) the tissue nitrogen content in % dw and (3) the total amino acid content in % dw (see Table 4.2 for definition of terms). When this data was only presented in a figure in an article it was obtained using the software DataThief III (Tummers 2006). All measurements were converted to % dw. Measurements expressed in terms of fresh weight or ash free dry weight were converted to % dw using moisture and ash contents, respectively. Protein measurements expressed as moles of nitrogen per unit biomass were converted using equation 4.1, assuming a protein nitrogen content of 16 % (Naldi and Wheeler 1999). Amino acid measurements expressed as moles per unit biomass were converted to % dw by using the sum of the molecular weight of individual amino acids. Total amino acid measurements expressed as % protein were converted into % dw using equation 4.2.

$$Protein\ (\%\ dw) = \frac{Protein\ (mol\ N\ g^{-1}\ dw) \times 14.007}{0.16} \qquad \textbf{Eq. 4.1}$$

$$Total\ amino\ acids\ (TAA)\ (\%\ dw) = \frac{TAA\ (\%\ protein) \times Protein\ (\%\ dw)}{100} \qquad \textbf{Eq. 4.2}$$

If a measurement could not be converted to % dw it was not included in the quantitative analysis of this meta-analysis.

Table 4.1. Defining criteria for allocating articles to a particular scientific discipline in qualitative assessment of literature

Discipline	Definition
1. Analytical	The study's main aim was to test analytical methods.
2. Cultivation/bioremediation	The study investigated the culture of seaweed using either seawater or wastewater.
3. Biochemical profiling	The study's main aim was to report biochemical profiles of seaweed in a non-physiological context.
4. Ecological	The study had an ecological context with its main aim to examine interactions between seaweed and other organisms.
5. Experimental/physiological	Any study which cultivated seaweed under experimental conditions where certain parameters were manipulated or where wild harvested seaweed was physiologically examined.
6. Feeding trial	Any study where the seaweed was used as a feed or feed ingredient in a feeding trial.
7. Protein extraction	The study's main aim was to test different protein extraction methods.
8. Protein digestibility	The study's main aim was to test the digestibility of protein from seaweeds.

For quantitative analysis between determination methods, all available data was used for N*6.25 and TAA methods – not just data from measurements where the respective method was used to officially determine protein. This meant that all the nitrogen content data was multiplied by 6.25 to obtain the quantitative N*6.25 data and all TAA (% dw) were used for the quantitative TAA data.

Table 4.2. Definition of commonly used terms in this chapter

Term	Definition
Protein content	The protein as a percentage of dry weight measured by any method.
Total nitrogen content	The nitrogen content as a percentage of dry weight measured by any method.
Extraction procedure	Protein content measured by extracting soluble protein and quantifying it with a colorimetric assay (mainly Bradford and Lowry).
N*6.25	Protein content determined by multiplying the nitrogen content by a factor of 6.25.
TAA	Protein content measured by quantifying and summing the proteomic amino acids (up to 20 amino acids, but most often 18 amino acids with cysteine and tryptophan excluded).
N-protein factor	The ratio of protein as measured by TAA to total nitrogen content.
TAA N	The concentration of nitrogen in the TAA fraction of the biomass (in g N 100 g^{-1} TAA, see Eq. 4.3).
Non-TAA N	The concentration of nitrogen in the non-TAA fraction of the biomass (in g N 100 g^{-1} TAA, see Eq. 4.4).

N-protein factors – the ratio of TAA content (% dw) to total nitrogen content (% dw) – were calculated from two different data sets in this meta-analysis for different reasons. First, as a way to compare direct extraction procedures with TAA analysis, N-protein factors were calculated for all measurements of protein determined using both direct extraction procedures and TAA analysis which had a corresponding tissue nitrogen content measurement. This included TAA measurements that were not originally used to determine protein content but were reported along with nitrogen and/or protein content. Second, N-protein factors were also calculated just for TAA data as this method is considered the most accurate method for determining protein (Heidelbaugh et al. 1975). These N-protein factors were calculated only from the 5^{th} to 95^{th} percentile so as not to represent extreme values and were used to determine seaweed-specific N-protein factors for an applied use and for correlations with internal N content. However, to determine seaweed-specific N-protein factors for an applied use, these N-protein factors were calculated using the means of each species so as not to over represent those species that had large numbers of measurements. However, for correlations with internal N content, raw N-protein factors (from the 5^{th} to 95^{th} percentile) calculated from all individual TAA data were used instead of the means of each species.

The concentration of nitrogen in both the TAA and non-TAA fractions were calculated for all seaweeds from which N-protein factors were calculated (with the exception of the data from five studies which did not report individual amino acid contents, n = 29 individual measurements and n = 2 species excluded). Furthermore, as with N-protein factors, the concentration of nitrogen in TAA and non-TAA acid fractions are reported

based on the means of each species (as explained in the previous paragraph). The concentration of TAA nitrogen and non-TAA nitrogen are expressed as g N 100 g^{-1} TAA and were calculated using Eq. 4.3 and 4.4, respectively, where D_i is the concentration of nitrogen in the *i*th AA per 100 g dw, AA_i is the concentration of the *i*th AA per 100 g dw and total N is the total concentration of nitrogen per 100 g dw.

$$TAA\ N = \frac{\sum D_i}{\sum AA_i} \times 100 \qquad \textbf{Eq. 4.3}$$

$$non\ TAA\ N = \frac{Total\ N - TAA_N}{TAA} \times 100 \qquad \textbf{Eq. 4.4}$$

4.2.4. Reporting of results and statistical analysis

All extracted quantitative and qualitative data were recorded in one Microsoft Excel 2007 spreadsheet with each column representing the qualitative and quantitative questions listed above and each row representing a unique measurement. Pivot tables were used to extract the qualitative meta-data. Qualitative data are presented in pie charts, bar graphs and tables. The % dw measurements of the major methods of protein determination (extraction, N*6.25 and TAA) and N-protein factors are presented using box and whisker plots overall, between phylum, between region and between cultivated and wild harvested seaweed using Statistica 12 (StatSoft Inc.). To standardise for nitrogen content, the methods of protein determination were also compared overall using box and whisker plots of N-protein factors. Boxplots were also used to compare the values and variation in TAA N and non-TAA N between- and within-species.

For all quantitative data: medians, means, inter-quartile ranges, 5^{th}/95^{th} percentile ranges and standard deviations were calculated using Statistica 12 and Microsoft Excel 2007.

As the data set for any specific combination of treatments was unbalanced, multivariate PERMANOVAs (PRIMER 6 & PERMANOVA+, PRIMER-E Ltd., Lutton, UK) were used to analyse the effect of determination method on protein content (% dw) between taxonomic groups (green, brown and red seaweeds), regions (temperate, tropical and polar) and cultivated vs. wild harvested seaweed. Differences in N determination method, N-protein factors between extraction and TAA methods, differences in N-protein factors between taxonomic groups (green, brown and red seaweeds), regions (temperate, tropical and polar) and cultivated vs. wild harvested seaweed were also all analysed using multivariate PERMANOVAs. Finally, differences in non-TAA N between the taxonomic groups were also analysed using multivariate PERMANOVAs.

N-protein factors of extraction and TAA methods were also each compared to 6.25 using one sample t-tests (Statistica 12; StatSoft Inc.).

Correlations were made between N content and N-protein factor using Statistica 12 (StatSoft Inc.) for all data as well as all combinations of taxonomic group, region and wild harvested or cultivated. A separate correlation was also made between N content and N-protein factor for data in Angell et al. (2014).

4.3. Results

My systematic approach retrieved 604 articles that were potentially relevant to the meta-analysis (Fig. 4.1). Of these, 345 articles did not have any measure of protein or amino acids of unprocessed seaweed in the title, abstract or materials and methods and were excluded from the meta-analysis. The remaining 259 articles were read in full to extract the relevant qualitative and quantitative data; however, 23 of these could not be retrieved as full text articles and were excluded. This resulted in a total of 236 articles. Of these, 31 articles had quantitative data that could not be standardised as % dw and were therefore only used in the qualitative section (Fig. 4.1). Reasons for this included measurements in wet weight with no moisture content reported (n = 17), no direct reporting of quantitative data (n = 7), measurements in ash-free dry weight with no ash content reported (n = 4, however, one of these was still used in the calculation of N-protein factors), amino acids reported as g 16 g^{-1} N with no N content reported (n = 1), amino acids reported as μmol g^{-1} dw with no individual amino acid contents reported (n = 1) and amino acids reported as % TAA with no TAA content reported (n = 2, however, one of these had usable protein content data). In addition to these, the method of protein determination could not be retrieved for one article.

The raw data set generated from this chapter has been made open access (Angell et al. 2015).

4.3.1. Qualitative results

The 236 articles that were included in the meta-analysis could be divided into eight disciplines (Fig. 4.2A) and were published across 90 journals. The major disciplines were biochemical profiling studies (44 %, n = 102), feeding trials (20 %, n = 46), experimental/physiological studies (19 %, n = 44), ecological (7 %, n = 16) and

cultivation/bioremediation (6 %, n = 14). The majority of the articles were found in the *Journal of Applied Phycology* (12 %, n = 29), *Food Chemistry* (7 %, n = 16), *Aquaculture* (6 %, n = 15), *Journal of Phycology* (6 %, n = 13), *Botanica Marina* (5 %, n = 12), *Marine Ecology Progress Series* (3 %, n = 7), *Aquaculture Research* (3 %, n = 6) and *Ecology* (2 %, n = 5), representing 44 % of all articles. The remaining journals (n = 82) had four or less articles with the majority having only one article (n = 51).

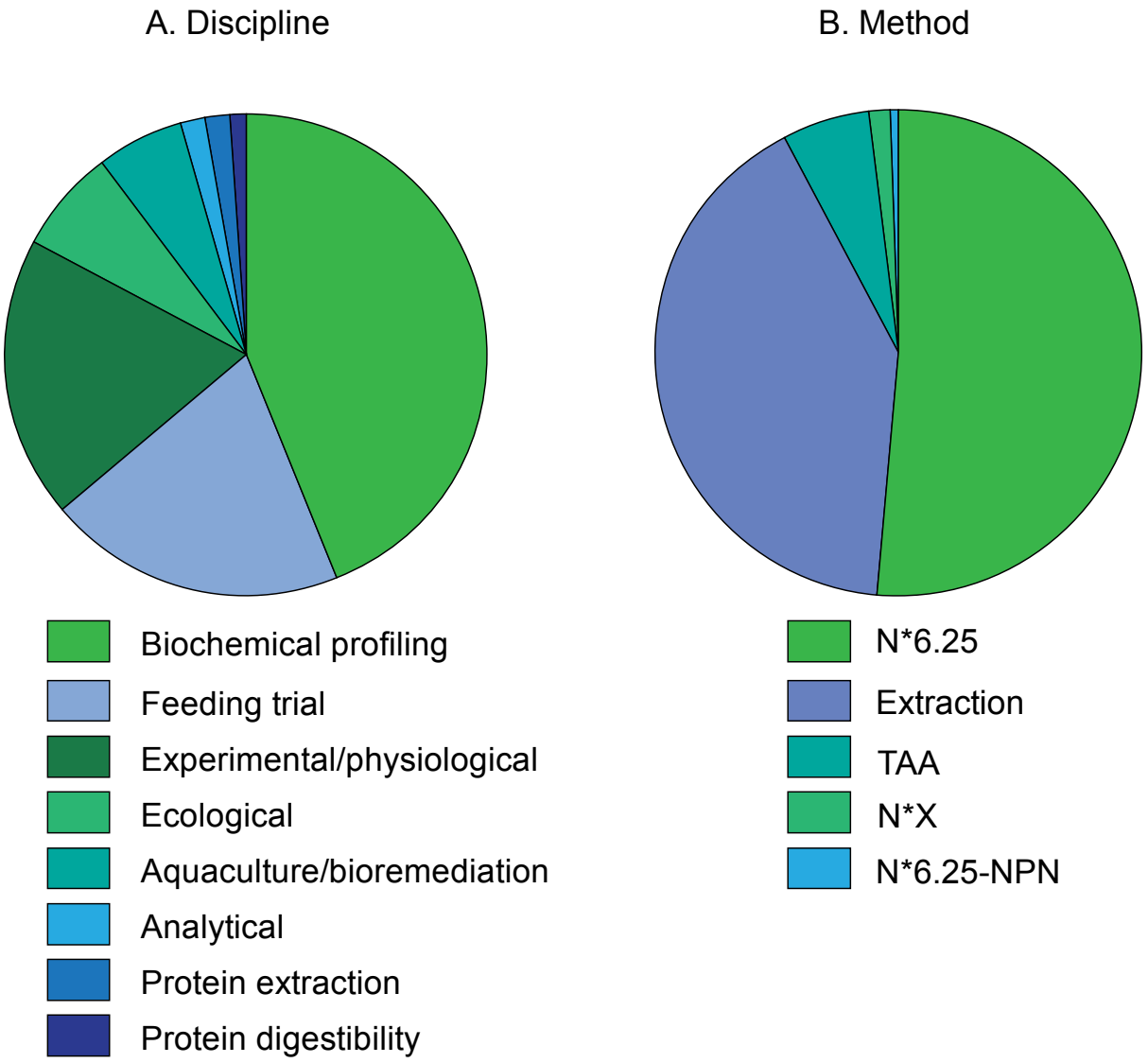

Figure 4.2. Proportion of (A) papers in different disciplines and (B) the methods used to determine protein in this chapter. Pie chart legends are listed in descending order of importance.

Overall, five broad methods of protein determination were found: multiplying tissue nitrogen content by 6.25 (N*6.25), protein extraction and quantification of soluble protein (via the Bradford, Lowry, Bicinchoninic acid assay (BCA) and UV absorption

methods) (Extraction), quantification of total proteomic amino acids (TAA), multiplying tissue nitrogen content by a unique factor determined specifically for seaweeds (N*X) and N*6.25 without including non-protein nitrogen (N*6.25 – NPN). Overall, the most commonly used method was N*6.25 (52 %) followed by direct extraction procedures (42 %) and TAA (6 %) (Fig. 4.2B). Almost 25 % of studies measured amino acids, however, over 70 % of these studies determined protein using either N*6.25 or direct extraction procedures. Within the major disciplines, N*6.25 was the most common method used in biochemical profiling studies (64 %), cultivation/bioremediation studies (64 %) and feeding trials (76 %). On the other hand, direct extraction procedures were most common in experimental/physiological studies (86 %) and ecological studies (75 %) (Table 4.3). Most extracted protein was quantified using the Bradford method (55 %), followed by the Lowry method (31 %) and the BCA method (11 %), with other methods making up less than 5 %.

Table 4.3. Number of papers within each discipline and the protein determination methods used. Note: some papers determined protein using more than one method.

	Extraction	N*6.25	N*6.25-NPN	N*X	TAA	Total papers
Analytical	1	2	0	1	2	4
Cultivation/bioremediation	4	9	0	0	2	14
Biochemical profiling	32	65	1	1	5	104
Ecological	12	2	0	0	2	16
Experimental/physiological	38	4	0	0	2	45
Feeding trial	11	35	0	1	1	47
Protein extraction	0	4	0	0	0	4
Protein digestibility	0	2	0	0	0	2
Total	98	123	1	3	14	236

Overall, protein data was recorded for 1841 measurements from 382 species. Red seaweeds were the most studied taxonomic group (highest number of measurements - 43 %, n = 792), followed by green seaweeds (32 %, n = 576) and brown seaweeds (25 %, n = 459). Red seaweeds were also the most diverse study group with 86 genera compared to brown seaweeds (50 genera) and green seaweeds (22 genera). Within the red seaweeds, the most studied genera were *Gracilaria* (29 %, n = 232), *Palmaria* (12 %, n = 98), *Gelidium* (9 %, n = 68), *Eucheuma* (8 %, n = 62) and *Pyropia* (formally *Porphyra* (6 %, n = 51)), with all remaining genera each representing less than 3 % (n = 81 additional genera). Within the brown seaweeds, the most studied genera were

Sargassum (19 %, n = 85), *Dictyota* (12 %, n = 54), *Macrocystis* (11 %, n = 49), *Laminaria/Saccharina* (9 %, n = 40), *Fucus* (6 %, n = 27) and *Padina* (6 %, n = 25), with all remaining genera representing less than 5 % (n = 33 additional genera). Within green seaweeds, the genus *Ulva* represented the vast majority of measurements (67 %, n = 385), followed by *Chaetomorpha* (12 %, n = 70) and *Codium* (6 %, n = 33), with all remaining genera representing less than 4 % (n = 19 additional genera).

The number of articles published according to our criteria increased with time (Fig. S4.1), although only a small number of studies (n = 11) were retrieved before 1995, owing to limited electronic database entries. The counts of articles retrieved since 1995 are an accurate representation of the size of the field. In 2010 and 2011 the total number of articles published per year increased dramatically and has remained high until the present. Before the year 2000, direct extraction procedures were generally the most common method for determining protein in seaweeds. However, more recently, the N*6.25 method has become the most widely utilised method, particularly in recent years (2009 – 2014). More recently (2012 – 2014), there has been a slight increase in the use of TAA as a method for determining protein, however, this method still represents a small proportion of studies (17 % in 2014).

4.3.2. Quantitative results - methods of protein determination

Overall, direct extraction procedures yielded the lowest protein contents and use of the N-protein conversion factor of 6.25 (N*6.25) resulted in the highest, while TAA content resulted in an intermediate measure of protein (Fig. 4.3A, PERMANOVA: Pseudo-$F_{2, 2616} = 504.16$, $p < 0.01$). Direct extraction procedures (n = 945) had the lowest protein measure (7.78 % dw) compared to both N*6.25 (PERMANOVA pair-wise test: $t = 31.53$, $p < 0.01$) and TAA ($t = 9.52$, $p < 0.01$) methods. In contrast, those protein contents determined using N*6.25 (n = 1411) had the highest mean protein measure (16.60 % dw) compared to extraction (PERMANOVA pair-wise test: $t = 31.53$, $p < 0.01$) and TAA ($t = 11.17$, $p < 0.01$) methods. Finally, where protein was determined by TAA (n = 299) it was an intermediate value compared to the other methods (11.60 % dw – see statistics above), with the protein contents of 90 % ($5^{th}/95^{th}$ percentile) of seaweeds between 3 and 27 % dw. Notably, the spread of the data for each method (standard deviations) were relatively similar for extraction and TAA (6.35 & 6.93 %

dw, respectively) but higher for N*6.25 (7.91 % dw) (see Table S4.1 for all descriptive statistics relating to methods of protein determination).

Protein contents were higher when using N*6.25, followed by TAA and direct extraction procedures, irrespective of the taxonomic group (Fig. 4.3B, PERMANOVA: Pseudo-$F_{2, 2610}$ = 489.16, $p < 0.001$), the region where the seaweed was collected (Fig. 4.3C, PERMANOVA: Pseudo-$F_{2, 2610}$ = 207.72, $p < 0.001$) or whether the seaweed was wild harvested or cultivated (Fig. 4.3D, PERMANOVA: Pseudo-$F_{2, 2613}$ = 375.14, $p < 0.001$).

4.3.2.1. Taxonomic groupings

Between taxonomic groups (Fig. 4.3B), brown seaweeds had the lowest mean protein content, followed by red seaweeds and green seaweeds (PERMANOVA pair-wise comparisons, $p < 0.01$). Relative to the overall mean brown seaweed protein content (10.00 % dw), red seaweeds had 33 % more protein (13.31 % dw) and green seaweeds had 45 % more protein (14.48 % dw). This pattern remained similar when taxonomic groups were standardised for wild harvested seaweeds – brown seaweeds with the lowest (10.00 % dw) (PERMANOVA pair-wise: $p < 0.01$) compared to green seaweeds (12.73 % dw) and red seaweeds (13.11 % dw). Within each determination method, brown seaweeds maintained the lowest protein content, but red and green seaweeds only differed in mean protein content for TAA method (PERMANOVA pair-wise: $p < 0.01$). The difference between brown seaweeds and green and red seaweeds was lowest for direct extraction procedures (means = 8.13, 5.98, 8.24 % dw, respectively). However, for N*6.25 and TAA methods, red and green seaweeds had much higher mean protein contents relative to brown seaweeds (see Table S4.1 for all descriptive statistics).

Based on the true proteomic (TAA) content, the green seaweeds had a $5^{th}/95^{th}$ percentile range of 4.6 – 32.2 % dw, the red seaweeds of 2.0 – 28.7 % dw and the brown seaweeds of 3.3 – 15.9 % dw.

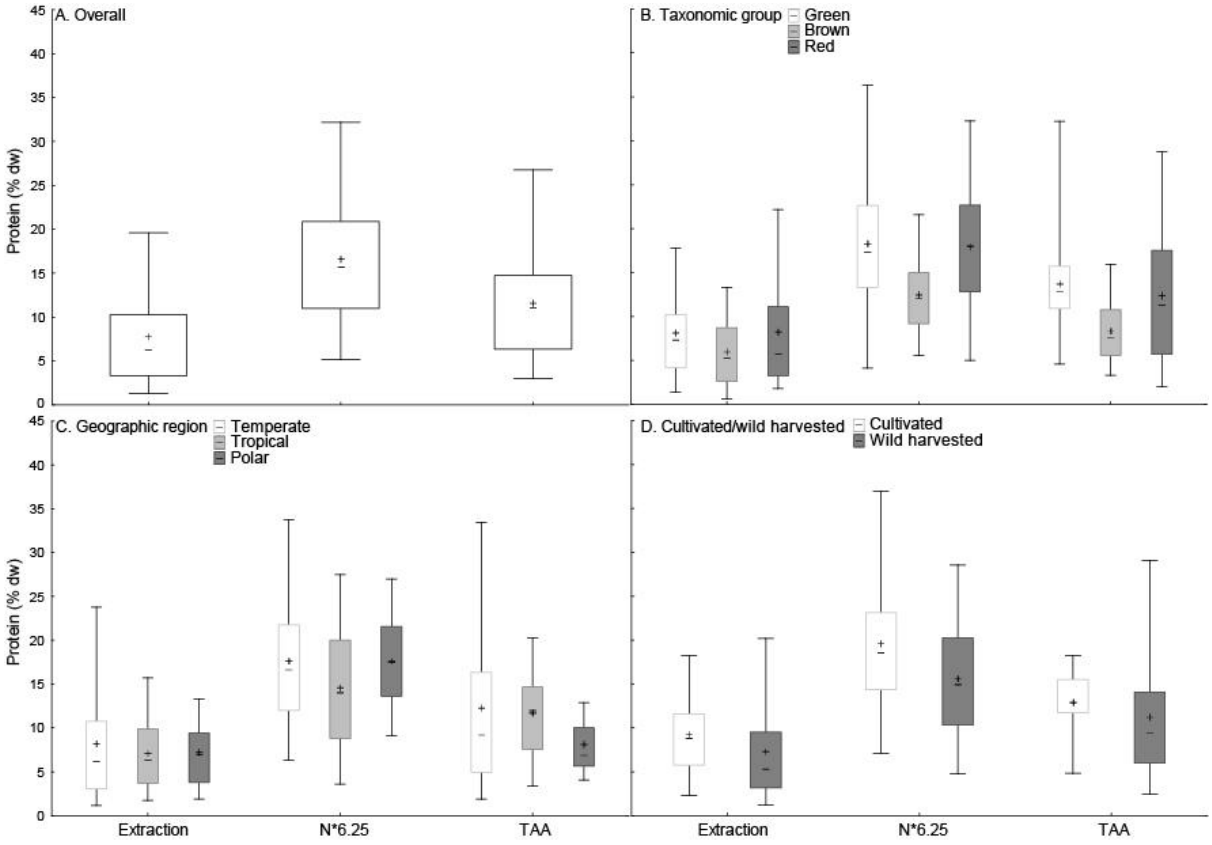

Figure 4.3. Quantitative protein measurements (% dw) of the papers examined in this chapter (A) overall, (B) among taxonomic groups, (C) among geographic regions and (D) among wild harvested and cultivated seaweed. Dashes represent medians, crosses represent means, boxes represent 25th percentiles and whiskers represent 5th/95th percentiles.

4.3.2.2. Geographic regions

The relationships between geographic regions varied with the different protein determination methods. In a similar result to the taxonomic groupings, the three regions had the most similar mean protein contents when determined using direct extraction procedures (8.21, 7.11 and 7.21 % dw for temperate, tropical and polar, respectively), although extreme measurements were more variable for temperate seaweeds (c.f. whiskers in Fig. 4.3C). In contrast, when determined using the N*6.25 method, mean protein content was slightly less for tropical seaweeds (14.57 % dw) compared to temperate (17.64 % dw) and polar (17.63 % dw – all of which were brown) seaweeds (PERMANOVA pair-wise comparisons: $p < 0.001$). However, variation was similar between regions for the N*6.25 method (SD = 7.98, 7.67 and 8.31 % dw for temperate, tropical and polar seaweeds, respectively). When determined using TAA methods, mean protein contents for tropical seaweeds (11.69 % dw) were lower than temperate

seaweeds (12.29 % dw) and higher than polar seaweeds (8.14 % dw) (PERMANOVA pair-wise comparisons: $p < 0.01$), with no significant difference between the mean measures of temperate and polar.

4.3.2.3. Cultivated vs. wild harvested

Protein contents were higher in cultivated seaweed (means = 9.26, 19.61 and 12.92 % dw for extraction, N*6.25 and TAA, respectively) compared to wild harvested seaweed (means = 7.29, 15.58 and 11.22 % dw for extraction, N*6.25 and TAA, respectively) for all three methods of determination (Fig. 4.3D, PERMANOVA pair-wise comparisons: $p < 0.01$).

4.3.2.4. N-protein conversion factor of different methods

Both extraction (One-sample t-test: $t_{537} = -19.85$, $p < 0.01$) and TAA ($t_{279} = -16.15$, $p < 0.01$) methods had mean N-protein conversion factors lower than 6.25 (Fig. S4.2). However, N-protein factors calculated using direct extraction procedures were lower and more variable (n = 538, mean = 3.51, median = 2.89, SD = 3.20) compared to the TAA measurements (n = 279, mean = 4.69, median = 4.87, SD = 1.62) (PERMANOVA: Pseudo-$F_{1,816} = 92.95$, $p < 0.0001$).

4.3.3. Determination of nitrogen

Of the studies that measured total tissue nitrogen content in addition to protein and/or amino acids, 64 % used a variant of the Kjeldahl method and 34 % determined nitrogen through combustion using CHN analysers. Determination by combustion had a higher mean (2.77 % dw) and smaller standard deviation (1.05 % dw) compared to determination by the Kjeldahl method (mean = 2.563 % dw, SD = 1.47 % dw) (Fig. S4.3, PERMANOVA: Pseudo-$F_{1, 1289} = 32.76$, $p < 0.001$).

4.3.4. Nitrogen-to-protein conversion factors

The nitrogen-to-protein conversion (N-protein) factors were determined for 110 species from 289 individual measurements (excluding within-article replication) but were calculated only for the $5^{th}/95^{th}$ percentile range (103 species, 260 individual measurements) (see Table S4.2 for all individual species N-protein factor data). Overall, the N-protein factors had a mean value of 4.76, a median of 4.97, an inter-quartile range of 3.83 – 5.68, a $5^{th}/95^{th}$ percentile range of 2.74 – 6.24 and a SD of 1.14 (Fig. 4.4A).

There was a significant albeit small difference in the mean N-protein factors between the different taxonomic groups (Fig. 4.4B, PERMANOVA: Pseudo-$F_{2,100}$ = 3.34, $p < 0.05$). Green and brown seaweeds had lower mean and median N-protein factors (PERMANOVA pair-wise comparisons: t = 2.390 and 2.259, $p < 0.05$, means = 4.49 and 4.56, medians = 4.68 and 4.81, for green [n = 26] and brown [n = 35] seaweeds, respectively) compared to red seaweeds (n = 42, mean = 5.10, median = 5.31). There was no significant difference in mean N-protein factors between the three regions (temperate: n = 30, mean = 4.89, median = 5.28, SD = 1.06; tropical: n = 74, mean = 4.79, median = 5.98, SD = 1.14). There were only 2 samples from the polar regions, however, these had noticeably lower N-protein factors (mean = 3.04, median = 3.04), (Fig. 4.4C). Finally, there was no significant difference in mean N-protein factor between cultivated and wild harvested seaweeds, although this was heavily weighted to wild harvested compared to cultivated seaweeds. Wild harvested seaweeds (n = 98) generally had higher and more variable N-protein factors (mean = 4.80, median = 5.05, SD = 1.15) than cultivated seaweeds (n = 6, mean = 4.25, median = 4.42, SD = 0.76) (Fig. 4.4D).

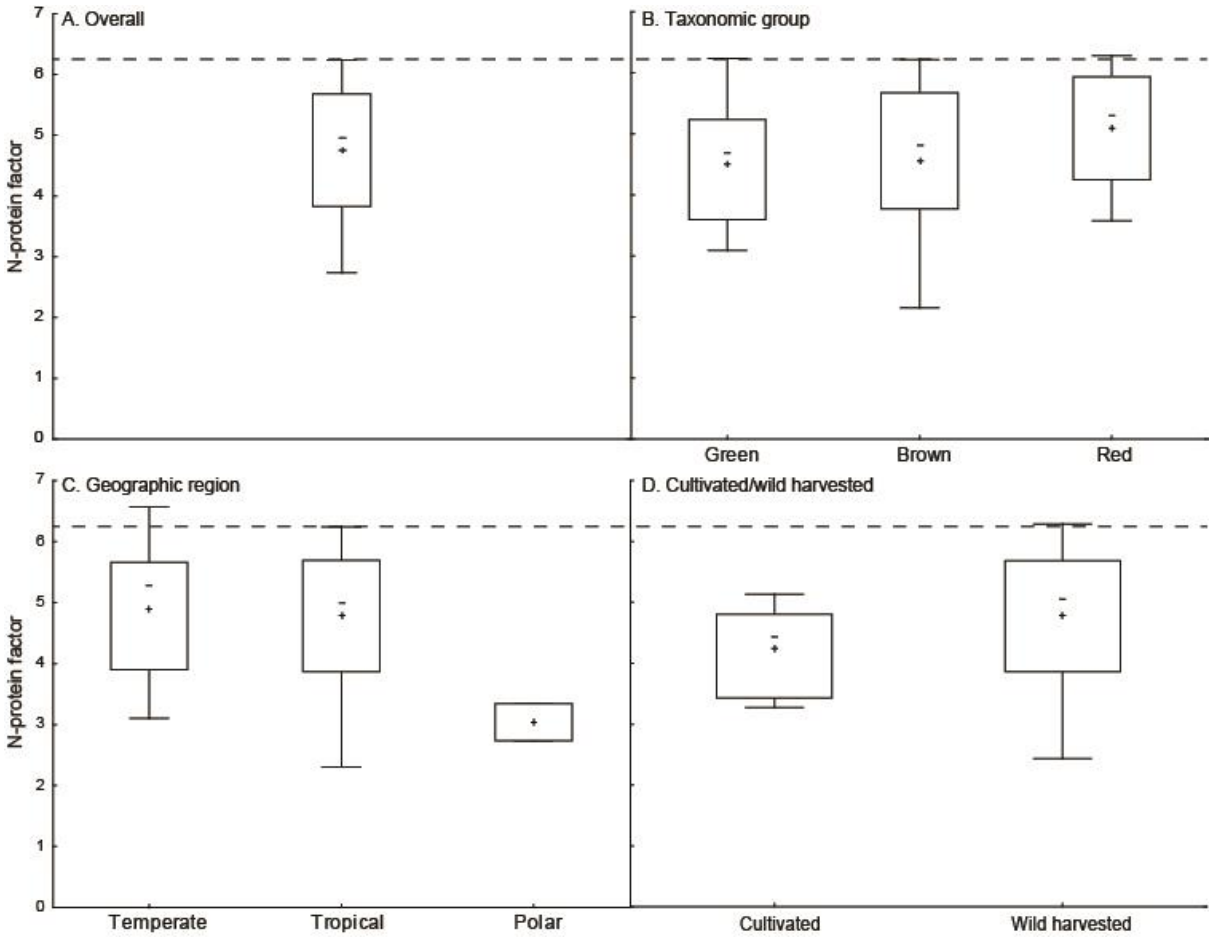

Figure 4.4. Nitrogen-to-protein conversion factors calculated from papers in this chapter (A) overall, (B) among taxonomic groups, (C) among geographic regions and (D) among wild harvested and cultivated seaweeds. Dashes represent medians, crosses represent means, boxes represent 25th percentiles and whiskers represent 5th/95th percentiles. Dashed lines indicate a conversion factor of 6.25.

4.3.4.1. Total amino acid versus non-total amino acid nitrogen

Overall, the total amino acid content represented considerably more nitrogen (TAA N; mean = 15.04 g N 100 g^{-1} TAA, median = 15.04 g N 100 g^{-1} TAA) compared to the non-TAA nitrogenous components (non-TAA N; mean = 7.51 g N 100 g^{-1} TAA, median = 4.72 g N 100 g^{-1} TAA). However, there was considerably more variation in the concentration of non-TAA N (SD = 7.32 g N 100 g^{-1} TAA) compared to TAA N (SD = 0.71 g N 100 g^{-1} TAA) (Fig. S4.4). For red seaweed, which was the only taxonomic group to have a significantly different N-protein factor, the non-TAA N was lower (mean = 5.28 g N 100 g^{-1} TAA) compared to green and brown seaweeds (8.98 and 9.17g N 100 g^{-1} TAA, respectively) (PERMANOVA pair-wise comparisons: $p < 0.05$), although for brown seaweeds this was not statistically significant.

4.3.4.2. Correlations between N content and N-protein conversion factors

Overall, there was no correlation between internal N content and N-protein factor (Table S4.3). However, there were significant correlations between various combinations of the categories. Many of these correlations were driven by Angell et al. (2014), which was the only study to measure N content and TAA content for a large number of individuals (n = 60) at a species level over a large range of internal N contents. As the study focused on the tropical green seaweed *Ulva ohnoi*, this resulted in relatively strong negative relationships between internal N content and N-protein factor for all sub-groups which encompassed tropical, green or cultivated seaweed (see Table S4.3 for R^2 and p values). However, there were still a number of other correlations, albeit weaker relationships, within other sub-groups. For example, brown seaweeds (all of which were wild harvested) showed a significant negative correlation as did red tropical seaweeds (all of which were wild harvested) and tropical wild harvested seaweeds. In contrast, temperate seaweeds and, more specifically, wild harvested temperate seaweeds had significant positive relationships between N content and N-protein factors, although these were very weak (R^2 = 0.062 and 0.081, respectively) (see Table S4.3 for all correlations).

4.3.4.3. Within-species variation in N-protein conversion factors

There was considerable variance in N-protein factors within the green seaweed *Ulva ohnoi*. *U. ohnoi* had a mean value of 5.14, a median value of 5.17 and a SD of 0.47 (Fig. S4.5A). Similar to the between-species results (Fig. S4.4), total amino acids represented considerably more nitrogen (mean = 15.10 g N 100 g^{-1} TAA, median = 14.86 g N 100 g^{-1} TAA) compared to non-TAA, nitrogenous components (mean = 4.51 g N 100 g^{-1} TAA, median = 4.48 g N 100 g^{-1} TAA). However, there was considerably more variation in the concentration of non-TAA nitrogen (SD = 1.57 g N 100 g^{-1} TAA) compared to TAA nitrogen (SD = 0.52g N 100 g^{-1} TAA) (Fig. S4.5B).

4.4. Discussion

A resurgence of interest in the industrial applications of seaweeds has led to a large (~ 200 – 300 %) increase in the number of studies published per year examining protein from 2009 to the present. However, there are considerable differences in approach

between these studies used to determine protein. Only a limited number of studies in the field measured the true protein content using the sum of proteomic amino acids (< 6 %) and the vast majority determined protein using either direct extraction procedures and the subsequent determination of soluble protein (42 % - mainly via Bradford and Lowry assays) or the indirect method of protein determination using the generic N-protein conversion factor of 6.25 translated from terrestrial animal and plant literature (52 %). The meta-analysis of the reported data for these methods demonstrates that direct extraction procedures generally underestimated protein content in seaweeds, and that the 6.25 N-protein factor overestimated protein content in seaweeds relative to protein determined by the sum of proteomic amino acids (TAA). However, the true proteomic amino acid analysis remains an expensive and technical method that is seldom used to determine protein contents in seaweeds. Therefore, it is suggest that a seaweed specific N-protein factor calculated from total amino acid analyses offers a simple, relatively inexpensive and easily reproducible method for protein determination. A consolidation of all nitrogen and total amino acid data shows that 95 % of N-protein factors for seaweeds are lower than 6.25. On the base of this evidence, I propose a universal N-protein factor for seaweeds of 5 in place of the commonly cited factor of 6.25 to be used when TAA is not calculated.

4.4.1. Methods of protein determination

The large majority of articles (96 %) analysed in this meta-analysis could be divided into the five main disciplines of biochemical profiling, feeding trial, experimental/physiological, ecological and cultivation/bioremediation studies. Of these, biochemical profiling, feeding trial and cultivation/bioremediation studies predominantly determined protein using the 6.25 N-protein factor, while experimental/physiological and ecological studies predominantly determined protein using direct extraction procedures. Further, over 70 % of articles (n = 41) which measured amino acids still determined protein using either extraction or N*6.25 methods. This latter point speaks to a desire by authors to present protein data, where possible, in a standardised manner, and this concept should be taken into consideration when recommending a unified approach for studies across disciplines.

I found that the choice between the proxy N*6.25 method and an extraction procedure is linked to the discipline that the research falls under. While the disciplines which were

primarily measuring protein for nutritional purposes predominantly used the N*6.25 method (66 % of the "nutritional" literature), the disciplines which examined the relative changes in protein content with respect to an experimental treatment or ecological process predominately used direct extraction procedures (82 % of the "physiological" and "ecological" literature). This suggests that the selection of the N*6.25 method may be biased when the purpose of reporting protein is to provide a nutritional assessment of the seaweed, although in the case of feeding trial studies the choice of N*6.25 is surprising considering the importance of optimal protein levels in animal feeds. In contrast, studies that examined protein in a physiological or ecological context were more likely to have used an extraction procedure over the proxy N*6.25 method because of a focus on the relative, within-study differences in protein rather than total nitrogen. Indeed, it is often acknowledged that direct extraction procedures typically underestimate protein in seaweeds. However, even within a study, differences can arise between algal species due to species-specific extraction efficiency (Fleurence et al. 1995; Barbarino and Lourenco 2005) and quantification accuracy (Crossman et al. 2000).

There were some clear trends in relation to the methods that simplified the primary outcomes of this meta-analysis, that is, protein contents determined using direct extraction procedures under-estimated protein and N*6.25 method over-estimated protein compared to the true value as the total content of the amino acids. These outcomes were true irrespective of whether seaweeds were categorised into taxonomic groups (greens, browns and reds), regions (temperate, tropical and polar) or whether the seaweed was wild harvested or cultivated. These results are in agreement with the limited number of empirical studies where this has been compared for seaweeds (Fleurence et al. 1995; Crossman et al. 2000; Lourenço et al. 2002; Barbarino and Lourenco 2005; Shuuluka et al. 2013). However, there were some differences between the protein determination methods within the various groupings of the categories. There was relatively little variability both overall and between taxonomic groups for direct extraction procedures, despite direct empirical evidence suggesting high variability in the efficacy of direct extraction procedures between different algal species (Fleurence 1999a; Crossman et al. 2000; Barbarino and Lourenco 2005). The determination by N*6.25 and TAA, which are both based on more standardised technical methods, showed more variation overall as well as more variation within green and red seaweeds,

within temperate and tropical seaweeds and between cultivated and wild harvested seaweeds. However, these categories are broad and this variation may simply reflect the plasticity of nitrogen content in some seaweeds (Hanisak 1983; Naldi and Wheeler 1999; Harrison and Hurd 2001; Angell et al. 2014). This is supported by the higher variability in N-protein factors calculated for direct extraction procedures compared to those calculated for TAA.

Green and red seaweeds had a protein content 33 – 45 % higher than brown seaweeds, irrespective of the method of determination. Some of these differences were attributable to most of the brown seaweed analysed in this meta-analysis being from wild populations, however, even when standardised for this factor, brown seaweeds still had a lower protein content than green and red seaweeds - although this was reduced to 27 – 31 %. Indeed, cultivated seaweeds generally had higher protein contents compared to wild harvested seaweeds in this meta-analysis, irrespective of the method of protein determination. Unlike many natural environments, cultivated seaweeds are often not nutrient limited as they are grown in nutrient-rich water in land-based systems. In contrast, the described differences in protein content of seaweeds between geographic regions varied depending on the method of determination used, indicating the inability to compare across methods for past work and the importance of using a universal approach to reporting protein. The lack of a defining pattern between seaweeds from different geographic regions across the different determination methods also suggests that this categorisation, unlike the others, does not have a strong link to protein physiology in seaweeds.

The highest protein content (as determined by TAA) was 32.2 % dw for green seaweeds, 28.7 % dw for red seaweeds and 15.9 % dw for brown seaweeds (95^{th} percentile). Values beyond these are possible, however, they are rare and potentially questionable data unless they represent some restricted taxonomic groups under specific physiological conditions (Chapters 2 & 3).

4.4.2. Beyond 6.25 – a seaweed-specific N-protein conversion factor

The traditional conversion factor of 6.25 overestimates protein contents in seaweeds. However, the use of a conversion factor is a standard approach that will remain a preferred method for the majority of studies because it is a simple means to estimate

protein based on the measurement of nitrogen in the tissue. Empirical studies that have addressed the notion of seaweed-specific factors are restricted to a limited number of species (n = 29) across a narrow geographic and nitrogen content range (Aitken et al. 1991; Lourenço et al. 2002; Diniz et al. 2011; Shuuluka et al. 2013).

Our synthesis of the available information in the literature calculated N-protein factors for a total of 103 species that spanned three taxonomic groups, multiple geographic regions and a range of physiological states. An overall median nitrogen-to-protein conversion factor of 4.97 was established and an overall mean nitrogen-to-protein conversion factor of 4.76. The mean N-protein factors for each of the categories were not statistically different for geographic regions (temperate, tropical and polar) and did not differentiate whether the seaweed was wild harvested or cultivated. This indicates that variation between species is far greater than any variation that exists within these categorisations and suggests that the use of specific N-protein factors for any of the geographic categories or whether the seaweed was wild harvested or cultivated is unnecessary. However, it is notable that between the taxonomic groups, red seaweeds had a higher mean N-protein factor (5.10) compared to green and brown seaweeds (4.49 and 4.59, respectively). Variation in N-protein factors stems from variation in the concentration of non-TAA nitrogen and variation in amino acid profiles (Mossé et al. 1985; Mossé 1990; Mariotti et al. 2008). While both of these factors varied among the seaweed species examined, the variation in non-TAA nitrogen varied considerably more than the variation in N content due to changing amino acid profiles (TAA N), supporting the former as the primary driver for between-species variation in N-protein factors. For red seaweeds, this non-TAA nitrogen was generally lower than in green and brown seaweeds and is likely the main reason behind their higher N-protein factors. This result is in contrast to Lourenço et al. (2002) which calculated a mean N-protein factor for red seaweeds (4.92) that was lower than green (5.13) and brown (5.38). However, given the considerable variation between species and the smaller number of species examined by Lourenço et al. (2002) (n = 19 compared to 103 in this study), these relative differences are likely a reflection of the local species used in that study. For simplicity, I consider that the variation in results and the small but significant difference between red seaweeds and green and brown seaweeds is not of critical importance.

On a broad scale, there was little correlation between total N content and N-protein factor due to considerable between-species variation within the categories examined. However, if only a single species is examined, the correlation between total N content and N-protein factor can be pronounced. A study by Angell et al. (2014) provides insight into within-species variation in N-protein factors (for the green seaweed *Ulva ohnoi*), measuring total N and TAA content for a large number of individuals (n = 60) over a large range of internal N contents. Within-species variation in N-protein factors was relatively high for *Ulva ohnoi* (SD = 0.47, with 90 % of the data falling between 4.42 – 5.83), with variation primarily driven by non-TAA N, as it was at the higher taxonomic levels, and an N-protein factor that was negatively correlated with total N content. A decrease in N-protein factor with increasing N content is a result of the increased luxury consumption of N and the storage of this N in the form of both non-TAA N and amino acids rich in N such as arginine (Chapter 2). Although there was some evidence for this in other taxonomic categories (namely brown wild harvested and red tropical sub-categories: Table S4.3), additional study of within-species variation is required to confirm this pattern. However, it is likely that a negative correlation between total N content and N-protein factor will occur for any seaweed that has the capacity to store excess N during luxury consumption, of which many examples exist (Hanisak 1983; McGlathery et al. 1996; Naldi and Wheeler 1999; Taylor et al. 2006).

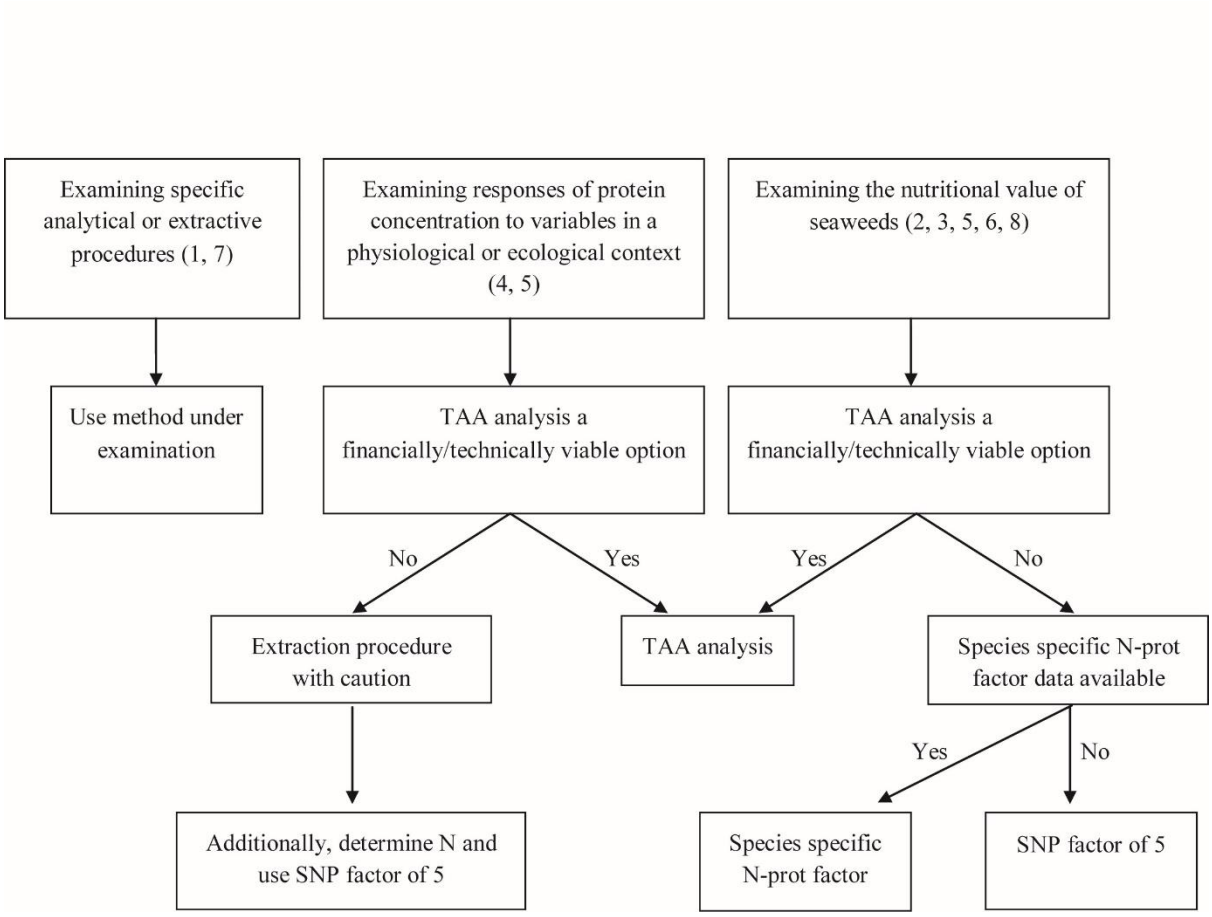

Figure 4.5. A decision tree for the selection of methods when determining seaweed protein content. Numbers in parenthesise in first row refer to disciplines in Table 4.1.

4.4.3. Conclusion

In this meta-analysis, I calculated an overall median N-protein conversion factor of 4.97 (with a mean factor of 4.76) based on the ratio of total proteomic amino acids and total nitrogen. There were some minor differences between the red seaweeds (mean = 5.10, median = 5.31) and the green and brown seaweeds (means = 4.49 and 4.56, medians = 4.68 and 4.81, respectively). However, considering the large between- and within-species variation, I suggest that these categorical factors can be avoided to streamline the data and simplify the results. Therefore I propose that the overall median factor of 4.97 be rounded to 5 and used as the default Seaweed N-Protein (SNP) factor where accurate data on amino acids is not available. Although the median and mean are close, the median most accurately represents the variance in N-protein factors as it is less susceptible to outliers and skewed data. An SNP factor of 5 is also a straightforward conversion factor for calculations. This new factor can be applied retrospectively for

previously presented N-content data where 6.25 has been used but, more importantly, can be a standard for protein measurements in place of direct extraction procedures and N*6.25, especially when reporting protein for nutritional purposes. Alternatively, order-, genera- or species-specific factors may also be applied (see Table S4.2), although I caution the use of factors outside the $5^{th}/95^{th}$ percentile range and those with low replication. In the case of many physiological or ecological studies where an interest lies in the changes to true protein rather than total N content (as a N-protein conversion represents), total amino acid analysis can be used to determine protein over direct extraction procedures for seaweeds, especially as there are many insights to be found in the changes to specific amino acids (Chapters 2 &3). I present a decision tree (Fig. 4.5) to demonstrate the benefits and limitations of alternative methods for protein determination in seaweed, with an overarching recommendation that the total N content is presented in addition to the protein content calculated using other methods.

Chapter 5: Seaweed as a protein source for mono-gastric livestock

5.1. Introduction

Protein for mono-gastric livestock, non-ruminants including chickens, swine and fish, is mostly provided through compound diets with soybean meal and fishmeal as the major sources (Boland et al. 2013). However, the food security of these protein ingredients is a growing concern due to an increasing world population (Godfray et al. 2010), increasing demand for ingredients to supply livestock protein (von Braun 2007), limited agricultural resources of arable land and fresh water (Pretty 2008), declining wild fish stocks (Tacon and Metian 2008) and competition with biofuels (Nigam and Singh 2011). Consequently, there is a critical role for alternative crops in securing the future supply of protein (Boland et al. 2013).

Seaweeds (marine macroalgae) are often proposed as an alternative protein crop for use in compound diets for animals as they have high biomass productivities per unit area (Bolton et al. 2009; Mata et al. 2010; Nielsen et al. 2012; Mata et al. 2016) and do not require arable land or fresh water. However, there has been no systematic analysis of which seaweeds should be targeted based on the quality (% amino acids as a proportion of protein) and concentration (% protein on a whole biomass basis) of protein, which to date are mostly reported independently (Fleurence 1999b; Fleurence et al. 2012). The synthesis of the quality and concentration of protein is critical in determining the nutritional value of whole seaweed biomass if it is to be used in its whole form as an ingredient. This synthesis is important as it provides the concentration of essential amino acids on a whole biomass basis and is the foundation for the nutritional assessment of a protein source. Furthermore, any new protein source needs to be assessed on the provision of the most-limiting essential amino acid on a whole weight basis relative to the requirements of the target livestock, preferably where ileal digestibility (amino acid digestibility at the end of the intestine, corrected for basal endogenous amino acid losses) is accounted for (Stein et al. 2007; Leser 2013). Finally, the low digestible energy content of seaweeds needs to be considered as an indirect factor as to whether the whole seaweed biomass can be used to replace other ingredients as a protein source in compound diets for mono-gastric livestock. The cumulative effect is that seaweeds cannot be assessed solely on the quality or concentration of protein, but

rather on the concentration of the limiting essential amino acids for each type of livestock.

Therefore, the aim of this chapter was to quantitatively assess the potential of seaweeds as a protein source for mono-gastric livestock. To do this, all available literature on the amino acids in seaweeds was systematically analysed to create, and make publically available (Angell et al. 2015), the first comprehensive data set on the concentration of amino acids in seaweeds. This data set contains 265 seaweed samples representing 121 species from 45 peer-reviewed articles. I compare the quality of protein and concentration of amino acids in these seaweeds to the two traditional protein sources – soybean meal and fishmeal. Subsequently, I compare each seaweed, and both traditional protein sources, to the requirements of mono-gastric livestock to determine the concentration of the limiting essential amino acid as a diet for chickens, swine and fish (salmon and tilapia). This quantitative data, in conjunction with a review of published feeding trials using seaweed, is used to assess positive and negative aspects of incorporating seaweeds in a whole form in the compound diets of mono-gastric livestock. Finally, I propose the isolation and concentration of protein to make seaweeds more accessible as an ingredient in compound diets and define a path for future research.

5.2. Materials and methods

The quantitative data examined in this chapter is based on the literature reviewed by Angell et al. (2016) (Chapter 4) and the publically available database Angell et al. (2015). Of the 236 articles included in the database Angell et al. (2015), only those that analysed amino acids were included in this chapter (58 articles).

5.2.1. Extraction of qualitative data

For each article included in this chapter, the following qualitative information was recorded: (1) the phylum, genus and species of each seaweed analysed (2) whether individual amino acids were reported, (3) the units that amino acids were reported in, and (4) the name of the first, second and third limiting amino acid relative to the requirements of chicken (0 – 3 and 6 – 8 week old), swine (5 - 7 and 100 – 135 kg), Atlantic Salmon and Tilapia (*Oreochromis* spp.) (see Table S5.3 and following section). The two age ranges for chickens and the two weight ranges for swine were selected as they represent the most extreme amino acid requirement levels over their respective

production cycles (i.e. fastest and slowest growing stages). The two fish, Atlantic salmon and tilapia, were selected as they are both widely cultured and represent distinct dietary requirements (carnivorous vs. omnivorous). Amino acid requirement data for each animal was sourced from the latest editions of the National Research Council's animal nutrition series (NRC 1994, 2011, 2012).

5.2.2. Extraction of quantitative data

The following quantitative information was recorded: (1) the protein content in % dry weight (dw), (2) the total amino acid content in % dw and the concentration of each measured proteomic amino acid (either as % dw or % protein). All measurements were converted to both % dw and % TAA. Measurements expressed in terms of fresh weight or ash free dry weight were converted to % dw using the reported moisture and ash contents, respectively. Protein measurements expressed as moles of nitrogen per unit biomass were converted using equation 5.1, assuming the proportion of nitrogen in protein to be 16 % (Naldi and Wheeler 1999). Individual and total amino acid measurements expressed as moles per unit biomass were converted to % dw using the molecular weights of individual amino acids. Total and individual amino acid measurements expressed as % protein were converted into % dw using equation 5.2.

$$Protein\ (\%\ DW) = \frac{Protein\ (mol\ N\ g^{-1}\ dw) \times 14.007}{0.16} \qquad \textbf{Eq. 5.1}$$

$$Amino\ acid\ (\%\ DW) = \frac{AA\ (\%\ protein) \times Protein\ (\%\ dw)}{100} \qquad \textbf{Eq. 5.2}$$

If a measurement could not be converted to % dw it was not included in the quantitative analysis.

All extracted quantitative and qualitative data were recorded in one Microsoft Excel 2007 spread sheet with each column representing the qualitative and quantitative questions listed above and each row representing a unique measurement. Pivot tables were used to extract the quantitative and qualitative meta-data.

5.2.3. Reporting of results and statistical analysis

Amino acid measurements in all seaweeds were assessed in three ways; as a proportion of total amino acids (% TAA), as a proportion of dry weight (% dw) and as a ratio to animal requirements for the limiting amino acid (see section 5.3.3. for details). The assessment of these quantitative data was done using the means of each species so as

not to over represent those species which had large numbers of measurements. However, prior to the calculation of the means of species, outliers were removed from the raw data using the outlier labelling method with a k value of 2.4 (Hoaglin and Iglewicz 1987). This method was applied to each essential amino acid based on the % TAA data because this metric standardises for protein concentration. The means for each species were then calculated for the % TAA and % dw data. These data were categorised overall and for each major taxonomic group (greens, browns and reds) and compared to currently used traditional protein sources (fishmeal and soybean meal) and the common feed ingredient, corn grain meal. These data were also compared to the amino acid requirements of major mono-gastric livestock (see below).

The quality and concentration of protein in seaweeds were assessed by directly comparing the ratio of each essential amino acid in the seaweed to the essential amino acid requirements of mono-gastric livestock (see Table S5.3). To do this, the concentration of each essential amino acid in the seaweed (% dw) was divided by each animal requirement (% dw) and ranked from lowest to highest. The lowest, second lowest and third lowest of these values were recorded as the first, second and third limiting amino acids, respectively. For each of these three limiting amino acids the name, percentage at which it was limiting and the concentration (% dw) in the seaweed was recorded for each animal. This assessment was applied to the means of species after all outliers were removed from all essential amino acids so that no individual measurement used in the calculation of a species' mean had an outlier measurement of any essential amino acid.

5.3. Results and discussion

5.3.1. The quality of protein in seaweeds

Mono-gastric livestock do not have a requirement for protein *per se* but rather for the amino acids from which proteins are made. It is those essential amino acids (Table 5.1) that cannot be synthesised by livestock that are critical in the diet and define the quality of a protein source. Seaweeds have a relatively high quality of protein (essential amino acids as a proportion of total amino acids (TAA)) compared to fishmeal and soybean meal. At the highest level of investigation, seaweeds have similar or higher proportions of total essential amino acids (EAA) (mean = 45.7 % TAA) compared to fishmeal (43.4

% TAA) and soybean meal (46.0 % TAA). More than 75 % of seaweeds have higher proportions of total EAA than fishmeal and 50% are higher than soybean meal (Fig. 5.1A).

The proportion of the essential amino acids methionine and lysine (% TAA), the limiting amino acids in most commercial diets and regularly supplemented artificially (NRC 2011, 2012), is comparable to traditional protein sources. Seaweeds (mean = 1.84 % TAA) are generally superior to soybean meal (1.25 % TAA) in the proportion of methionine, but generally have a lower proportion of methionine than fishmeal (2.8 % TAA) (Fig. 5.1B). More than 75 % of seaweeds have a higher proportion of methionine than soybean meal. On the other hand, most seaweeds have a lower proportion of lysine than soybean meal (6.66 % TAA) and fishmeal (7.3 % TAA), although some species have higher values than either of these protein sources (25 species with a proportion of lysine > 7.3 % TAA) (Fig. 5.1C).

Red seaweeds typically have a higher quality of protein than brown and green seaweeds. Red seaweeds have the highest mean proportion of total EAA and lysine and the second highest mean proportion of methionine compared to brown and green seaweeds (Fig. 5.1A-C). However, there is substantially more variation between species of seaweeds within the taxonomic groups (red, green or brown seaweed) than between the taxonomic groups. Therefore, broad taxonomic groupings provide little certainty in selecting species with a high quality of protein.

The data for the quality of protein in this chapter confirms the conclusions reached by many authors that seaweeds generally have a comparable, if not superior, quality of protein to traditional protein sources.

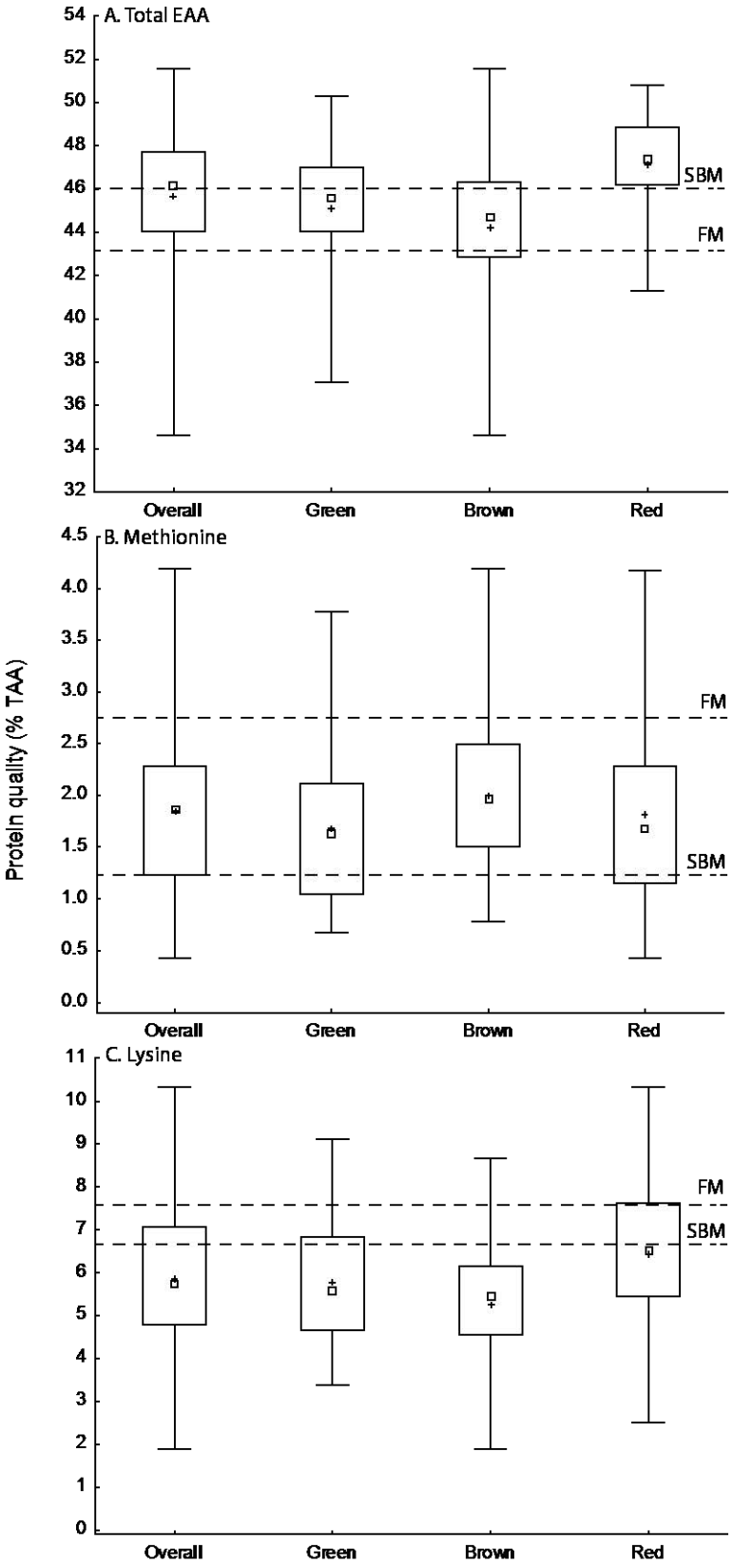

Figure 5.1. The proportion of (A) total essential amino acids, (B) lysine and (C) methionine of TAA for seaweeds analysed in this chapter compared to concentrations in major feed ingredients. Squares represent medians, crosses represent means, boxes represent 25th percentiles and whiskers represent minimum/maximum. FM = fishmeal and SBM = soybean meal.

5.3.2. The concentration of essential amino acids in seaweeds

The assessment of a protein source to provide nutritional value relies on a synthesis of the quality of protein and the concentration of protein if the seaweed is to be used in a whole form as a feed for any particular livestock. The concentration of essential amino acids as a % of the whole biomass on a dry weight basis (dw) takes into account the quality of protein (essential amino acids as a proportion of protein or TAA) and the concentration of protein in the biomass.

The concentration of total essential amino acids (total EAA % dw) in seaweeds (5.49 %) is substantially lower than in soybean meal (22.34 %) and fishmeal (31.19 %) (Table 5.1, Fig. 5.2A). However, there is considerable variation in the concentration of total EAA among seaweeds, with the maximum value reported (16.35 % dw) three-times that of the mean (5.49 % dw). Similarly, the concentration of the essential amino acids methionine and lysine (% dw) are substantially lower in seaweeds than in soybean meal and fishmeal. More than 80 % of seaweeds have less than half the concentration of methionine and lysine than that of soybean meal and fishmeal (Table 5.1, Fig. 5.2B & C). These differences are consistent for all individual essential amino acids for soybean meal and fishmeal – with the exception of threonine (Table 5.1).

The low concentration of EAAs in seaweeds (% dw) highlights the problem of generalising that whole seaweed can be used effectively as a protein source. I acknowledge this contradicts the accepted paradigm that seaweeds have a high concentration of protein with high proportions of essential amino acids (Fleurence 1999b; Fleurence et al. 2012; Garcia-Vaquero and Hayes 2016). However, protein concentration calculated for seaweeds are often overestimated by the use of the animal-based 6.25 N-to-protein conversion factor, and more accurately should be determined using a N-to-protein factor of 5.0 (Chapter 4). In many instances the low concentration of protein is rarely identified when presenting amino acid profiles as a ratio to protein or TAA (Table 5.1). Furthermore, the true value of seaweeds as a protein source needs to be assessed on a per livestock (domesticated species) basis based on the provision of the limiting essential amino acid. This is because the essential amino acid requirements vary substantially between mono-gastric livestock and between different stages of their production (Table S5.3).

Table 5.1. Concentration of protein and essential amino acids (% dw) of traditional protein sources, other feed ingredients and seaweeds. The concentration of essential amino acids, specifically those that are often limiting in the diets of mono-gastric livestock (i.e. lysine and methionine), determine how much protein can be utilised by mono-gastric livestock.

% dw	Fish meal[a]	Soybean meal[b]	Corn grain meal[c]	Seaweed	
				Mean	Range
Protein	68.70	48.00	10.20	11.60[d]	2.98 – 26.76[d]
Arginine	3.71	3.60	0.40	0.73	0.02 – 2.99
Histidine	1.55	1.30	0.25	0.24	0.00 – 1.02
Isoleucine	3.35	2.60	0.29	0.54	0.01 – 1.78
Leucine	4.85	3.80	1.00	0.92	0.03 – 3.18
Lysine	6.21	2.24	0.26	0.69	0.02 – 2.12
Methionine	2.08	0.70	0.18	0.20	< 0.01 – 0.69
Methionine + cystine	3.19	1.41	0.37		
Phenylalanine	2.67	2.70	0.42	0.61	0.02 – 1.83
Phenylalanine + tyrosine	4.80	3.95	-		
Threonine	2.66	2.00	0.30	0.61	0.02 – 2.11
Tryptophan	0.72	0.70	0.07	0.10	0.00 – 0.27
Valine	3.39	2.70	0.42	0.68	0.02 – 2.45
Total EAA (% dw)	31.19	22.34	3.59	5.49	0.15 – 16.35

[a]NRC (2011) – Mean of anchovy and herring fishmeal, protein value is crude protein.
[b]NRC (2011) – Solvent extracted without hulls, protein value is crude protein.
[c]NRC (2011) – Protein value is crude protein.
[d]Chapter 4 – mean and range based on the 5th/95th percentile range of protein determined by total amino acid analysis (n = 299 red, green and brown seaweeds).

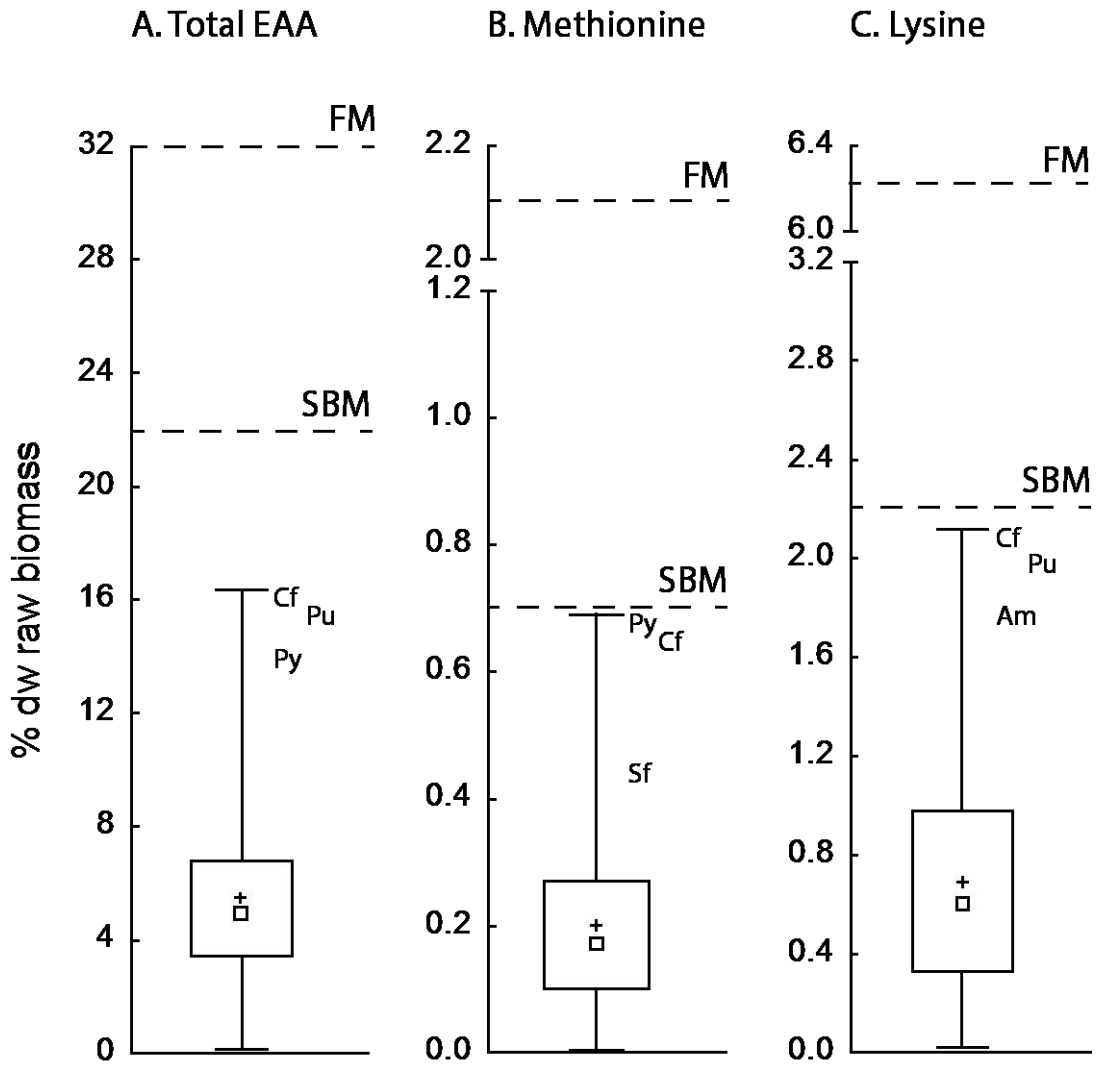

Figure 5.2. The dry weight content (% dw) of (A) total essential amino acids, (B) methionine and (C) lysine of seaweeds analysed in this chapter compared to concentrations in major feed ingredients (see Table 5.1). These amino acids are most often artificially supplemented in plant-based diets for domesticated livestock. Squares represent medians, crosses represent means, boxes represent 25th percentiles and whiskers represent minimum/maximum. Dashed lines represent amino acid scores for major feed ingredients and high protein sources: SBM = soybean meal and FM = Fishmeal. Seaweeds with the highest quantities indicated by Cf = *Capsosiphon fulvescens*, Pu = *Porphyra umbilicalis*, Py = *Pyropia yezoensis*, Sf = *Solieria filiformis* and Am = *Amansia multifidi*.

5.3.3. Limiting essential amino acids

The amount of each essential amino acid in a protein source (on a % dw basis) relative to that of the livestock requirements (% dw basis) can be used to calculate the maximum potential (before digestibility is accounted for) of the protein source to provide the essential amino acids for that particular livestock. The amino acid score, defined as the smallest ratio of any of the 10 essential amino acids, sets the limiting essential amino acid and this ratio determines how much protein or TAA can be utilised by the livestock (amino acid score – Eq. 5.1). An amino acid score was calculated for each seaweed for

each livestock (chicken, swine, salmon and tilapia) based on using seaweed as the sole protein source.

$$Amino\ acid\ score = \frac{Quantity\ of\ 1st\ limiting\ EAA\ in\ protein\ source\ (\%\ dw)}{Livestock\ requirement\ of\ limiting\ EAA\ in\ diet\ (\%\ dw)} \qquad \textbf{Eq. 5.1}$$

The five essential amino acids methionine, arginine, histidine, tryptophan and lysine are the limiting amino acids for 96 % of seaweed species for chickens, swine, salmon and tilapia. Furthermore, these amino acids also account for more than 80 % of the second limiting amino acid and more than 30 % of the third limiting amino acid. Methionine is most frequently the limiting essential amino acid for all major mono-gastric livestock (33 – 49 % of seaweed species), with the exception of tilapia which is most commonly limited by histidine (53 % of species) (Fig. 5.3). This contrasts with terrestrial plant sources that are commonly limited by lysine (NRC 2011, 2012). In contrast, lysine is rarely limiting for seaweeds, especially for chickens and tilapia where methionine, arginine, histidine and tryptophan are the main limiting amino acids for these livestock (Fig. 5.3). Notably threonine, which is often artificially supplemented in commercial diets with traditional protein sources (NRC 2011, 2012), is rarely limiting for seaweeds for any livestock. Tryptophan, which was only measured in 37 % of seaweeds, is the limiting amino acid for between 15 % (salmon) and 38 % of species (mature swine). This supports that tryptophan should be reported even if its measurement requires a different analysis.

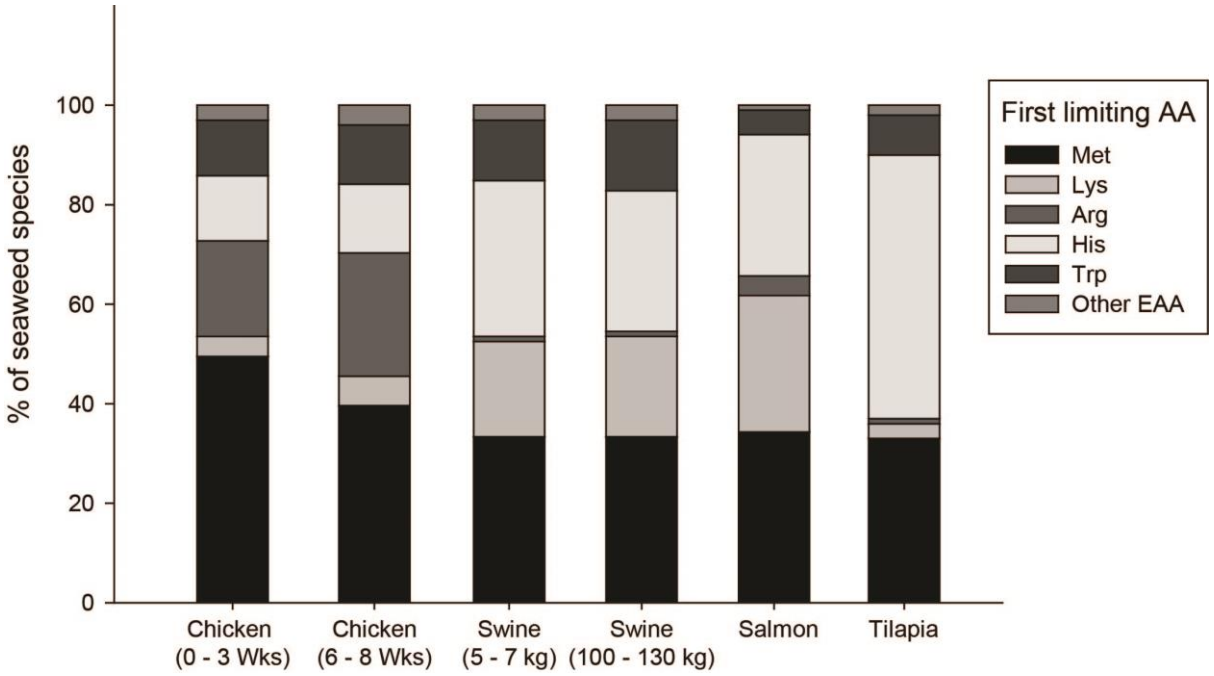

Figure 5.3. Proportion of seaweed species that were limiting in each essential amino acid for mono-gastric livestock. Amino acid requirement data for chickens, swine and fish from NRC (1994), NRC (2012) and NRC (2011), respectively. Met = methionine, Lys = lysine, Arg = arginine, His = histidine, Trp = tryptophan and EAA = essential amino acid.

Ideally, the amino acid scores calculated above should be adjusted for ileal digestibility, that is, the amino acid outflow at the end of the intestine, corrected for basal endogenous amino acid losses (Stein et al. 2007). This would then enable the calculation of a Digestible Indispensible Amino Acid Score – DIAAS (Leser 2013). However, standardised ileal digestibility data for amino acids in seaweeds have not been reported so I provide a best case scenario (assuming 100 % digestibly) to assess the potential of seaweeds to provide protein nutrition to mono-gastric livestock. While it is difficult to predict what these scores would be after accounting for digestibility, amino acid scores above 1.50 are presumed to be high enough to balance losses due to digestibility inefficiencies, considering that the true ileal digestibility of essential amino acids for the majority of commonly used feed ingredients for swine range from 60 – 95 % (NRC 1998).

Amino acid scores, calculated based on the assumption of 100 % digestibility (Eq. 5.1), for the majority of seaweeds are low for all livestock and lower than traditional protein sources (Fig. 5.4). This suggests that these seaweeds cannot satisfy the amino acid requirements of mono-gastric livestock even in the best case scenario for digestibility. This was most evident for fish (salmon and tilapia) where no seaweed species has an amino acid score above 0.70 (70 % of essential amino acid requirements met before

losses due to digestibility). Furthermore, only a few seaweeds have amino acid scores above 1.00 (100 % of essential amino acid requirements met) for young chickens, young swine and mature chickens, and none have a score greater than 1.50 (150 % of essential amino acid requirements met) (see Table S5.4). The best outcome is for mature swine where seven seaweed species have amino acid scores higher than 1.50 (Table S5.4). However, these scores also assume 100 % inclusion in the diet and does not account for the inclusion of an energy source (e.g. corn) that usually represents approximately 75 % of the diet for swine (NRC 1998) and has a poor concentration of essential amino acids (Table 5.1). It is also evident that the amino acid scores of the best seaweeds (1.52 – 2.50) are still considerably lower than those for soybean meal (3.67) and fishmeal (6.55) (Fig. 5.4). This does not mean that they should be overlooked, however, considerable effort will need to be focussed on the industrial production and processing of seaweeds to make them competitive as a feedstock. This would be bolstered by the uncertainty in the future security and price of these traditional sources (Aiking 2011), but the targeted development of seaweeds as a protein resource would also need to facilitate innovations in the concentration or extraction of proteins (see below).

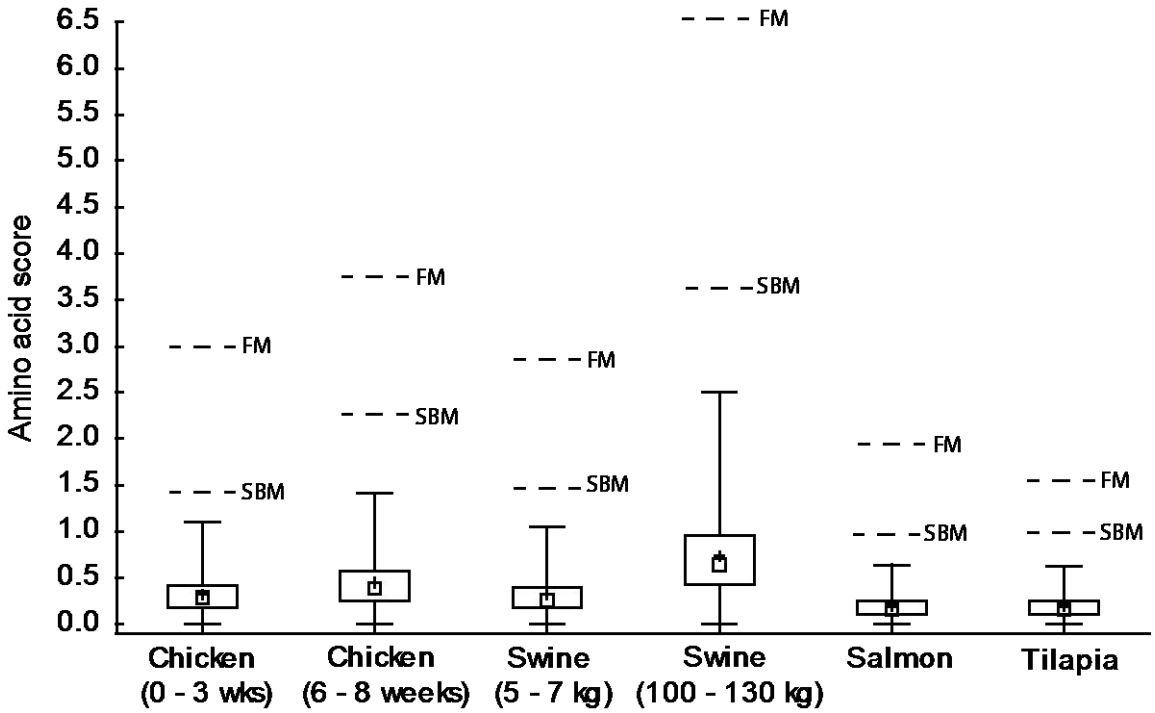

Figure 5.4. Amino acid scores – defined as the ratio of the limiting AA (% dw) to the livestock requirement of the same amino acid - of seaweeds analysed in this chapter for major domesticated livestock. Squares represent medians, crosses represent means, boxes represent 25th percentiles and whiskers represent minimum/maximum. SBM = soybean meal and FM = Fishmeal (NRC 2011). Amino acid requirement data for chicken, swine and fish from NRC (1994), NRC (2012) and NRC (2011), respectively.

5.3.4. The use of whole seaweeds in mono-gastric livestock diets

Most of the research on incorporating seaweeds into livestock diets as a source of protein has focused on aquatic livestock, especially commercial marine herbivores that feed naturally on seaweeds (abalone and sea urchins). Seaweeds can provide complete or partial protein nutrition for abalone (Bautista-Teruel et al. 2001; Viera et al. 2011; Bilbao et al. 2012; Mulvaney et al. 2013; Kemp et al. 2015), sea urchins (Cook and Kelly 2007) and shrimp (Cruz-Suarez et al. 2009; da Silva and Barbosa 2009; Felix and Brindo 2013). In contrast, the inclusion of seaweed in diets of commercial fish (herbivores or carnivores) at levels greater than 10 % results in reduced growth and feed utilisation (Table 5.2), although there are some promising results for tilapia (Stadtlander et al. 2013). There is little literature on incorporating seaweeds as a protein source into the diets of poultry and swine. In contrast, there are many studies examining the functional effects of seaweed and their extracts on immune function, gut health, and meat and egg quality (Katayama et al. 2011; Michalak et al. 2011; Walsh et al. 2013a; Walsh et al. 2013b; Kulshreshtha et al. 2014). However, these studies use low inclusion levels (< 5 %) that contribute little protein to the diet or, its corollary, that the seaweeds do not displace traditional protein sources in the feed. The few studies that have incorporated seaweeds into livestock diets at levels higher than 5 % report some positive results (no negative effect on growth or feed utilisation) up to 10 % for chickens (Ventura et al. 1994; Zahid et al. 1995) and up to 15 % for ducks (El-Deek and Brikaa 2009a), but reduced performance at higher inclusion levels for chickens (Ventura et al. 1994).

The potential of substituting seaweeds as a protein source into compound diets (a diet composed of multiple ingredients) is based on the assumption that it should displace a substantial proportion of the existing protein source (usually soybean meal) while maintaining the concentration of essential amino acids and the digestible energy level. This can be modelled using a theoretical example based on changes to the overall amino acid score of the diet. As a best case example, I used mature swine (the livestock with the highest amino acid scores, i.e. the lowest amino acid requirements), and focused on the three seaweeds with the highest concentration of the amino acid (% dw) that limits the swine compound diet (*Capsosiphon fulvescens, Porphyra umbilicalis* and *Amansia*

multifida). A typical swine compound diet consists of an energy component (corn) and a protein component (soybean meal). These two ingredients make up approximately 97.5 % of the diet, with corn typically 74.1 % and soybean meal 23.4 % (NRC 1998). This combination of corn and soybean meal gives an amino acid score for mature swine of 1.17 and lysine as the limiting amino acid (note that, in practice, this score will change based on losses due to digestibility and gains due to supplementation with artificial lysine). Only three species of seaweed, of the 119 examined (Table S5.2), have quantities of lysine comparable to soybean meal (2.24 % dw) (*Capsosiphon fulvescens* (2.12 % dw)*, Porphyra umbilicalis* (2.05 % dw) and *Amansia multifida* (1.85 % dw)) (see Fig. 5.2C). Therefore, these three seaweeds were used, as best case examples, to examine what effect substituting soybean meal with whole seaweed has on the overall amino acid score of the theoretical swine compound diet (74.1 % corn and 23.4 % soybean meal) with substitution levels from 5 to 100 % of soybean meal.

Table 5.2. A summary of feeding trial research that has incorporated whole seaweeds as part of the diets of mono-gastric livestock

Livestock examined	Inclusion levels tested (% of diet)	Seaweed examined (Genus)	Major findings	Reference
Fish & shrimp				
Asian seabass (*Lates calcarifer*)	5	*Kappaphycus, Eucheuma, Sargassum*	• No effect on growth performance • *Sargassum* improved feed intake • Seaweeds used to replace commercial feed binders	Shapawi and Zamry 2016
Asian seabass (*Lates calcarifer*)	6, 10, 14, 18, 22 (cooked) and 6 (raw)	*Kappaphycus*	• 6 % inclusion of cooked seaweed improved growth and FCR compared to control and other treatments • Reduced growth and increased FCR with increasing inclusion levels above 6 % • Note: seaweed replaced tapioca starch rather than the protein sources used (fishmeal and soybean meal)	Shapawi et al. 2015
European seabass (*Dicentrarchus labrax*)	5, 10 and 15	*Pterocladia, Ulva*	• Seaweed inclusion had no significant effect on growth or PER. • 5 % inclusion increased performance, nutrient composition and stress resistance compared to control • Note: seaweed primarily replaced wheat flour rather than fishmeal - the major protein source	Wassef et al. 2013
European seabass (*Dicentrarchus labrax*)	5 and 10	*Gracilaria, Ulva*	• FCR improved for both inclusion levels • Note: 10% reduced growth and digestibility	Valente et al. 2006
Nile tilapia (*Oreochromis niloticus*)	5 and 10	*Gracilaria*	• No difference in growth, FCR and PER between control and 5 %, but these were negatively affected at 10 % • 5 % increased innate immune response	Araújo et al. 2016
Nile tilapia (*Oreochromis niloticus*)	13.6 and 27.2	*Porphyra*	• No effect on growth or feed utilisation	Stadtlander et al. 2013
Nile tilapia (*Oreochromis niloticus*)	10, 15 and 20	*Ulva*	• Inclusion above 10 % decreased growth performance, protein utilisation and protein retention	Marinho et al. 2013
Nile tilapia (*Oreochromis niloticus*)	30	*Gracilaria, Porphyra, Sargassum, Ulva*	• Diet protein digestibility decreased compared to control in all seaweed inclusion diets except for *Gracilaria*	Pereira et al. 2012
Rainbow trout (*Oncorhynchus mykiss*)	5 and 10	*Gracilaria*	• No effect on growth of FCR at 5 %, but these were negatively affected at 10 % • Increased iodine and moisture content and higher colour intensity at 5 %	Valente et al. 2015
Rainbow trout (*Oncorhynchus mykiss*)	30	*Gracilaria, Porphyra, Sargassum, Ulva*	• Diet protein digestibility decreased compared to control in all seaweed inclusion diets	Pereira et al. 2012
Rabbitfish (*Siganus canaliculatus*)	33	*Gracilaria*	• Reduced growth and feed utilisation in seaweed inclusion diet compared to control (fishmeal protein source)	Xu et al. 2011

			• Note: some immunity parameters improved in seaweed diet	
Mullet (*Chelon labrosus*)	16.5 and 33	*Porphyra*	• Increased inclusion levels of seaweed resulted in reduced growth and compromised FCR, PER and NPU.	Davies et al. 1997
Large yellow croaker (*Pseudosciaena crocea*)	5, 10 and 15	*Ulva*	• Inclusion up to 15 % had no effect on growth or survival • Note: seaweed replaced wheat meal not fishmeal or soybean	Asino et al. 2011
Atlantic cod (*Gadus morhua*)	5.5 and 11	*Porphyra*	• No effect on survival, growth or hepasomatic index • Note: diets were isonitrogenous and isocaloric through additional blood meal	Walker et al. 2009
Rainbow trout (*Oncorhynchus mykiss*)	10	*Ulva*	• Reduced growth, feed intake and feed utilisation	Yildirim et al. 2009
Atlantic salmon (*Salmo salar*)	5, 10 and 15	*Palmaria*	• No effect on red coloration of fillets • Positive effect on yellow/orange colour of fillets	Moroney et al. 2015
Japanese flounder (*Paralichthys olivaceus*)	3, 6 and 9	*Eucheuma*	• 3 % inclusion improved growth and feed efficiency • Reduced growth and feed efficiency at inclusion levels above 6 %	Ragaza et al. 2015
Shrimp (*Litopenaeus vannamei*)	3.3	*Ulva, Macrocystis, Ascophyllum*	• No effect on feed intake or survival, • For *Ulva* only, small increase in growth, improved FCR and PER	Cruz-Suarez et al. 2009
Shrimp (*Litopenaeus vannamei*)	13, 26 and 39	*Hypnea, Crytonemia*	• Increased survival • No difference in biomass and SGR • Note: fishmeal also increased with increasing amounts of seaweed	da Silva and Barbosa 2009
Freshwater prawn (*Macrobrachium rosenbergii*)	10, 20 and 30	*Kappaphycus* (whole and fermented)	• No effect on growth, digestibility and flesh quality with whole seaweed (up to 20 %) and fermented seaweed (up to 30 %)	Felix and Brindo 2013
Terrestrial livestock				
Chicken	10, 20 and 30	*Ulva*	• Reduced growth and feed intake as seaweed inclusion level increased	Ventura et al. 1994
Chicken	1 and 3	*Ulva*	• No effect on feed intake, growth or feed utilisation • Positive effects of 3 % diet on muscle yield and serum quality	Abudabos et al. 2013
Duck	1.5 and 3	*Polysiphonia*	• No significant effect of seaweed on growth parameters or carcass traits • Note: pellet hardness increased with seaweed inclusion	El-Deek and Brikaa 2009b
Duck	5, 10 and 15	*Polysiphonia*	• No effect on growth or FCR	El-Deek and Brikaa 2009a
Swine	0.8	Not specified	• Improved immune function response	Katayama et al. 2011
Swine	1 and 2	*Ascophyllum*	• Improved gut health at 1 %	Dierick et al. 2009

FCR = feed conversion ratio, PER = protein efficiency ratio, NPU = net protein utilisation

By substituting the soybean meal protein source with increasing amounts of the three seaweeds with the highest lysine concentration, there is little change to the overall amino acid score of the diet as long as lysine remains the limiting amino acid (see Fig. S5.1 and supplementary material S5.1 – Annex to Chapter 5). However, the amino acid score of the compound diet drops dramatically as tryptophan, the limiting amino acid of the seaweed, becomes more limiting than lysine. This demonstrates that when seaweeds with high quantities of lysine are partially substituted for soybean meal there may be some potential to use specific seaweeds in a whole form in the compound diets of livestock with low amino acid requirements, provided tryptophan is artificially supplemented. However, the majority of seaweeds contain substantially less lysine than soybean meal (Fig. 5.2C) and, if added to a compound diet already limiting in lysine, will substantially decrease the amino acid and digestible indispensable amino acid scores of the diet. Moreover, as seaweeds contain large concentrations of fibre and ash (Ventura et al. 1994; Marrion et al. 2005; Al-Harthi and El-Deek 2012), the digestible energy content of the compound diet will also decrease as soybean is substituted with seaweed. This suggests that whole seaweeds are too diluted by fibre and ash to maintain or improve the amino acid content of traditional compound diets without negatively affecting their energy content for even one of the most promising livestock (mature swine) when 100 % digestibility is assumed.

I have established that there are clear limitations in the concentration of essential amino acids and fibre and ash of seaweed when considering whole seaweeds as an alternative to traditional protein sources of soybean meal and fishmeal feed ingredients. This dictates that the pathway forward is to concentrate the protein either by the removal of non-protein components or the extraction and isolation of protein. If this can be done then the concentration of essential amino acids (low in seaweeds) can be separated from the quality of protein (high in seaweeds). This provides a renewed focus on developing processing methods to concentrate protein in seaweeds.

5.3.5. Developing an alternative protein source from seaweeds

In the previous sections I established that the key limitation for the use of seaweed protein is the concentration of essential amino acids on a whole basis not the quality of the total amino acids or protein. Effectively, the large proportion of non-amino acid material (indigestible carbohydrates/fibre and ash) dilutes the high quality protein of

seaweed. The processing methods for soybeans provide a model for the production of concentrated protein products for mono-gastric livestock. After extraction of lipids, residual soybean biomass (soybean meal) is further processed to concentrate protein by the removal of non-protein components (soybean protein concentrate), or by the direct extraction and isolation of proteins (soybean protein isolate) (Berk 1992). For most seaweeds, the aim of the processes would be to concentrate protein by a factor of 75 - 200 % to provide a comparable protein source to soybean meal.

5.3.5.1. Removal of non-protein components to concentrate protein

The simplest form of processing to remove non-protein material from seaweeds and concentrate protein is rinsing biomass with freshwater to reduce the content of ash. Ash contents are comprised of external and internal salts and usually constitute between 20 – 50 % of the dry weight (Chapter 3) (McDermid and Stuercke 2003; McDermid et al. 2007). Therefore, processing with freshwater has the potential to increase the concentration of protein in seaweeds by an equivalent amount. This may only be suitable for those seaweeds that have a high concentration of essential amino acids as the polysaccharides, which can represent up to 76 % of the dry weight (Kraan 2012), are typically not affected by simple rinsing. However, few studies have quantified the effect of rinsing on the concentration of ash and protein. Notably, rinsing increased the concentration of protein in the siphonous green seaweed *Derbesia tenuissima* by 34%, and the green blade seaweed *Ulva ohnoi* by 15 % (Neveux et al. 2014). Similarly, optimised rinsing further increased the concentration of protein from 23.4 to 27.4 % (17 % increase) for *Ulva ohnoi* and 15.2 % to 19.5 % for *Ulva tepida* (28 % increase) (Magnusson et al. 2016).

The extraction of polysaccharides has the potential to further increase the amino acid concentration in the seaweed biomass. Most of these polysaccharides are structural cell wall material (e.g. cellulose in green seaweed), however, unlike terrestrial plants, structural and storage polysaccharides in seaweeds are predominantly species-specific. For example, green seaweeds contain cellulose, sulfated galactans (ulvans), sulphated polysaccharides and xylans, brown seaweeds contain alginic acid, fucoidan, laminarin and sargassan, and red seaweeds contain agars, carrageenans, xylans, floridean starch, sulfated galactan and porphyran (Percival 1979; Ray and Lahaye 1995; Chiovitti et al. 1997; Kraan 2012). This diversity of polysaccharides means that the extraction yield

and methodology is varied and often species-specific. Extractible polysaccharide content in seaweeds range from 6.5 to 38 % dw (Maciel et al. 2008; Kraan 2012; Barros et al. 2013) and have been extracted using water-soluble extraction at room temperature (Kolender and Matulewicz 2002; Maciel et al. 2008; Alves et al. 2013) or high temperatures (Yamamoto 1980; Barros et al. 2013), enzymatic digestion (BobinDubigeon et al. 1997; Melo et al. 2002), and acidic- (fucans) and alkaline-soluble extractions (Ray 2006). However, the protein fraction has rarely been considered and it will now be important to quantify the effects the solvents have on the extraction of proteins. For example, the by-product of the agar extraction process from *Gracilaria* potentially represents an underutilised protein resource, however, extraction procedures involve an alkaline extraction using sodium hydroxide (Armisen 1995) that is critical in solubilising a large proportion of total soluble protein during protein extraction processes for seaweeds (Fleurence et al. 1995; Wong and Cheung 2001b, a; Kandasamy et al. 2012; Kumar et al. 2014). Although it has not yet been a commercial focus for seaweeds, it is promising that the extraction of soluble polysaccharides with minimal protein losses is routinely done for soybeans, providing a model for seaweeds (Berk 1992).

5.3.5.2. Direct isolation of protein

The isolation of total protein from seaweeds is impeded by cell wall mucilage (neutral polysaccharides) and phenolic compounds (Jordan and Vilter 1991; Fleurence et al. 1995; Wong and Cheung 2001b). Chemical binding between protein and compounds such as polysaccharides and phenolic compounds limits the solubility of protein and reduces the yield of the soluble protein fraction (Loomis and Battaile 1966; Jordan and Vilter 1991; Harnedy and FitzGerald 2011). Nonetheless, yields of 36.1 – 48.0 % of total protein have been obtained using an initial aqueous extraction followed by an alkaline extraction (Wong and Cheung 2001b, a; Kandasamy et al. 2012; Kumar et al. 2014). However, these yields are considerably lower than those reported for terrestrial plant sources such as rice (97.4 % - Ju et al. (2001)) which implies that isolation protocols are not yet optimised for seaweeds. For example, seaweed isolation protocols have only focused on procedures that use dry, milled biomass based on those for terrestrial seed crops such as soybean (Berk 1992), rice (Ju et al. 2001; Agboola et al. 2005) and canola (Tan et al. 2011). Terrestrial seed crops generally have relatively low concentrations of insoluble polysaccharides and most of the protein is in the form of

storage proteins. In many ways seaweeds are more physiologically and biochemically similar to leaves with high concentrations of insoluble structural polysaccharides and a diverse range of proteins, many of which are associated with chloroplasts and photosynthesis, such as the enzyme RuBisCO. RuBisCO alone can represent up to 65 % of total soluble leaf protein (Ellis 1979; Spreitzer and Salvucci 2002). The potential therefore exists to optimise protein isolation procedures for seaweeds by incorporating elements of leaf protein isolation protocols that use fresh biomass and have a more mechanical focus (Sinclair 2009; Bals and Dale 2011; Chiesa and Gnansounou 2011).

The crude protein (determined from the N content using the 6.25 N-protein conversion factor) of seaweed protein isolates has been determined in a limited number of studies (range = 33.4 to 86.3 % dw) (Wong and Cheung 2001b, a; Kandasamy et al. 2012; Kumar et al. 2014). If the concentration of essential amino acids as a proportion of the isolate is calculated (for those studies that also determined the quality of protein – amino acids as a proportion of protein), it can be seen that the total essential amino acids, methionine and lysine increase substantially in protein isolates compared to the whole seaweed (Table 5.3). Protein isolates have between 3.4 – 14.0 times more total essential amino acids, 2.0 – 9.5 times more methionine and 2.7 – 13.6 times more lysine than seaweed on a whole weight basis. These isolates have higher quantities of total essential amino acids and lysine than soybean meal, and, similar quantities of total essential amino acids to fishmeal, although lower quantities of lysine than the latter. In contrast, the concentration of methionine does not increase to the same extent and is similar to that of soybean meal. Notably, the other sulfur-containing amino acid cysteine (which spares methionine in nutrition) was absent from all isolates, suggesting that it may be destroyed by the high pH of the extraction process (Berk 1992).

Table 5.3. Crude protein, total essential amino acid, methionine and lysine concentration in whole seaweed and protein isolates (% dw) for different seaweeds as well as for high protein sources. [1]Wong and Cheung (2001b), [2]Wong and Cheung (2001a) and [3]NRC (2011).

Seaweed	Yield*	Crude protein		Total EAA concentration		Met concentration		Lys concentration	
		Whole	PI	Whole**	PI	Whole**	PI	Whole**	PI
Red									
Hypnea charoides[1]	46.3	18.13	83.1	8.12	27.92	0.31	1.35	1.19	3.26
Hypnea japonica[1]	45.4	19.40	85.0	8.37	31.54	0.35	1.67	1.26	3.79
Brown									
Sargassum hemiphyllum (oven-dried)[2]	9.5	5.33	85.0	2.28	31.96	0.12	1.11	0.33	4.48
Sargassum hemiphyllum (freeze-dried)[2]	7.8	5.03	75.6	2.28	28.20	0.12	0.86	0.33	3.85
Sargassum henslowianum (oven-dried)[2]	33.1	11.33	86.3	3.80	31.50	0.20	0.70	0.68	3.82
Sargassum henslowianum (freeze-dried)[2]	27.0	11.93	76.9	3.80	27.99	0.20	0.63	0.68	4.08
Sargassum patens (oven-dried)[2]	48.0	7.53	84.4	2.85	33.51	0.14	0.79	0.46	4.88
Sargassum patens (freeze-dried)[2]	37.8	8.20	75.0	2.85	27.68	0.14	0.71	0.46	4.25
Green									
Ulva lactuca[1]	36.4	7.13	76.3	4.89	29.83	0.23	0.47	0.56	3.54
Other protein sources									
Soybean meal[3]	-	48.00	-	22.34	-	0.70	-	2.24	-
Fishmeal[3]	-	68.70	-	31.39	-	2.08	-	6.21	-
Spirulina[3]	-	57.50	-	27.77	-	1.15	-	3.03	-

*% of total protein
**Data based on the mean from this chapter
Whole = Whole seaweed biomass as dry weight
PI = Protein isolate of seaweed

In addition to the high quantities of essential amino acids, seaweed protein isolates also have high *in vitro* digestibility (Wong and Cheung 2001a & b) and functional properties (emulsifying and foaming properties and water- and oil-holding capacities) that are comparable to other protein concentrates (Kandasamy et al. 2012; Kumar et al. 2014). Moreover, seaweed protein isolates have comparable effects to casein controls on the growth and health parameters in rats (Wong et al. 2004). These qualities are strong indicators that seaweed protein isolates can be used more broadly as a protein source in the compound diets of mono-gastric livestock.

5.3.6. Future research

Seaweeds provide an opportunity to supply a novel source of protein for mono-gastric animals but only through the concentration of protein from carefully selected species. Notably, the commercially produced red seaweeds *Gracilaria* and *Porphyra,* brown seaweeds *Hizikia* and *Laminaria* (*Saccharina*), and also a tropical species of the green seaweed *Ulva* (Mata et al. 2016), all have a high quality of protein (Table S5.5). These seaweeds also have relatively high quantities of essential amino acids on a whole biomass basis (see Table S5.5). There is also the possibility to complement the traditional methods of protein concentration and isolation (Fleurence et al. 1995; Ju et al. 2001; Wong and Cheung 2001b, a) with industrial processing procedures employed for leaves. This research should focus on optimising the yields (proportion of total protein extracted) and the concentration of essential amino acids in the isolate or concentrate (as a % of dw), and examine the functional properties, toxicity, digestibility, and performance in *in vivo* growth trials.

5.3.7. Conclusion

The quality of protein in seaweeds, including that of the commercially-available species, is comparable to or better than that of the traditional protein sources of soybean or fishmeal. Seaweeds generally contain more total essential amino acids and methionine as a proportion of protein than soybean meal, the most widely used protein source. However, the concentration of essential amino acids in seaweeds on a whole biomass basis is considerably lower than traditional sources and is not adequate as a protein component of compound diets for mono-gastric livestock. This does not detract from their positive health benefits to humans (Mabeau and Fleurence 1993; Holdt and Kraan 2011; Fleurence et al. 2012) and livestock (at low inclusion levels - Dierick et al.

(2009), Katayama et al. (2011), where a low calorific value and high mineral content can be desirable. If seaweed protein is to be used for commercial mono-gastric livestock production it needs to be concentrated by the removal of fibre and ash components, or selectively extracted so that the concentration of essential amino acids can be increased to comparable levels with other protein products. This may ultimately be a stepping stone for the processing of seaweed protein to enter the targeted human protein supplement market.

Chapter 6: A comparison of techniques for isolating and concentrating protein from seaweed

6.1. Introduction

Seaweeds (marine macroalgae) have potential as a novel protein crop to reduce pressure on traditional agricultural resources as they have high productivities (Bolton et al. 2009; Mata et al. 2010; Nielsen et al. 2012; Mata et al. 2016) and their culture does not require arable land or fresh water. Proteins from seaweeds contain essential amino acids at proportions comparable to traditional protein sources, such as soybean meal and fishmeal. However, the essential amino acid content of seaweeds on a whole weight basis is low, resulting in a comparatively low "quantitative" protein resource even though it is a high "qualitative" protein resource. As an example, methionine, as a proportion of total amino acids, is approximately 50 % higher in seaweeds than in soybean meal yet the concentration of methionine (concentration per unit weight) is less than a third in seaweeds compared to soybean meal (Chapter 5). Consequently, seaweeds are only used in the livestock feed industry for their functional benefits based on a high mineral and fibre content (Dierick et al. 2009; Katayama et al. 2011; Evans and Critchley 2014). Utilising seaweeds as a protein resource in compound feeds of mono-gastric livestock will therefore require the processing of biomass to deliver a more concentrated form of this high quality protein. Concentrating the protein content of plant material has traditionally been achieved directly by extracting and isolating the protein, or indirectly by extracting non-protein components to increase the protein content in the remaining biomass. These processing methods are routinely performed for commodity crops such as soy (Berk 1992), rice (Ju et al. 2001; Agboola et al. 2005) and canola (Tan et al. 2011), yielding between 75 to 97 % of total protein.

In contrast, the isolation and concentration of protein from seaweeds is relatively unexplored and has focused on extraction methods used for the dried and milled seed crops of soybean (Berk 1992), rice (Ju et al. 2001; Agboola et al. 2005) and canola (Tan et al. 2011). Yields of up to 48.0 % of total protein have been obtained for seaweeds using combined solvent (aqueous + alkaline) extractions (Fleurence et al. 1995; Wong and Cheung 2001b, a; Kandasamy et al. 2012; Kumar et al. 2014) of dried biomass. However, these yields are comparatively low because the efficiencies of extraction and

isolation of protein from seaweeds are hindered by neutral polysaccharides and phenolic compounds that interact with the proteins and limit their solubility in solvent extractions (Jordan and Vilter 1991; Fleurence et al. 1995; Wong and Cheung 2001b; Harnedy and FitzGerald 2011). Notably, seed crops and seaweeds are physiologically and biochemically distinct, with seeds having relatively low concentrations of insoluble polysaccharides and most protein in the form of storage proteins. In contrast, leaves and seaweeds are physiologically and biochemically similar with high concentrations of insoluble polysaccharides and a diverse range of physiological proteins, many of which are associated with photosynthesis, including the enzyme RuBisCO, which represents up to 65 % of the total soluble protein in leaves (Ellis 1979; Spreitzer and Salvucci 2002). This suggests that protein extraction and isolation procedures for leaves should be suitable for seaweeds, but notably have not yet been examined. In these procedures, a mechanical protein extraction method is applied to fresh biomass (Sinclair 2009; Bals and Dale 2011; Chiesa and Gnansounou 2011) making it a potentially viable method for the extraction and isolation of protein in seaweeds.

An alternative processing method to improve the concentration of protein in seaweeds is the extraction of non-protein components. Protein concentration through the extraction of non-protein components is a relatively simple method commonly employed for terrestrial crops (Berk 1992). In seaweeds, ash (external and internal salts and minerals) and soluble carbohydrates are the major non-protein material that could be removed. Ash represents between 20 – 50 % of the dry weight of seaweed (McDermid and Stuercke 2003; McDermid et al. 2007) and can be removed using freshwater rinsing and soaking (Neveux et al. 2014; Magnusson et al. 2016). Soluble carbohydrates represent between 6.5 – 38 % of dry weight (Maciel et al. 2008; Kraan 2012; Barros et al. 2013) and can be extracted using aqueous extraction at room temperature (Kolender and Matulewicz 2002; Maciel et al. 2008; Alves et al. 2013) and high temperatures (Yamamoto 1980; Barros et al. 2013), enzymatic digestion (BobinDubigeon et al. 1997; Melo et al. 2002), and acidic- (fucans) and alkaline-soluble extractions (Ray 2006). The key to successfully concentrating protein in seaweeds using this approach is to remove these components whilst minimising the amount of protein that is co-extracted.

Consequently, understanding the apportioning of protein and non-protein material in all components through multiple extraction processes is needed to develop the most suitable pathways for the concentration of proteins from seaweeds. Therefore, this

chapter aims to quantify key procedural variables to optimise the isolation (through the extraction of the protein component) or concentration (through the extraction of the non-protein component) of protein for the green seaweed *Ulva ohnoi*. To do this, the 'seed method' and 'leaf method' were simultaneously examined for their efficacy in the isolation and concentration of protein using a factorial design in order to cross some of the unique elements from both methods.

6.2. Materials and methods

6.2.1. Sample preparation

The green seaweed *Ulva ohnoi* M. Hiroka and S. Shimada (Lawton et al. 2013b) (GenBank accession numbers KFI195501 and KFI95536) was collected from bioremediation cultures at an aquaculture facility in Ayr, Queensland, Australia (19°35'0" S, 147°24'0" E) in August 2015 and held overnight in a recirculating system at the Marine and Aquaculture Research Facility Unit (MARFU), James Cook University, Queensland before harvesting for experiments. Separate collections were made for each experimental replicate (n = 3 collections – all protocols performed on each collection) as it was not logistically possible to perform all extractions simultaneously. Raw biomass was centrifuged to remove excess water and then either oven dried (55 ˚C for 48 hours) and milled (< 1 mm) for processing using the 'seed method' and for ash, nitrogen and amino acid analysis of the original material, or immediately processed using the 'leaf method'.

6.2.2. Experimental design

The two methods for protein extraction, as described below, were examined for both the isolation and concentration of protein using a factorial design with three factors – starting material, solvent to biomass ratio and incubation time for the initial aqueous extraction. The starting materials were dry and milled biomass (seed method), or fresh and pulped biomass (leaf method). The solvent to biomass ratios in the initial aqueous extraction step were 20:1 (v/w) or 5:1. The incubation times were 16 hours (seed method) or < 1 min. (leaf method). The 5:1 aqueous solvent volume to biomass ratio was not practical for the seed method as it formed a thick, dry paste that could not be

centrifuged. Therefore, the 5:1 aqueous solvent volume to biomass ratio was not examined for the seed method.

6.2.3. Seed method

The seed method has been most commonly applied to seaweeds and is based on methods for the extraction of protein from seed crops. The method uses dried, milled biomass (DM), a high biomass to aqueous solvent ratio (20:1 v/w - volume to dry weight ratio) and a long incubation time for the aqueous extraction (16 h) and is based on that described in Wong and Cheung (2001b) with slight modification. In brief, 20 g of dried, milled *U. ohnoi* was suspended in de-ionised water (20:1 v/w) and stirred overnight (16 h) at 30˚C, or not incubated (stirred for 15 seconds at ambient temperature). Subsequently, for each treatment, the suspension was centrifuged at 3,200 x g for 30 minutes at 4˚C. The supernatant was then collected for protein precipitation (see below) and discarded after protein precipitation. The pellet was retained (hereafter referred to as the aqueous DM pellet) and re-suspended in de-ionised water and the pH adjusted to 12 using 1 M NaOH. The mixture was then stirred for 2 h (30˚C) before centrifugation as above. In contrast to Wong and Cheung (2001b), the reducing agent 2-mercaptoethanol was not used in the alkaline extraction step as it cannot be used in food grade quality processes (Turhan et al. 2003). The supernatant was then collected for protein precipitation (see below) and discarded after the precipitation of protein. The pellet was retained (hereafter referred to as the alkaline DM pellet) and dried in an oven at 55 ˚C, milled (< 1 mm) and stored at - 20 ˚C for ash, nitrogen and amino acid analysis (see below) (Fig. 6.1). The alkaline DM pellet is the alkaline DM total residual (Fig. 6.1).

In addition, a duplicate process was conducted and truncated after the initial aqueous extraction. This resulted in an aqueous supernatant and aqueous DM pellet. The aqueous DM pellet was dried in an oven at 55 ˚C, milled (< 1 mm) and stored at - 20 ˚C for ash, nitrogen and amino acid analysis (see below). This process was used to provide a quantitative compositional analysis of the seaweed biomass post-aqueous extraction (Fig. 6.1), which was not possible in the complete process as the aqueous DM pellet undergoes alkaline extraction (Fig. 6.1). The aqueous DM pellet is the aqueous DM total residual (Fig. 6.1).

6.2.4. Leaf method

The leaf method has traditionally been applied to the leaves of higher plants. The method uses fresh, pulped biomass (FP), a low biomass to solvent ratio (5:1 v/w) and no incubation time for the aqueous extraction (< 1 min.) and is based on that described in Pirie (1969) with slight modification. In brief, 120 g (≈ 20 g dw) of freshly harvested *U. ohnoi* was pulped in de-ionised water at 20:1 (v/w), or a minimum amount of de-ionised water (5:1 v/w), using a stick blender (HB724 700 W, Kenwood) and stirred overnight (16 h) at 30˚C, or not incubated (stirred for 15 seconds at ambient temperature). Subsequently for each treatment, the slurry was pressed and filtered through a 100 µm mesh to provide a pressed cake (hereafter referred to as the aqueous pressed cake), which was retained, and a suspension. For each treatment, the suspension was centrifuged as above. The supernatant was then collected for protein precipitation (see below) and discarded after the precipitation of protein. The pellet was retained (hereafter referred to as the aqueous FP pellet), combined with the aqueous pressed cake to give the aqueous FP total residual and re-suspended in de-ionised water and the pH adjusted to 12 using 1 M NaOH. The mixture was then stirred for 2 h (30˚C) before centrifugation as above. The slurry was then pressed and filtered through a 100 µm mesh to provide a pressed cake (hereafter referred to as the alkaline pressed cake), which was retained, and a suspension. The retained alkaline pressed cake was dried in an oven at 55°C, milled (< 1 mm) and stored at -20°C for ash, nitrogen and amino acid analysis (see below). The suspension was centrifuged as above. The supernatant was then collected for protein precipitation (see below) and discarded after the precipitation of protein. The pellet was retained (hereafter referred to as the alkaline FP pellet) and dried in an oven at 55 ˚C, milled (< 1 mm) and stored at - 20 ˚C for ash, nitrogen and amino acid analysis (see below). Together the alkaline pressed cake and the alkaline FP pellet are the alkaline FP total residual (Fig. 6.1).

In addition, a duplicate process was conducted and truncated after the aqueous extraction. This resulted in an aqueous supernatant, aqueous pressed cake and aqueous FP pellet. The aqueous pressed cake and aqueous FP pellet were dried in an oven at 55 ˚C, milled (< 1 mm) and stored at - 20 ˚C for ash, nitrogen and amino acid analysis (see below). This process was used to provide a quantitative compositional analysis of the seaweed biomass post-aqueous extraction (Fig. 6.1), which was not possible in the complete process as the pressed cake and aqueous FP pellet undergo alkaline extraction

(Fig. 6.1). Together the aqueous pressed cake and the aqueous FP pellet are the aqueous FP total residual (Fig. 6.1).

6.2.5. Protein precipitation

Extracted protein was isolated from each supernatant by adjusting the pH to its isoelectric point (Ip) using HCl. The Ips were determined by subjecting supernatants to incremental decreases in pH (using HCl) and determining turbidity (optical density at 750 nm) with a spectrophotometer (SPECTROstar Nano, BMG Labtech). The pH that gave the highest turbidity was taken as the Ip (Ju et al. 2001). This procedure was performed on aqueous and alkaline extracted supernatants for both seed and leaf methods. Each mixture containing the precipitated proteins were then centrifuged using the methods described above. The precipitated proteins were then oven dried at 55°C, milled (< 1 mm) and stored at -20˚C for ash, nitrogen and amino acid analysis. The resulting dry protein powders from the aqueous and alkaline extractions are referred to as the aqueous and alkaline protein isolates (PIs), respectively.

6.2.6. Mass Balance

All dried components for the aqueous and alkaline extractions for each treatment combination in the factorial experiment were weighed. These components were the aqueous and alkaline PIs and the aqueous and alkaline total residuals. The mass of all PIs, total residuals and supernatants are expressed as a percentage of the original biomass (mass yield) to give a mass balance of 100 %. The mass yield of the extracted material in the aqueous supernatant was determined by deducting the mass yield of the aqueous PI and the aqueous total residual from the original biomass. The mass yield of the extracted material in the alkaline supernatant was determined by deducting the mass yield of the alkaline PI and the alkaline total residual from mass yield of the aqueous total residual. The total PI for each treatment combination was determined by the addition of aqueous and alkaline PIs.

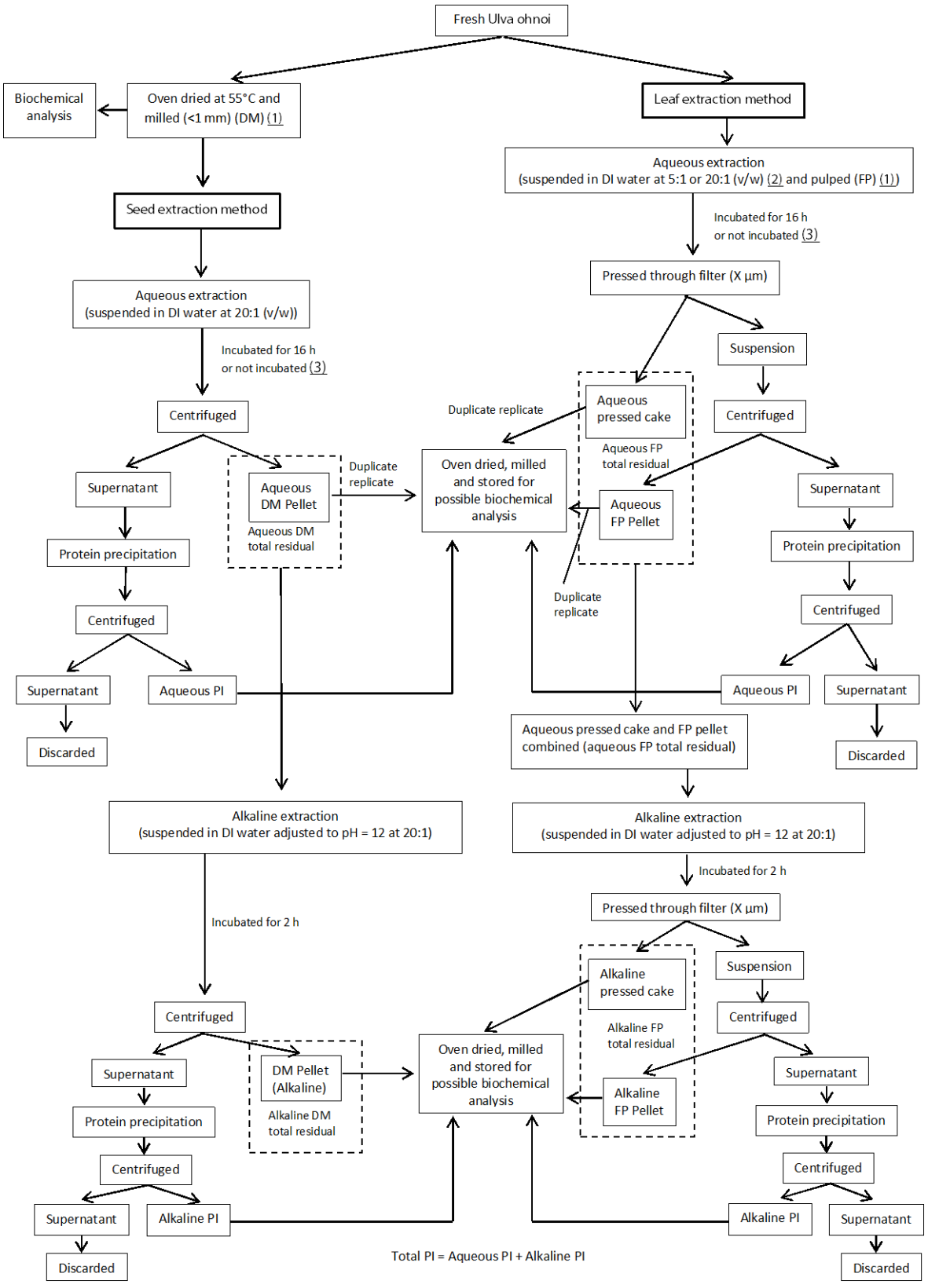

Figure 6.1. An overview of the seed and leaf extraction methods and the factorial experimental design examining factors in protein isolation or concentration efficiency between the two methods. The factors and treatment levels were **(1)** the starting material as dry and milled biomass (seed method) or fresh and pulped biomass (leaf method), **(2)** an aqueous solvent to biomass ratio in the aqueous extraction step of 20:1 (v/w) (seed and leaf method) or 5:1 (leaf method) and **(3)** an incubation time of 16 hours at 30°C or no incubation time (< 1 min.).

6.2.7. Ash analysis

The concentration of ash (% dw) in the original biomass and all aqueous and alkaline total residuals from each treatment combination was determined by incinerating samples at 550˚C for 6 h. The ash in all residuals is expressed as the percentage of the quantity of ash in the original biomass (% ash yield) and as a percentage of their dry weight (% dw). The difference between the ash yield in the original biomass and the aqueous and alkaline total residuals was used to determine the ash yield in the aqueous and alkaline supernatants, respectively. Due to insufficient sample sizes, the concentration of ash was not measured in any of the PIs and these were assumed to contain a negligible concentration of ash.

6.2.8. Nitrogen analysis

The concentration of nitrogen (% dw) in the original biomass and all dried components (aqueous and alkaline PIs and total residuals) for a selected sub-set of treatment combinations was analysed using an elemental analyser (OEA Laboratory Ltd., Callington, UK). Treatment combinations were selected based on a high potential to isolate or concentrate protein. Treatment combinations that have the most potential as protein isolation procedures will have relatively high mass yields in the total PI (combined aqueous and alkaline PIs). In contrast, treatment combinations that have the most potential to concentrate the protein in the original biomass will have relatively high mass yields in the supernatant after precipitation (i.e. high non-protein mass extracted). The concentration of N in all PIs, total residuals and supernatants are expressed as a percentage of the quantity of N in the original biomass (% N yield) to give a N balance of 100 % and also as a percentage of their dry weight (% dw). The N yield in the aqueous supernatant was determined by deducting the quantity of N in the aqueous PI and the aqueous total residual from the original biomass. The N yield in the alkaline supernatant was determined by deducting the N yield in the alkaline PI and the alkaline total residual from the N yield in the aqueous total residual. The amount of N extracted by the aqueous extraction was determined by deducting the N yield in the aqueous total residual from the original biomass. The amount of N extracted by the alkaline extraction was determined by deducting the N yield in the alkaline total residual from the aqueous total residual. The total amount of N extracted (from both

aqueous and alkaline extractions) was determined by deducting the N yield in the alkaline total residual from the original biomass.

6.2.9. Protein and amino acid analysis

The concentration of amino acids (% dw) in the original biomass and selected PIs and aqueous residuals was analysed for a sub-set of treatment combinations based on the selection criteria for N analysis (above). Amino acids were quantified after 24 h liquid hydrolysis in 6 M HCl at 110 ˚C using a Waters ACQUITY UPLC at the Australian Proteome Analysis Facility, Macquarie University, Sydney using procedures based on the Waters AccQTag amino acid methodology (Cohen 2000; Bosch et al. 2006). The following amino acids were analysed: aspartic acid, asparagine, glutamic acid, glutamine, serine, histidine, glycine, threonine, alanine, arginine, valine, methionine, phenylalanine, isoleucine, leucine, lysine, and proline. As asparagine is hydrolysed to aspartic acid and glutamine to glutamic acid during analysis, the sum of these amino acids were reported as asparagine/aspartic acid or glutamine/glutamic acid. The two remaining proteome amino acids, cysteine and tryptophan, were not analysed as they are minor constituents in *Ulva* spp. (Angell et al. 2012; Angell et al. 2014). The TAA content was calculated based on the sum of the above amino acids and was used as the measure of protein. Total essential amino acid (TEAA) content was taken as the sum of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine. Protein, TEAA, methionine and lysine in the selected PIs and residuals are expressed as a percentage of the quantity of protein, TEAA, methionine and lysine in the original biomass (% yield) and also as a percentage of their dry weight (% dw).

6.2.10. Data analysis

As the factorial experimental design was incomplete because the 5:1 aqueous solvent volume to biomass ratio was not examined for the seed method, multivariate PERMANOVAs (PRIMER 6 & PERMANOVA+, PRIMER-E Ltd, UK) were used to analyse the effect of starting material (milled & dry or pulped & fresh), aqueous solvent to biomass ratio (20:1 or 5:1) and incubation time in aqueous solvent (16 h incubation time at 30°C or no incubation time (< 1 min.) on mass yields of aqueous and alkaline PIs and total residual biomass (mass extracted). For all N and amino acid data, where only three factor combinations were analysed (DM.20.I (dry and milled starting material, 20:1 aqueous solvent to biomass ratio and 16 h incubation time in aqueous

solvent), FP.20.I (fresh and pulped starting material, 20:1 aqueous solvent to biomass ratio and 16 h incubation time in aqueous solvent) and FP.5.NI (fresh and pulped starting material, 5:1 aqueous solvent to biomass ratio and no incubation time in aqueous solvent)), the three treatment combinations were analysed as a single factor using PERMANOVAs. All PERMANOVA analyses were conducted using Bray-Curtis dissimilarities on fourth root transformed data and 9 999 unrestricted permutations of raw data. Tukey's multiple comparison was used to determine any differences between treatments. The proportion of variation (%) of the total variation of the independent variable explained by a particular factor or factor interaction was calculated by eta-squared (% variance explained, η^2) = $MS_{factor}/MS_{total} \times 100$, where MS_{factor} and MS_{total} are the mean sum of squares of a particular factor and the total mean sum of squares, respectively (Anderson and Gorley 2007). For pair-wise tests, Monte Carlo tests were also applied when the number of possible permutations was low. For these situations Monte Carlo *P*-values (p (Monte Carlo)) were used to assess significance (Anderson and Gorley 2007).

6.3. Results

6.3.1. Protein precipitation

Turbidity in all protein solutions increased to a maximum with decreasing pH until a pH of approximately 2.25 (Fig. 6.2). Therefore, a pH of 2.25 was taken as the isoelectric point for all the soluble protein fractions in this study.

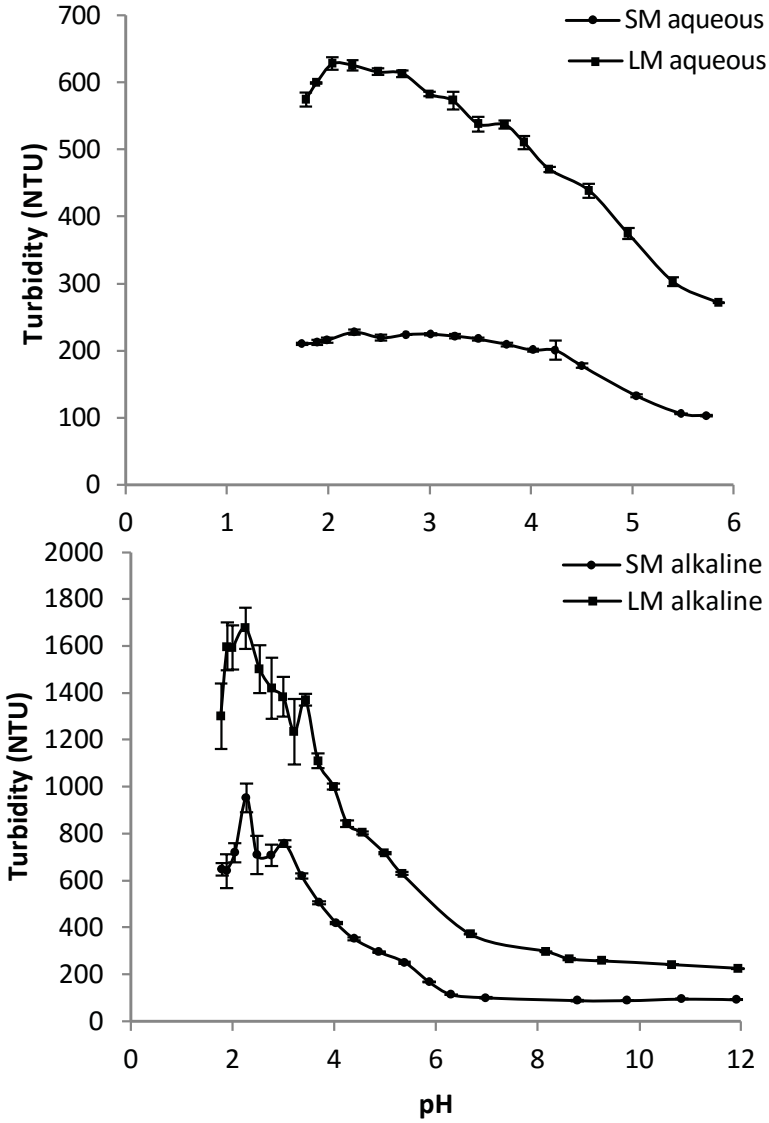

Figure 6.2. Turbidity changes (mean ± SE) of *U. ohnoi* proteins extracted using (A) water (albumin) and (B) alkaline solution (pH = 12, glutelin) for the seed method (SM) and leaf method (LM) with changing pH.

6.3.2. Mass balance and ash

For both the aqueous and alkaline extraction steps, the mass of the starting material is divided into three components; (1) the total residual biomass (the DM pellet for dry and milled starting material and the pressed cake and FP pellet for the fresh and pulped starting material – see Fig. 6.1), (2) the protein isolate (PI) and (3) the supernatant after protein precipitation (supernatant) (Fig. 6.3). As described above, treatment combinations with potential as protein isolation procedures will have high mass yields in the total PI (combined aqueous and alkaline PIs) while treatment combinations with potential to concentrate the protein in the original biomass will have high mass yields in

the supernatant after precipitation (i.e. high non-protein mass extracted). On this basis, a sub-set of treatment combinations in the factorial experiment was selected for N and amino acid analysis (Fig. 6.3) (see below).

Aqueous PI mass yields ranged from 0.25 ± 0.03 to 2.18 ± 0.02 % and alkaline PI mass yields ranged from 2.11 ± 0.18 to 4.56 ± 0.24 % (Fig. 6.4A). For aqueous PIs, aqueous extraction incubation time (Pseudo-$F_{1,17}$ = 110.74, p = 0.0001) and aqueous solvent volume to biomass ratio (Pseudo-$F_{1,17}$ = 17.007, p = 0.0004) had significant effects on mass yields, explaining 82.95 % and 12.74 % of the variance, respectively. Treatments with no incubation time (mean = 1.54 ± 0.06 %) had higher aqueous PI mass yields compared to those that were incubated (mean = 0.34 ± 0.01 %). A lower aqueous solvent volume to biomass ratio also resulted in a higher aqueous PI mass yield (mean = 1.33 ± 0.38 %) compared to a higher ratio (mean = 0.75 ± 0.16 %). The highest overall aqueous PI mass yield resulted when fresh and pulped biomass was used with a 5:1 aqueous solvent volume to biomass ratio and no incubation (2.18 ± 0.02 %, Fig. 6.4A). The aqueous PI of this treatment was therefore analysed for N and amino acids.

For alkaline PIs, starting material (Pseudo-$F_{1,17}$ = 83.187, p = 0.0001) and the interaction between the aqueous solvent volume to biomass ratio and aqueous extraction incubation time (Pseudo-$F_{1,17}$ = 24.589, p = 0.0006) had significant effects on PI mass yields, with these effects explaining 69.21 % and 20.46 % of the variance, respectively. Alkaline PI mass yields were higher when fresh and pulped biomass was used (mean = 3.78 ± 0.21 %) compared to dry and milled biomass (mean = 2.54 ± 0.23 %). When a high aqueous solvent volume to biomass ratio was used, higher alkaline PI mass yields resulted when the aqueous extraction was incubated (mean = 3.76 ± 0.39 %) compared to when it was not (mean = 2.90 ± 0.37 %) (t = 5.284, p = 0.0008). However, when a low aqueous solvent volume to biomass ratio was used, higher alkaline PI mass yields resulted when the aqueous extraction was not incubated (mean = 4.08 ± 0.16 %) compared to when it was incubated (mean = 2.81 ± 0.19 %) (t = 3.611, p (Monte Carlo) = 0.0233). The highest alkaline PI mass yields resulted when fresh and pulped biomass was used and either incubated at a high aqueous solvent volume to biomass ratio (mass yield = 4.56 ± 0.24 %) or not incubated at a low aqueous solvent volume to biomass ratio (4.08 ± 0.16 %). The highest alkaline PI mass yield when dry and milled biomass was used resulted when a high aqueous solvent volume to biomass ratio was used with

an incubation time (2.96 ± 0.22 %). Therefore the alkaline PIs of these three treatment combinations were analysed for N and amino acids (see Table 1 and Fig. 6.3 & 6.4).

Aqueous supernatants yielded between 19.50 ± 1.26 and 43.41 ± 0.28 % of the original biomass and alkaline supernatants yielded between 4.65 ± 0.54 and 17.07 ± 2.26 % of the original biomass (Fig. 6.4B). For the aqueous supernatant mass yields, there was a significant effect of starting material (Pseudo-$F_{1,17}$ = 15.220, p = 0.0031) and a significant interaction effect between aqueous solvent volume to biomass ratio and incubation time (Pseudo-$F_{1,17}$ = 65.584, p = 0.0001), however, aqueous solvent volume to biomass ratio explained most of the variance (79.93 %). The highest mass yield in the aqueous supernatant occurred when an aqueous solvent volume to biomass ratio of 20:1 was used compared to a ratio of 5:1 for both incubated (t = 15.153, p = 0.0013, mean = 41.71 ± 0.80 and 19.50 ± 1.26 % for 20:1 and 5:1, respectively) and non-incubated treatments (t = 12.170, p = 0.0018, mean = 35.54 ± 1.30 and 27.63 ± 0.18 % for 20:1 and 5:1, respectively), however, this difference was greater when the aqueous solvent was incubated compared to when it was not incubated (Pseudo-$F_{1,17}$ = 65.584, p = 0.0001). In contrast, higher alkaline supernatant mass yields resulted when the lower aqueous solvent volume to biomass ratio was used (Pseudo-$F_{1,17}$ = 50.033, p = 0.0001, 61.02 % of variance explained). The proportion of the mass yielded in the aqueous supernatant that consisted of ash was between 43.80 ± 2.53 – 62.77 ± 3.43 %, with supernatant mass yields from extractions that had an aqueous solvent volume to biomass ratio of 5:1 (mean = 59.80 ± 2.23 %) having a higher proportion of ash compared to those that had a ratio of 20:1 (mean = 48.80 ± 1.23 %) (Pseudo-$F_{1,17}$ = 14.211, p = 0.0037). However, aqueous supernatants from treatments with an aqueous solvent volume to biomass ratio of 20:1 yielded significantly more total ash from the original biomass (mean = 65.99 ± 2.27 %) compared to those with a ratio of 5:1 (mean = 48.86 ± 2.82 %) (Pseudo-$F_{1,17}$ = 193.45, p = 0.0001), with this factor explaining most of the variance (67.79 %) in the proportion of total ash removed from the original biomass. The highest supernatant mass and ash yields were in the aqueous supernatant when the aqueous solution was incubated in a aqueous solvent volume to biomass ratio of 20:1 for both types of starting material (supernatant mass yield = 40.02 ± 0.51 % and 43.41 ± 0.28 %, ash yield = 61.47 ± 3.27 % and 75.17 ± 1.26 % for dry and milled and fresh and pulped, respectively). Therefore, these treatment combinations were assessed

for protein concentration efficiency by analysing N and amino acids in the aqueous total residual (Fig. 6.3 & 6.4B).

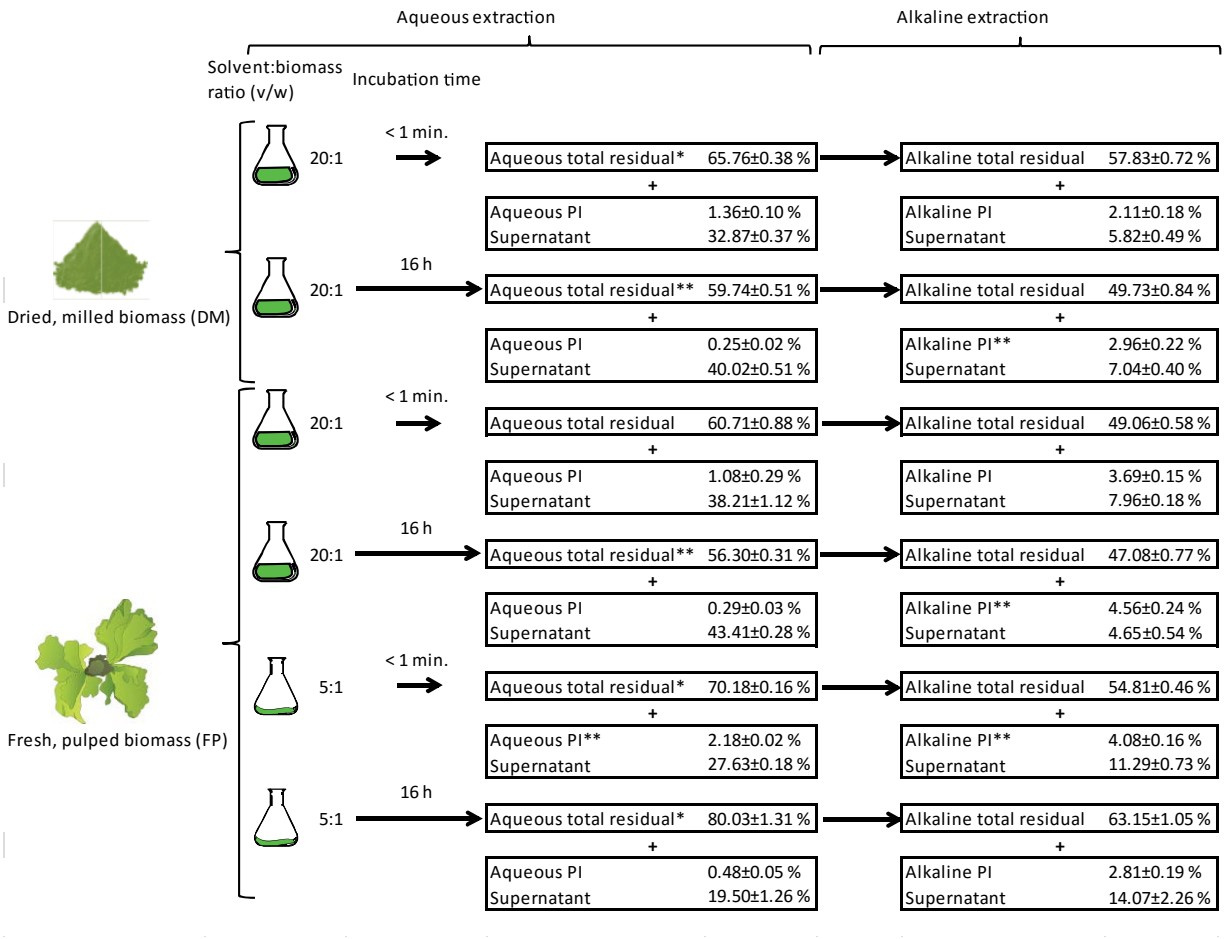

Figure 6.3. Mass balance (as % of original seaweed biomass) of all treatment combinations in the factorial experimental design (mean ± SE) (Fig. 6.1). The mass of the starting material is divided into three components after each extraction; the total residual biomass (the DM pellet for dry and milled starting material and the pressed cake and FP pellet for the fresh and pulped starting material), the protein isolate (PI) or the residual supernatant after protein precipitation (supernatant). * = analysed for N, ** = analysed for N and amino acids. Note: the 5:1 aqueous solvent volume to biomass ratio was not practical for the seed method as it formed a thick, dry paste that could not be centrifuged. Therefore, the 5:1 aqueous solvent volume to biomass ratio was not examined for the seed method.

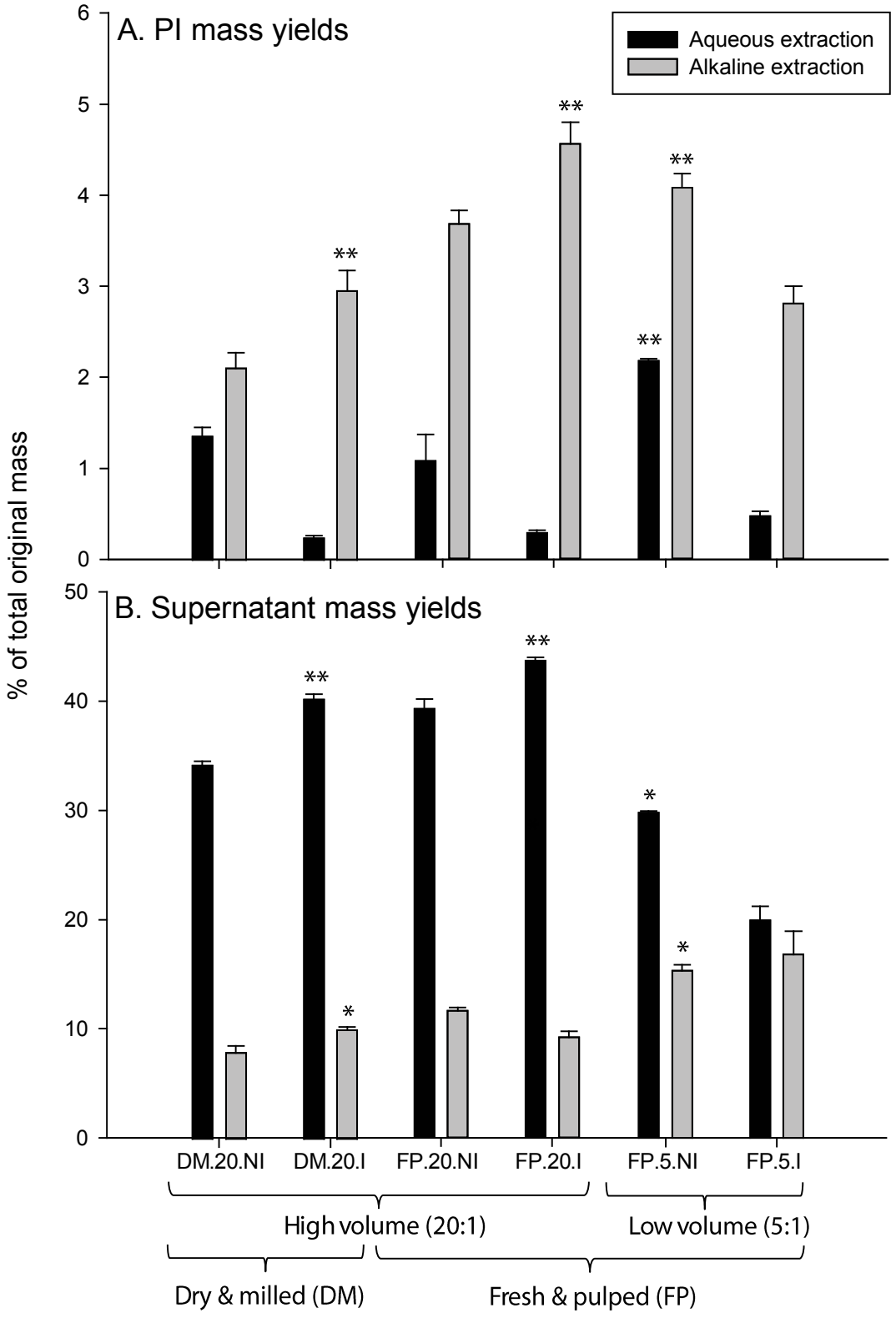

Figure 6.4. (A) The proportion of the original biomass in the aqueous and alkaline protein isolates (PIs) and (B) the total mass extracted (mean ± SE) for aqueous and alkaline extractions for the six treatment combinations examined in the factorial design (see Fig. 6.1 & 6.3). * and ** indicate PIs and total residuals (for extracted mass values) that were analysed for total N or total N and amino acids, respectively.

The concentration of ash in all total residuals was lower than the original biomass (28.50 ± 0.52 %) and ranged from 12.55 ± 0.41 to 20.43 ± 0.33 % for aqueous total residuals and from 16.81 ± 0.20 to 24.09 ± 0.08 % for alkaline total residuals (Fig. 6.5). For aqueous total residuals, aqueous solvent volume to biomass ratio (Pseudo-$F_{1,17}$ = 60.119, p = 0.0001) and starting material (Pseudo-$F_{1,17}$ = 44.668, p = 0.0001) had significant effects on the concentration of ash, explaining 52.69 % and 39.15 % of the variance, respectively. There was also a significant interaction between starting material and incubation time (Pseudo-$F_{1,17}$ = 5.802, p = 0.0333) but this explained only 5.09 % of the variance. A higher aqueous solvent volume to biomass ratio resulted in a lower concentration of ash in the aqueous total residual (mean = 15.88 ± 0.88 %) compared to a lower ratio (mean = 19.34 ± 0.54 %). The concentration of ash in the aqueous total residual was also lower when fresh and pulped material was used (mean = 16.35 ± 0.98 %) compared to dry and milled biomass (mean = 18.40 ± 0.73 %), with this effect greater when the aqueous solvent was incubated. Therefore, the aqueous total residual with the lowest concentration of ash resulted when fresh and pulped biomass was used with a 20:1 aqueous solvent volume to biomass ratio with an incubation time of 16h at 30°C (12.55 ± 0.41 %).

For alkaline total residuals there was a similar pattern. Aqueous solvent volume to biomass ratio (Pseudo-$F_{1,17}$ = 68.413, p = 0.0001) and starting material (Pseudo-$F_{1,17}$ = 43.649, p = 0.0001) had significant effects on the concentration of ash, with these effects explaining 48.40 % and 30.88 % of the variance, respectively. There was also a significant interaction between aqueous solvent volume to biomass ratio and incubation time (Pseudo-$F_{1,17}$ = 21.771, p = 0.0012) and an interaction between starting material and incubation time (Pseudo-$F_{1,17}$ = 4.573, p = 0.0359), but these explained only 15.40 % and 3.23 % of the variance, respectively. A higher aqueous solvent volume to biomass ratio resulted in a lower concentration of ash in the alkaline total residual (mean = 20.84 ± 0.84 %) compared to a lower ratio (mean = 23.72 ± 0.18 %), with this effect greater when the aqueous solvent was incubated. The concentration of ash in the alkaline total residual was also lower when fresh and pulped material was used (mean = 21.34 ± 0.86 %) compared to dry and milled biomass (mean = 22.72 ± 0.86 %), with this effect being slightly greater when the aqueous solvent was incubated. Therefore, the alkaline total residual with the lowest concentration of ash resulted when fresh and

pulped biomass was used with a 20:1 aqueous solvent volume to biomass ratio with an incubation time of 16h at 30°C (16.81 ± 0.20 %).

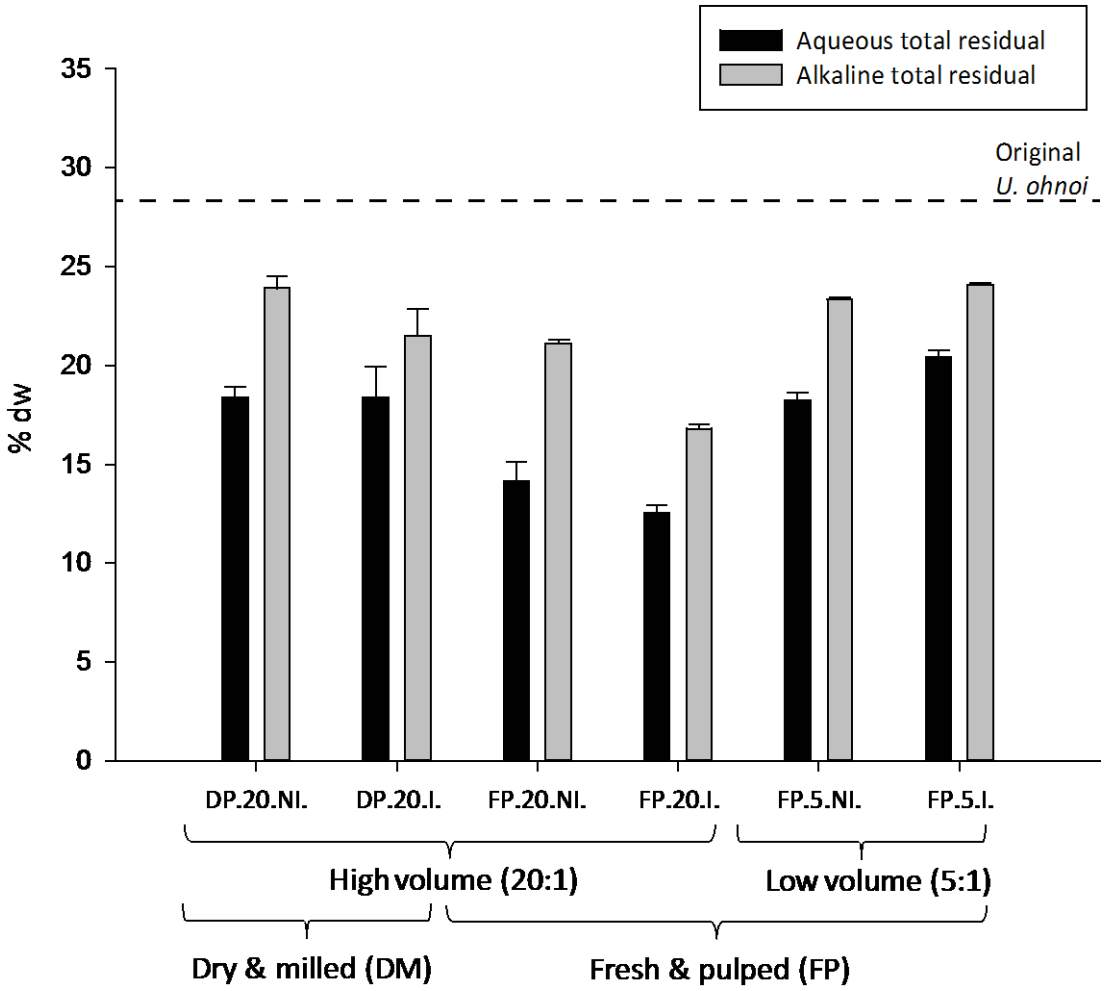

Figure 6.5. The concentration of ash in the aqueous and alkaline total residuals (mean ± SE) for the six treatment combinations examined in the factorial design (see Fig. 6.1 & 6.3). Dashed line represents the concentration of ash in the original biomass.

Table 6.1. The combination of treatments that were analysed for N and amino acids.

ID	Starting material	Aqueous solvent to biomass ratio (v/w)	Incubation time of aqueous extraction
DM.20.I	Dry and milled (DM)	20:1 (20)	16 h (I)
FP.20.I	Fresh and pulped (FP)	20:1 (20)	16 h (I)
FP.5.NI	Fresh and pulped (FP)	5:1 (5)	Not incubated (< 1 min.) (NI)

6.3.3. Nitrogen balance

The aqueous solvent extracted between 7.65 ± 1.05 and 23.77 ± 0.21 % of the total nitrogen from the original biomass (N yield, Fig. 6.6A). Significantly more N was extracted by the aqueous extraction for treatment combinations FP.20.I (23.77 ± 0.21 %,

t = 8.4088, p (Monte Carlo) = 0.001) and DM.20.I (20.58 ± 3.19 %, t = 5.0288, p (Monte Carlo) = 0.0067) compared to treatment combination FP.5.NI (7.65 ± 1.05 %). However, almost no N was isolated for the FP.20.I or the DM.20.I treatment combinations (PI mass yields were < 0.29 % of original biomass (Fig. 6.3)) and were therefore not analysed for N. In contrast, the FP.5.NI treatment combination, which extracted the least amount of N from the original biomass during the aqueous extraction (7.65 ± 1.05 %), recovered the most N in the aqueous PI (5.98 % - Fig. 6.6A).

The alkaline solvent extracted similar amounts of the total N from the original biomass for all three treatment combinations (proportion of original N extracted (N yield) = 19.27 ± 0.27, 19.82 ± 1.75 and 24.09 ± 0.83 % for FP.20.I, DP.20.I and FP.5.NI, respectively) and all yielded alkaline PIs (Fig. 6.6A). Of this alkaline extracted N, treatment combination FP.20.I recovered proportionally more N in the alkaline PI (78.19 ± 2.67 % of alkaline extracted N recovered in alkaline PI) compared to treatment combinations FP.5.NI (57.70 ± 3.95 %, %, t = 4.0385, p (Monte Carlo) = 0.0153) and DM.20.I (54.13 ± 4.62 %, %, t = 3.9123, p (Monte Carlo) = 0.0189), which recovered similar proportions. Treatment combination FP.5.NI and FP.20.NI recovered similar amounts of the total N from the original biomass in the alkaline PI (13.83 ± 0.47 % and 15.08 ± 0.73 %, respectively), but only FP.20.NI had a significantly higher alkaline PI N yield compared to DM.20.I (10.66 ± 1.05 %, t = 3.3277, p (Monte Carlo) = 0.0328).

Combined, the aqueous and alkaline solvents extracted less than half of the total N from the original biomass, leaving most of the N in the final residual biomass (i.e. the alkaline DM pellet for DM.20.I or the combined alkaline press cake and alkaline FP pellet for FP.20.I and FP.5.NI treatment combinations) (Fig. 6.6A). The FP.5.NI treatment combination extracted significantly less N in total (31.74 ± 0.25 % of total N extracted from the original biomass) compared to FP.20.I (43.05 ± 0.69 %, t = 14.32, p (Monte Carlo) = 0.0004) and DM.20.I (40.40 ± 2.35 %, t =3.3976, p (Monte Carlo) = 0.0286), which extracted similar amounts of N. However, the FP.5.NI treatment combination recovered the most extracted N in the total PI (62.41 ± 0.25 % of total extracted N) compared to FP.20.I (35.01 ± 0.69 %, t = 16.992, p (Monte Carlo) = 0.0001) and DM.20.I (26.76 ± 2.35 %, t = 6.0557, p (Monte Carlo) = 0.0035) (Fig. 6.6A). The proportion of total N lost to the total residual supernatant and not recovered in either the total residual biomass or total PI was lower for the FP.5.NI treatment

combination (11.93 ± 0.13 %) compared to the FP.20.I and DM.20.I treatment combinations (27.96 ± 0.21 and 29.75 ± 3.19 %, respectively).

6.3.4. The concentration of nitrogen in PIs and residual biomass

The concentration of N (% dw) in all recovered components (DM pellets, FP pressed cakes, FP pellets and PIs) was higher compared to the original seaweed biomass for aqueous and alkaline extractions across all three treatment-combinations analysed (Fig. 6.6B). The aqueous PI from the FP.5.NI treatment combination had a N concentration of 7.94 ± 0.17 % dw and was 173.79 ± 8.25 % higher than the original biomass (2.90 ± 0.07 %). The concentration of N in the alkaline PIs of all three treatment combinations were significantly higher than the aqueous PI from the FP.5.NI treatment combination (PERMANOVA pair-wise comparisons, $p < 0.05$) and ranged from 9.60 ± 0.23 % dw to 10.40 ± 0.41 % dw (230.78 ± 1.77 to 258.43 ± 7.99 % higher than original biomass).

The concentration of N in the residual DM pellets and pressed cakes ranged from 3.48 ± 0.17 to 3.86 ± 0.17 % dw and were 19.78 ± 3.57 to 32.96 ± 4.55 % higher than the original biomass, with all DM pellets and pressed cakes for all treatments having a similar concentration of N for both aqueous and alkaline extractions (Fig. 6.6B). Further, with the exception of the FP.20.I treatment combination ($t = 3.8519$, p (Monte Carlo) = 0.0184), there was no difference in concentration of N between aqueous DM pellets or pressed cakes and alkaline DM pellets or pressed cakes. The concentration of N in the FP.20.I and FP.5.NI aqueous FP pellets (5.36 ± 0.38 and 6.55 ± 0.13 % dw, respectively) were 85.20 ± 14.97 and 125.97 ± 1.82 % higher, respectively, than the original biomass and significantly higher than the alkaline FP pellets (3.50 ± 0.06 and 3.60 ± 0.06 % dw for FP.20.I and FP.5.NI, respectively) (Pseudo-$F_{1,11} = 161.73$, $p = 0.0002$) (Fig. 6.6B).

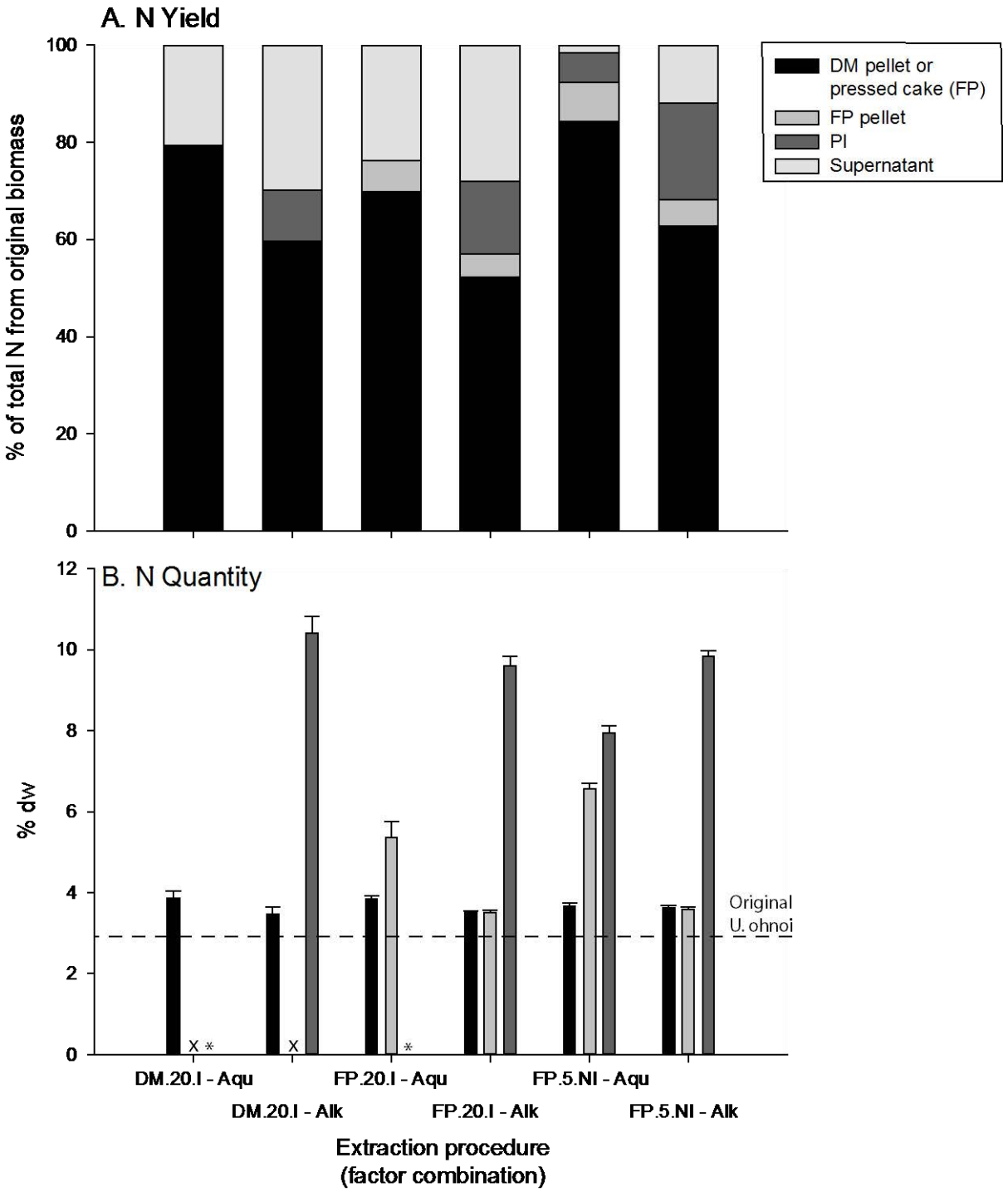

Figure 6.6. (A) The distribution of N from the original seaweed biomass and (B) concentration of N in recovered components (mean ± SE). N from the original biomass is divided among the residual biomass (the pellet for dry and milled starting material (DM pellet) and the pressed cake and pellet (FP pellet) for the fresh and pulped starting material), protein isolate (PI) and residual supernatant after protein precipitation (supernatant). DM = dry and milled biomass, FP = fresh and pulped biomass, 20 or 5 = 20:1 or 5:1 aqueous solvent to biomass ratio for aqueous extraction step, I = Incubated for 16h and NI = no incubation (< 1 min.). Note: the PIs for aqueous extractions for DM.20.I and FP.20.I did not yield sufficient mass for N analysis and were assumed to contribute a negligible amount of N to the N yield balance. Dashed line represents the concentration of N in the original biomass.

6.3.5. Protein isolation

The protein yields (determined by TAA) of PIs ranged from 12.28 ± 1.32 to 21.57 ± 0.57 % for the three treatment combinations, with the alkaline PI providing all of the yield for treatment combinations FP.20.I and DM.20.I and most of the yield for FP.5.NI (70.89 %) (Table 6.2). The protein yield for the alkaline PI for all three treatment combinations were similar, however, FP.5.NI had a significantly higher protein yield compared to the others due to the addition of the aqueous PI, which was not obtained for the other treatment combinations. This pattern was the same for TEAA yield. Methionine yields in the alkaline PI were similar across the three treatment combinations, however, the alkaline PI of FP.5.NI was significantly higher than that of DM.20.I (Table 6.2). In total, FP.5.NI had the highest methionine yield due the addition of the aqueous PI. Lysine yields in the alkaline PI were significantly higher for the FP.20.I treatment compared to the DM.20.I and FP.5.NI treatments, which were similar. However, due to the addition of the aqueous PI, treatment FP.5.NI had the highest lysine yield in total (Table 6.2).

The concentration of protein (TAA), TEAA, methionine and lysine in all PIs were substantially higher compared to the original biomass for all three treatment combinations (Table 6.2). The alkaline PIs for the three treatments had similar concentrations of protein, and TEAA, however, the DM.20.I alkaline PI had significantly higher concentrations of methionine compared to the FP.5.NI alkaline PI and significantly higher concentrations of lysine compared to both FP.20.I and FP.5.NI alkaline PIs (Table 6.2). The aqueous PI of treatment combination FP.5.NI had significantly lower concentrations of protein, TEAA, methionine and lysine compared to all alkaline PIs. As a result, the total PI for the DM.20.I and FP.20.I treatments had significantly higher methionine and lysine concentrations compared to the total PI for the FP.5.NI treatment, but there was no difference for the concentration of protein or TEAA (Table 6.2).

Table 6.2. The yield (% of original material) and concentration (% dw) of protein (TAA), total essential amino acids (TEAA), methionine (Met) and lysine (Lys) for protein extracts obtained for the three treatment combinations analysed in this study (mean ± SE) as well as those for other studies. Common superscript letters within variables indicate no significant difference (PERMANOVA pair-wise comparisons, p (Monte Carlo) > 0.05). The concentration of protein, TEAA, Met, Lys and ash are also shown for the starting material (*Ulva ohnoi*). [1]Wong and Cheung (2001b), [2]Wong and Cheung (2001a). Aq = Aqueous PI, Alk = Alkaline PI, * = Crude protein (N*6.25), NA = not applicable.

	Protein		TEAA		Met		Lys	
	% Yield	% dw	% Yield	% dw	% Yield	% dw	% Yield	% dw
Original *Ulva ohnoi*	NA	13.58 ± 0.42^{a}	NA	6.33 ± 0.21^{a}	NA	$0.23 \pm <0.01^{a}$	NA	0.84 ± 0.03^{a}
PIs (this study)								
DM.20.I (Alk only)	12.28 ± 1.32^{a}	56.04 ± 2.35^{b}	12.97 ± 1.4^{a}	27.56 ± 1.16^{b}	15.2 ± 1.15^{a}	1.18 ± 0.03^{b}	11.22 ± 0.86^{a}	3.17 ± 0.01^{b}
FP.20.I (Alk only)	17.13 ± 1.39^{ab}	50.87 ± 2.39^{bc}	18.17 ± 1.48^{ab}	25.13 ± 1.21^{bc}	21.22 ± 1.98^{abc}	1.07 ± 0.07^{bc}	15.17 ± 0.83^{b}	2.78 ± 0.07^{c}
FP.5.NI (Aq + Alk)	21.57 ± 0.57^{b}	47.43 ± 0.78^{c}	22.61 ± 0.59^{b}	23.16 ± 0.39^{c}	27.6 ± 0.63^{b}	1.03 ± 0.01^{c}	18.92 ± 0.23^{c}	2.54 ± 0.04^{d}
Aq	6.28 ± 0.07^{c}	39.07 ± 0.32^{d}	6.59 ± 0.08^{c}	19.08 ± 0.16^{d}	8.14 ± 0.29^{d}	0.86 ± 0.03^{d}	6.22 ± 0.15^{d}	2.38 ± 0.03^{e}
Alk	15.29 ± 0.64^{a}	50.85 ± 0.87^{b}	16.03 ± 0.67^{a}	24.83 ± 0.43^{b}	19.47 ± 0.85^{c}	1.1 ± 0.01^{b}	12.71 ± 0.35^{a}	2.61 ± 0.05^{cd}
Other studies								
Ulva lactuca[1]	36.4*	73.9	NA	29.83	NA	0.47	NA	3.54
Hypnea charoides[1]	46.3*	78.7	NA	27.92	NA	1.35	NA	3.26
Hypnea japonica[1]	45.4*	78.7	NA	31.54	NA	1.67	NA	3.79
Sargassum hemiphyllum[2]	9.5*	81.2	NA	31.96	NA	1.11	NA	4.48
Sargassum hemiphyllum [2]	7.8*	70.6	NA	28.20	NA	0.86	NA	3.85
Sargassum henslowianum [2]	33.1*	69.2	NA	31.50	NA	0.70	NA	3.82
Sargassum henslowianum [2]	27.0*	71.2	NA	27.99	NA	0.63	NA	4.08
Sargassum patens[2]	48.0*	81.2	NA	33.51	NA	0.79	NA	4.88
Sargassum patens[2]	37.8*	70.6	NA	27.68	NA	0.71	NA	4.25

6.3.6. Protein concentration

The aqueous total residual yielded between 73.9 to 89.6 % of the protein, TEAA, methionine and lysine from the original biomass, with no difference between the two treatment combinations analysed (DM.20.I and FP.20.I, Table 6.3). For fresh and pulped biomass, where the total residual was divided into a cake and suspended material, the cake yielded most of the protein, TEAA, methionine and lysine (Table 6.3).

The aqueous total residual from both the DM.20.I and FP.20.I treatments had significantly higher concentrations of protein, TEAA, methionine and lysine compared to the original seaweed biomass. Concentrations of protein, TEAA, methionine and lysine were 36.17 ± 4.15, 39.88 ± 4.25, 42.03 ± 5.23 and 33.12 ± 3.62 % higher for the DM.20.I treatment, respectively, and 44.70 ± 1.21, 50.46 ± 1.46, 63.75 ± 3.41 and 32.24 ± 2.06 % higher for the FP.20.I treatment, respectively, compared to the original seaweed biomass. The total residuals for the DM.20.I and FP.20.I treatments had similar concentrations of protein, TEAA and lysine, however, FP.20.I had a significantly higher concentration of methionine compared to DM.20.I (Table 6.3). The pressed cake portion of the FP.20.I aqueous total residual was similar in protein, TEAA, methionine and lysine concentration compared to the aqueous total residual of the DM.20.I treatment. However, the aqueous FP pellet portion of the FP.20.I aqueous total residual had significantly higher concentrations of protein, TEAA, methionine and lysine (Table 6.3). Concentrations of protein, TEAA, methionine and lysine were 104.30 ± 5.14, 117.59 ± 5.21, 163.77 ± 5.23 and 71.84 ± 3.76 % higher in the aqueous FP pellet compared to the original seaweed biomass.

Table 6.3. The yield (% of original material) and concentration (% dw) of protein (TAA), total essential amino acids (TEAA), methionine (Met), lysine (Lys) and ash in the residual biomass of the aqueous extraction step for DM.20.I and FP.20.I (mean ± SE). The concentration of protein, TEAA, Met, Lys and ash are also shown for the starting material (*Ulva ohnoi*). Common superscript letters within variables indicate no significant difference (PERMANOVA pair-wise comparisons, p (Monte Carlo) > 0.05). Note: the aqueous total residual for FP.20.I consisted of the aqueous pressed cake and aqueous FP pellet. These are reported separately and as a total residual. * = pooled sample due to insufficient sample mass, NA = not applicable.

	Protein		TEAA		Met		Lys		Ash	
	% Yield	% dw	% Yield	% dw	% Yield	% dw	% Yield	% dw	% Yield	% dw
Ulva ohnoi	NA	13.58 ± 0.42^{a}	NA	6.33 ± 0.21^{a}	NA	0.23 ± <0.01^{a}	NA	0.84 ± 0.03^{a}	NA	28.50 ± 0.52^{a}
Aqueous residual biomass										
DM.20.I (DM pellet)	81.34 ± 2.47^{a}	18.49 ± 0.62^{b}	83.55 ± 2.48^{a}	8.85 ± 0.29^{b}	84.83 ± 2.99^{a}	0.33 ± 0.02^{b}	79.5 ± 1.87^{a}	1.12 ± 0.03^{bc}	38.53 ± 3.27^{a}	18.38 ± 1.54^{b}
FP.20.I (total)	80.48 ± 0.46^{a}	19.65 ± 0.21^{b}	83.49 ± 0.54^{a}	9.52 ± 0.11^{b}	89.6 ± 2.61^{a}	0.38 ± 0.01^{c}	73.98 ± 0.75^{a}	1.11 ± 0.01^{b}	24.83 ± 1.26^{b}	12.55 ± 0.41^{c}
Pressed cake	73.35 ± 0.32^{b}	18.86 ± 0.27^{b}	75.9 ± 0.33^{b}	9.09 ± 0.13^{b}	80.39 ± 2.69^{a}	0.35 ± 0.02^{bc}	67.99 ± 0.63^{b}	1.08 ± 0.01^{c}	23.48 ± 1.21^{b}	12.65 ± 0.44^{c}
FP pellet	7.13 ± 0.22^{c}	27.72 ± 0.22^{c}	7.59 ± 0.25^{c}	13.75 ± 0.08^{c}	9.21 ± 0.41^{b}	0.61 ± 0.02^{d}	6.00 ± 0.17^{c}	1.44 ± 0.01^{d}	1.35 ± 0.05^{c}	11.05^{*d}

6.4. Discussion

Yields of protein isolate from the green seaweed *Ulva ohnoi* were higher for the 'leaf method' than the 'seed method' demonstrating, for the first time, an innovative approach to isolating protein from seaweed biomass inspired by the physiological and morphological similarities between seaweeds and leaves. Importantly, the quality of the protein isolate produced using the leaf method was also high, containing similar concentrations of protein, total essential amino acids and methionine to the seed method, and a slightly lower concentrations of lysine. This supports methods used primarily for the isolation of protein in leaves as an alternative for seaweeds.

The total protein isolate (PI) for each extraction included an aqueous solvent extracted PI and an alkaline solvent extracted PI. For the aqueous PI, no incubation time resulted in significantly higher mass yields than a 16 hour incubation at 30°C. This effect of incubation time was consistent across both types of starting material (dry and milled vs. fresh and pulped) and both aqueous solvent to biomass ratios (20:1 and 5:1). Lower PI mass yields when aqueous extractions were incubated for 16 hour at 30°C may be due to heat-related denaturing of proteins over this time. Indeed, long incubation times (16 hours) at low temperatures (4 and 22 °C) resulted in no difference in aqueous protein isolate yields for the red seaweed *Palmaria palmata* compared to shorter incubation times (4 and 7 hours) (Harnedy and FitzGerald 2013). Similarly, for leaves, aqueous solvent extractions are either processed immediately after pulping or incubated at low temperatures to prevent proteins from deteriorating (Fernández et al. 1999; Chiesa and Gnansounou 2011). However, more N was extracted from those treatment combinations that were incubated at 30°C (DM.20.I and FP.20.I) compared to those that were not (FP.5.NI treatment combination), demonstrating that lower PI yields were a result of lower protein precipitation rates rather than lower protein extraction rates. This suggests that if the extracted proteins were denatured, this did not reduce their solubility but rather their rate of precipitation. Previous studies on the extraction of protein from seaweeds have also used long incubation times at above ambient temperatures (Fleurence et al. 1995; Wong and Cheung 2001a, b). However, these studies precipitated proteins using ammonium sulphate, rather than acid, and did not distinguish between extracted and isolated protein, making it difficult to elucidate any denaturing effects on protein precipitation. For the alkaline PI, the use of fresh and pulped biomass resulted in significantly higher mass yields compared to when dry and milled biomass

was used. This effect has been established for leaves (Bals and Dale 2011; Chiesa and Gnansounou 2011) and suggests that the proteins extracted from *Ulva ohnoi* are similar in nature to those in leaves and are likely denatured during the drying process. However, as with the aqueous PI, differences in N yields in the alkaline PI stemmed from different protein precipitation yields of extracted N rather than differences in the amount of total N extracted by the alkaline solvent, suggesting that any denaturing of proteins limits precipitation, rather than extraction. A study by Wong and Cheung (2001a) showed that PI yields were higher when *Sargassum* spp. were oven-dried compared to when they were freeze-dried. As *Ulva ohnoi* was oven-dried in this study, the reduced PI yields that resulted from the use of dry and milled biomass compared to fresh and pulped biomass may be directly due to the drying process rather than heat.

Overall, the PI protein yields obtained in this study (12.28 – 21.57 %) were moderate compared to other studies on seaweeds, which range from 7.8 % to 48.0 % (Fleurence et al. 1995; Wong and Cheung 2001b, a) and lower than those reported for other species of *Ulva* (26.8 – 36.4 %) (Fleurence et al. 1995; Wong and Cheung 2001b) (Table 6.2). These studies applied methodologies that used dry and milled biomass, had high aqueous solvent to biomass ratios and a 16 h incubation time for the aqueous extraction. However, the key differences between these studies and this one were that extractions were repeated 5 – 6 times, the reducing agent 2-mercaptoethanol was used during the alkaline extraction to increase protein solubility by breaking disulphide linkages, and proteins were precipitated using ammonium sulphate. While any or all of these may have been responsible for the higher protein yields, repeating extraction protocols 5 – 6 times and the use of 2-mercaptoethanol are unlikely to be transferred to larger scales for food or feed production. While repeating extraction protocols on the same biomass would yield diminishing returns, the use of 2-mercaptoethanol is not permissible for the extraction of proteins for human or livestock consumption (Turhan et al. 2003). Alternative food grade reducing agents (cysteine-hydrochloride-monohydrate and N-acetyl-l-cysteine) have been successfully used to improve protein isolation yields from seaweeds (Harnedy and FitzGerald 2013) and could be incorporated into protein isolation protocols, warranting their viability at scale. Consequently, protein isolation yields may be increased beyond those reported here if a food grade reducing agent is used in conjunction with the leaf method.

As PI protein yields for seaweeds (up to 48 %) are considerably lower than those that are routinely achieved for terrestrial seed crops (> 75 %) (Berk 1992; Ju et al. 2001), there is considerable scope to improve the PI protein yields of seaweeds. Further increases in PI protein yields will result from increasing protein precipitation efficiencies (increasing the proportion of extracted protein that is precipitated and isolated) and/or increasing protein extraction efficiencies (total protein removed from original biomass). As this study is the first to quantify protein precipitation yields for a seaweed, through the examination of N and protein in the total residual biomass and PI, it is difficult to elucidate which parameters examined in other studies affect protein precipitation rates. However, there is evidence to suggest that further improvements in extraction efficiencies of fresh biomass methods can be achieved. For example, novel cell disruption techniques, such as enzyme preparations, microwave and ultrasound, have improved the extraction efficiencies of protein and other cellular-bound components in dry seaweed biomass (Harnedy and FitzGerald 2013; Kadam et al. 2013) and fresh terrestrial leaves (Barba et al. 2015; Šic Žlabur et al. 2016). These could provide similar improvements for fresh seaweed biomass. Indeed, Le Guillard et al. (2016) extracted 54 % of protein (as TAA) from the red seaweed *Grateloupia turuturu* using a combination of enzymes and ultrasound on fresh biomass. Alternatively, other methodologies that are based on completely different principles to traditional solvent extractions could be explored for seaweeds. For example, hydrothermal liquefaction (HTL), which is a thermochemical process that chemically and physically transforms biomass in liquid water at high temperature and pressure, has been used under mild conditions (150 – 220 ° C) to extract over 50 - 70 % of the N from microalgae (Yu et al. 2011; Jazrawi et al. 2015).

The concentrations of protein in the total PIs in this study (47.43 – 56.04 % dw) were approximately 20 - 40 % lower compared than those of other studies on seaweeds (range from 69.2 – 81.2 % dw as TAA), which included a PI from *Ulva lactuca* (73.9 % dw) (Table 6.2). Despite this lower concentration of protein, PIs from this study had similar concentrations of total essential amino acids (1 – 30 % lower in this study) (Table 6.2). Furthermore, they had 20 – 90 % more methionine than PIs obtained from *Sargassum* spp. (Wong and Cheung 2001a) and 120 – 150 % more methionine than the PI obtained from *Ulva lactuca* (Wong and Cheung 2001b) (Table 2). In contrast, the concentration of lysine was lower compared to other studies (3 – 50 % lower) (Table

6.2). These differences in the concentration of protein, total essential amino acids, methionine and lysine may be associated with the use of the reducing agent 2-mercaptoethanol, which is used to improve protein solubility by breaking di-sulphur bonds, or the use of ammonium sulphate for protein precipitation, as these were the two major differences between the extraction methods used by Wong and Cheung (2001a & b). Irrespective, the concentration of protein, total essential amino acids, methionine and lysine in the PIs in this study were 250 – 400 % higher than the original seaweed biomass.

Protein in the original seaweed biomass can be concentrated in the total residual biomass after aqueous and alkaline extractions if more non-protein material is removed relative to protein. The aqueous extraction removed considerably more non-PI mass (supernatant mass yield) than the alkaline extraction for all treatment combinations. For the aqueous extraction, a higher aqueous solvent volume to biomass ratio (20:1) and a 16 hour incubation time extracted the most non-PI mass for both types of starting material. Most of this non-PI mass was ash (~ 50 %) and non-precipitated protein (~ 30 %). The remaining extracted material (~ 20 %) represented approximately 10 % of the original biomass and was likely soluble fibre, which has been reported to be 11.3 – 12.6 % dw in *Ulva ohnoi* (Magnusson et al. 2016; Mata et al. 2016). The co-extraction of protein with ash and soluble fibre during the aqueous extraction step resulted in only a moderate (~ 30 – 50 %) increase in the concentration of protein and essential amino acids in the aqueous total residual relative to the original biomass (Table 3). Correspondingly, the concentration of protein, total essential amino acids, methionine and lysine in the aqueous total residuals were 55 – 70 % less than the concentrations in the PIs. These findings demonstrate that protein isolation methods are more suited to seaweeds with low concentrations of protein, such as *Ulva ohnoi*, compared to protein concentration methods.

One novel outcome for the concentration of protein resulted from the analysis of the aqueous FP pellet portion of the total residual for the leaf method treatments, which was high in N, protein and essential amino acids. Any protein-rich residual components could be combined with aqueous PIs to improve yields, without detracting from the quality of the PI. Alternatively, as there was little difference in the concentration of N and only a small increase in the concentration of ash between the aqueous and alkaline total residuals, the aqueous total residual could likely undergo an alkaline extraction

step without a significant reduction in the nutritional value of the final residual biomass. This would allow for co-production of an alkaline PI along with a protein-concentrated residual biomass (alkaline total residual). However, as the yields for protein isolation improve, it follows that the concentration of protein and essential amino acids in the final alkaline residual biomass will decrease. A similar process has been proposed for terrestrial leaves (Sinclair 2009), where relatively low protein extraction yields ensure that enough protein is retained in the residual to be utilised as a feed. However, this is a new concept for seaweeds and there are clearly many methods and stepwise procedures to consider when processing seaweed biomass, with a caveat that these should be viable at scale.

Both PIs and total residuals had higher concentrations of protein, TEAA, methionine and lysine compared to the original seaweed biomass, however, only the PIs are suitable as protein sources for mono-gastric livestock. This is because PIs had concentrations of protein, TEAA, methionine and lysine that were 5 – 70 % higher than soybean meal, but total residuals had concentrations that were 45 – 60 % lower than soybean meal (Chapter 5). Instead, the total residual biomass after either aqueous or alkaline protein extractions may be suitable as a feed for ruminant livestock due to their reduced ash (salt) content (Masters et al. 2007).

6.4.1. Conclusion

The protein isolation methods in this study increased the concentration of protein and essential amino acids 3 to 5-fold compared to whole *Ulva ohnoi* and were considerably more effective than protein concentrating methods, which only increased protein and amino acid concentrations by 30 – 50 %. The use of fresh and pulped biomass over dry and milled biomass as the starting material, no incubation time over a 16 h incubation time at 30 °C, and a low aqueous solution volume resulted in the highest protein isolate yield of 22 % of the protein found in the whole seaweed. This chapter has demonstrated that proteins from the green seaweed *U. ohnoi* were most effectively isolated by adopting methodologies for terrestrial leaves. However, the best protein isolate yields in this chapter are lower than other studies for seaweeds, and together they are considerably lower than those obtained for terrestrial seed crops, both of which infer that the physiology and morphology of the seaweeds will need to be considered on a case by case scenario. However, regardless of species-specific responses to protein

isolation methods, this chapter supports the development of innovative cell-disruption and extraction techniques to improve protein yields to deliver on the paradigm of using seaweeds as an alternative protein crop.

Chapter 7: General discussion

Seaweeds have the potential to become an alternative and sustainable protein crop as they do not require traditional agricultural resources (arable land, fresh water or fertilisers) and have high biomass productivities (Bolton et al. 2009; Mata et al. 2016). This thesis examined the potential of seaweeds as an alternative protein crop by examining (1) within-species variation in the concentration and quality of protein and its relationship with biomass production, (2) between-species variation in the concentration and quality of protein in seaweeds and their suitability as a protein source for mono-gastric livestock, and (3) processing methods to improve the concentration of protein in seaweeds post-harvest.

In Chapters 2 and 3, I established that there was considerable within-species variation in the concentration and quality of protein in the green seaweed *Ulva ohnoi,* which was strongly related with growth rate. These chapters demonstrated the limited extent to which the concentration of protein and essential amino acids in seaweeds can be increased in culture if high biomass productivities are also targeted. The dynamic relationship between N content in biomass, and the concentration and quality of protein, also highlighted the importance of accurate methods for measuring protein in seaweeds, specifically when a nitrogen-to-protein conversion factor is used. Consequently, the seaweed literature was reviewed in Chapter 4 to systematically assess the methods used in measuring the protein content of seaweeds. This resulted in the recommendation of a seaweed specific N-protein conversion factor, for the first time, of 5. This work also highlights that much of the quantitative reporting of protein in the literature are overestimates because of the use of a generic N-protein conversion factor of 6.25. Therefore in Chapter 5, the potential of seaweeds as a protein source for mono-gastric livestock was re-assessed by examining the concentration of essential amino acids in seaweeds using meta-analysis, in what was a first for the field. This meta-analysis demonstrated that seaweeds contain insufficient protein and, more specifically, insufficient essential amino acids compared to traditional protein sources and are unable to meet the nutritional requirements of most mono-gastric livestock if used in the whole form. Instead, it was highlighted that the isolation or concentration of protein from seaweeds is the critical step if they are to be developed as an alternative protein crop for mono-gastric livestock. Therefore in Chapter 6, different methods were examined to

isolate and concentrate protein in *Ulva ohnoi*. The isolation of protein using a method for terrestrial leaves was tested for the first time on seaweeds and was identified as the most promising pathway to improving the nutritional value of seaweeds as a protein source.

A major outcome of this thesis is that seaweeds, without exception, do not have high enough concentrations of protein, and more specifically the essential amino acids, in the whole form to be utilised as a protein source for mono-gastric livestock. This has implications for the future direction of research and development for developing seaweeds as a protein crop and its commercial use in such applications. These are (1) identifying suitable species for the production of protein, (2) optimising cultivation protocols for the production of protein and (3) post-harvest processing of seaweed biomass for the production of protein. These three factors are now discussed in the context of the major findings of this thesis.

7.1. Identifying suitable species for the production of protein

Identifying suitable species of seaweed for the production of protein is the essential first step in establishing seaweeds as a protein crop for mono-gastric livestock. A logical way to begin this selection process is by identifying seaweeds with high concentrations of protein and essential amino acids on a whole biomass basis.

The highest reported concentration of protein for a seaweed is 47.8 % dw (*Gracilaria cornea*) (Angell et al. 2015). This number drops to approximately 35 % dw when revised using the N-protein conversion factor established for seaweeds in Chapter 4 (N*5) and extreme outliers are removed (Chapter 5). Therefore, a protein concentration of ~ 35 % is probably the upper limit of what we will find in any seaweed across the globe. Similarly, the highest concentrations for the essential amino acids methionine and lysine of 0.69 and 2.12 % dw, respectively, can be considered the upper limits for these amino acids (Chapter 5). These upper limits for protein, methionine and lysine (protein = 35 %, methionine = 0.69 %, lysine = 2.12 %) are slightly lower than the concentrations found in soybean meal (protein = 40 %, methionine = 0.70 %, lysine = 2.24 %), which suggests that the species of seaweed that have concentrations close to these limits (of which there are only 6 in the literature from the following genera: *Amansia, Capsosiphon*, *Pyropia* (formerly *Porphyra*), *Solieria* and *Ulva* – note that the

Gracilaria reported above was labelled as an extreme outlier by the outlier labelling rule (see section 5.2.) and therefore deemed erroneous and removed from the dataset) are the most promising candidates for protein production. However, because seaweeds typically have low *in vitro* protein digestibility (Fleurence 1999b; Maehre et al. 2016) and low digestible energy, even the best species are unlikely to be a viable source of protein in their whole form. In this sense, the concentration of protein or amino acids on a whole biomass basis is less relevant when selecting species for the production of protein for mono-gastric livestock. However, the concentration of protein and essential amino acids should not be discounted completely as high whole biomass concentrations will play an additional role in the post-harvest processing of seaweeds (see subsequent section 7.3).

The necessity to concentrate the protein in seaweeds post-harvest supports the selection of species with a high quality of protein (proportion of protein as essential amino acids). However, because most species of seaweed have a quality of protein comparable to, or higher than, soybean meal (75 % of seaweeds have higher proportions of methionine and over 25 % had higher concentrations of lysine (Chapter 5)), this selection criterion is less critical. Instead, selecting species that can be cultivated with high productivities at scale is arguably more important. In this sense, it is not the concentration or quality of protein that is important but rather the rate of protein production (g of protein m^{-2} d^{-1}).

Of those genera currently produced at commercial scales, *Pyropia* (formerly *Porphyra*) has the highest concentration of protein (N ×5 mean = 21 % dw, range = 11 – 35 % dw), followed by *Undaria* (mean = 14 % dw, range = 10 – 18 % dw), *Gracilaria* (mean = 13 % dw, range = 4 – 26 % dw) and *Hizikia* (mean = 8 % dw, range = 6 – 10 % dw) (Angell et al. 2015) (Chapters 4 and 5). *Pyropia* species also have among the highest concentration of methionine and lysine on a whole biomass basis, with concentrations of these being comparable to soybean meal (Chapter 5). This would suggest that species from the genera *Pyropia* have the most potential to become a protein crop among seaweeds that are produced commercially. However, *Pyropia* is produced in off-shore systems and used exclusively for human food where it is sold as a high-value product in a whole form (Paul et al. 2012). In this situation, *Pyropia* not only provides a negligible amount of protein to humans (see section 1.4.2. in Chapter 1), but the competition with other uses may restrict its development as a protein source for mono-gastric livestock in the short term. This is because production costs are high due to the labour intensive

nature of the off-shore cultivation techniques that are utilised, with these costs off-set by their high value as a whole food for humans. Similarly, *Gracilaria* is also produced in labour intensive systems and it is sold as a high-value feedstock for the production of the phycocolloid agar. In this situation, the production of protein from *Gracilaria* has the potential to be integrated with the extraction of phycocolloids under a biorefinery process (see section 7.3 below), however, this remains to be investigated.

In addition to those seaweeds that are commercially produced, there are a number of other species of seaweed that can be cultivated at scale and have a high potential to be utilised as a protein crop for mono-gastric livestock. Green seaweed species from the genus *Ulva* are among the best candidates for the production of protein for mono-gastric livestock. These species have high biomass productivities (Chapters 2 and 3) (Bolton et al. 2009; Mata et al. 2016), high environmental tolerances (Chapter 3, (Cohen and Fong 2004; Larsen and Sand-Jensen 2006) and are competitively dominant in cultivation. Importantly, *Ulva* species can be grown in intensive-land-based systems (Neori et al. 2003; Bolton et al. 2009; Mata et al. 2016), which allows for the utilisation and remediation of wastewater from aquaculture and agriculture (Bolton et al. 2009; Nielsen et al. 2012). *Ulva* species have a highly variable concentration of protein that ranges from ~ 1 – 35 % (Chapter 4). Notably, if high biomass productivities are targeted in intensive cultivation systems, when N is not limiting, then the concentration of protein will not reach the within-species maximum due to the dilution effect identified in Chapters 2 and 3 of this thesis. For high growth situations, the protein concentration used for modelling should, therefore, be the moderate values (~ 13 - 25 % dw) not the extreme ones (Chapter 2, 3 and 6, (Nielsen et al. 2012; Shuuluka et al. 2013; Mata et al. 2016). However, irrespective of the concentration of protein in the harvested biomass, *Ulva* species, like other seaweeds with a high concentration of protein, will need to be processed post-harvest to concentrate it as a protein source for mono-gastric livestock (Chapter 5).

7.2. Optimising cultivation protocols for the production of protein

The intensive land-based cultivation of seaweed provides the opportunity to tailor cultivation protocols for the production of protein for mono-gastric livestock. This thesis has established that seaweeds will need to be processed post-harvest to increase

the concentration of protein and that the cultivation protocols should target the maximum productivity of protein (g of protein m^{-2} d^{-1}) rather than the concentration of protein.

Protein productivity is a function of the concentration of protein in the biomass and the biomass productivity. The biomass productivity is in turn a function of growth rate (specific growth rate – SGR) and the stocking density. However, for any given stocking density, the growth rate is directly analogous to biomass productivity. The intensive land-based cultivation of seaweeds will ideally utilise wastewater and provide conditions under which growth is not limited by nitrogen (N) (de Paula Silva et al. 2008; de Paula Silva et al. 2012; Nielsen et al. 2012; Cole et al. 2015b). In Chapters 2 and 3 I demonstrated that a clear trade-off between the concentration of protein (or TAA) and growth rate (biomass productivity) under non-N limiting conditions, with the highest rates of protein production coinciding with the highest rates of biomass productivities. This highlights that the primary objective for maximising protein productivity in non-N limiting systems should be to maximise biomass productivity. This has also been highlighted at an inter-species level where the more productive species with a lower concentration of protein (*Ulva ohnoi*) had a higher protein productivity, compared to slower growing species with a higher concentration of protein (*Derbesia tenuissima*) (Mata et al. 2016). Furthermore, this focus on biomass productivity over the concentration of protein becomes even more important if other components of the biomass are also utilised for alternative uses, e.g. salts and polysaccharides, which will be facilitated by the necessity to process biomass post-harvest (see section 7.3 below).

Although I have recommended that the short term focus should be on biomass productivity, methods to increase the concentration of protein without limiting protein productivity will likely remain an important research goal in the longer term. For example, maintaining high rates of protein production through increases in the concentration of protein in the biomass would yield less biomass for the same amount of protein and, in the future, reduce transport and processing requirements. Further, higher concentrations of protein in harvested biomass may also allow for higher protein isolate yields and higher concentrations of protein in residual biomass after the removal of non-protein material (see section 7.3). In species of *Ulva*, maintaining high protein productivities through increasing the concentration of protein in the harvested biomass

may be achieved by optimising stocking densities. This is because as stocking density increases, the concentration of protein in the biomass will increase, provided N supply is sufficient (Chapters 2 and 3). In Angell et al. (2014), a higher stocking density resulted in reduced growth rates and a higher concentration of N in the biomass, but because there was more total stocked biomass, biomass productivities were not decreased. That is similar quantities of biomass can be produced with more stock growing at lower rates. This concept, however, would need to be examined directly and will be dependent on the nexus between species and cultivation system.

Protein production rates for seaweeds are highly variable and depend on species and cultivation system. This makes it difficult to generalise more broadly from any individual empirical study. In this thesis, protein production rates from small scale cultures of *Ulva ohnoi* (Chapters 2 and 3) reached a maximum of 2.8 g m^{-2} d^{-1}, which is equivalent to ~ 10 t ha^{-1} $year^{-1}$. For larger scale land-based systems (10 000 L) over a longer period (6 months) a protein production rate of 18 t ha^{-1} $year^{-1}$ has been achieved for *Ulva ohnoi* and a rate of 14 t ha^{-1} $year^{-1}$ for *Derbesia tenuissima* (Mata et al. 2016). For commercial species in off-shore systems, protein production rates are likely to be considerably lower, in the order of ~ 3 t ha^{-1} $year^{-1}$, for *Pyropia* species, as estimated based on a wet weight production of 150 t ha^{-1} $year^{-1}$ for off-shore cultivation systems (Gao and Mckinley 1994; Lüning and Pang 2003; Titlyanov and Titlyanova 2010), a wet:fresh weight ratio of 2:1, a fresh:dry weight ratio of 5:1 (Ramus et al. 1976) and a mean protein concentration of 21 % (Chapter 4). Despite this variability, seaweeds have favourable protein production rates compared to soybean, which has a protein production rate of ~ 1.2 t ha^{-1} $year^{-1}$ (based on a dry weight production of ~ 3 t ha^{-1} $year^{-1}$ and a protein concentration of 40 % (Grieshop and Fahey 2001; Boye et al. 2010)). This highlights that seaweeds do have the capacity to produce more protein per unit area than traditional crops without the use of traditional agricultural resources. However, from a nutritional perspective, seaweeds will need to be processed post-harvest before they can be viably included into the compound diets of mono-gastric livestock, which is also what is done to produce soybean meal from a de-fatting process to remove the vegetable oil product (Berk 1992).

7.3. Post-harvest processing for the production of protein

Increasing the concentration of protein and essential amino acids in whole seaweed biomass through post-harvest processing will be the most critical factor in establishing seaweeds as a protein source for mono-gastric livestock. This processing can involve the direct extraction and isolation of protein (protein isolation) or the removal of non-protein material so that the protein in the original biomass is concentrated (protein concentration). In Chapter 6, protein isolation techniques produced a more concentrated source of protein and essential amino acids (protein = 47 – 56 %, methionine = 1.03 – 1.18 %, lysine = 2.54 – 3.17 %) compared to concentration techniques (protein = 18 - 20 %, methionine = 0.33 – 0.38 %, lysine = 1.11 – 1.12 %) for *Ulva ohnoi* (protein = 14 %, methionine = 0.23 %, lysine = 0.84 %). Consequently, the isolation of protein should be prioritised, especially in seaweeds with low concentrations of protein on a whole biomass basis, over protein concentration through the removal of non-protein components. However, the residual biomass after the isolation of protein also represents a potentially concentrated protein source (Chapter 6) and should not be overlooked.

The nutritional barriers that will prevent seaweeds from becoming a protein source for mono-gastric livestock are akin to those preventing the utilisation of protein from terrestrial leaves. This is because seaweeds and leaves are biochemically similar, with low concentrations of protein and high concentrations of insoluble polysaccharides. Indeed, it was established in Chapter 6 that isolation methods based on those for leaves are more suited to *Ulva ohnoi* than those methods based on seeds. However, in a broader context, the biorefinery process models that have been proposed for terrestrial leaves provide insights as to how seaweed proteins may be most effectively utilised. For leaves, protein isolates could be used as a feed ingredient for either mono-gastric livestock, or for human nutrition, and the residual biomass could be used as either a feed for ruminant livestock (Sinclair 2009) or as a feedstock for bioenergy (Dale et al. 2009; Bals and Dale 2011). A similar model is possible for seaweeds. The protein isolates obtained from seaweeds (Chapter 6) (Fleurence et al. 1995; Wong and Cheung 2001b, a) have higher concentrations of protein and essential amino acids than soybean meal and, therefore, are viable protein sources for mono-gastric livestock (Chapter 5 and 6). Further, the residual biomass of seaweeds after protein isolation (Chapter 6), the composition of which had not previously been assessed prior to this thesis, could be used as a feed for ruminant livestock. This biomass had slightly higher concentrations of protein than the original whole biomass. However, more importantly the residual

biomass also had a lower concentration of ash (salts), which can act as a feed deterrent or decrease the digestibility of feed (Masters et al. 2007). Alternatively, the residual biomass could be utilised as a feedstock for bioenergy, where low concentrations of ash are also desirable (Neveux et al. 2014).

Together, the protein isolate and the subsequent residual biomass allows for most of the protein produced by the seaweed to be utilised. However, there is also the potential to integrate the isolation process with the extraction and isolation of other non-protein components so that the whole biomass is efficiently utilised. The green seaweed *Ulva ohnoi*, a focus of this thesis, is a promising example as to how different post-harvest processes may be integrated in a biorefinery approach. In addition to protein, *Ulva* species also contain salts, which have beneficial Na:K ratios (Magnusson et al. 2016), and ulvans, which have been shown to have a number of bioactive properties (Lahaye and Robic 2007; Holdt and Kraan 2011), that could be extracted in addition to protein in an integrated process. Both salt (Magnusson et al. 2016) and ulvans (Lahaye and Robic 2007) can be extracted using aqueous solvents and therefore integrated with the aqueous protein extraction. Indeed, in Chapter 6, up to ~ 65 % of the total ash and an estimated ~ 80 % of the soluble polysaccharides (ulvans) were extracted from *Ulva ohnoi* during the aqueous protein extraction step. While the extracted ulvans and water-soluble proteins could be sequentially separated from the aqueous solvent using ultrafiltration and/or precipitation methods (Fleurence et al. 1995; Wong and Cheung 2001b; Lahaye and Robic 2007), the salts could be concentrated through evaporation. Subsequently, alkaline soluble proteins could then be extracted and isolated from the resulting residual biomass (see Chapter 6). Alternatively, salts, ulvans and proteins could be sequentially extracted separately to avoid the separation of these products post-extraction. The aim in this case would be to minimise the amount of non-target material that is co-extracted at each step. This integrated biorefinery process would yield four distinct products: (1) salts, which have the potential to be used in human health, (2) ulvans, which have the potential to be utilised in the nutraceutical industry, (3) protein isolates, which could be used as a feed ingredient for mono-gastric livestock or for human nutrition, and (4) a residual biomass, which could be utilised as a feed for ruminant livestock (Fig. 7.1). This approach to processing seaweed biomass not only allows for the seaweed proteins to become nutritionally available, but also increases the value of the whole biomass (Williams and Laurens 2010; Jung et al. 2013). However, developing and optimising

this integrated biorefinery process will require considerable research effort, moving forward.

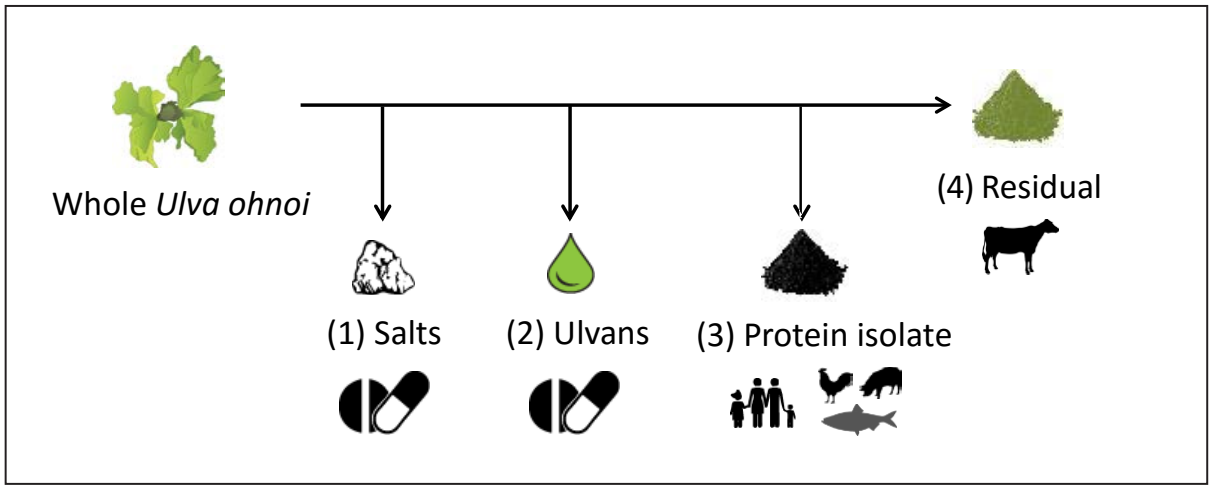

Figure 7.1: A proposed biorefinery process for the green seaweed *Ulva ohnoi* that would allow for the production of four distinct products: (1) salts, which have the potential to be used in human health, (2) ulvans, which have the potential to be utilised in the nutraceutical industry, (3) protein isolates, which could be used as a feed ingredient for mono-gastric livestock or for human nutrition, and (4) a residual biomass, which could be utilised as a feed for ruminant livestock

7.4. General conclusions and future research

There has until now been a widely accepted paradigm that seaweeds have a high concentration and quality of protein. Correspondingly, research into developing seaweeds as a protein source has focused on the whole seaweed biomass. However, this thesis has established that seaweeds, irrespective of cultivation conditions and species, are not viable as a protein source for mono-gastric livestock in a whole form and will need to be processed post-harvest to concentrate their protein. It is proposed, therefore, that research efforts shift from a focus on whole seaweed biomass to the development of protein isolates and concentrates from seaweeds produced under intensive cultivation. The outcomes of this thesis should encourage people to re-assess the paradigm of the use of proteins from seaweeds and hopefully set out a new research direction for establishing seaweeds as a protein crop.

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Annex 1

Annex to chapter 2

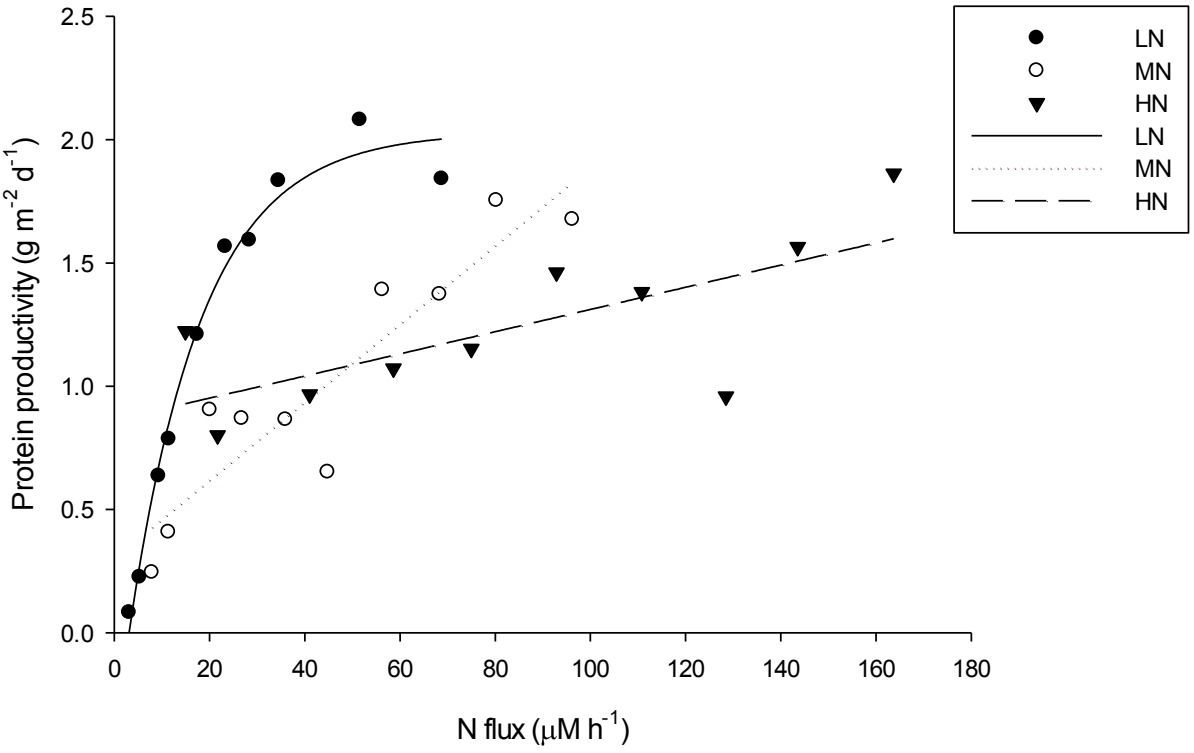

Figure S2.2. Protein (TAA) productivities (g m^{-2} d^{-1}) against N flux for low (LN – 20.65 μM), medium (MN – 86.41 μM) and high (HN – 183.15 μM) water nitrogen concentration treatments.

Table S2.1. The culture conditions and compositional analysis for all cultures in the N flux experiment. Water N concentrations: LN = 20.65 µM, MN = 86.41 µM and HN = 183.15 µM. N = nitrogen, C = carbon, TAA = total amino acids, His = histidine, Ser = serine, Arg = arginine, Gly = Glycine, Asp = aspartic acid, Asn = asparagine, Glu = glutamic acid, Gln = glutamine, Thr = threonine, Ala = alanine, Pro = proline, Lys = lysine, Tyr = tyrosine, Met = methionine, Val = valine, Ile = isoleucine, Leu = leucine and Phe = phenylalanine.

Treatment parameters			Compositional analysis (g $100g^{-1}$, dry weight basis)																		
N Flux (µM h^{-1})	Water	Water renewal (% h^{-1})	N	C	TAA	His	Ser	Arg	Gly	Asp & Asn	Glu & Gln	Thr	Ala	Pro	Lys	Tyr	Met	Val	Ile	Leu	Phe
3.10	LN	15.00	0.61	23.66	2.98	0.04	0.19	0.15	0.20	0.34	0.37	0.18	0.32	0.13	0.17	0.11	0.06	0.22	0.13	0.21	0.16
5.30	LN	25.64	0.66	21.78	3.15	0.05	0.21	0.15	0.21	0.37	0.38	0.19	0.33	0.15	0.20	0.09	0.05	0.23	0.14	0.23	0.18
9.27	LN	44.87	0.83	20.80	4.58	0.08	0.28	0.24	0.31	0.54	0.54	0.26	0.44	0.22	0.25	0.18	0.09	0.33	0.21	0.36	0.25
11.41	LN	55.26	0.92	21.55	4.82	0.08	0.30	0.26	0.33	0.56	0.58	0.28	0.46	0.23	0.27	0.18	0.10	0.34	0.22	0.38	0.27
17.38	LN	84.18	1.25	19.27	7.04	0.13	0.41	0.40	0.48	0.84	0.83	0.38	0.63	0.34	0.38	0.30	0.13	0.49	0.33	0.56	0.40
23.27	LN	112.67	1.64	20.94	8.76	0.16	0.50	0.52	0.57	1.07	1.03	0.46	0.77	0.45	0.52	0.37	0.17	0.58	0.39	0.70	0.50
28.32	LN	137.12	2.28	22.72	11.52	0.23	0.66	0.73	0.77	1.44	1.30	0.60	0.94	0.60	0.63	0.54	0.21	0.79	0.53	0.91	0.65
34.49	LN	167.00	2.46	22.80	11.95	0.24	0.67	0.78	0.77	1.53	1.35	0.61	0.95	0.66	0.69	0.54	0.22	0.77	0.53	0.96	0.68
51.54	LN	249.56	2.53	21.27	12.23	0.25	0.70	0.80	0.81	1.64	1.37	0.63	0.98	0.67	0.68	0.47	0.20	0.83	0.55	0.97	0.68
68.74	LN	332.85	2.75	21.39	12.87	0.27	0.69	0.86	0.84	1.65	1.44	0.62	1.03	0.78	0.76	0.44	0.21	0.85	0.60	1.08	0.75
7.89	MN	9.13	2.91	24.44	15.12	0.23	0.73	2.17	0.84	1.58	3.10	0.62	0.99	0.64	0.80	0.44	0.18	0.77	0.51	0.87	0.65
11.37	MN	13.15	3.12	24.01	16.14	0.28	0.85	2.09	0.96	1.79	2.97	0.72	1.09	0.67	0.80	0.50	0.20	0.92	0.59	0.98	0.73
20.06	MN	23.21	3.55	25.71	16.89	0.28	0.85	2.07	0.98	1.84	3.37	0.73	1.14	0.74	0.86	0.47	0.20	0.91	0.61	1.06	0.78
26.77	MN	30.98	3.54	25.94	16.72	0.27	0.85	1.86	0.97	1.94	3.20	0.74	1.16	0.79	0.91	0.37	0.21	0.96	0.63	1.06	0.80
35.92	MN	41.57	3.33	25.32	15.87	0.27	0.85	1.50	0.95	2.00	2.73	0.75	1.15	0.80	0.87	0.38	0.20	0.96	0.62	1.06	0.78
44.80	MN	51.85	3.23	24.30	14.26	0.26	0.74	1.47	0.87	1.66	2.46	0.66	1.02	0.68	0.73	0.40	0.20	0.88	0.58	0.95	0.70
56.26	MN	65.11	3.11	23.73	13.79	0.28	0.79	1.08	0.91	1.81	1.79	0.70	1.07	0.71	0.74	0.41	0.19	0.92	0.60	1.03	0.76
68.31	MN	79.05	3.03	23.65	12.87	0.26	0.71	0.98	0.85	1.58	1.74	0.62	1.02	0.67	0.71	0.39	0.19	0.88	0.59	0.98	0.70
80.22	MN	92.83	3.17	24.15	14.35	0.29	0.79	1.07	0.91	1.82	1.88	0.71	1.11	0.79	0.84	0.42	0.23	0.93	0.63	1.11	0.82
96.25	MN	111.38	2.91	23.48	13.84	0.26	0.76	1.06	0.88	1.82	1.78	0.68	1.10	0.75	0.78	0.50	0.21	0.87	0.59	1.05	0.75
14.89	HN	8.13	4.23	27.87	18.72	0.31	0.95	2.38	1.07	2.09	3.68	0.80	1.27	0.83	0.97	0.52	0.21	0.99	0.67	1.13	0.85
21.67	HN	11.83	3.84	25.57	18.2	0.31	0.91	2.33	1.02	2.03	3.71	0.77	1.17	0.79	0.96	0.45	0.20	0.99	0.65	1.07	0.84
41.01	HN	22.39	3.56	26.70	17.41	0.30	0.89	1.91	1.00	1.99	3.37	0.77	1.19	0.81	0.94	0.44	0.22	0.99	0.66	1.09	0.84
58.63	HN	32.01	3.11	26.29	15.51	0.27	0.85	1.39	0.94	1.88	2.41	0.74	1.18	0.78	0.87	0.54	0.23	0.93	0.63	1.08	0.79
74.96	HN	40.93	3.09	25.41	15.85	0.29	0.88	1.33	0.97	1.99	2.38	0.79	1.23	0.82	0.91	0.47	0.22	0.99	0.65	1.11	0.82
92.86	HN	50.70	2.92	23.68	13.23	0.27	0.75	0.95	0.87	1.67	1.62	0.65	1.04	0.71	0.74	0.48	0.23	0.89	0.59	1.03	0.74
110.81	HN	60.50	3.03	24.24	14.78	0.29	0.85	1.10	0.96	1.90	1.89	0.76	1.18	0.79	0.85	0.46	0.22	0.98	0.65	1.10	0.80
128.47	HN	70.14	3.02	23.91	14.27	0.29	0.81	1.02	0.94	1.81	1.79	0.74	1.14	0.77	0.81	0.47	0.21	0.97	0.64	1.08	0.78
143.54	HN	78.37	2.97	23.76	14.31	0.29	0.79	0.98	0.95	1.89	1.73	0.72	1.11	0.79	0.84	0.48	0.22	0.96	0.65	1.11	0.80
163.71	HN	89.38	3.26	24.93	16.16	0.31	0.91	1.11	1.05	2.15	2.00	0.83	1.33	0.87	0.95	0.49	0.24	1.05	0.71	1.25	0.91

Annex 2

Annex to chapter 3

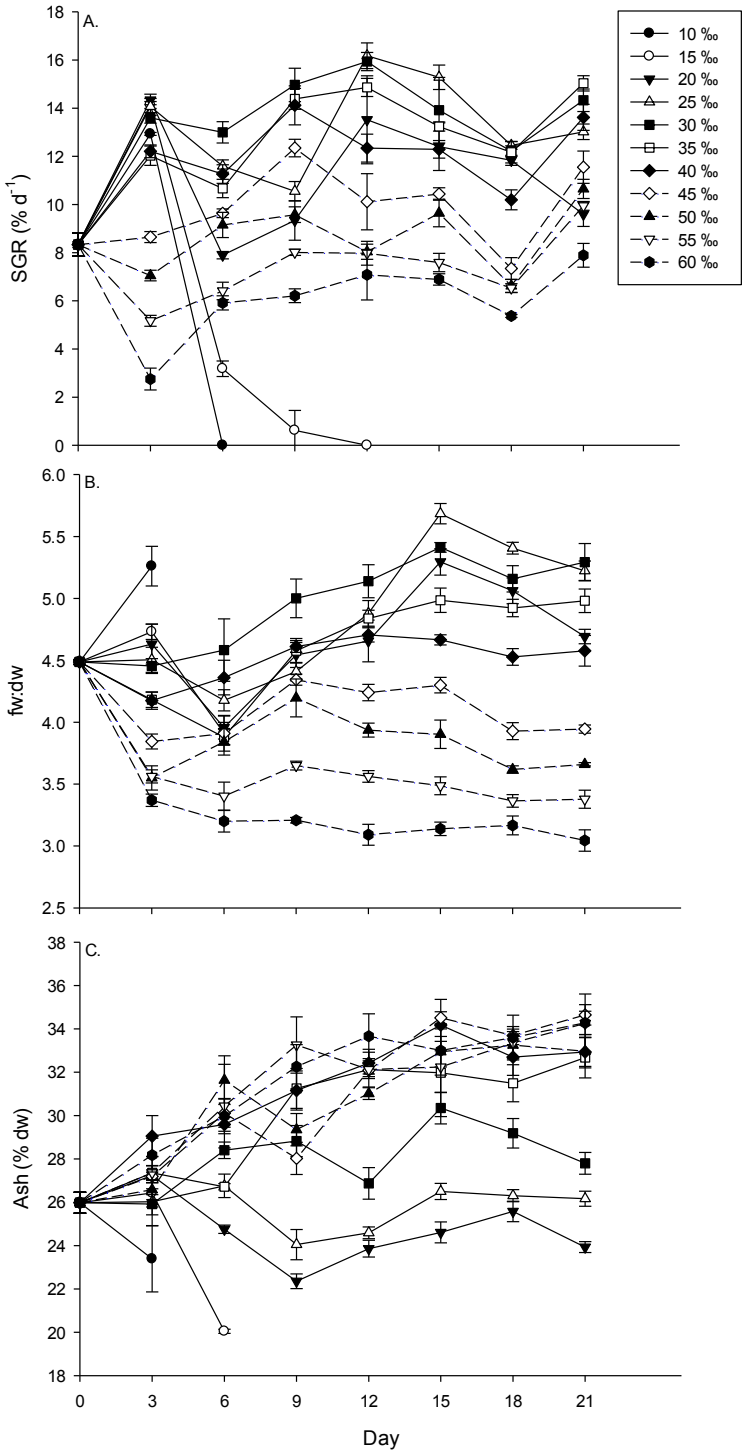

Figure S3.1. (A) The fresh weight specific growth rate (SGR), (B) fresh to dry weight ratio (fw:dw) and (C) ash content over time of *U. ohnoi* cultured at various salinities (mean ± SE, n = 4) for 21 days. Cultures were harvested and restocked back to initial density (4 g L^{-1} fw) every 3 days.

Annex 3

Annex to chapter 4

Table S4.1. Descriptive statistics for protein content data (% dw) overall and for each categorisation. Data shown for all and each method of protein determination.

Category	Method	n	Mean	Median	SD	Inter-quartile range	$5^{th}/95^{th}$ percentile range
Overall	All	2655	12.90	11.69	8.35	6.26 – 17.76	2.28 – 28.29
	Extraction	945	7.78	6.24	6.35	3.35 – 10.25	1.30 – 19.60
	N*6.25	1411	16.60	15.69	7.91	11.00 – 20.88	5.19 – 32.20
	TAA	299	11.60	11.04	6.93	6.35 – 14.73	2.98 – 26.76
Green	All	865	14.48	13.46	8.96	7.59 – 19.10	2.50 – 33.09
	Extraction	271	8.13	7.32	5.93	4.18 – 10.23	1.4 – 17.76
	N*6.25	477	18.28	17.31	8.76	13.31 – 22.63	4.13 – 36.30
	TAA	117	13.72	12.84	6.89	10.93 – 15.72	4.58 – 32.20
Brown	All	635	10.00	10.00	5.39	6.05 – 13.04	1.50 – 20.38
	Extraction	179	5.98	5.30	4.26	2.60 – 8.72	0.61 – 13.31
	N*6.25	359	12.45	12.13	4.29	9.20 – 14.99	5.60 – 21.56
	TAA	97	8.34	7.59	3.97	5.57 – 10.75	3.33 – 15.94
Red	All	1141	13.31	12.10	8.86	5.26 – 19.59	2.30 – 28.85
	Extraction	495	8.24	5.75	7.07	3.28 – 11.10	1.80 – 22.16
	N*6.25	561	17.92	18.13	7.84	12.80 – 22.69	5.00 – 32.25
	TAA	85	12.40	11.30	8.22	5.70 – 17.52	2.02 – 28.71
Temperate	All	1538	13.79	12.56	9.07	6.50 – 19.10	2.00 – 31.20
	Extraction	573	8.21	6.18	7.35	3.07 – 10.76	1.15 – 23.76
	N*6.25	869	17.64	16.64	7.98	12.00 – 21.75	6.36 – 33.75
	TAA	96	12.29	9.22	9.97	4.97 – 16.35	1.87 – 33.44
Tropical	All	968	11.65	10.93	7.09	6.01 – 15.54	2.74 – 24.38
	Extraction	310	7.11	6.33	4.34	3.70 – 9.86	1.74 – 15.70
	N*6.25	479	14.57	14.06	7.67	8.80 – 20.00	3.60 – 27.50
	TAA	179	11.69	12.00	4.88	7.56 – 14.65	3.39 – 20.24
Polar	All	149	11.77	12.21	6.85	6.15 – 17.19	2.40 – 24.06
	Extraction	62	7.21	7.00	4.33	3.80 – 9.40	1.90 – 13.31
	N*6.25	63	17.63	17.50	5.31	13.63 – 21.56	9.10 – 27.00
	TAA	24	8.14	6.87	3.58	5.63 – 10.03	4.05 – 12.87
Cultivated	All	667	15.19	14.20	8.82	9.00 – 19.44	2.96 – 34.96
	Extraction	237	9.26	8.81	6.25	5.75 – 11.59	2.32 – 18.20
	N*6.25	356	19.61	18.59	8.54	14.36 – 23.13	7.13 – 37.00
	TAA	74	12.92	12.87	3.84	11.70 – 15.51	4.82 – 18.20
Natural	All	1966	12.11	10.78	8.05	5.59 – 16.90	2.02 – 27.00
	Extraction	708	7.29	5.31	6.32	3.16 – 9.50	1.22 – 20.19
	N*6.25	1038	15.58	14.94	7.42	10.33 – 20.25	4.75 – 28.56
	TAA	220	11.22	9.41	7.68	5.98 – 14.08	2.42 – 29.08

Table S4.2. Mean N-protein factors for all species examined (raw data) and those included in the 5th/95th percentile range, which were used in the meta-analysis. Numbers in parenthesises indicate medians for N-protein factors.

	Raw mean N-protein factors	n	Mean N-protein factors (5th/95th percentiles)	n (5th/95th percentile)
Green	**4.65 (4.75)**	**114 (26 spp.)**	**4.49 (4.68)**	**110 (26 spp.)**
Temperate				
Culture				
Ulva lactuca	4.64	7	4.64	7
Wild				
*Capsosiphon fulvescens**	6.20	6	5.67	5
Codium fragile	3.10	1	3.10	1
Ulva capensis	5.29	1	5.29	1
Ulva lactuca	5.36	3	5.36	3
*Ulva prolifera**	7.17	2	6.72	1
Ulva rigida	4.68	2	4.68	2
Tropical				
Culture				
Chaetomorpha linum	3.27	1	3.27	1
Cladophora coelothrix	3.43	1	3.43	1
Derbesia tenuissima	4.80	1	4.80	1
Ulva clathrata	4.20	2	4.20	2
Ulva ohnoi	5.13	61	5.13	61
Wild				
Caulerpa fastigiata	3.83	2	3.83	2
Caulerpa lentillifera	4.44	2	4.44	2
*Caulerpa racemosa**	6.11	2	4.83	1
Caulerpa sertularioides	6.24	1	6.24	1
Chaetomorpha aerea	4.69	1	4.69	1
Cladophora vagabunda	3.60	1	3.60	1
Codium decorticatum	5.24	2	5.24	2
Codium hawaiiense	3.38	1	3.38	1
Codium reediae	3.09	1	3.09	1
Codium spongiosum	5.48	1	5.48	1
Codium taylorii	5.00	1	5.00	1
Rhizoclonium implexum	2.12	1	2.12	1
Ulva fasciata	5.26	5	5.26	5
*Ulva flexuosa**	5.75	2	4.02	1
Ulva lactuca	5.58	1	5.58	1
Ulva sp.	4.43	2	4.43	2
Brown	**5.08 (5.34)**	**100 (40 spp.)**	**4.56 (4.81)**	**92 (35 spp.)**
Polar				
Wild				
*Ascoseira mirabilis**	2.65	16	2.74	15
Desmarestia menziesii	3.34	6	3.34	6
Temperate				
Wild				
Ascophyllum nodosum	4.70	3	4.70	3
Colpomenia sinuosa	2.43	1	2.43	1
Dictyota dichotoma	3.27	1	3.27	1
Eisenia arborea	3.90	10	3.90	10
*Fucus spiralis***	9.49	1		
*Fucus vesiculosus***	7.41	1		
Himanthalia elongate	5.87	1	5.87	1

Hizikia fusiforme	5.68	1	5.68	1
Macrocystis integrifolia	5.73	4	5.73	4
Macrocystis pyrifera	4.06	3	4.06	3
*Nereocystis luetkeana**	5.53	11	4.95	9
Padina pavonica	6.56	1	6.56	1
Saccharina/Laminaria digitata	3.44	1	3.44	1
Saccharina/Laminaria japonica	5.66	1	5.66	1
Saccharina/Laminaria sp.	5.41	1	5.41	1
Saccharina/Laminaria latissima	3.88	1	3.88	1
Undaria pinnatifida	5.60	3	5.60	3
Tropical				
Wild				
Chnoospora minima	5.43	2	5.43	2
Cystoseira trinodis	5.28	1	5.28	1
Dictyota acutiloba	3.77	1	3.77	1
Dictyota menstrualis	3.86	2	3.86	2
Padina australis	5.88	1	5.88	1
Padina gymnospora	5.68	3	5.68	3
*Sargassum dentifebium***	10.28	1		
Sargassum echinocarpum	3.95	1	3.95	1
Sargassum filipendula	4.93	1	4.93	1
*Sargassum flavicans***	7.20	1		
Sargassum fluiians	6.22	1	6.22	1
Sargassum hemiphyllum	4.76	5	4.76	5
Sargassum henslowianum	5.71	2	5.71	2
Sargassum mangarevense	2.15	1	2.15	1
Sargassum mcclurei	4.45	1	4.45	1
Sargassum naozhouense	4.81	1	4.81	1
Sargassum patens	5.41	2	5.41	2
*Sargassum polycystum***	8.84	1		
Sargassum vulgare	5.75	3	5.75	3
Turbinaria ornate	2.31	1	2.31	1
Turbinaria ornate/ Sargassum mangarevense	1.83	1	1.83	1
Red	**5.23 (5.35)**	**75 (44 spp.)**	**5.10 (5.31)**	**58 (42 spp.)**
Temperate				
Wild				
Gracilaria chilensis	3.73	1	3.73	1
Gracilaria lemaneiformis	5.65	1	5.65	1
Gracilaria salicornia	5.56	1	5.56	1
*Gracilaria tikvahiae**	5.28	5	4.72	4
Osmundea pinnatifida	5.34	1	5.34	1
Porphyra sp.*	1.84	18	3.66	4
Porphyra purpurea	5.27	1	5.27	1
Porphyra umbilicalis	5.78	1	5.78	1
Porphyra yezoensis	5.41	1	5.41	1
Tropical				
Wild				
Acanthococcus spicifera	5.46	2	5.46	2
Aglaothamnion uruguayense	3.58	2	3.58	2
Ahnfeltiopsis concinna	3.16	1	3.16	1
Amansia muliipda	6.07	1	6.07	1
Asparagopsis taxiformis	5.36	2	5.36	2
Bryothamnion seaforthii	5.94	1	5.94	1
Bryothamnion triquetrum	5.93	1	5.93	1

Centroceras clavulatum	4.97	1	4.97	1
Corallina officinalis	6.22	1	6.22	1
Cryptonemia seminervis	3.75	1	3.75	1
Digenea simplex	6.10	1	6.10	1
Enantiocladia duperreyi	6.12	1	6.12	1
Eucheuma cottonii	3.38	1	3.38	1
Gelidiella acerosa	6.89	1	6.89	1
Gracilaria bailinae	5.99	1	5.99	1
Gracilaria domingensis	5.39	1	5.39	1
Gracilaria lemaneiformis	6.09	1	6.09	1
Gracilaria salicornia	4.53	1	4.53	1
Gracilariopsis tenuifrons	5.14	1	5.14	1
Hypnea charoides	5.50	1	5.50	1
Hypnea japonica	5.69	1	5.69	1
Hypnea musciformis	4.52	2	4.52	2
*Hypnea pannosa***	9.03	1		
Hypnea valentiae	6.38	1	6.38	1
*Jania crassa***	8.22	1		
Kappaphycus alvarezii	4.25	1	4.25	1
Laurencia flagellifera	5.11	1	5.11	1
Laurencia majuscule	5.05	1	5.05	1
Laurencia obtuse	6.29	1	6.29	1
Ochtodes secundiramea	3.67	1	3.67	1
Plocamium brasiliense	3.92	2	3.92	2
Porphyra acanthophora	3.75	2	3.75	2
Porphyra crispate	4.76	1	4.76	1
Pterocladiella capillacea	3.64	3	3.64	3
Solieria flliformis	6.19	1	6.19	1
Spyridia hypnoides	5.20	1	5.20	1
Vidalia obiusiloba	6.08	1	6.08	1
Grand Total	**5.04 (5.22)**	**289 (110 spp.)**	**4.76 (4.97)**	**260 (103 spp.)**

*Species whose means were adjusted after removing data outside of $5^{th}/95^{th}$ percentile range

**Species which were completely removed after removing data outside of $5^{th}/95^{th}$ percentile range

Table S4.3. Correlations between N content and N-protein factor for all seaweeds overall and all possible sub-groups. R2 values, direction and p-value given. Bold text indicates a significant correlation ($p < 0.05$). Sub-groups not shown had no data available.

	n	R^2	Direction	p-value
Overall	260	0.015	-	0.056
Green	110	0.027	-	0.086
Brown[a]	**92**	**0.081**	**-**	**0.006**
Red[b]	58	0.043	-	0.118
Temperate	**74**	**0.062**	**+**	**0.031**
Tropical	**165**	**0.114**	**-**	**< 0.001**
Polar[c]	21	0.032	+	0.435
Natural	187	0.003	-	0.442
Cultivated[d]	**73**	**0.297**	**-**	**< 0.001**
Green*Temperate	20	0.067	+	0.286
Green*Tropical	**90**	**0.190**	**-**	**< 0.001**
Green*Natural	37	0.010	+	0.557
Green*Cultivated[d]	**73**	**0.297**	**-**	**< 0.001**
Brown*Temperate[e]	39	0.014	+	0.464
Brown*Tropical[f]	32	0.023	-	0.428
Brown*Polar[c]	21	0.032	+	0.435
Brown*Natural[a]	**92**	**0.081**	**-**	**0.006**
Red*Temperate[g]	15	0.219	+	0.079
Red*Tropical[h]	**43**	**0.178**	**-**	**0.005**
Red*Natural[b]	58	0.043	-	0.118
Temperate*Natural	**67**	**0.081**	**+**	**0.0.011**
Temperate*Cultivated[i]	7	0.024	-	0.742
Tropical*Natural	**99**	**0.081**	**-**	**0.005**
Tropical*Cultivated[j]	**66**	**0.416**	**-**	**< 0.001**
Polar*Natural[c]	21	0.032	+	0.435
Green*Temperate*Natural	13	0.113	+	0.286
Green*Tropical*Natural	24	0.086	-	0.164
Brown*Temperate*Natural[e]	39	0.014	-	0.464
Brown*Tropical*Natural[f]	32	0.023	-	0.428
Brown*Polar*Natural[c]	21	0.032	+	0.435
Red*Temperate*Natural[g]	15	0.219	+	0.079
Red*Tropical*Natural[h]	**43**	**0.178**	**-**	**0.005**
Green*Temperate*Cultivated[i]	7	0.024	-	0.742
Green*Tropical*Cultivated[j]	**66**	**0.416**	**-**	**< 0.001**
Angell et al. (2014) – *U.ohnoi*	**60**	**0.309**	**-**	**< 0.001**

Note: Correlation combinations with common superscripts are from the same data.

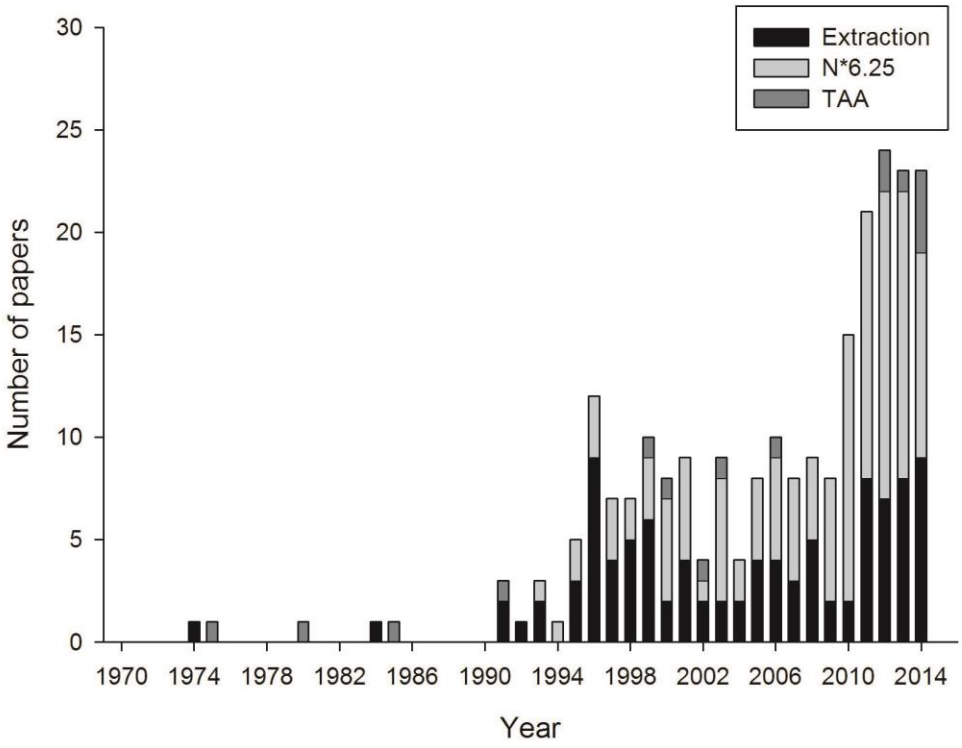

Figure S4.1. Number of papers identified in this chapter per year of publication as the sum of the three main determination methods – Extraction, N×6.25 and total amino acids (TAA).

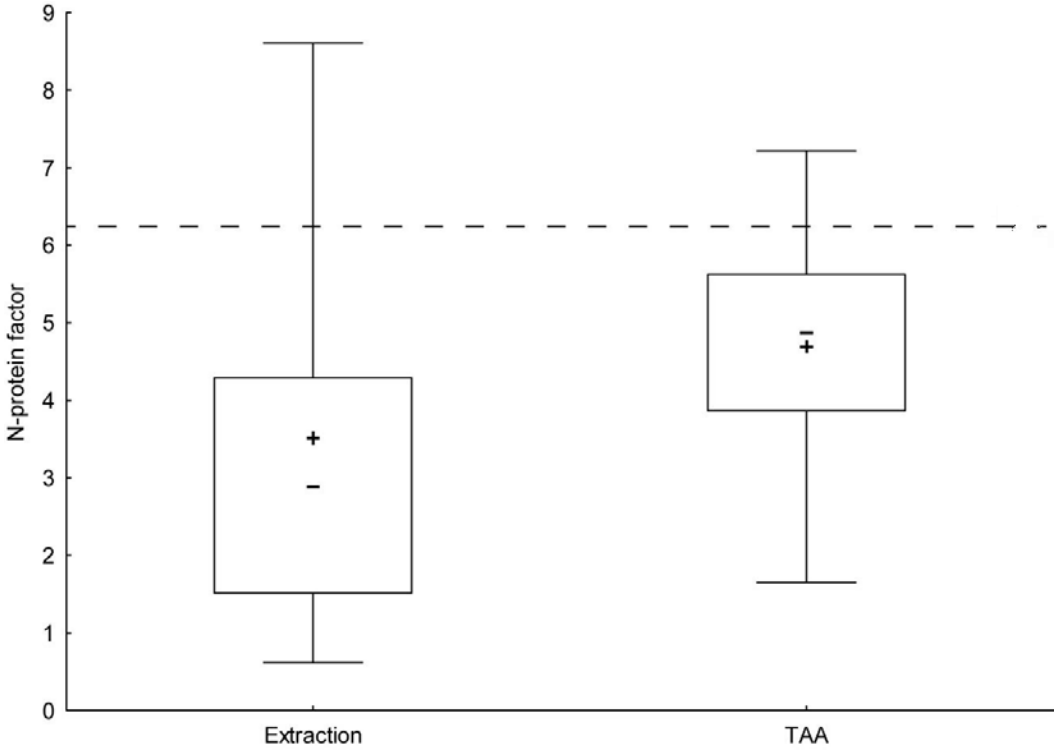

Figure S4.2. Nitrogen-to-protein conversion factors calculated for extraction and total amino acid (TAA) determination methods. Dashes represent medians, crosses represent means, boxes represent 25th percentiles and whiskers represent 5th/95th percentiles. The horizontal dashed line represents a ratio of 6.25.

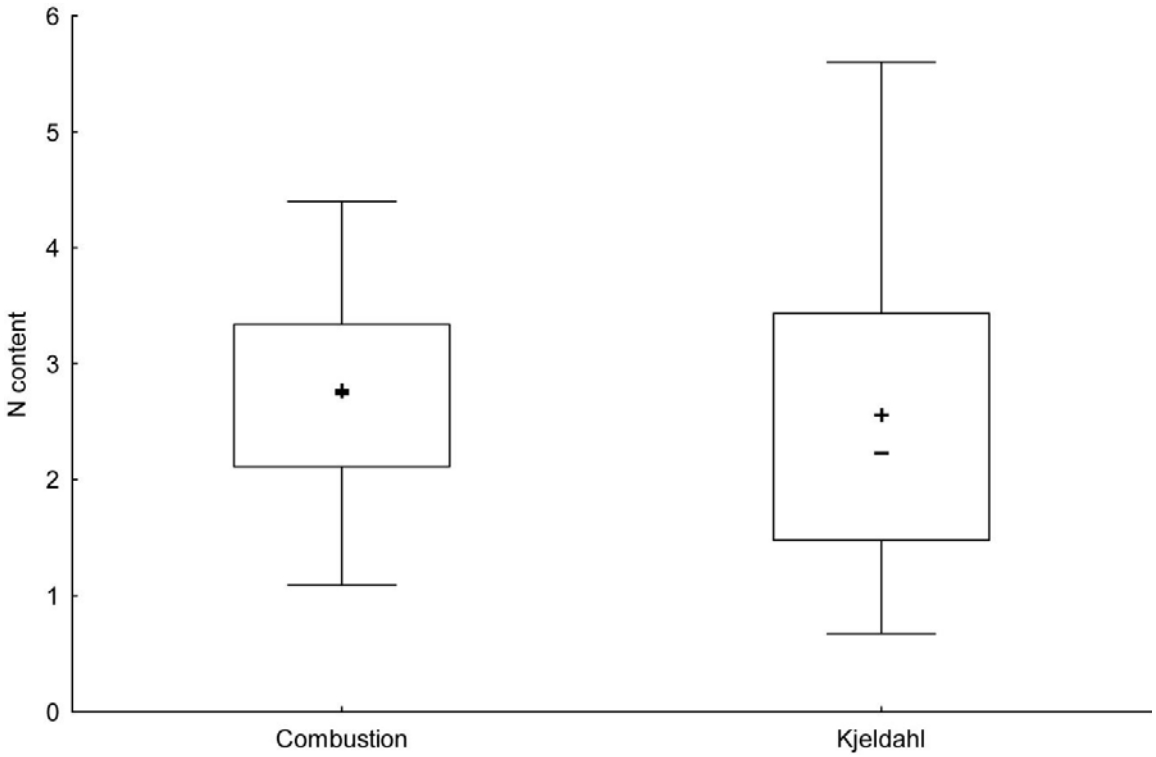

Figure S4.3. Quantitative tissue nitrogen measurements (% dw) of papers examined in Chapter 4 analysed using the two main methods; analysis by combustion and by variants of the Kjeldahl method. Dashes represent medians, crosses represent means, boxes represent 25th percentiles and whiskers represent 5th/95th percentiles.

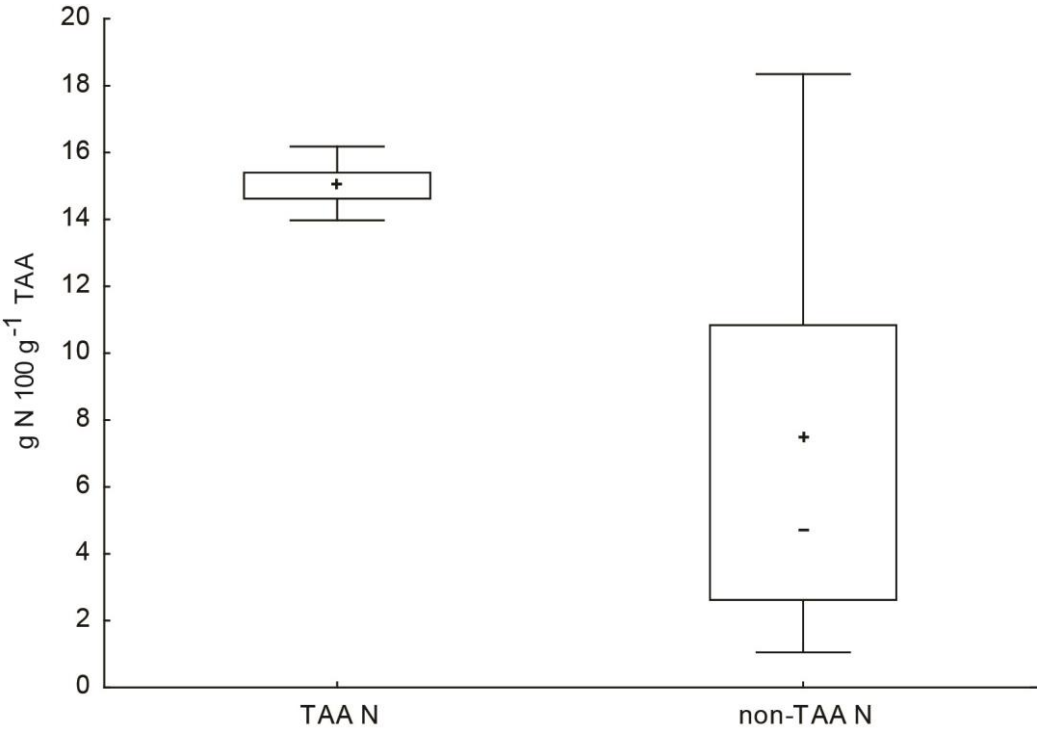

Figure S4.4. Concentration of nitrogen in the total amino acid (TAA) and non-TAA fractions of seaweeds analysed for N-protein factors in Chapter 4. Dashes represent medians, crosses represent means, boxes represent 25th percentiles and whiskers represent 5th/95th percentiles.

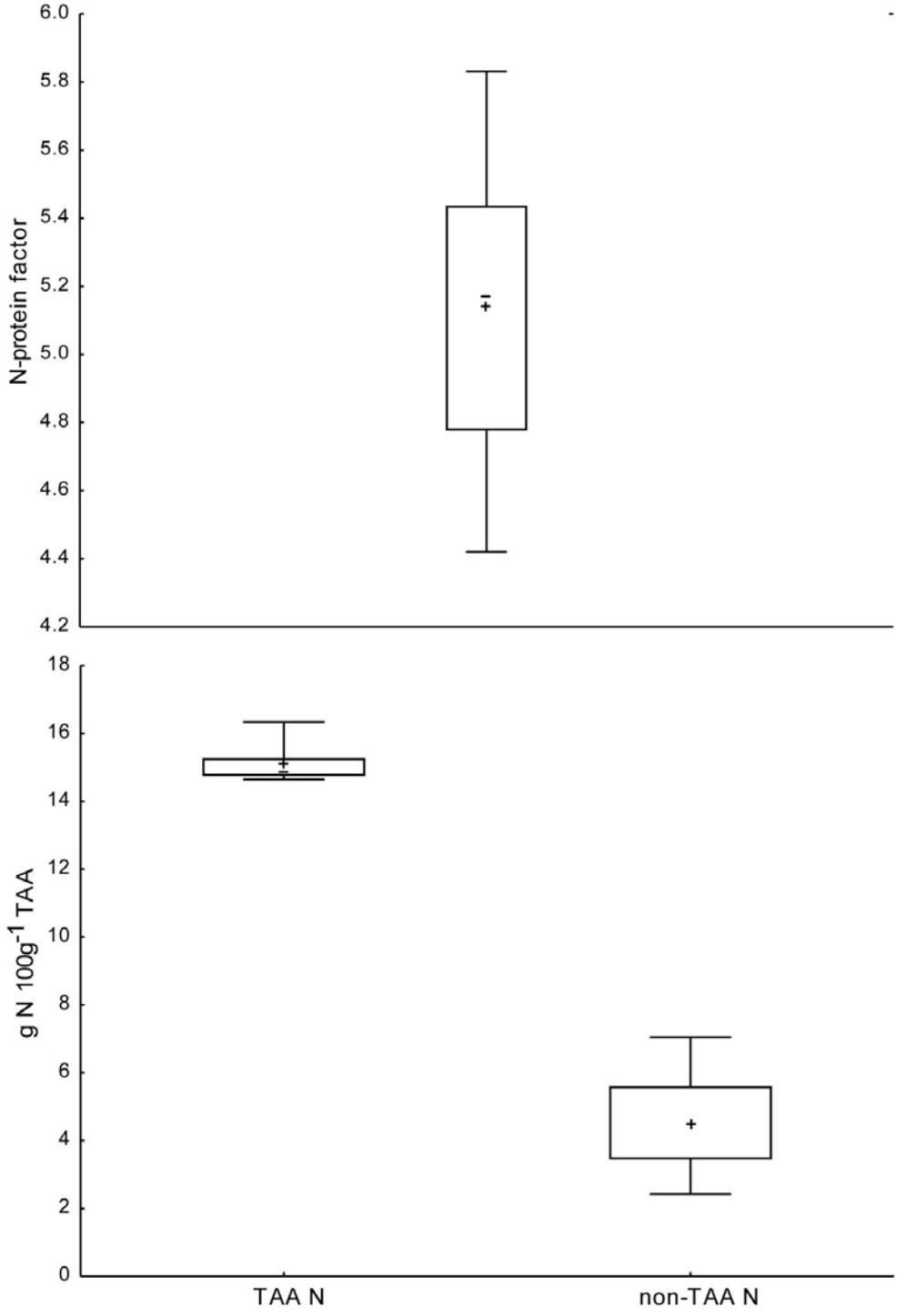

Figure S4.5. The within-species variation in (A) nitrogen-to-protein conversion factors and (B) the concentration of nitrogen in the total amino acid (TAA) and non-TAA fractions of the green seaweed *Ulva ohnoi*. All data is from Angell et al (2014). Dashes represent medians, crosses represent means, boxes represent 25th percentiles and whiskers represent 5th/95th percentiles.

Supplementary information S4.1. Additional articles included in meta-analysis that were not retrieved by search string as well as the list of all articles examined in qualitative and quantitative meta-analysis.

Additional articles included in the meta-analysis that were not retrieved by search string (n = 17)

Dawes et al. 1974; Rosell and Srivastava 1985; Kopczak et al. 1991; Hurtadoponce 1995; Navarro-Angulo and Robledo 1999; Ramos et al. 2000; Lourenço et al. 2002; Orduna-Rojas et al. 2002; Zubia et al. 2003; Boulus et al. 2007; Chakraborty and Santra 2008; Angell et al. 2012; Pereira et al. 2012a; Westermeier et al. 2012; Cazon et al. 2014; Liu et al. 2014; Neveux et al. 2015.

Articles included in qualitative meta-analysis only (n = 31)

Kopczak et al. 1991; Mcglathery 1992; Vergara and Niell 1993, 1995; Hernandez 1996; Kennish 1997; Rico and Fernandez 1997; Sturm and Horn 1998; Jayasankar and Kulandaivelu 1999; Mercado et al. 1999; Costanzo et al. 2000; Bischof et al. 2006; Gordillo et al. 2006; Dworjanyn et al. 2007; Margret et al. 2008; Martins et al. 2008; Zou and Gao 2009; Martins et al. 2011; Ramlov et al. 2011; Moreda-Pineiro et al. 2012; Romaris-Hortas et al. 2012; Yildiz et al. 2012; Ben Chekroun et al. 2013; Garcia-Sartal et al. 2013; Gouveia et al. 2013; Kim et al. 2013; Ribeiro et al. 2013; Kumari et al. 2014; Misurcova et al. 2014; Yildiz et al. 2014; Zou and Gao 2014.

Articles included in quantitative and qualitative meta-analysis (n = 205)

Dawes et al. 1974; Horn and Neighbors 1984; Rosell and Srivastava 1985; Buchsbaum et al. 1991; Duffy and Hay 1991; Neighbors and Horn 1991; Mercer et al. 1993; Mouradigivernaud et al. 1993; Lightfoot and Raghavan 1994; Mai et al. 1994; Figueroa et al. 1995; Gomez and Westermeier 1995; Hurtadoponce 1995; Vergara et al. 1995; Bolser and Hay 1996; Castro-Gonzalez et al. 1996; Cronin and Hay 1996c, a, b; Kaehler and Kennish 1996; McGlathery et al. 1996; Rico and Fernandez 1996; Shpigel et al. 1996; Smit et al. 1996; Westermeier and Gomez 1996; Chan et al. 1997; Davies et al. 1997; Renaud et al. 1997; Robledo and Pelegrin 1997; Wahbeh 1997; Foster and Hodgson 1998; Gojon-Baez et al. 1998; Gomez et al. 1998; Gomez and Wiencke 1998; Rijstenbil et al. 1998; Ventura and Castanon 1998; Andria et al. 1999; Bautista-Teruel and Millamena 1999; Galland-Irmouli et al. 1999; Gunnarsson et al. 1999; McGlathery and Pedersen 1999; Naldi and Wheeler 1999; Navarro-Angulo and Robledo 1999; Shpigel et al. 1999; Cruz-Rivera and Hay 2000; Norziah and Ching 2000; Ramos et al.

2000; Rosen et al. 2000; Vadas et al. 2000; Wong and Cheung 2000; Bautista-Teruel et al. 2001; Boarder and Shpigel 2001; Cruz-Rivera and Hay 2001; Gordillo et al. 2001; Granado and Caballero 2001; Liu and Dong 2001; Ruperez and Saura-Calixto 2001; Wong and Cheung 2001a; Wong and Cheung 2001b; Goni et al. 2002; Lourenço et al. 2002; Martinez and Rico 2002; Orduna-Rojas et al. 2002; Dere et al. 2003; Marrion et al. 2003; McDermid and Stuercke 2003; Neori et al. 2003; Reyes and Fermin 2003; Risso et al. 2003; Sanchez-Machado et al. 2003; Schuenhoff et al. 2003; Zubia et al. 2003; Barile et al. 2004; Hemmi and Jormalainen 2004; Rodde et al. 2004; Sanchez-Machado et al. 2004; Aguilera-Morales et al. 2005; Barbarino and Lourenco 2005; Fayaz et al. 2005; Israel et al. 2005; Mamelona and Pelletier 2005; Marrion et al. 2005; Peters et al. 2005; Viera et al. 2005; Freile-Pelegrin and Robledo 2006; Jacquin et al. 2006; Lartigue and Sherman 2006; Marinho-Soriano et al. 2006; Ortiz et al. 2006; Renaud and Luong-Van 2006; Valente et al. 2006; Boulus et al. 2007; Cook and Kelly 2007; Dawczynski et al. 2007; Hong et al. 2007; Mallo et al. 2007; Mamatha et al. 2007; Marsham et al. 2007; McDermid et al. 2007; Wang et al. 2007; Chakraborty and Santra 2008; Cho et al. 2008; Hwang et al. 2008; Msuya and Neori 2008; Nelson et al. 2008; Polat and Ozogul 2008; Yu and Yang 2008; Banerjee et al. 2009; Cruz-Suarez et al. 2009; de Oliveira et al. 2009; Hernandez-Carmona et al. 2009; Hernandez et al. 2009; Matanjun et al. 2009; Ortiz et al. 2009; Cho et al. 2010; Cirik et al. 2010; Cofrades et al. 2010; Cruz-Suarez et al. 2010; Denis et al. 2010; Gomez-Ordonez et al. 2010; Gressler et al. 2010; Ktita et al. 2010; Kumar et al. 2010; Misurcova et al. 2010; Msuya and Neori 2010; Schaal et al. 2010; Smith et al. 2010; Taboada et al. 2010; Zhang et al. 2010; Akkoz et al. 2011; Asino et al. 2011; Badrinathan et al. 2011; Chen 2011; Diniz et al. 2011; Duarte et al. 2011; Frikha et al. 2011; Gressler et al. 2011; Murakami et al. 2011; Nguyen et al. 2011; Patarra et al. 2011; Pato et al. 2011; Pena-Rodriguez et al. 2011; Perez-Estrada et al. 2011; Senthil et al. 2011; Viera et al. 2011; Xu et al. 2011; Yaich et al. 2011; Yildiz et al. 2011; Al-Harthi and El-Deek 2012; Ambreen et al. 2012; Angell et al. 2012; Bilbao et al. 2012; Cho and Kim 2012; Eddy et al. 2012; El Din and El-Sherif 2012; Hofmann et al. 2012; Kandasamy et al. 2012; Madden et al. 2012; Nielsen et al. 2012; Pereira et al. 2012a; Pereira et al. 2012b; Pise et al. 2012; Prado et al. 2012; Rohani-Ghadikolaei et al. 2012; Suarez-Alvarez et al. 2012; Sun et al. 2012; Tabarsa et al. 2012a; Tabarsa et al. 2012b; Westermeier et al. 2012; Borell et al. 2013; Francavilla et al. 2013; Jard et al. 2013; Khairy and El-Shafay 2013; Marinho et al. 2013; Mulvaney et al. 2013; Munier et al. 2013; Peng et al. 2013;

Polat and Ozogul 2013; Shuuluka et al. 2013; Stadtlander et al. 2013; Taboada et al. 2013; Teichberg et al. 2013; Trigui et al. 2013; Uslu et al. 2013; Wassef et al. 2013; Yu et al. 2013; Angell et al. 2014; Astorga-Espana and Mansilla 2014; Baghel et al. 2014; Baumgartner et al. 2014; Blanco-Pascual et al. 2014; Cazon et al. 2014; Cian et al. 2014; Duarte et al. 2014; Hafezieh et al. 2014; Johnson et al. 2014; Liu et al. 2014; Manns et al. 2014; O'Mahoney et al. 2014; Orozco et al. 2014; Paiva et al. 2014; Turan and Tekogul 2014; You et al. 2014; Yu et al. 2014; Neveux et al. 2015.

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Annex 4

Annex to chapter 5

Table S5.1. Basic statistics of essential amino acid data after outlier removal using the outlier-labelling rule (k = 2.4). These data were used in the calculation of the means of species (Table S5.2).

Amino acid	Units	Non-outlier data							
		n (measurements)	Species	Mean	Median	Min.	Max.	Lower quartile	Upper quartile
Arg	% DW	247	113	0.74	0.68	0.02	2.99	0.41	0.94
	% TAA	247	113	5.98	5.81	1.19	11.63	4.98	6.88
His	% DW	250	114	0.25	0.24	0.00	1.02	0.12	0.31
	% TAA	250	114	2.08	1.93	0.00	4.85	1.56	2.34
Ile	% DW	258	117	0.57	0.53	0.01	2.04	0.34	0.70
	% TAA	258	117	4.64	4.57	2.00	7.81	4.08	5.16
Leu	% DW	254	116	0.96	0.92	0.03	3.60	0.59	1.11
	% TAA	254	116	7.82	7.78	3.92	11.47	7.23	8.56
Lys	% DW	260	119	0.70	0.65	0.02	2.60	0.40	0.87
	% TAA	260	119	5.82	5.63	1.90	10.34	5.01	6.56
Met	% DW	244	111	0.22	0.20	< 0.01	0.80	0.12	0.27
	% TAA	244	111	1.89	1.97	0.16	4.34	1.35	2.27
Phe	% DW	251	115	0.64	0.64	0.02	2.15	0.33	0.80
	% TAA	251	115	5.21	5.33	2.13	8.00	4.63	5.75
Thr	% DW	255	118	0.63	0.61	0.02	2.11	0.40	0.75
	% TAA	255	118	5.13	5.14	2.58	7.63	4.76	5.65
Trp	% DW	55	48	0.10	0.10	0.00	0.27	0.03	0.16
	% TAA	55	48	1.06	0.94	0.00	2.95	0.60	1.49
Val	% DW	257	117	0.73	0.70	0.02	2.50	0.41	0.91
	% TAA	257	117	5.92	5.98	3.13	8.48	5.54	6.52
Cumulative	%DW	212	93						
	% TAA	212	93						

Table S5.2. Basic statistics of quantitative and qualitative essential amino acid data for the means of each species. The means, medians and total ranges for the overall data are presented in table 1 of the main article and these statistics for EAA, methionine and lysine are presented as boxplots in figures 5.1 and 5.2. For qualitative data (% TAA), EAA, methionine and lysine are also graphed based on taxonomic groups in figure 5.1.

	Units	n (species)	Mean	Median	Min.	Max.	Lower quartile	Upper quartile	Std. Dev.
Overall									
Arg	% DW	113	0.73	0.60	0.02	2.99	0.40	0.89	0.52
	% TAA	113	5.87	5.72	1.19	10.89	4.89	6.56	1.61
His	% DW	113	0.24	0.21	0.00	1.02	0.11	0.34	0.17
	% TAA	113	2.09	1.98	0.00	4.68	1.42	2.61	0.94
Ile	% DW	117	0.54	0.46	0.01	1.78	0.31	0.68	0.33
	% TAA	117	4.52	4.55	2.00	6.30	4.01	5.05	0.80
Leu	% DW	115	0.92	0.82	0.03	3.18	0.52	1.14	0.55
	% TAA	115	7.83	7.89	3.92	11.47	7.08	8.60	1.34
Lys	% DW	119	0.69	0.60	0.02	2.12	0.33	0.98	0.44
	% TAA	119	5.88	5.72	1.90	10.34	4.79	7.07	1.64
Met	% DW	110	0.20	0.17	< 0.00	0.69	0.10	0.27	0.14
	% TAA	110	1.84	1.86	0.43	4.19	1.23	2.28	0.85
Phe	% DW	114	0.61	0.56	0.02	1.83	0.32	0.79	0.37
	% TAA	114	5.26	5.27	2.13	8.00	4.69	5.86	1.02
Thr	% DW	117	0.61	0.59	0.02	2.11	0.36	0.77	0.39
	% TAA	117	5.15	5.31	2.58	7.63	4.61	5.76	0.95
Trp	% DW	47	0.10	0.09	0.00	0.27	0.03	0.16	0.08
	% TAA	47	1.08	0.95	0.00	2.95	0.60	1.54	0.69
Val	% DW	116	0.68	0.61	0.02	2.45	0.36	0.88	0.45
	% TAA	116	5.75	5.92	3.23	8.48	5.07	6.31	0.91
EAA	% DW	93	5.49	4.93	0.15	16.35	3.43	6.81	3.08
	% TAA	93	45.65	46.20	34.61	51.59	44.04	47.72	3.25
Green									
Arg	% DW	27	0.77	0.75	0.14	1.85	0.42	1.03	0.43
	% TAA	27	5.48	5.39	3.34	8.38	4.71	6.24	1.22
His	% DW	27	0.27	0.28	0.00	0.71	0.16	0.37	0.15
	% TAA	27	2.03	1.83	0.00	4.68	1.50	2.38	0.92
Ile	% DW	28	0.60	0.54	0.12	1.78	0.41	0.73	0.35
	% TAA	28	4.34	4.38	3.59	5.23	4.05	4.71	0.48
Leu	% DW	26	1.16	0.96	0.20	3.18	0.79	1.45	0.64
	% TAA	26	8.04	8.14	5.12	11.00	7.25	8.80	1.28
Lys	% DW	27	0.77	0.68	0.14	2.12	0.54	1.02	0.43
	% TAA	27	5.77	5.58	3.39	9.10	4.67	6.84	1.50
Met	% DW	24	0.22	0.21	0.02	0.66	0.09	0.28	0.15
	% TAA	24	1.68	1.62	0.67	3.77	1.04	2.11	0.76
Phe	% DW	27	0.75	0.66	0.13	1.83	0.48	0.94	0.41
	% TAA	27	5.46	5.41	4.15	8.00	4.83	5.89	0.86
Thr	% DW	28	0.68	0.62	0.11	1.82	0.44	0.80	0.39
	% TAA	28	4.97	5.10	3.01	6.75	4.55	5.57	0.89
Trp	% DW	11	0.08	0.06	0.00	0.19	0.04	0.16	0.07
	% TAA	11	0.81	0.60	0.00	2.40	0.46	1.13	0.72
Val	% DW	27	0.86	0.77	0.18	2.34	0.56	1.03	0.48

	% TAA	27	6.21	6.14	4.70	7.14	6.00	6.54	0.53
EAA	% DW	22	6.71	5.94	3.35	16.35	4.89	7.81	3.04
	% TAA	22	45.13	45.56	37.08	50.30	44.04	47.02	3.04
Brown									
Arg	% DW	39	0.43	0.43	0.02	1.14	0.24	0.59	0.25
	% TAA	39	5.41	5.32	3.17	10.89	4.60	5.88	1.43
His	% DW	37	0.18	0.17	0.01	0.59	0.08	0.26	0.12
	% TAA	37	2.23	2.13	0.34	4.24	1.70	2.89	0.99
Ile	% DW	39	0.34	0.31	0.01	0.93	0.19	0.45	0.20
	% TAA	39	4.37	4.40	2.00	6.30	3.84	4.93	0.88
Leu	% DW	40	0.63	0.55	0.03	1.50	0.38	0.89	0.35
	% TAA	40	7.94	8.05	3.92	10.25	7.44	8.75	1.38
Lys	% DW	40	0.42	0.36	0.02	1.22	0.21	0.57	0.28
	% TAA	40	5.24	5.44	1.90	8.66	4.54	6.15	1.47
Met	% DW	36	0.16	0.15	< 0.00	0.62	0.09	0.21	0.12
	% TAA	36	2.00	1.96	0.78	4.19	1.51	2.49	0.69
Phe	% DW	37	0.41	0.42	0.02	0.95	0.24	0.55	0.23
	% TAA	37	5.10	5.26	2.49	6.47	4.62	5.70	0.85
Thr	% DW	38	0.40	0.40	0.02	1.05	0.19	0.54	0.23
	% TAA	38	4.91	5.06	2.70	7.15	4.30	5.52	0.97
Trp	% DW	9	0.05	0.03	0.00	0.10	0.00	0.10	0.05
	% TAA	9	0.84	0.81	0.00	1.58	0.47	1.39	0.62
Val	% DW	39	0.45	0.38	0.02	1.08	0.27	0.60	0.25
	% TAA	39	5.74	5.79	4.08	8.48	4.94	6.27	0.96
EAA	% DW	32	3.51	3.78	0.15	6.26	2.25	4.65	1.62
	% TAA	32	44.24	44.69	34.61	51.59	42.88	46.32	3.69
Red									
Arg	% DW	47	0.95	0.83	0.09	2.99	0.54	1.27	0.61
	% TAA	47	6.47	6.19	1.19	10.67	5.44	7.56	1.78
His	% DW	49	0.27	0.20	0.03	1.02	0.12	0.41	0.20
	% TAA	49	2.03	1.77	0.75	4.39	1.35	2.57	0.92
Ile	% DW	50	0.66	0.66	0.08	1.44	0.40	0.85	0.34
	% TAA	50	4.75	4.75	2.71	6.18	4.23	5.52	0.84
Leu	% DW	49	1.02	0.87	0.15	2.80	0.70	1.33	0.53
	% TAA	49	7.64	7.66	5.08	11.47	6.57	8.23	1.35
Lys	% DW	52	0.85	0.78	0.08	2.05	0.51	1.18	0.46
	% TAA	52	6.43	6.52	2.52	10.34	5.45	7.62	1.67
Met	% DW	50	0.21	0.18	0.03	0.69	0.10	0.30	0.14
	% TAA	50	1.80	1.68	0.43	4.18	1.15	2.28	0.98
Phe	% DW	50	0.69	0.65	0.13	1.68	0.46	0.91	0.38
	% TAA	50	5.28	5.27	2.13	7.98	4.61	6.15	1.20
Thr	% DW	51	0.74	0.65	0.11	2.11	0.47	0.94	0.42
	% TAA	51	5.43	5.58	2.58	7.63	5.04	5.84	0.90
Trp	% DW	27	0.12	0.12	0.01	0.27	0.04	0.20	0.08
	% TAA	27	1.28	1.16	0.15	2.95	0.77	1.76	0.67
Val	% DW	50	0.76	0.68	0.07	2.45	0.45	1.00	0.48
	% TAA	50	5.52	5.59	3.23	7.65	4.93	6.18	0.96
EAA	% DW	39	6.43	5.67	1.13	15.84	4.27	8.12	3.26

% TAA	39	47.10	47.33	41.32	50.83	46.19	48.85	2.32

Table S5.3. Amino acid requirements of major domesticated mono-gastric livestock (% diet) used to determine the limiting amino acid (Figure 5.3) and the rate of limitation (amino acid scores – Figure 5.4) for all seaweeds for each livestock.

	Chicken[a]		Swine[b]		Fish[c]	
Amino acid (% diet)	0 to 3 weeks	6 to 8 weeks	5 to 7 kg	100 to 135 kg	Atlantic Salmon (*Salmo salar*)	Tilapia (*Oreochromis* spp.)
Arginine	1.25	1.0	0.68	0.28	1.8	1.2
Glycine + serine[e]	1.25	0.97	NR[f]	NR	NR	NR
Histidine	0.35	0.27	0.52	0.21	0.8	1.0
Isoleucine	0.80	0.62	0.77	0.33	1.1	1.0
Leucine	1.20	0.93	1.50	0.62	1.5	1.9
Lysine	1.10	0.85	1.50	0.61	2.4	1.6
Methionine	0.50	0.32	0.43	0.18	0.7	0.7
Methionine + cystine	0.90	0.60	0.82	0.36	1.1	1.0
Phenylalanine	0.72	0.56	0.88	0.37	0.9	1.1
Phenylalanine + tyrosine	1.34	1.04	1.38	0.58	1.8	1.6
Proline[e]	0.60	0.46	NR	NR	NR	NR
Threonine	0.80	0.68	0.88	0.40	1.1	1.1
Tryptophan	0.20	0.16	0.25	0.11	0.3	0.3
Valine	0.90	0.70	0.95	0.41	1.2	1.5

[a]NRC (1994)
[b]NRC (2012)
[c]NRC (2011)
[d]NRC (2006)
[e]Non-essential amino acids which cannot be synthesised at rates required for maximum growth.
[f]NR, not required

Table S5.4. The highest amino acid scores and respective limiting amino acid (in parentheses) for seaweeds examined in Chapter 5.

Amino acid score (the limiting AA in seaweed: AA requirement)	Chicken		Swine		Fish	
	0 - 3 weeks	6 - 8 weeks	5 - 7 kg	100 - 130 kg	Salmon	Tilapia
Highest ranked for all animals						
Pyropia yezoensis	1.10 (Trp)	1.38 (Trp)	0.88 (Trp)	2.00 (Trp)	0.64 (His)	0.51 (His)
Solieria flliformis	0.96 (Met)	1.39 (Lys)	0.79 (Lys)	1.93 (Lys)	0.49 (Lys)	0.57 (His)
Capsosiphon fulvescens	0.94 (Trp)	1.17 (Trp)	0.75 (Trp)	1.70 (Trp)	0.62 (Trp)	0.62 (Trp)
Derbesia tenuissima	0.92 (Met)	1.26 (Arg)	0.90 (His)	2.24 (His)	0.59 (His)	0.47 (His)
Porphyra umbilicalis	0.90 (Met)	1.41 (Met)	1.05 (Met)	2.50 (Met)	0.64 (Met)	0.57 (His)
Additional animal-specific seaweeds						
Chicken (0 – 3 weeks)						
Chaetomorpha aerea	0.81 (His)	1.04 (His)	0.54 (His)	1.34 (His)	0.35 (His)	0.28 (His)
Chicken (6 - 8 weeks)						
Chaetomorpha aerea	0.81 (His)	1.04 (His)	0.54 (His)	1.34 (His)	0.35 (His)	0.28 (His)
Ulva prolifera	0.79 (Trp)	0.99 (Trp)	0.63 (Trp)	1.44 (Trp)	0.43 (Lys)	0.39 (His)
Ulva clathrata	0.71 (Arg)	0.89 (Arg)	0.63 (His)	1.56 (His)	0.41 (His)	0.33 (His)
Macrocystis integrifolia	0.65 (Arg)	0.83 (Val)	0.61 (Val)	1.42 (Val)	0.45 (Lys)	0.39 (Val)
Sargassum fluiians	0.65 (Arg)	0.81 (Arg)	0.53 (Met)	1.30 (Lys)	0.33 (Lys)	0.29 (His)
Swine (100 - 130 kg)						
Ulva clathrata	0.71 (Arg)	0.89 (Arg)	0.63 (His)	1.56 (His)	0.41 (His)	0.33 (His)
Codium taylorii	0.42 (Arg)	0.52 (Arg)	0.63 (Met)	1.52 (Met)	0.29 (Arg)	0.37 (His)
Nereocystis luetkeana	0.51 (Arg)	0.64 (Arg)	0.65 (His)	1.44 (His)	0.35 (Arg)	0.34 (His)
Ulva prolifera	0.79 (Trp)	0.99 (Trp)	0.63 (Trp)	1.44 (Trp)	0.43 (Lys)	0.39 (His)
Macrocystis integrifolia	0.65 (Arg)	0.83 (Val)	0.61 (Val)	1.42 (Val)	0.45 (Lys)	0.39 (Val)
Porphyra columbina	0.78 (Trp)	0.97 (Trp)	0.60 (His)	1.41 (Trp)	0.39 (His)	0.31 (His)
Chaetomorpha aerea	0.81 (His)	1.04 (His)	0.54 (His)	1.34 (His)	0.35 (His)	0.28 (His)
Plocamium brasiliense	0.48 (Met)	0.74 (Met)	0.55 (Met)	1.32 (Met)	0.34 (Met)	0.34 (Met)
Sargassum fluiians	0.65 (Arg)	0.81 (Arg)	0.53 (Met)	1.30 (Lys)	0.33 (Lys)	0.29 (His)
Caulerpa fastigiata	0.44 (Met)	0.69 (Met)	0.51 (Met)	1.22 (Met)	0.31 (Met)	0.31 (Met)
Ulva ohnoi	0.45 (Met)	0.71 (Met)	0.44 (Lys)	1.07 (Lys)	0.27 (Lys)	0.25 (His)
Cryptonemia seminervis	0.38 (Met)	0.60 (Met)	0.44 (Met)	1.06 (Met)	0.27 (Met)	0.27 (Met)
Gracilariopsis tenuifrons	0.37 (Val)	0.58 (Met)	0.43 (Val)	1.03 (Met)	0.26 (Met)	0.26 (Met)
Padina gymnospora	0.36 (Met)	0.57 (Met)	0.42 (Met)	1.01 (Met)	0.26 (Met)	0.26 (Met)
Cladophora coelothrix	0.36 (Met)	0.56 (Met)	0.42 (Met)	1.00 (Met)	0.26 (Met)	0.26 (Met)

Table S5.5. Quantity and quality of total essential amino acids, methionine and lysine as well as amino acid scores for mono-gastric livestock of seaweed genera that are commercially produced.

Genera	Amino acid quality (% TAA)			Amino acid quantity (% dw)			Amino acid score					
							Chicken		Swine		Fish	
	TEAA	Met	Lys	TEAA	Met	Lys	0 - 3 wks	6 - 8 wks	5 - 7 kg	100 - 130 kg	Salmon	Tilapia
Red												
Gracilaria (n = 6 species)	47.40 ± 1.04	2.82 ± 0.44	5.69 ± 0.60	4.59 ± 0.56	0.26 ± 0.04	0.56 ± 0.10	0.32 ± 0.07	0.45 ± 0.10	0.30 ± 0.05	0.73 ± 0.12	0.20 ± 0.04	0.18 ± 0.03
Porphyra (n = 5 species)	45.50 ± 1.34	1.63 ± 0.29	6.12 ± 0.30	10.31 ± 2.25	0.37 ± 0.10	1.36 ± 0.27	0.67 ± 0.17	0.91 ± 0.22	0.61 ± 0.16	1.44 ± 0.37	0.41 ± 0.10	0.36 ± 0.08
Brown												
Saccharina/Laminaria (n = 3)	39.26 ± 2.32	1.76 ± 0.35	5.20 ± 0.27	2.74 ± 1.30	0.14 ± 0.07	0.36 ± 0.17	0.25 ± 0.12	0.31 ± 0.15	0.23 ± 0.11	0.58 ± 0.28	0.15 ± 0.07	0.15 ± 0.08
Undaria	48.00	1.76	5.62	4.75	0.15	0.52	0.15	0.18	0.12	0.27	0.10	0.10
Hizikia	43.14	1.89	3.66	0.52	0.02	0.04	0.03	0.04	0.02	0.05	0.02	0.02
Green												
Ulva (n = 9 species)	43.14 ± 1.12	2.25 ± 0.34	5.15 ± 0.57	6.21 ± 0.88	0.27 ± 0.03	0.70 ± 0.07	0.47 ± 0.06	0.62 ± 0.08	0.40 ± 0.05	0.96 ± 0.12	0.26 ± 0.03	0.25 ± 0.03
Other protein sources												
soybean meal	46.00	1.25	6.66	22.34	0.70	2.24	1.40	2.19	1.49	3.67	0.93	1.00
Fishmeal	43.40	2.80	7.30	31.19	2.08	6.21	2.97	3.71	2.88	6.55	1.94	1.55
Spirulina	49.70	2.40	4.60	27.77	1.15	3.03	2.30	3.56	2.02	4.97	1.26	1.09

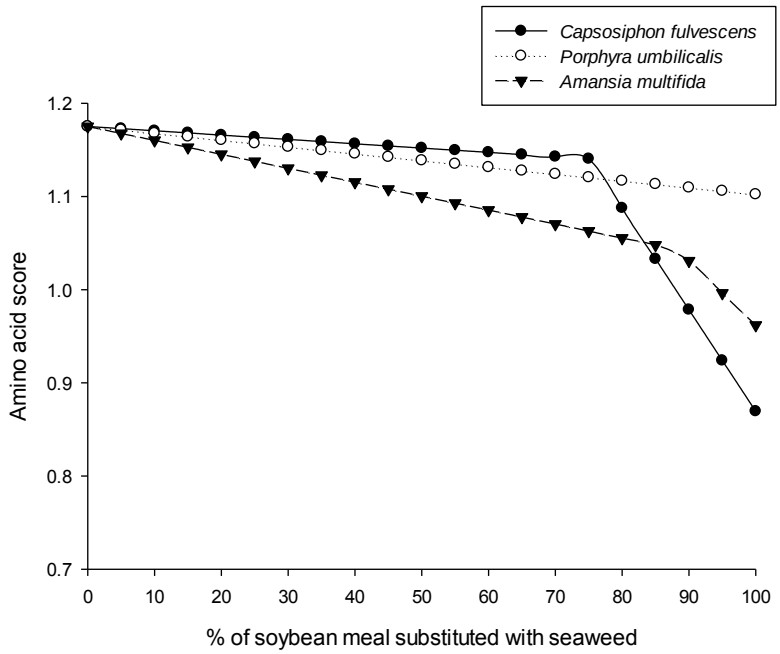

Figure S5.1. Modelled change in amino acid score of a typical corn/soybean meal based diet (74.1 % corn/23.4 % soybean meal) for mature swine when soybean meal is theoretically substituted for three seaweeds with the highest quantities of lysine – the limiting amino acid for a corn/soybean meal compounded diet.

Supplementary information S5.1: Supplementary results for Chapter 5

Of the 58 articles retrieved from the literature search that measured the amino acid content in seaweeds, the concentration of individual amino acids (as a % dw) could not be retrieved for 13 articles. Reasons for this included measurements as % TAA with no TAA content reported (2 articles, 14 measurements), measurements as g 16 g^{-1} N with no N content reported (1 article, 1 measurement) and individual amino acid measurements not presented (10 articles, 61 measurements). These articles were not analysed further. The remaining 45 articles which were included in Chapter 5 represented 265 individual seaweed samples from 121 different species. Of these articles, amino acid measurements were most often reported on a % dry weight (% dw) basis (49 % of articles) followed by a % protein basis (44 %), with only 1 article (2 %) each reporting amino acids in the units % TAA, g 16 g^{-1} N and µmol g^{-1} dw.

Outlier identification and removal

The raw data set of 265 individual seaweed measurements from 121 species contained a number of extreme % TAA values for essential amino acids. These measurements were identified using the outlier labelling method with a k value of 2.4 and were removed

from the raw data. The total number of measurements and species after outliers were removed for each essential amino acid are shown in Table S1. This data was used to calculate each species mean (Table S2) that was used to assess the quality (% TAA) and concentration (% dw) of essential amino acids in seaweeds. Once all measurements that had an extreme value in were removed, 212 measurements representing 93 species remained. This data set was used to assess amino acid concentration and quality relative to mono-gastric livestock requirements – identification of the limiting amino acid and the calculation of the amino acid score (ratio of the limiting amino acid in the seaweed to the animal requirement), based on the means of species.

The amino acids cysteine and tryptophan were the least reported amino acids, measured in only 64 and 27 % of articles, respectively. All other amino acids were measured in all articles, with the exception of methionine and tyrosine which were measured in 96 and 93 % of articles.

Substituting seaweeds in compound swine diet

By theoretically substituting the soybean meal protein source in a standard compound diet for mature swine (74.1 % corn/23.4 % soybean meal) with increasing amounts (from 5 to 100 %) of the three seaweeds with the highest lysine concentration, there is little change to the overall amino acid score of the diet as long as lysine remains the first limiting amino acid (Fig. S1). However, for the seaweed that has the highest lysine concentration (*Capsosiphon fulvescens*), there was a critical point (80 % substitution) where the amino acid score of the compound diet dropped dramatically as the limiting amino acid of the seaweed (tryptophan) became more limiting than lysine. This is because corn is severely deficient in tryptophan (Table 1) and is unable to for fill tryptophan requirements alone for mature swine if soybean meal is removed. This situation does not occur for the other two species as their limiting amino acid (methionine) is always supplemented by the corn. Tryptophan was not measured in these two seaweeds but may well have been also first limiting as tryptophan is the most frequently limiting amino acid for mature swine when it is measured (38 % of species).

Annex 5

List of publications from this thesis

Angell, A. R., Mata, L., de Nys, R. & Paul, N. A. 2014. Variation in amino acid content and its relationship to nitrogen content and growth rate in *Ulva ohnoi* (Chlorophyta). *Journal of Phycology* **50**:216-26.

Angell, A. R., Mata, L., de Nys, R. & Paul, N. A. 2015. Indirect and direct effects of salinity on the quantity and quality of total amino acids in *Ulva ohnoi* (Chlorophyta). *Journal of Phycology* **51**:536-45.

Angell, A. R., Mata, L., de Nys, R. & Paul, N. A. 2016. The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five. *Journal of Applied Phycology* **28**:511-524.

Angell, A. R., Angell, S. F., de Nys, R. & Paul, N. A. 2016. Seaweeds as a protein source for mono-gastric livestock. Under review in *Trends in Food Science and Technology*.

Angell, A. R., Paul, N. A. & de Nys, R. 2016. A comparison of multiple techniques for concentrating and isolating protein in *Ulva ohnoi*. In Prep.

Contributed to

Cole, A. J., Angell, A. R., de Nys, R., & Paul, N. A. 2015. Cyclical changes in biomass productivity and amino acid content of freshwater macroalgae following nitrogen manipulation. *Algal Research* **12**:477-486.