Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics

Seaweed proteins – how to get to them?

Effects of processing on nutritional value, bioaccessibility and extractability

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Hanne K. Mæhre

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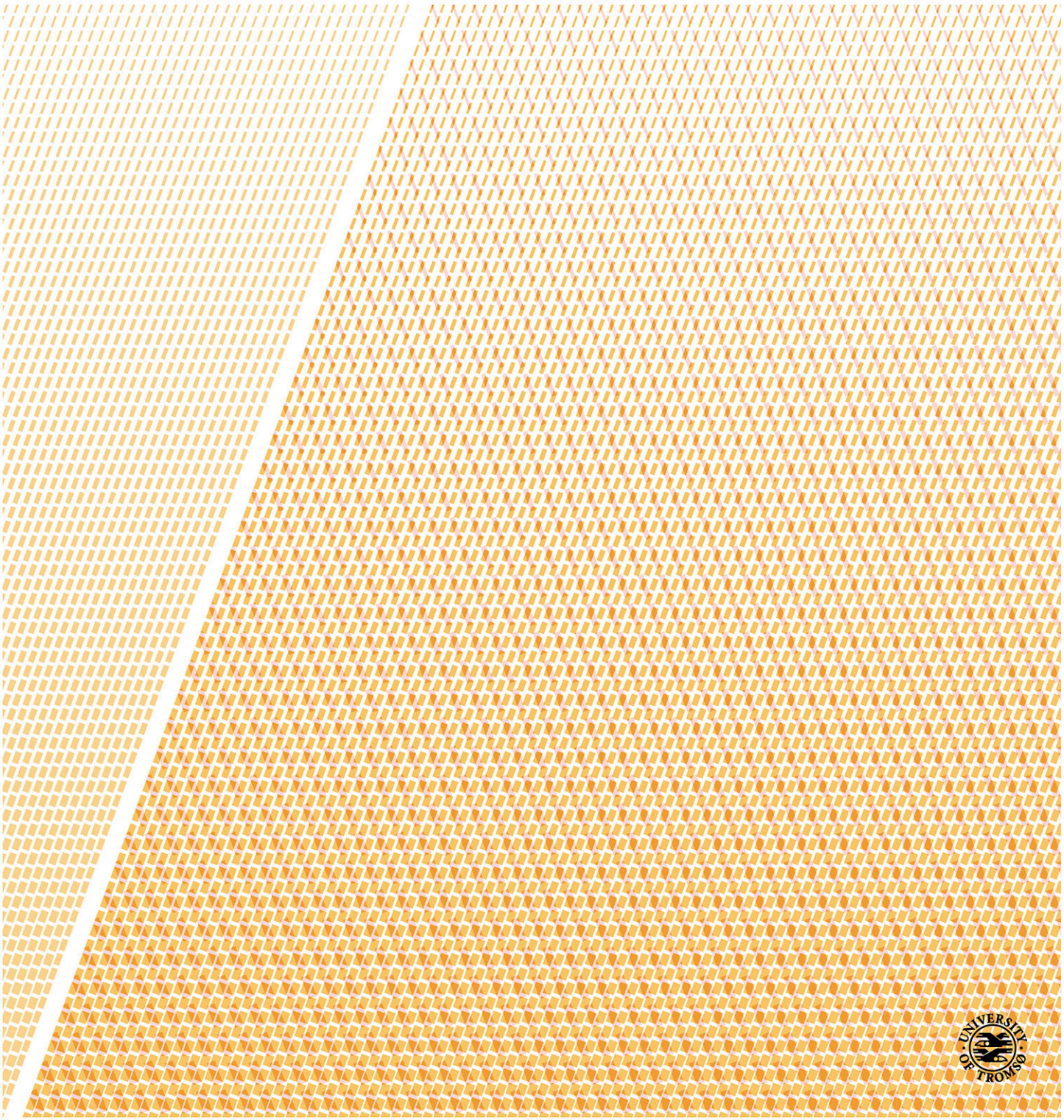

Acknowledgements

Ay, think of it – wish it done – will it to boot, - but do it…..
(Henrik Ibsen: Peer Gynt, 3rd act, 1st scene, translation by William and Charles Archer)

Now I have done it - written a PhD thesis. Almost five years of hard work, disappointments and joy, exhaustion and excitement. Well, it was worth it!

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Summary

As a consequence of the expected population growth towards 2050, the demand for food, and in particular proteins, will increase. Due to limited resources of arable land and freshwater, this increase cannot come in the agricultural sector alone. At present, the utilization of marine environments for food production is low and should be increased. Seaweeds are fast-growing plants occurring in marine environments worldwide and some of them have proven to be rich in proteins. Living in oceans, they neither need arable land nor freshwater in order to grow and as primary producers they absorb inorganic compounds from their surroundings and convert them into macronutrients.

The overall aims of this project were to examine the nutritional quality, along with effects of processing on bioaccessibility and extractability of seaweed proteins, and to evaluate their suitability as food, feed or ingredients in such. The specific goals were limited to i) document the nutritional composition in ten different seaweed species, ii) study the impact of heat treatment on the bioaccessibility of seaweed proteins, and iii) study the effect of enzymatic treatment on the bioaccessibility and extractability of seaweed proteins.

The lipid contents in seaweeds were generally low, while mineral contents were high. The protein contents were generally higher in red seaweeds than in the other classes. However, the variation in protein contents within the brown and green seaweeds were large. The protein contents were generally lower than presented in many other studies, most likely due to methodological differences. However, also seasonal and geographical variations may have influenced this. The proteins in several of the analysed seaweeds were found to be complete proteins, as they contained sufficient amounts of the essential amino acids in order to cover the human requirements. Both protein content and quality of dulse (*Palmaria palmata*) were higher compared to that of wheat, corn and rice.

In *P. palmata*, heat treatment increased the amount of accessible amino acids and the amount of amino acids liberated during a simulated gastrointestinal digestion model. The contents of essential amino acids were not negatively affected as a result of the process. For winged kelp (*Alaria esculenta*), no equivalent changes were observed. Also enzymatic treatment of *P. palmata* increased the amount of accessible amino acids and the amount of amino acids liberated during a simulated gastrointestinal digestion model. Enzymatic treatment also increased the protein extractability.

Sammendrag

Som en konsekvens av den forventede befolkningsveksten frem mot 2050 vil behovet for mat, og spesielt for proteiner, øke. På grunn av begrenset dyrkbart landareal og begrensede ferskvannsressurser, kan ikke denne veksten utelukkende komme innenfor landbrukssektoren. Utnyttelsen av marine ressurser for produksjon av mat er og har vært lav og bør derfor økes. Alger, også kjent som tang og tare, er hurtigvoksende planter som vokser i marine områder over hele verden og noen av dem har vist seg å være proteinrike. Siden de lever i havet, trenger de verken landareal eller ferskvann for å vokse. De er dessuten primærprodusenter som kan absorbere uorganisk materiale fra sine omgivelser og omdanne dem til næringsstoffer.

Hovedmålene i dette prosjektet var å undersøke biokjemisk sammensetning, samt effekter av prosessering på biotilgjengelighet og ekstraheringsutbytte av algeproteiner, samt å vurdere utnyttelsesmulighetene for alger som mat, fôr og ingredienser. De spesifikke delmålene var i) å dokumentere biokjemisk sammensetning i ti ulike tang- og tarearter, ii) å studere effekten av varmebehandling på biotilgjengeligheten til algeproteiner og iii) å studere effekten av enzymatisk behandling på biotilgjengeligheten og ekstraheringsutbyttet av algeproteiner.

Fettinnholdet i algene var generelt lavt, mens mineralinnholdet var høyt. Proteininnholdet var generelt høyere i rødalger enn i de andre klassene. Imidlertid varierte proteininnholdet mye innad i de brune og grønne algene. Proteininnholdet var generelt lavere enn presentert i mange andre studier, mest sannsynlig på grunn av metodeforskjeller. Imidlertid kan sesongvariasjoner og geografiske forskjeller også ha påvirket dette. Proteinene i flere av de analyserte algene kan karakteriseres som komplette proteiner fordi de inneholder tilstrekkelige mengder av de essensielle aminosyrene til å dekke de humane behovene. Både proteininnholdet og – kvaliteten i søl (*Palmaria palmata*) var høyere enn i hvete, mais og ris.

For *P. palmata*, førte varmebehandling til økt mengde tilgjengelige aminosyrer og økt mengde aminosyrer frigjort gjennom en simulert mage-tarmfordøyelsesmodell. Innholdet av essensielle aminosyrer ble ikke negativt påvirket av prosessen. For butare (*Alaria esculenta*) ble ikke tilsvarende endringer observert. Enzymatisk behandling av *P. palmata* førte også til økt mengde tilgjengelige aminosyrer frigjort gjennom den simulerte mage-tarmfordøyelsesmodellen, samt økt ekstraheringsutbytte.

Contents

Paper I, II and III

List of papers

The following papers form the basis of this thesis. They are referred to by their roman numerals in the text.

Paper I:

Mæhre HK, Malde MK, Eilertsen K-E & Elvevoll EO (2014) Characterization of protein, lipid and mineral contents in common Norwegian seaweeds and evaluation of their potential as food and feed. *Journal of the Science of Food and Agriculture* 94: 3281-3290.

Paper II:

Mæhre HK, Edvinsen GK, Eilertsen K-E & Elvevoll EO (2015) Heat treatment improves the protein bioaccessibility in the red seaweed dulse (*Palmaria palmata*), but not in the brown seaweed winged kelp (*Alaria esculenta*). *Journal of Applied Phycology*. DOI: 10.1007/s10811-015-0587-4

Paper III:

Mæhre HK, Elvevoll EO & Eilertsen K-E (manuscript) Enzymatic pre-treatment increases the protein bioaccessibility and extractability in dulse (*Palmaria palmata*).

Additional scientific contributions

During the course of my PhD project I have contributed to several other scientific articles and book chapters, covering various topics within the fields of seafood and health, food science and analytical biochemistry. However, these are not directly linked to this project and are thus not considered as parts of this thesis.

Scientific articles:

Mæhre HK, Jensen I-J, Elvevoll EO & Eilertsen K-E (2015) Omega-3 fatty acids and cardiovascular diseases – Effects, mechanisms and dietary relevance. *International Journal of Molecular Sciences*, 16:22636-22661.

Kristiansen RG, Rose CF, Fuskevag OM, **Maehre H**, Revhaug A, Jalan R & Ytrebo LM (2014) L-Ornithine Phenylacetate reduces ammonia in pigs with acute liver failure through phenylacetylglycine formation: a novel ammonia-lowering pathway. *American Journal of Physiology – Gastrointestinal and Liver Physiology*, 307: G1024-1031.

Jensen I-J, **Mæhre HK** & Eilertsen K-E (2013) Marine bioactive peptides from digestion and their relation to cardiovascular health promotion. *Agro Food Industry Hi-Tech*, 24, 36-40.

Vang B, **Mæhre HK**, Jensen I-J & Olsen RL (2013) Detection of tropomyosin and determination of proteins in crustacean oils. *Food Chemistry*, 141, 72-76.

Mæhre HK, Hamre K & Elvevoll EO (2013) Nutrient evaluation of rotifers and zooplankton: feed for marine fish larvae. *Aquaculture Nutrition*, 19, 301-311.

Jensen I-J, **Mæhre HK**, Tømmerås S, Eilertsen K-E, Olsen RL & Elvevoll EO (2012) Farmed Atlantic salmon (*Salmo salar* L.*)* is a good source of long chain omega-3 fatty acids. *Nutrition Bulletin*, 37, 25-29.

Eilertsen K-E, **Mæhre HK**, Jensen I-J, Devold H, Olsen JO, Lie RK, Brox J, Berg V, Elvevoll EO & Østerud B (2012) Wax ester and astaxanthin-rich extract from the marine copepod *Calanus finmarchicus* attenuate atherogenesis in female apolipoprotein E-deficient mice. *Journal of Nutrition*, 142, 508-512.

Triumf EC, Purchas RW, Mielnik M, **Mæhre HK**, Elvevoll E, Slinde E & Egelandsdal B (2012) Composition and some quality characteristics of the longissimus muscle of reindeer in Norway compared to farmed New Zealand red deer. *Meat Science*, 90, 122-129.

Eilertsen K-E, **Mæhre HK**, Cludts K, Olsen JO & Hoylaerts MF (2011) Dietary enrichment of apolipoprotein E-deficient mice with extra virgin olive oil in combination with seal oil inhibits atherogenesis. *Lipids in health and disease*, 10:41 DOI: 10.1186/1476-511X-10-41

Book chapters:

Mæhre HK, Jensen I-J & Eilertsen K-E (2016) Fish: Dietary Importance and Health Effects. In: Caballero, B., Finglas, P., and Toldrá, F. (eds.) *The Encyclopedia of Food and Health* vol. 2, pp. 699-705. Academic Press, Oxford, UK.

Larsen R, Eilertsen K-E, **Mæhre H**, Jensen I-J & Elvevoll EO (2014) Taurine content in marine foods - Beneficial health effects. In: Hernández-Ledesma B & Herrero M (eds.) *Bioactive compounds from marine foods: plant and animal sources*, pp. 249-268. Wiley-Blackwell, Oxford, UK.

Jensen I-J, Eilertsen K-E, **Mæhre HK**, Elvevoll EO & Larsen R (2013) Health effects of antioxidative and antihypertensive peptides from marine sources. In: Kim SKS (ed.) *Marine proteins and peptides: Biological activities and applications*, pp. 297-322. Wiley & Sons, Oxford, UK.

Eilertsen K-E, Larsen R, **Mæhre HK**, Jensen I-J & Elvevoll EO (2012) Anticholesterolemic and antiatherogenic effects of taurine supplementation is model dependent. In: Frank S & Kostner G (eds.) *Lipoproteins – Role in health and disease*, pp. 269-288. InTech Europe, Rijeka, Croatia.

List of figures and tables

Figures:

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Abbreviations

AA: Arachidonic acid

ALA: α-linolenic acid

DIAAS: Digestible Indispensable Amino Acid Score

DHA: Docosapentaenoic acid

DW: Dry weight

EAA: Essential amino acids

ECM: Extracellular matrix

EPA: Eicosapentaenoic acid

FAO: Food and Agriculture Organization of the United Nations

FID: Flame Ionization Detector

G3P: Glyceraldehyde-3-phosphate

ICP/MS: Inductively Coupled Plasma Mass Spectrometry

JECFA: Joint Expert Committee on Food Additives

LA: Linoleic acid

LC-PUFA: Long-chained polyunsaturated fatty acid

MeHg: Methyl mercury

MS: Mass spectrometer

NPN: Non-protein nitrogen

PDCAAS: Protein Digestibility Corrected Amino Acid Score

PTWI: Provisional Tolerable Weekly Intake

SDA: Stearidonic acid

Sec: Selenocystein

WHO: World Health Organization

1 Introduction

The world population is continuously growing and according to the latest prospects, it will reach 9.7 billion by 2050 [1]. Consequently, there will be an increased demand for food, in particular protein. The single most important food energy sources worldwide today are cereals and among them, wheat, rice and corn are the major species. In 2011, cereals made up 45 % of the total daily energy consumption (kcal capita^{-1} day^{-1}) globally. The equivalent values for Africa and Asia, counting for 75 % of the world's population, were 49 % and 52 %, respectively [2]. Despite their relatively low protein content, cereals also account for almost 40 % of the global human daily protein intake. In addition, plant proteins are increasingly utilized as feed ingredients for animals and fish. The efficiency of converting plant proteins into animal proteins for human consumption is, however, low and as much as 85 % may be lost during the process [3]. For instance, production of 1 kg of beef protein requires approximately 60 kg of grain, while the equivalent for pork protein is 38 kg of grain [4].

The agricultural sector is a big contributor to the environmental challenges the world is facing, accounting for 30% of greenhouse gas emissions [5]. Increased use of mineral fertilizers in the sector has also led to increased emissions of nitrogen and phosphorus [6, 7]. Moreover, 30 % of the world's total arable land area and 70 % of the available freshwater is already used by the agricultural sector. Other environmental effects associated with agriculture are flooding, erosion, deforestation and loss of biodiversity [5].

In order to meet food requirement for the estimated population growth, the necessary growth in the agriculture sector has been estimated to be 70 % towards 2050, where of one billion extra tonnes of cereals and 200 million extra tonnes of meat [8]. Even with improving cultivation and irrigation technologies, such an increase will intensify the environmental challenges. Thus, discovering, exploring and implementing alternative protein sources for food and feed is important.

Despite approximately 70% of the earth's surface being covered by water, only 1-2 % (based on energy) of the world food production is ocean-based [2]. Based on proteins, this corresponds to approximately 6.5 % of the total food protein production, or 17 % of the animal protein production [4]. The dominating species group is fish. The total production was 173 million tonnes in 2011 (capture and aquaculture), where of 131 million tonnes were used for direct human consumption. Fish and shellfish have been and are the fastest growing food-supply industry in the world, growing by a factor of 8 since 1950. In the same period, the aquaculture industry has increased from being negligible to making up approximately 50 % of the total fish production [4].

In order to ensure food security, and considering the large ocean area available and the low utilization at present, there should be a shift in the food production systems, from land-based to ocean-based. In particular, the relative amount of marine food proteins should increase. Besides fish there are many other organisms in the ocean capable of contributing to the future protein needs. Among these are seaweeds, or macroalgae, a very diverse group of fast growing plants that occur in marine environments worldwide. Botanically they are classified after phylum, class, order, family and genus (species). Colloquially they are usually divided into three main groups corresponding to the phylum; rhodophyta (red algae), chlorophyta (green algae) and phaeophyta (brown algae). Worldwide more than 20 000 different species have been identified [9] and the species diversity is more evident in tropical and temperate climates than in the polar regions [10].

Growing in oceans, seaweeds neither need arable land nor freshwater in order to grow. They are characterized as primary producers, as they efficiently take up inorganic compounds from the seawater and convert them to macronutrients, such as carbohydrates, lipids and proteins. Globally, seaweeds account for 5-10 % of marine primary production [11]. Being photosynthetic organisms, they take up light and carbon dioxide (CO_2), along with bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), from their surroundings and utilize it for production of sugar and oxygen. This capacity makes them efficient for sequestrating anthropogenic carbon, and in fact, marine primary producers (bacteria, micro- and macroalgae) have been shown to be responsible for at least 50 % of the carbon fixation and 71 % of carbon storage, worldwide [9]. In addition, they take up and utilize other organic and inorganic substances, such as nitrogen and phosphorus, also reducing possible environmental pollution from these compounds.

1.1 Use of seaweeds today

1.1.1 Worldwide

In 2012, 24.9 million tonnes of aquatic plants were harvested worldwide. In these figures are included both macroalgae and microalgae. Around 95 % of the aquatic plant production originate from aquaculture and as many as 33 countries are registered as having some degree of production. However, China and Indonesia are the major contributors, accounting for 81.4 % of the total production. It is estimated that around 36 % of the cultivated algae are used for direct human consumption, mainly in East Asia [12]. Some major products produced from seaweeds are the hydrocolloids alginates, agars and carrageenans, used as gel-forming additives in the food, pharmaceutical and biotechnological industries. Alginates are extracted from brown seaweed and are

salts of alginic acid, composed of the two subunits β-D-mannopyranosyluronic acid (mannuronic acid) and α-L-gulopyranosyluronic acid (guluronic acid). Both carrageenans and agars are sulphated galactans derived from red seaweed. For carrageenans the main genera are *Kappaphycus* and *Eucheuma*, with small contributions also from *Chondrus*, *Hypnea* and *Gigartina*, while *Gracilaria* and *Gelidium*, with small contributions from *Gracilariopsis* and *Gelidella* are the main sources of agars [2, 13]. The global production of these products is around 100 000 tonnes per year, with an estimated market value of 1.1 - 1.2 billion US dollar per year [14].

1.1.2 In Norway

In Norway, as in most Western European countries, over 99 % of the utilized seaweeds originate from wild resources. The predominating species is *Laminaria hyperborea* (tangle), but also some *Ascophyllum nodosum* (knotted wrack) is being harvested. Both species are used for extraction of alginates. In 2014, around 150 000 tonnes of wild seaweeds were harvested, having an export value of 1- 1.5 billion Norwegian kroner (NOK) [15].

During the last decade, there has been a great interest in developing aquaculture of seaweeds, also in Norway. Due to the high content of carbohydrates in seaweeds, the main field of interest was originally production of biofuel and thus the main species was *Laminaria saccharina* (sugar kelp). However, contents of other nutrients and bioactive molecules make seaweeds interesting candidates for a range of products such as food and feed, fertilizers and nutraceuticals [16] and several projects including other species, such as *Alaria esculenta* and *Palmaria palmata*, have recently been initiated in order to investigate optimal exploitation of seaweeds.

1.2 Problem outline

In order to make use of beneficial compounds in seaweeds, knowledge about their biochemical properties, including composition and bioaccessibility, is important. Biochemical composition has already been described for many algae species. However, the variation is large between different studies. In particular, protein content varies significantly, both within and between species. Seasonal and geographical variations are partly responsible for these variations, but also methodological differences are significant. Another important issue concerning algae proteins is their bioaccessibility, i.e. the potential of the proteins to be absorbed in the body, and thus, their potential for utilization as food, feed and ingredients in such. Bioaccessibility of plant proteins, including algae, is recognized as being inferior to that of animal protein, mostly due to strong interactions between complex

polysaccharides and proteins, along with the presence of anti-nutritional factors in plants. However, also in this field the choice of analytical methods influences the outcome. Effects of different processing techniques have been studied in a range of important food plants, but when it comes to seaweeds more studies are needed. In order to achieve optimal utilization of this biomass, increasing protein bioaccessibility is of utmost importance.

1.3 Project aims

The overall aims of this project were to examine the nutritional quality, along with effects of processing on bioaccessibility and extractability of seaweed proteins, and to evaluate their suitability as food, feed and ingredients in such. The specific goals were limited to the following:

- Document differences in nutritional composition between different seaweed species
- Study the impact of heat treatment on the bioaccessibility of seaweed proteins
- Study the effect of enzymatic pre-treatment on bioaccessibility and extractability of seaweed proteins

1.4 Research design

Figure 1 shows a schematic overview of the research design of this project. In **paper I**, a screening of the biochemical composition of ten different seaweeds commonly found in Norwegian water was performed. Based on the protein content and the amino acid composition found in this screening, two species (*Alaria esculenta* and *Palmaria palmata*) were chosen for assessment of effects of heat treatment on the bioaccessibility of seaweed proteins (**paper II**). One of these species (*P. palmata*) was also subjected to enzymatic treatment in order to examine the effects on protein bioaccessibility and extractability (**paper III**).

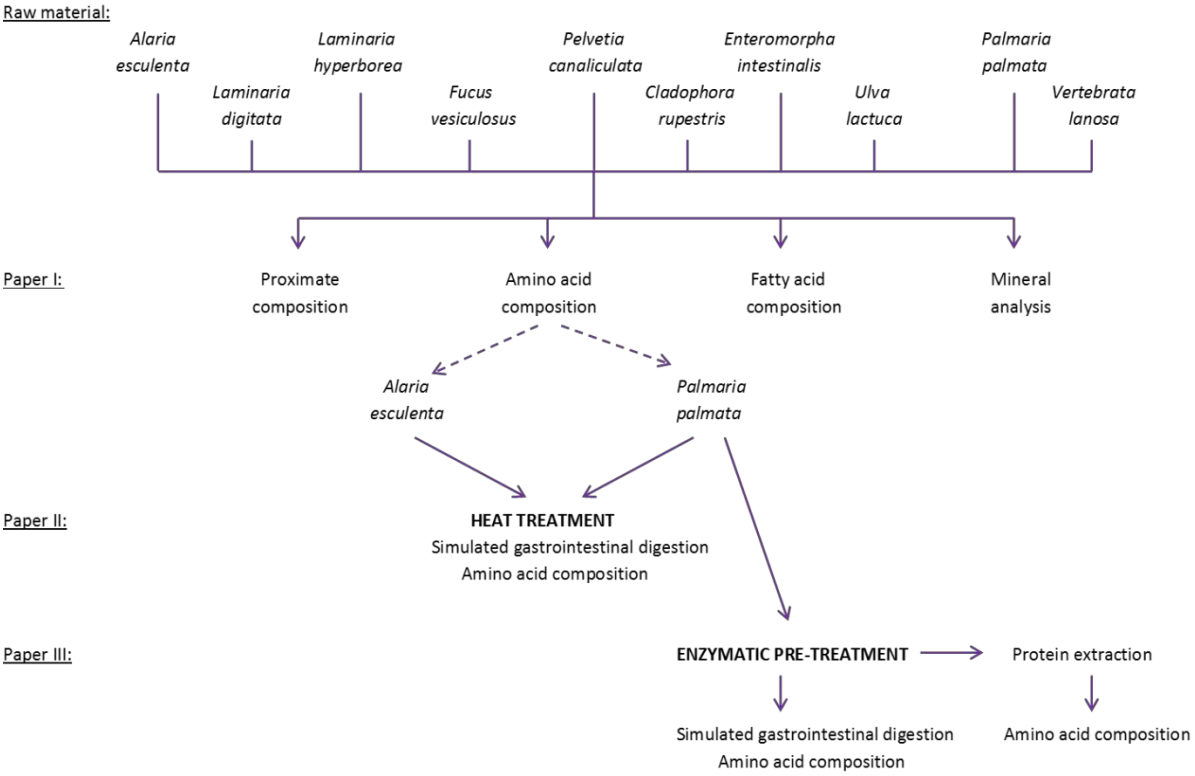

Figure 1: Schematic overview of the research design of this project.

2 Background

2.1 Primary production of nutrients

As mentioned in the introduction section, seaweeds are primary producers of macronutrients such as carbohydrates, lipids and proteins. The process of turning inorganic compounds into nutrients involves a wide range of biochemical reactions (mainly enzymatic) in several cell organelles. Carbon, nitrogen and phosphorus are recognized as the main elements for nutrient production and are present in seaweeds at a ratio of 550:30:1 [17]. Some of the processes involving these three elements will be highlighted here.

2.1.1 Carbon

Carbon dioxide is the main carbon source for nutrient production by terrestrial plants. In seawater the concentration of HCO_3^- and CO_3^{2-} are significantly higher than that of CO_2 and the amount of CO_2 may be too low to fulfil the carbon demands. As a means to ensure a sufficient amount of carbon for production of sugars, marine algae have developed carbon concentrating mechanisms and are thus able to utilize HCO_3^- and CO_3^{2-} as carbon sources by converting them to CO_2 [18].

Photosynthesis is a process where light energy converts CO_2 and water into sugar and oxygen. This process takes place in chloroplasts, which are cell organelles unique for plants. Within the chloroplasts, light energy is absorbed by photoreceptor proteins and converted to chemical energy in the form of nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP). The photoreceptor proteins contain different pigments, where of chlorophylls are the most abundant in all green plants, including green algae. The other algae classes also contain chlorophylls, along with other pigments, such as phycocyanin, carotenes, xanthophylls, phycoerythrin and fucoxanthin, ensuring uptake of light over a wide range of wavelengths. The newly formed NADPH and ATP are utilized in the fixation of CO_2 in the Calvin cycle producing glyceraldehyde-3-phosphate (G3P). This process is catalysed by the enzyme ribulose bisphosphate carboxylase/oxygenase (RuBisCO), which is assumed to be the most abundant protein in the world [19].

The G3P produced in the Calvin cycle is a very versatile compound and is utilized in a wide range of metabolic pathways in cells, either directly or as precursor for other metabolically active compounds, such as pyruvate. Among the pathways in which G3P is active are glycolysis/gluconeogenesis, fatty acid synthesis and Krebs' cycle. These pathways are, however, not limited to plant cells.

2.1.2 Nitrogen

Nitrogen is one of the major constituents in amino acids and proteins. In addition it is present in a wide range of other metabolically important molecules, such as nucleic acids and chlorophylls. The main sources of nitrogen for synthesis by seaweeds are nitrate (NO_3^-) and ammonium (NH_4^+), but also nitrite (NO_2^-), urea and amino acids may be absorbed from seawater in smaller amounts [11]. Ammonium is absorbed by facilitated diffusion, demanding less energy than the other nitrogen sources, who depend on active transport across cell membranes [20]. Ammonium may also be incorporated directly in amino acids, while nitrate depend on reduction first to nitrite and then to ammonium prior to entering into amino acids.

All seaweeds are able to store nitrogen intracellularly, but the storage capacity varies between species. The main storages are located in fluid-filled organelles called vacuoles. The size and function of these organelles vary between species. Besides nitrogen storage, vacuoles possess a range of important metabolic functions in cells, including regulation of turgor pressure, protein turnover, waste disposal and cell growth [21].

There are six main enzymatic pathways for amino acid biosynthesis in plant cells. These have been described in detail in a review article by Bromke [22]. The cellular carbon metabolism pathways, such as the glycolysis, photosynthesis, oxidative pentose pathway, photorespiration and Krebs' cycle, provide carbon backbones for the different amino acids [22]. In macroalgae, the first amino acid synthesized is glutamine, catalysed by the enzyme glutamine synthetase, followed by glutamate catalysed by glutamine-oxoglutarate aminotransferase [11]. Not many studies have been performed regarding the biosynthesis of essential amino acids in macroalgae. However, Angell *et al*. [23] showed that the contents of all amino acids in the macroalga *Ulva ohnoi* (chlorophyta) were affected by varying N fluxes in seawater, suggesting that macroalgae possess the necessary enzymes for biosynthesis of all amino acids [23].

2.1.3 Phosphorus

Phosphorus is a constituent in many biologically important molecules, such as nucleic acids, phospholipids and coenzymes (including coenzyme A). Another important physiological role of phosphorus is energy transfer as high-energy phosphate compounds, such as ATP, in photosynthesis and respiration [11].

Phosphorus, mainly as the inorganic ions PO_4^{3-}, HPO_4^{2-} and $H_2PO_4^-$, is actively transported from seawater across the cellular membrane of algae. Inside the cell they are incorporated in polyphosphate

vesicles or phosphorylated metabolites in cytosol or stored in the vacuoles [11]. The phosphorus storage capacity of different algae varies depending on their growth pattern. Fast-growing ephemeral seaweeds seem to have lower storage capacity than do slower-growing perennial species [24].

2.2 Nutrients and their impact on human health

Proteins, lipids and carbohydrates are, along with water, the main nutrients in food. In addition, other compounds, such as vitamins and minerals, are normally present, although in smaller amounts. The distribution of the different nutrients varies between food items and the actual composition determine their nutritional properties. Factors such as seasonality, geographical location and soil composition vary and also affect the nutritional properties. Compositional analyses are therefore very important in all nutritional studies.

2.2.1 Proteins

Proteins are large molecules consisting of chains of amino acids. There are 20 common amino acids that may enter into proteins, where of nine are essential to humans. An essential amino acid was originally defined as one that cannot be synthesized by the animal out of materials ordinarily available to the cells at a speed commensurate with the demands for normal growth [25], but is more commonly recognized as one that cannot be synthesized *de novo*. The essential amino acids for humans are threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine and tryptophan. Some of the other amino acids, such as proline, arginine, cysteine and glycine, may be essential in some periods of life or during illness and recovery of such. These are termed conditionally essential amino acids [26]. Proteins pose a wide range of important functions in the body, among them growth and maintenance, enzymatic activity and transport of nutrients and other biochemical compounds across cellular membranes [27]. In order to maintain these important functions, it is essential to provide the body with good quality proteins through diet. Muscle is the main reservoir of bodily protein. Inadequate amounts of dietary proteins or essential amino acids will lead to increased turnover of muscular proteins in order to provide sufficient amounts of amino acids to other organ systems. This may, over time, lead to reduced growth and loss of muscle mass and subsequently reduced immunity, reduced hormonal and enzymatic activity [28].

In developed countries malnutrition due to protein deficiency is not common in most population groups. However, studies have shown that the frequency of malnutrition among elderly people,

particularly those institutionalized, is high [29, 30]. Inadequate protein intake relative to estimated average requirements has been shown for up to 40 % of the elderly populations [30, 31].

In developing countries the situation is somewhat different. Around 795 million people are estimated to be undernourished in the world today, around 94 % of these living in sub-Saharan Africa and Southern and Eastern Asia [32]. In these areas, the staple foods are often cereal based, being low in protein and some essential amino acids. Young children have very high protein demands relative to their body size (table 1) and are thus particularly at risk for developing protein deficiency disorders, in particular in the transition period from weaning to eating regular food.

Table 1: Estimated essential amino acid and total protein requirements for infants, young children (0.5 – 3 years), older children (3 – 18 years) and healthy adults, given as mg g^{-1} protein for amino acids and mg kg^{-1} body weight d^{-1} for total protein, respectively. Adapted from FAO/WHO/UNU [33].

Essential amino acid	Infants (< 0.5 years)	Young children (0.5 – 3 years)	Older children (3 – 18 years)	Healthy adults
Histidine	21	20	16	15
Isoleucine	55	32	30	30
Leucine	96	66	60	59
Lysine	69	57	48	45
Methionine (+ Cysteine)	33	28	23	22
Phenylalanine (+ Tyrosine)	94	52	41	38
Threonine	44	31	25	23
Tryptophan	17	8.5	6.6	6
Valine	55	43	40	39
Total protein (mg kg^{-1} BW d^{-1})	1.14-1.77	0.9-1.31	0.82-0.9	0.66

However, not only the amount, but also the quality, of protein is important. The quality of proteins is often determined by their ability to cover the requirements of essential amino acids, along with their absorption and utilization in the body. A reference protein, containing the minimum requirements of essential amino acids has been defined for comparison purposes [33]. This reference protein resembles the demands given for healthy adults in table 1.

Chemical score is a simple and frequently used term for estimating protein quality and equals the lowest value returned when calculating the ratio between each essential amino acid in the food protein and the corresponding essential amino acid in the reference protein. The amino acid posing the lowest value is termed the (first) limiting amino acid of the protein. Generally, proteins of animal origin is considered to be of good quality, posing a chemical score of around 1.0. Cereal proteins are often deficient in one or more essential amino acids and commonly have a chemical score of 0.4-0.6. Legumes, beans and nuts normally range between these [34].

The limiting amino acid in most cereals is lysine, but some cereals are also low in threonine. Both of these two amino acids are synthesized through the aspartate pathway [22]. Lysine is a highly water soluble, basic amino acid, with the chemical formula $C_6H_{14}O_2N_2$ (figure 2). It is positively charged at physiological pH. Lysine is the most abundant essential amino acid in human muscle, accounting for 9.8 %, indicating its essential role in amino acid metabolism [35]. Most metabolic functions specific for lysine are related to the ε-amino group as this group is highly reactive [36].

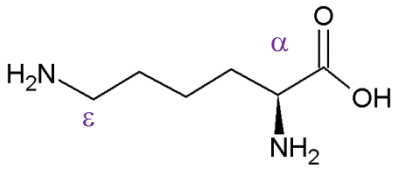

Figure 2: Lysine

The symptoms of lysine deficiency mainly resemble those of general protein deficiency [37], indicating that protein deficiency basically is a result of inadequate lysine intake. Supplementation of lysine has shown to positively affect treatment of recurrent Herpes Simplex, increase calcium absorbance, reduce incidence of stroke and prevent hypertension [38].

2.2.2 Carbohydrates

Carbohydrates comprise most of the dry matter in plant materials (up to 90 %). They consist of monosaccharides, or simple sugars, joined together to form larger structures. Chains of 2 to 20 monosaccharides are termed oligosaccharides, while those containing more than 20 are termed polysaccharides. The human digestive tract contain enzymes able to cleave smaller oligosaccharides, such as sucrose, maltose and lactose, along with a few polysaccharides, namely starch amylose and amylopectin. Longer, and more complex, polysaccharides are not digestible for humans [39].

The cell wall of plant materials, including seaweeds, consist mainly of different polysaccharides. Among the polysaccharides is cellulose, which is a linear high-molecular weight chain of glucose molecules joint together by (1-4)-β-D-glycosidic linkages. Cellulose molecules associate with each other via hydrogen bonds and form strong fibrous bundles, insoluble in water. In addition to cellulose, seaweed cell walls contain a range of other polysaccharides, which are specific for each main class. Both green and red seaweed contain (1-3), (1-4)-β-D-glucans. In addition, green algae contain ulvans, while red algae contain agars, carrageenans and lignin and lignin-like compounds. In brown algae the major cell

wall polysaccharides, besides cellulose, are fucoidans and alginates [40]. The human digestion system lack the enzymes necessary to break down these polysaccharides to their constituent sugars (glucose, galactose, mannose etc.) and they are thus recognized as indigestible. Agars, carrageenans and alginates are, as mentioned in the introduction section, extracted to make hydrocolloids that are used as gel-forming additives in the food, pharmaceutical and biotechnological industries.

2.2.3 Lipids

Lipids hold a variety of functions in the body and are present in different structures, or lipid classes, depending on their specific function. Fatty acids are integral parts of all lipids and their common chemical structure is an aliphatic hydrocarbon chain, normally ranging between 4 and 22 carbon atoms. At one end, they have a carboxylic acid group and at the other end, there is a methyl group. The carbon atoms are covalently bound together with only single bonds, or with one to six carbon-carbon double bonds. Depending on the number of double bonds in the chain, fatty acids are classified as saturated (only single bonds), monounsaturated (one double bond) or polyunsaturated (2 – 6 double bonds). Unsaturated fatty acids are further classified by the so-called “omega” denotation, which indicates the placement of the first double bond in the chain, counted from the methyl end of the carbon chain.

Linoleic acid (LA, C18:2, n-6) and α-linolenic acid (ALA, C18:3, n-3) are essential to humans and animals, due to the lack of the enzymes Δ12- and Δ15-desaturase, which insert double bonds in the omega-3 position and omega-6 position, respectively. Longer and more unsaturated fatty acids (LC-PUFA) may be derived from these through a series of enzymatic processes, involving elongases and desaturases [41]. Arachidonic acid (AA, C20:4, n-6) is derived from LA, while ALA is precursor for eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3). These two conversion pathways compete for the same enzymes and synthesis of the respective fatty acid thus reflects the contents of LA and ALA in the diet. In a Western diet, the content of LA is normally 10-20 times higher than the content of ALA [42] and this favours the synthesis of AA over EPA and DHA in the body. In addition, the conversion of ALA to EPA and DHA is less efficient than that of LA to AA, due to ALA having the highest oxidation rate among unsaturated fatty acids [41]. There are also gender differences in the conversion rate from ALA to EPA and DHA, where the rate is higher in woman where up to 21% of ALA is converted to EPA and up to 9 % further to DHA [43]. In men, the conversion rates are 8 % to EPA and < 4% to DHA, respectively [44].

The mentioned AA, EPA and DHA are all origin for a range of compounds, namely eicosanoids, resolvins and protectins, which have impact on acute and chronic inflammatory responses [45]. Resolvins and

protectins may be derived from all of these three fatty acids. These compounds participate in the resolution phase following an inflammatory response and the effects of them are more or less similar independent of the originating fatty acid [46]. Eicosanoids, however, may be derived from AA and EPA and have very different properties and potencies depending on the fatty acid of origin. The eicosanoids derived from AA are potent blood platelet aggregators and induce inflammation, leukocyte chemotaxis and adherence. The mechanisms of EPA-derived eicosanoids are quite similar, but their signalling is much weaker, and thus the adverse effects are not as pronounced. Eicosanoids, and in particular those derived from AA, may thus act as stressors for development and progression of a range different medical conditions, such as atherosclerosis, inflammatory bowel diseases and rheumatoid arthritis [47].

2.2.4 Vitamins and minerals

Vitamins and minerals are micronutrients essential for humans and lack of these nutrients may lead to impaired development and health. Deficiency in vitamin A may lead to blindness, while lack of vitamin D may cause osteoporosis or rickets. In earlier days, vitamin C deficiency was the primary cause of scurvy, especially affecting people with low access to fruit and vegetables.

Various seaweeds are good sources of vitamins A, B-series, C and E [48, 49]. Also vitamins whose levels are usually not very high in plant materials, such as D_3 and B_{12}, have been found in some species, namely *Fucus spiralis* (D_3), *Enteromorpha intestinalis* (B_{12}) and *Porphyra sp.* (D_3 and B_{12}) [50, 51].

Minerals are metallic elements that may be present in their elemental form, as inorganic compounds (salts) or bound in organic complexes. Their functions in the human body are widespread from bone mineralization, via blood pressure regulation to protection from oxidative stress. Seaweeds are efficient in absorbing and accumulating minerals from seawater and although the absorption capacity vary between different seaweeds [52], mineral type and physiochemical states [53], they may be very good dietary sources for a range of minerals. The efficient mineral absorption and accumulation in seaweeds is however not entirely advantageous. Also toxic minerals, such as arsenic, cadmium, lead and mercury may accumulate in seaweeds and cause adverse health effects, such as peripheral vascular disease, renal tubular dysfunction, anaemia and kidney damage and impaired mental development.

In particular two trace minerals are associated with deficiency disorders, iodine and selenium. Iodine is essential for the synthesis of thyroid hormones, which in turn are essential for regulation of metabolism. Lack of this mineral may cause serious illnesses, such as goitre and cretinism. Iodine

deficiency is one of the commonest deficiency disorders worldwide. The recent decades, large efforts have been made to fight this condition, the main instrument being iodization of salt. Although progress have been made, iodine deficiency still remains a significant global health issue [54]. Selenium may be incorporated in a range of proteins, selenoproteins, where the sulphur in cysteine or methionine has been replaced by selenium. The selenoproteins are involved in a range of metabolic processes, one of them being the thyroid hormone metabolism, by deiodination of the inactive thyroxin T4 to the active triiodothyronine T3. Selenium deficiency may thus lead to, or even intensify, some of the same conditions associated with iodine deficiency [55]. In addition, being a constituent in glutathione peroxidases, a class of antioxidant enzymes, selenium is regarded as one of the most important endogenous antioxidants [56]. Selenium also ameliorate the toxic effects of methylmercury (MeHg). The mechanism has been suggested to be that MeHg binds to selenocysteine (Sec), forming MeHg-Sec, a molecularly non-reactive compound. This reduces the toxicity of MeHg, but at the same time removes Sec from circulation and reduces the formation of selenoproteins and – enzymes. A selenium-rich diet, or alternatively selenium supplement, may offset the selenium sequestered by Hg and thus maintain the selenoprotein activity [57].

2.2.5 Bioactive compounds and anti-nutritional factors

A bioactive compound is defined as a food compound that, in addition to its nutritional value and at a physiologically relevant concentration, positively affects biological processes in the human body [58]. Bioactivity is normally linked to definite chemical groups or structures in the food matrix. For instance, phenolic groups may scavenge free radicals and thus act as antioxidants. Other groups that have been associated with bioactivity are peptides, polysaccharides, carotenoids and sterols. Algae has been found to contain many bioactive compounds exerting a range of bioactive effects. These include fucoxanthin showing anti-oxidative, anti-cancer and anti-diabetic effects [59], sterols showing anti-microbial effects [60] and fucoidan showing anti-inflammatory effects [61]. Most of the studies documenting health benefits from seaweed bioactive compounds are based on *in vitro* models, cell lines and animal studies. However, more and more human studies are performed and recently a comprehensive review evaluating these human studies was published [62].

An anti-nutritional factor is defined as any natural or synthetic compound that interferes with the absorption of a nutrient [63]. Examples of such compounds may be enzyme inhibitors, glucosinolates, phytic acid, lectin, tannins and other polyphenolic compounds [64]. Terrestrial plant foods are particularly rich in anti-nutritional factors as these are a part of their defence against predators, but also in seaweeds such compounds may be found. Both lectins and phytic acid have been found in

seaweeds [65], but among the anti-nutritional factors, polyphenolic compounds are more frequent, especially in brown seaweeds. The anti-nutritional effect of polyphenolic compounds is associated with their binding to food macromolecules (proteins and polysaccharides), forming insoluble high molecular complexes and thus reducing the bioavailability of the macromolecules [66]. Polyphenols may also bind to digestive enzymes, reducing their effects in the gastrointestinal tract and further reduce the utilization of macromolecules.

Interestingly, some of the anti-nutritional factors may also possess bioactive properties. For instance, tannins and phytic acid are associated with reduced protein digestion and mineral uptake, respectively. However, these compounds have also been shown to have anti-microbial and anti-cancer effects [67].

2.3 Digestion and bioaccessibility of proteins

Protein digestion *in vivo* involves a series of enzymes and hormones interacting in a complex feedback process. As a response to food entering the stomach, chief cells and parietal cells are stimulated and hydrochloric acid (HCl) and pepsinogen are released. Pepsinogen is converted into its active form, pepsin, and the proteins are hydrolysed into polypeptide chains. As the food moves into the duodenum, HCl is neutralised by bicarbonate and a range of proteases, including trypsin and chymotrypsin, are released from the pancreas and continue the cleavage of the polypeptide chains. Further down the small intestine, aminopeptidases and carboxypeptidases cleave off the N-terminal and C-terminal amino acids, respectively, liberating free amino acids that are absorbed in the enterocytes [68].

The digestibility and bioaccessibility of proteins are affected by the food matrix, i.e. levels and types of fat, carbohydrates and anti-nutritional factors [69]. The bioaccessibility, or digestibility, of plant proteins is normally lower than that of animal proteins. There are two main reasons for this. One is the presence of anti-nutritional factors as described in section 2.2.5. The other is strong interactions between proteins and complex polysaccharides in the cell walls of plants. The algae cell wall is built up of a network of water-insoluble cellulose microfibrils, water-soluble polysaccharides, such as xylans and alginates, along with small amounts of proteins [70]. The protein fraction of the cell wall form ionic bonds with the charged side-groups of the soluble polysaccharides, leaving them inaccessible to the gastrointestinal enzymes. Due to the inability of human gastrointestinal enzymes to hydrolyse the bonds within polysaccharides, the cell wall is resistant to degradation in the human gastrointestinal tract and thus the access of intracellular proteins is also hindered.

2.3.1 Evaluation of protein digestibility

Several methods for evaluation of protein digestibility have been developed and applied throughout the years. Traditional methods, such as true digestibility, biological value and net protein utilization are rather simple and evaluate protein quality based solely on the absorption of nitrogen. In 1991, the Food and Agriculture Organization of the United Nations (FAO) recommended the protein digestibility corrected amino acid score (PDCAAS) as the preferred method for protein quality evaluation. Here, the contents of the indispensable (essential) amino acids and the digestibility of crude protein are taken into account and assessed against the human requirements of these amino acids [71], given as the requirements of young children (0.5 – 3 years). Later, this method has been modified and the method currently recommended for protein quality assessment is the digestible indispensable amino acid score (DIAAS) [72]. This method attends to the digestibility of each individual essential amino acid, as opposed to digestibility of crude protein used in PDCAAS. In addition, the digestibility in DIAAS is evaluated as essential amino acids absorbed at the end of the ileum instead of over the whole gastrointestinal tract (measured in faeces), thus avoiding the amino acid contribution from microbial activity in the colon.

2.3.2 Effects of processing/heat treatment

Processing of foods have several effects, both positive and negative. Improvement of taste and texture, food quality, safety and preservation of food products and ingredients, along with increased bioaccessibility and inhibition of anti-nutritional factors are among the positive traits of processing [73-76]. Of negative effects can be mentioned loss of free amino acids [77, 78] and some vitamins [79, 80].

For proteins, processing has shown both positive and negative effects. A gentle heat treatment (70 - 90°C) normally increases protein digestibility and bioaccessibility in foods. This is due to partial protein denaturation by heat, resolving the tertiary structure and unmasking accession sites for gastrointestinal enzymes. Heat may also inactivate proteinaceous anti-nutritional factors, such as enzyme inhibitors and lectins. However, some processing procedures may negatively affect protein digestibility and accessibility. Heating at high temperatures (> 200°C) and alkaline pH, acidic hydrolyzation and roasting may lead to racemization of amino acids from L-amino acids to D-amino acids. As only L-amino acids are biologically utilizable for humans, racemization will lead to reduced amino acid accessibility. Another chemical alteration induced by heat and/or alkaline pH is cross-linking of proteins. A highly reactive compound, dehydroalanine (DHA), may be formed, a compound that readily reacts with nucleophilic groups in amino acids, such as the ε-amino group of lysine or the thiol group of cysteine, forming lysinoalanine and lanthionine, respectively. Formation of these

compounds reduces the bioaccessibility of the involved proteins because gastrointestinal enzymes are not able to break the peptide bond in lysinoalanine. If absorbed, lysinoalanine is not utilized in protein metabolism and thus, the bioavailability of lysine is reduced. A third possible negative effect of processing on amino acid availability is the formation of carbonyl-amine compounds, which is a reaction between an amino acid and a reducing sugar (Maillard reaction). Also in this reaction, lysine with its ε-amino group is frequently involved. The Maillard products are not readily absorbed in the intestine and hence, the bioaccessibility of lysine is reduced [81].

3 Summary of papers

Paper I:

Characterization of protein, lipid and mineral contents in common Norwegian seaweeds and evaluation of their potential as food and feed.

In this paper the aim was to study the nutritional composition of ten species of marine seaweeds commonly found in Norwegian waters in order to evaluate their potential as alternatives for cereals in food and feed. Samples from all of the three main seaweed groups (two red, three green and five brown, where of three kelps and two wracks) were harvested in May and June of 2010 and 2012. The samples were subjected to analysis of proximate composition (lipids, proteins, water and ash), fatty acid composition, amino acid composition and contents of essential minerals and heavy metals. All samples were freeze-dried prior to analysis. The protein content ranged from 34 g kg^{-1} dry weight (DW) in *Cladophora rupestris* to 123 g kg^{-1} DW in *Palmaria palmata*. Proteins from several species from all of the three groups contained sufficient amounts of essential amino acids in order to cover both human and salmonid requirements for these compounds. Lipid contents were generally low, ranging from 6 to 58 g kg^{-1} DW. Substantial species variability was evident in the fatty acid composition. The relative content of long-chained n-3 fatty acids was highest in the red algae containing 32-34 % EPA, followed by the kelps (brown) containing 7-11 % EPA. The wracks (brown) and the green algae contained only small amounts of these health beneficial fatty acids, ranging from 1-4 % EPA. None of the algae contained DHA. Iodine content was high in all species and especially in *Laminaria hyperborea*, *Laminaria digitata* and *Vertebrata lanosa*, where the content was more than 1000 times higher than the mean value of marine fish. Of the heavy metals analyzed, only arsenic levels were high enough to be of concern. In conclusion, the total composition of the red algae *P. palmata* makes it the best candidate for utilization in food and feed, while the green algae *C. rupestris* seems to be least suitable for utilization. In the other species, the combination of health beneficial and potential health threatening compounds is more evident, making utilization of the whole product more difficult. For these species it seems like extraction of single compounds such as proteins and/or minerals for use in the ingredient market is a better strategy.

Paper II:

Heat treatment increases the protein bioaccessibility in the red seaweed dulse (*Palmaria palmata*), but not in the brown seaweed winged kelp (*Alaria esculenta*).

The aim of this study was to investigate the effects of heat treatment on the protein bioaccessibility of the red seaweed dulse (*Palmaria palmata*) and the brown seaweed winged kelp (*Alaria esculenta*). The seaweeds were harvested on the south coast of Iceland, flushed with seawater and dehydrated at 40°C for 24 hours using electrical fans driven by geothermal energy. Samples from the two seaweed species were boiled in water for 15, 30 and 60 minutes and, along with a raw sample, they were subjected to an *in vitro* gastrointestinal digestion model. Samples were collected after 5, 120 and 240 minutes of the digestion process, simulating the mouth, stomach and intestinal phases, respectively. Following acidic hydrolysis, all samples were subjected to amino acids analysis. In *P.palmata*, the content of bioaccessible amino acids increased by 86-109% after heat treatment. Following the simulated gastrointestinal digestion, the amount of amino acids liberated in the liquid phases was 64-96% higher in the heat-treated samples compared to their raw counterparts. The increase was largest in the samples boiled for 15 and 30 minutes. No deterioration of single amino acids was observed and hence, the amount of available essential amino acids was increased accordingly. In *A. esculenta*, no equivalent changes were observed. In conclusion, a short heat treatment may be a simple means to increase the bioaccessibility of seaweed proteins and thus increase their utilization potential in food and feed. However, there are species differences and the effects observed in the *in vitro* digestion model need to be confirmed in clinical studies.

Paper III:

Enzymatic pre-treatment increases the protein bioaccessibility and extractability in dulse (*Palmaria palmata*).

The aims of this study were to further develop and optimize existing protein extraction protocols and to examine the effect of enzymatic pre-treatment on bioaccessibility and extractability of seaweed proteins. Dulse (*Palmaria palmata*) was harvested on the south coast of Iceland, flushed with seawater and dehydrated at 40°C for 24 hours using electrical fans driven by geothermal energy, before packing in airtight bags. In the first part of this study several modifications of existing protein extraction protocols were tested on seaweed samples in order to find the most efficient combination of type and concentration of extraction solvents, extraction times and agitation during extraction. The extraction

efficiency was examined using amino acid analysis. In the second part of the study, alga samples were subjected to enzymatic treatment with the purpose of disrupting the cell wall and thus release intracellular proteins. Following enzymatic treatment, some samples were subjected to an *in vitro* gastrointestinal digestion model, while others were subjected to alkaline protein extraction. During the gastrointestinal model, samples for amino acid analysis were collected after 5, 120 and 240 minutes, simulating the mouth, stomach and intestinal phases, respectively. Samples for amino acid analysis were also collected after alkaline extraction. Enzymatic treatment of seaweed samples increased the amino acids available for extraction 2.5-fold compared to untreated alga. Combining enzymatic treatment with alkaline extraction increased the protein extraction yield 1.7-fold compared to a protocol based on mechanical degradation of cell wall followed by alkaline extraction. A simulated gastrointestinal digestion model showed that enzymatic treatment of seaweed increased the amount of amino acids available for intestinal absorption 2.5-fold compared to an untreated sample. In conclusion, enzymatic pre-treatment of seaweeds is effective for increasing the amount of amino acids available for utilization. However, both the enzymatic treatment protocol and the protein extraction protocol need further optimization in order to obtain maximal cost-benefit.

4 Methodological considerations

4.1 Raw material

Norwegian waters serves as habitats for approximately 500 different species, where of 100 green, 200 brown and 210 red algae [82]. In **paper I**, the nutritional composition of a selection of ten species commonly found in Norwegian waters, and covering all of the three main classes, were analyzed (table 2).

Table 2: Summary of the macroalgae species included in the study.

Species	Common name	Class	Harvest area
Alaria esculenta	Winged kelp	Brown (kelp)	Sommarøy (69°N, 18°E)
Laminaria digitata	Oarweed	Brown (kelp)	Sommarøy (69°N, 18°E)
Laminaria hyperborea	Tangle	Brown (kelp)	Sommarøy (69°N, 18°E)
Fucus vesiculosus	Bladderwrack	Brown (wrack)	Sommarøy (69°N, 18°E)
Pelvetia canaliculata	Channeled wrack	Brown (wrack)	Brensholmen (69°N, 18°E)
Cladophora rupestris	Mekong weed	Green	Skjerstadfjorden (67°N, 14°E)
Enteromorpha intestinalis	Gut weed	Green	Skjerstadfjorden (67°N, 14°E)
Ulva lactuca	Sea lettuce	Green	Trondheimsfjorden (63°N, 9°E)
Palmaria palmata	Dulse	Red	Voldsfjorden (62°N, 5°E)
Vertebrata lanosa	Wrack siphon weed	Red	Oldervik (69°N, 19°E)

Both seasonal and geographical variations, such as water temperature and light amount and intensity, has been shown to affect the biochemical composition of seaweeds [83-85]. In order to reduce these factors as much as possible, the intention in this study was to narrow the harvesting area as much as possible. Most of the species were harvested between 67-69°N, but during the sampling period *P. palmata* and *U. lactuca* could not be retrieved in these locations and thus, these species had to be collected further south (62-63°N). All of the samples were harvested at the same time of the year, in May and June. This harvesting time was chosen based on aquaculture studies from Trondheimsfjorden (63°N), which showed that this is the optimal harvesting time, due to it being the end of the growth period and that the rise in ocean temperature later in summer increase epiphytic fouling and reduces the quality of seaweeds [86]. Epiphytic fouling has later been shown to be less of a problem in aquaculture sites further north (S. Mattson (Akvaplan-Niva), personal communication).

Based on the high content of protein and essential amino acids found in **paper I**, *P. palmata* was chosen as the primary species for examination of effects of processing in **paper II** and **III**. In Norway, there is currently a great interest in aquaculture of seaweeds, mostly of brown seaweeds. One of the species

considered for aquaculture is *A. esculenta*. This species was also found to be quite high in protein and essential amino acids. Thus, it was decided to include this alga in some further studies. In order to ensure a stable delivery of raw materials, it was decided that commercially available seaweeds were to be used in the studies on effects of processing.

As intra-thallus variations has been documented in several algae species [87-89], it was decided that all analyses were to be performed on entire plants, after drying and milling.

4.2 Plant cell structure and impact on extraction of intracellular nutrients

All living tissues contain cells and extracellular matrix (ECM). Animal and plant cells have some common constituents, such as endoplasmic reticulum, Golgi apparatus, ribosomes, mitochondria and a nucleus. All of these constituents are surrounded by cytoplasm and a cell membrane. Still, there are some important differences between animal and plant tissues that may affect chemical analyses, along with extractability and bioaccessibility of various nutrients.

A major difference between plant cells and animal cells is the presence of a cell wall in plant cells. The cell wall is a part of the ECM and in plant physiology the term ECM and cell wall are often used interchangeably. As stated in section 2.2.2, the cell wall of seaweeds, consist mainly of different large and complex polysaccharides (up to 90 % of the dry material). Proteins normally account for less than 10 % of the dry fraction of ECM and most of the proteins are associated with the polysaccharides, forming glycoproteins [90-92]. Many of the cell wall polysaccharides contain highly charged side-groups and thus, they have good water-binding capacities, forming viscous gels that are stable over a wide pH-range. The charged side-groups also make ionic interactions with the proteins, hindering protein extraction [93].

In marine environments variation of salinity is frequent. In order to counter these variations, seaweed cells possess an osmotic stress response [94]. As a response to lower salinity, water will flow into the intracellular vacuoles in which the ionic concentration is much higher than in the outside environment. As a rapid response, the size of the vacuole will increase and push the cell membrane towards the cell wall, increasing the turgor pressure of the cell. The cell wall will exert opposite pressure, hindering the cell from bursting. A secondary, slower response will release ions and low-molecular organic compounds from the vacuole to the cytosol, regaining the normal turgor pressure inside the cell. When exposed to higher salinity, the response will be reversed [94].

As stated in section 2.1., the algal cell is metabolically active, possessing a large amount of intracellular enzymes and stored proteins. Extraction and utilisation of these proteins is dependent on disruption of the cell wall. In most protein extraction protocols, tissues are exposed to hypotonic solutions, i.e. water or weak buffers. In order to level the osmotic difference between the cell and the environment, water or buffer will flow into the cells. In cells with no cell wall, i.e. animal cells, this sudden change in intracellular pressure will cause the cell to swell until it bursts, releasing its intracellular content into the solution. In plant and algae cells, on the other hand, the inherent osmotic stress response will hinder the cell from bursting, preventing extraction of intracellular proteins and enzymes. In order to ensure extraction of these compounds, it is thus necessary to apply additional stress factors, such as mechanical power or enzyme treatment, on the cell walls.

4.3 Biochemical analyses

For every nutrient there exists a set of different analytical methods, each of them having different qualities. There is no single answer to which method is the optimal and in the following section some of the analytical principles and choice of methods will be discussed.

4.3.1 Proteins and amino acids

The commonest method for determination of protein in foods is the Kjeldahl method [95], where the food sample is digested in concentrated sulphuric acid at high temperature, liberating the total nitrogen from the sample. Crude protein content may be calculated from the nitrogen content using a conversion factor. The original, and still quite frequently used, conversion factor is 6.25, assuming an average nitrogen content of 16 % in food proteins. However, the amino acid composition vary substantially between different food proteins and the relative nitrogen content in different amino acids varies from 8 – 32 % [96]. In addition, not all nitrogen in a food sample is protein-bound. A wide range of non-protein nitrogen (NPN) compounds, such as nitrate, ammonia, nucleic acids, free amino acids, urea, chlorophylls and alkaloids [97] may also be present. The content of NPN is normally greater in plant foods than in foods of animal origin [98]. These factors have to be taken into account before direct conversion of nitrogen to protein. Already in 1941, Jones [99] suggested that specific conversion factors should be adapted for different food proteins, where proteins from animal sources in general should have a higher conversion factor than proteins from plant sources. Throughout the years, specific conversion factors have been applied for more food proteins and as a result of more precise analytical methods they have also been adjusted [100]. Also for seaweeds, specific conversion factors have been calculated. Due to species variability in protein and NPN content between the different

seaweed classes, separate factors have been suggested for each class, namely 5.38 for brown algae, 5.13 for green algae and 4.59 for red algae, respectively [101]. Another possible drawback of the Kjeldahl method is that the experimental conditions are very harsh and that all nitrogen present in the sample is extracted, whether or not it is available for digestion. Potential differences in proteins available for utilization as a result of pre-treatment, such as heating, will therefore not be easily detected.

Although the Kjeldahl method has been modified and improved over the years, the recommended method for protein determination is amino acid analysis [102]. Here, the proteins are broken down to their constituent amino acids by hydrolysis of the peptide bonds. The amino acids are then analysed, most often chromatographically, and proteins are calculated as the sum of individual amino acid residues after deduction of water. As only amino acids are included in the calculation of protein content, this method is considered the most accurate. However, this method is quite time consuming and involves costly equipment and reagents and hence, it is not applicable to all laboratories. Another possible drawback concerning this method is the hydrolysis of proteins prior to analysis. Several protocols have been developed for this purpose, most of them including strong acids or bases and high temperatures [103], the commonest being hydrolysis for 24 hours at 110°C in 6M hydrochloric acid (HCl) [104]. This method is also the established method in our laboratory. These analytical conditions are, however, detrimental to some amino acids and may therefore underestimate the total amino acid content in the sample. Tryptophan is completely destroyed during acidic hydrolysis, but may be analysed after alkaline hydrolysis [105]. The loss of threonine, serine and tyrosine may be in the range of 5-20 %, while sulphur-containing amino acids are easily oxidized under acidic conditions. In addition, asparagine and glutamine will be deaminated during the process and will therefore be present as aspartic acid and glutamic acid, respectively. Some of these losses may be prevented or reduced by adding protectants such as 2-mercaptoethanol or phenol to the sample. Peptide bonds between groups of aliphatic amino acids such as alanine, valine and isoleucine has been shown to be resistant to hydrolysis and may need longer hydrolysis time or higher temperature in order to be completely hydrolysed [106].

In this thesis (**paper I-III**), the protein content of the seaweeds was reported based on the sum of amino acid residues, according to the FAO recommendations [102]. Several protein hydrolysis protocols were tested, but in the end it was decided that the established method was to be used. Following hydrolysis, the amino acids were chromatographically separated on an ion-exchange column, exposed to ninhydrin for post-column derivatization and UV-detection at 570nm (440 nm for proline and hydroxyproline). Identification of the amino acids was performed by comparison to commercially available physiological amino acid standards.

4.3.2 Extraction of lipids and analysis of fatty acid composition

The main function of food lipids is to provide energy and the main lipid class is triglycerides, i.e. three fatty acids bound to a glycerol backbone. In its report in 2003, FAO therefore stated that the preferred method for lipid determination is through analysis of fatty acids and further conversion to triglyceride equivalents. Gravimetrical determination is also acceptable [102].

There are several approved methods for extraction of lipids, the main difference between them being the choice of and ratios between solvents [107]. Lipid extraction yields from the same sample may differ substantially depending on the method used. This is mostly due to the different polarity of the solvents and the distribution of different lipid classes in the sample, as polarity differs greatly between different lipid classes.

Due to lack of knowledge on the distribution of different lipid classes in seaweeds, two different lipid extraction methods were applied on the seaweed samples (**paper I**), namely the AOAC method 946.15 [108] and the method described by Folch *et al.* [109]. The former uses the non-polar petroleum ether as solvent and is efficient for extraction of neutral lipids only, while the latter uses the more polar mixture chloroform/methanol (2:1, v/v) as solvent and thereby also extracts polar lipids. Two adaptations were applied to the Folch's method, the first being replacement of chloroform with dichloromethane as this solvent is regarded less toxic [110, 111]. Previous in-house testing has shown that this replacement does not affect either extraction yield or fatty acid composition. Secondly, the samples were homogenized with an Ultra Turrax T25 during extraction in order to increase cell wall disruption and hence, increase lipid extraction yield. No significant difference in extraction yield was observed between the two different extraction methods (Student's t-test).

Lipids extracted by the Folch's method were submitted to analysis of fatty acid composition. Also for this analysis there are several methods available. One of the most common methods is separation of fatty acids by capillary gas chromatography, followed by detection by either a flame ionization detector (FID) or a mass spectrometer (MS) and finally identification and quantification of the fatty acids. Performing analysis by FID is simpler and less time-consuming than by MS, but also less specific and selective. As identification of the fatty acids by FID is solely based on comparisons of retention times, it is also crucial to have access to suitable standards. Still, due to its simplicity, FID is the preferred method for routine analysis of simple mixtures of common fatty acids [112]. However, for complex samples, FID may be too unspecific and MS will be a better alternative. In this study (**paper I**), only FID detection was performed and this proved to be sufficient for fatty acid identification of the brown algae and the red alga *V. lanosa*. In the green algae and the red alga *P. palmata*, however, the fatty acid compositions were more complex and contained a large proportion of uncommon fatty acids. The

lack of commercial standards made the identification process difficult and hence, the proportion of unidentified fatty acids was as high as 10-15 % in these species.

4.3.3 Water, ash and minerals

Water and ash, which is a collective term for the content of inorganic matter in a food item, was determined gravimetrically, using slightly modified versions of the AOAC official methods 946.50B and 938.08, respectively [108].

All mineral analyses in this thesis (**paper I**) were performed using inductively coupled plasma mass spectrometry (ICP/MS) and reported as the total content of each mineral. As earlier mentioned, the properties of the minerals vary depending on their physiochemical state and especially when examining heavy metals, knowledge of physiochemical status may be important. For instance, arsenic is more toxic in its inorganic forms, while mercury is more toxic when present as methyl mercury (organic). Speciation of the heavy metals could thus have given additional information on potential risks of consumption.

4.3.4 Carbohydrates

The commonest way of reporting total carbohydrate content in foods is by difference, i.e. [100 % - (sum of protein, lipid, water, alcohol and ash)]. There are margins of error associated with all analytical methods. These will be accumulated when summing up results from several analyses and included in the estimation. In addition, this estimate does not give any information on carbohydrates available for energy [102].

Carbohydrates may be divided into available or resistant, depending on their digestibility in the human gastrointestinal tract. Available carbohydrates comprise sugars and starch, while resistant carbohydrates comprise resistant starch, non-starch polysaccharides, resistant short-chain carbohydrates and sugar alcohols [113]. The optimal method for determination of carbohydrates in foods would be by analysing the monosaccharide composition of each of these carbohydrate classes, following suitable pre-treatments for liberation. This is, however, laborious and time-consuming and is not applicable for routine analysis.

Dietary fibre, defined as intrinsic plant cell wall polysaccharides [114], have been found to have health beneficial properties and an increased intake of them is recommended. A precise determination of this class of carbohydrates is thus desired. The traditional, and official, method for determination of dietary

fibre (AOAC 985.29) is based on enzymatic degradation of available carbohydrates, followed by several washing steps and finally gravimetric determination of fibre, after deduction of contents of Kjeldahl protein and ash [108].

This method was tested and considered used for analysis of dietary fibres in the seaweeds in **paper I**. Due to a very time-consuming analytical procedure this was eventually omitted since the major aims in the thesis were focussed on algae proteins.

4.3.5 Gastrointestinal digestion

As previously described, gastrointestinal digestion is a complicated process and in order to examine digestibility or bioaccessibility of proteins or other food components, pre-clinical (animal) or clinical (human) intervention studies are thus optimal. However, such studies are very expensive and time consuming, and may also be associated with ethical concerns. In order to establish a fundament for *in vivo* studies, a series of basal studies, using either biochemical or cell-based *in vitro* models, should be performed. These study types are inexpensive and rapid and although very simplified, they may provide useful information on for instance reaction mechanisms and modes of action.

A variety of *in vitro* model systems have been developed and are being used in order to study hydrolysis of proteins. There are large variations in study setups among the different models, most often depending on the main purpose of the study. Care should thus be taken when comparing results from studies using different model systems.

Type and concentration of enzymes, reaction times, salts, buffers and pH-adjustments, mechanical stressors etc. are factors that vary between studies [115]. For instance, different proteolytic enzymes have different pH- and temperature optimums, and buffering or adjusting pH are factors that may affect the reaction. Enzymes may be endo-proteases or exo-proteases, cleaving peptide bonds within the protein or at the end of the proteins, respectively. Some enzymes depend on different metals as co-factors for optimal function and addition of salts in buffers may thus accelerate the process. And finally, mechanical or physical energy may also ease the reaction.

In our laboratory, several *in vitro* gastrointestinal digestion models have already been established [116, 117]. The model described by Dragnes *et al*. is quite simple, using purified proteolytic enzymes (pepsin, trypsin and chymotrypsin) dissolved in water. Two pH-adjustments are done during the experiment, the first (to pH 2.0, using HCl) concurrent with addition of pepsin and the second (to pH 6.5, using NaOH) after two hours of incubation and just prior to the addition of trypsin and chymotrypsin. Samples for analysis are collected 2.5 hours after addition of trypsin and chymotrypsin. The model

described by Sannaveerappa *et al*. is more complex, involving mixtures of enzymes and salts (pepsin, pancreatin and bile salts). In addition, this model involves multiple pH-adjustments, first gradually from pH 5.5 via pH 3.8 to pH 2.0, using HCl, during the first hour of the experiment and then gradually from pH 2.0 via pH 5.0 to pH 6.5, using NaOH during the final 90 minutes of the experiment.

Both of these methods were tested for digestion of seaweeds and none of them were considered suitable. In particular, the pH-adjustments were shown to be challenging in that the times used for pH-adjustment from 2.0 to 6.5 were long and variable between samples (up to 30 minutes for each sample). This resulted in reduced reaction times at optimal pH and temperature, and probably also to variable reaction times between samples.

The model described by Versantvoort *et al.* [118] was thus chosen (figure 3). In this model, the enzymes are dissolved in buffers mimicking the physiological conditions in the human gastrointestinal tract. First, salivary juice at pH ≈ 6.8 containing amylase is added to the seaweed sample. The mixture is homogenized and incubated at 37°C with constant stirring for 5 minutes. After collection of a sample for analysis, gastric juice at pH ≈ 1.3 containing pepsin is added, followed by incubation for two hours at 37°C with constant stirring and collection of a new sample for analysis. Then, pancreatic juice at pH ≈ 8.1 containing pancreatin and lipase and bile juice at pH ≈ 8.2 containing bile salts, along with bicarbonate is added. After a final incubation for two hours at 37°C with constant stirring, another sample is collected for analysis. The main advantages of this method compared to the others previously described is that it involves a mixture of all enzymes relevant for human digestion, the reaction times are physically relevant and no pH-adjustments apart from addition of buffers are necessary.

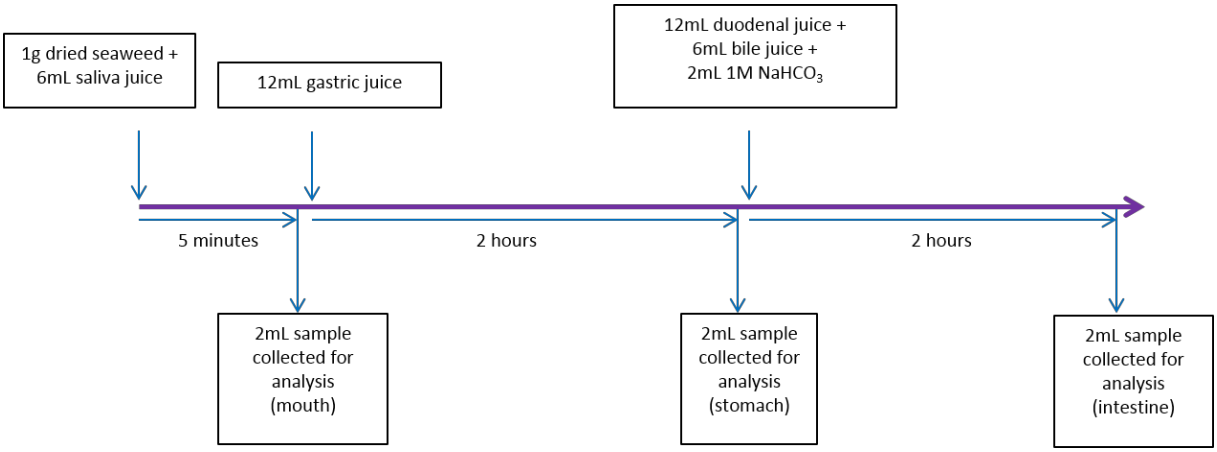

Figure 3: Schematic illustration of the simulated gastrointestinal digestion model.

4.3.6 Enzymatic treatment

There are large variations in types and concentrations of enzymes used in previous studies on enzymatic degradation of seaweed cell walls and no general protocol has been developed [93, 119, 120]. Thus, the protocol in this study was designed as a combination of several protocols. Due to the cell wall of *P. palmata* containing a large amount of xylans in addition to cellulose, both cellulase and xylanase, and a mixture of the two were tested for degrading the cell wall. The enzyme mixture was found to be most effective in increasing the available amino acids and thus, this was chosen for further experiments. The concentration of each enzyme was set to 100 U g^{-1} alga material, while incubation temperature and pH were decided based on the manufacturer's specifications on optima of the different enzymes.

4.3.7 Protein extraction

Proteins were originally divided into four main classes based on their solubility. These classes are albumins that are soluble in water, globulins that are soluble in salt solutions, glutelins that are soluble in dilute acids or bases and prolamins that are soluble in 70 % alcohol [81]. A thorough literature search did not reveal a complete characterisation of seaweed proteins and the choice of different extraction solvents in **paper III** was thus based on this classification, As Potter homogenisation had already been proved to increase the amount of accessible amino acids, all of these different extraction solutions were tested on homogenised samples.

Table 3: Overview of different extraction solvents, extraction times and frequency of agitation during protein extraction.

	0.01 M NaOH	0.05 M NaOH	0.1 M NaOH	0.2 M NaOH	0.5 M NaOH	1.0 M NaOH	0.1 M HCl	70 % ethanol	4 M Guanidine HCl
1h, intermittent agitation	X	X	X	X	X	X			
1h, constant agitation	X	X	X			X	X	X	X
3h, intermittent agitation	X	X	X						
3h, constant agitation	X	X	X			X	X	X	X
6h, constant agitation			X						
24h, constant agitation			X						

5 General discussion and main results

The overall aims of this project were to examine the nutritional quality, along with effects of processing on bioaccessibility and extractability of proteins from seaweeds, and to evaluate their suitability as food, feed and ingredients in such. In **paper I**, a screening of the biochemical composition of ten different seaweeds commonly found in Norwegian waters was performed. Based on the protein content and the amino acid composition found in this screening, two species (*Alaria esculenta* and *Palmaria palmata*) were chosen for assessment of effects of heat treatment on the bioaccessibility of seaweed proteins (**paper II**). One of these species (*P. palmata*) was also subjected to enzymatic pre-treatment in order to examine the effects on protein bioaccessibility and extractability (**paper III**).

5.1 Nutritional composition

Seaweeds are, as previously stated, a very heterogeneous group of plants and their nutritional composition is equally heterogeneous. Both geographical and seasonal variations within species have been described for most nutrients [83-85]. As seaweeds are photosynthetic organisms and primary producers of carbohydrates, lipids and proteins, the seasonal variations are largely due to variations in physical conditions such as light intensity, water temperature and nutrient availability. For instance, carbon uptake and utilization, and consequently carbohydrate synthesis, is strongly depending on light amount and intensity and will therefore vary greatly over the year. Likewise will geographical and seasonal fluctuations in the seawater contents of nitrogen and phosphorus affect synthesis of phospholipids, amino acids and proteins [121, 122].

5.1.1 Lipids

The lipid level of seaweeds is generally low, ranging from approximately 0.5 to 5 % on a dry weight basis [123, 124]. The fatty acid composition varies greatly, both between and within the different phyla. In general, green algae have higher content of saturated fatty acids, lower content of PUFA and higher n-6: n-3 ratio than brown and red algae. In addition, their content of fatty acids longer than 18 C-atoms is low, making them more similar to terrestrial plants than the other phyla. Both brown and red algae have a high relative content of LC-PUFA, ARA and EPA being the dominant fatty acids. The relative EPA content is generally higher in red algae than in brown and may be as high as 40 % in some species. The n-6: n-3 ratio is also generally lower in red algae than in brown [125, 126].

In this project, lipid content and fatty acid composition was a part of the screening performed in **paper I**. The results compared well with the general description given above. When evaluating health effects related to fatty acids, the contents of the two long-chained n-3 PUFA EPA and DHA, along with the content of the n-6 PUFA AA, is the main focus. This is due to them being the precursors of a range of compounds involved in acute and chronic inflammatory responses, namely eicosanoids, resolvins and protectins. Inflammation processes are central in a range of medical conditions, such as cardiovascular diseases, inflammatory bowel disease and rheumatoid arthritis [47]. The primary production of EPA and DHA takes place in the marine environment, mainly by phytoplankton and microalgae, making organisms higher up in the marine food web good sources of these fatty acids. However, also photosynthetic macroalgae are able to produce EPA and DHA to a certain extent [127].

The two red seaweeds *P. palmata* and *V. lanosa* had the largest relative content of EPA, 34% and 32% of the total fatty acids, respectively. The kelps contained between 7% and 11% of this fatty acid, while the wracks and the green seaweeds all had an EPA content < 5%. None of the algae contained DHA. In the kelps and the green algae, the level of the precursors of EPA and DHA, ALA and stearidonic acid (SDA, C18:4, n-3), were quite high. However, as stated in section 2.2.3., the conversion rate from the precursors to EPA and DHA is very low in humans and a high level of the precursors is thus no guarantee for increasing the human load of EPA and DHA. The contents of AA and its precursor LA were highest in brown algae, ranging between 4 to 8 % and 5 to 13 %, respectively. Green algae contained 5 – 10 % LA and no AA, while red algae were low in both, containing 0.5 – 6 % of LA and AA.

As mentioned in section 2.2.3., the ratio between n-6 and n-3 fatty acids in the Western diet today is between 10:1 and 20:1, mainly due to a very high dietary intake of LA, along with a low intake of ALA. This imbalance directly affects the endogenous conversion of LA and ALA into AA, EPA and DHA, and indirectly the formation of eicosanoids, resolvins and protectins. Thus, EPA and DHA should be provided through diet. Lowering the n-6: n-3 ratio has been suggested as a means to decrease the incidence of diseases related to inflammation and an optimal ratio is believed to be 2-5:1, depending on the disease in question [42]. Most of the algae analysed in **paper I** had n-6: n-3 ratios below 1, the exceptions being the wracks, having ratios of 2:1 and 2.6:1, respectively. Although some algae have high relative contents of EPA and favourable n-6: n-3 ratios, the lipid content of macroalgae is very low and thus, they cannot be characterized as very good dietary sources of LC n-3 PUFA.

5.1.2 Minerals

Ash content and a selection of minerals, including some heavy metals, were also analysed in **paper I**. Several studies have concluded that seaweeds are good sources of a range of minerals compared to

other common food products [49, 50, 128]. Seaweeds are especially known to contain large amounts of iodine [129], a trace mineral essential for the regulation of thyroid hormones and one of the trace minerals most commonly associated with deficiency disorders. The results from **paper I** confirmed the high contents of this mineral in all of the analysed species. Recommended daily intake of iodine is 150 µg for adults [130]. In order to achieve this, a daily intake of one gram or less would be sufficient for all of the seaweed species analysed in **paper I**, except for the two green algae *C. rupestris* and *U.lactuca*, where the sufficient amount would be 2 and 7 grams, respectively. However, like in most metabolic processes in the body, the regulation of thyroid hormone production is tightly controlled and as iodine is a central compound in this process, excess intake may disturb this regulation. An upper tolerable intake limit of 1100 µg day^{-1} for adults has therefore been established for this mineral [131]. For *L. digitata*, *L. hyperborea* and *V.lanosa* an intake of less than one gram would exceed this limit.

As stated in section 2.2.4., the other element associated with deficiency disorders is selenium. Like iodine, selenium is important for the regulation of thyroid hormones. In addition, being a central part of the glutathione peroxidase enzymes, it is recognized as one of the major endogenous antioxidants. Of the major sources of selenium in the Norwegian diet are fish and seafood, containing on average 300 µg kg^{-1} edible product [132]. In **paper I**, *P. palmata* and *V. lanosa* were shown to contain 140 and 530 µg kg^{-1} dried seaweed, respectively, which is in accordance with other studies [133, 134]. Recommended daily intake of selenium is 50 – 60 µg [130]. In order to achieve this by consuming these seaweeds, the consumption of them would have to be around 400 and 100 g dried seaweed day^{-1}, respectively, which would be highly unlikely.

The efficient mineral uptake of seaweeds is not entirely advantageous, as also heavy metals are taken up by similar mechanisms. These are associated with some adverse health effects, such as various cancers or impaired mental development. Limits for assumed safe intakes of these metals and other contaminants, so-called tolerable weekly intake (TWI) values and Benchmark Dose Limits (BMDL), have been set by the European Food Safety Association (EFSA). For arsenic, BMDLs have been calculated for various cancers, and the current limits are between 0.3 and 8 µg kg^{-1} body weight day^{-1}. These limits are set for inorganic arsenic, as this is the most toxic form of this metal [135]. For cadmium, the current TWI is 2.5 µg kg^{-1} body weight $week^{-1}$ [136] and for mercury, the TWIs are 4 and 1.3 µg kg^{-1} body weight $week^{-1}$ for inorganic and organic mercury, respectively [137]. In the analysed species, only arsenic levels were high enough to be of concern, as a weekly intake of 2.3 - 61 g of *L. digitata* could lead to exceeding the BMDL limits for a 70 kg person.

5.1.3 Proteins and amino acids

Brown seaweeds are generally lower in proteins than are green and red seaweeds. Most brown seaweeds contain less than 15 % DW of protein, the exception being *Undaria pinnatifida* (or wakame), which has been reported to contain up to 24 % DW. The variation in protein content among the green species is large, ranging between 5 % and 27 %, where of the *Ulva* family have the highest content (23-27 %). Also among the red seaweeds, the variation is large, ranging between 8 % and 44 %, *Porphyra spp*. (nori) being the richest ranging from 39 to 44 % [138].

In this project, protein content (given as sums of TAA) and amino acid composition were analysed in all three papers. Although being in the lower range, the results of the screening of different algae in **paper I**, showed a pattern similar to the one described above. Protein content in brown algae ranged between 5 and 9 %, in red algae it was around 12 % in both species, while the variation was greatest in green algae, ranging between 3 and 11 %.

As previously stated, there are several analytical methods for determination of protein content. The commonest is determination of total nitrogen with subsequent conversion to crude protein using a specific conversion factor, most often 6.25, as first described by Kjeldahl [95]. Also for seaweeds this method is frequently used and in a recent meta-analysis regarding protein determination in seaweeds, this was shown to have been used in 52 % of all studies [139]. The main drawback of this method, especially when used on plant materials, is that the conversion factor overestimates the protein content, mainly due to their high content of non-protein nitrogen. According to Angell *et al*. [139], this overestimation may be as high as 43 % compared to amino acid analysis, which was the method used in this project.

Based on the protein content and amino acid composition found in **paper I**, two species were chosen for further analyses, namely *A. esculenta* and *P. palmata*. For *P. palmata* there were no significant difference in total or essential amino acids between papers. This species is one of the best studied red seaweeds and the protein content vary greatly between studies, from 8 to 35 % has been reported, and the commonest value is around 20 % [140]. Comparing to the average value, the protein content in this study is in the lower range, but when taking the seasonal variations into consideration and comparing to another sample collected in June, it is similar [83]. Also the relative distribution of amino acids is comparable to other studies [83, 141]. Neither in *A. esculenta* the total amino acid content was different between papers and also for this species, the level was comparable to other studies [85]. The relative amount of essential amino acids was, however, significantly lower in **paper I** compared to **paper II**, which mainly reflected a much lower level of lysine.

As stated in section 2.2.1., essential amino acids have to be provided through the diet and intake of good quality protein is thus important. Normally, plant proteins are deficient in one or more essential amino acids, while proteins of animal origin are recognized as complete. In **paper II**, *P. palmata* protein and proteins of some important cereals were compared to the reference protein set by FAO as defined in table 1 [33]. This comparison showed that *P. palmata* and rice proteins contain sufficient amounts of all essential amino acids, different from corn and wheat who both are deficient in lysine (figure 4 in **paper II**). Considering the higher protein content of *P. palmata* compared to the three cereals, intake of this seaweed will provide significantly more essential amino acids than intake of equivalent amounts of cereals (figure 5 in **paper II**). Thus, seaweeds may be a valuable supplement of good quality proteins in cases where cereals are the main protein source.

Figure 4 shows an illustration of the essential amino acid composition (mg EAA g^{-1} protein) of the proteins of *P. palmata*, wheat and two common animal foods, namely cod and pork, compiled from two papers by Jensen *et al.* [142, 143] and **paper II**. This shows that for most essential amino acids, seaweed protein is also comparable to proteins of animal origin. The protein content in seaweeds is, however, much lower than that of animal foods and seaweeds will therefore not be a good replacement for animal proteins.

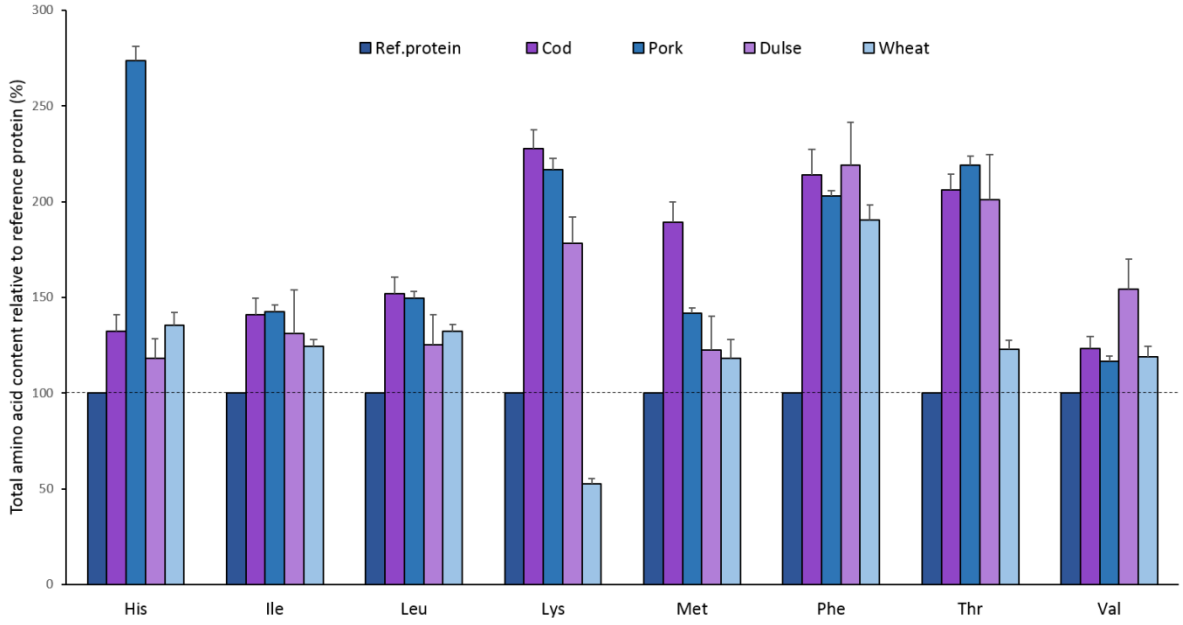

Figure 4: Compiled illustration of the essential amino acid composition (mg EAA g^{-1} protein) in cod (*Gadus morhua*), pork (*Sus scrofa domesticus*), dulse (*Palmaria palmata*) and wheat (*Triticum aestivum*) proteins relative to the reference protein set by FAO/WHO/UNU [33]. The values are given in % of the reference protein. Values for cod and pork are retrieved from Jensen *et al.* [143] and Jensen *et al.* [142], respectively and presented as mean ± SE (n = 10). Values for dulse and wheat are retrieved from paper II and presented as mean ± SD (n = 5).

5.2 Effects of processing

Several studies on protein rich plant material have shown that processing improves the protein bioaccessibility [144-146]. A thorough literature search has not revealed any studies regarding effects of processing on bioaccessibility of seaweed proteins. Two processing techniques were chosen for this project, heat treatment as a representative for a method commonly used in food preparation, and mechanical and enzymatic degradation of cell wall as a representative for a method more specific for seaweeds. The effects of different processing methods on the bioaccessibility of seaweed proteins were examined in **paper II** and **III**. In **paper II** the chosen method was heat treatment, while mechanical disruption and enzymatic degradation of cell walls were tested in **paper III**. The effects of processing were evaluated as amino acid accessibility, liberation of amino acids through an *in vitro* gastrointestinal digestion model and protein extractability.

5.2.1 Amino acid accessibility

In this project the amino acid accessibility was defined as the amount of amino acids liberated after acidic hydrolysis performed as described by Moore & Stein [104].

The total amino acid content in *P. palmata* raw material was 143.6 ± 3.7, 129.8 ± 11.4 and 165.7 ± 14.6 mg g^{-1} DW in **paper I, II and III**, respectively. In *A. esculenta*, the total amino acids were 107.2 ± 6.6 and 106.1 ± 9.1 mg g^{-1} DW in **paper I and II**, respectively. In **paper II**, the seaweeds were boiled in water for 15, 30 and 60 minutes. Heat treatment did not affect the amount of accessible amino acids in *A. esculenta* significantly, whereas in *P. palmata* the amino acid accessibility increased 88 – 109 % as a result of boiling. Boiling for 30 minutes resulted in the highest increase and is thus the treatment included in comparisons between processing methods.

In **paper III**, the processing methods chosen for increasing the amount of accessible amino acids were mechanical disruption of the cell wall by Potter homogenisation and enzymatic degradation of the cell wall polysaccharides. Both methods resulted in an increase in accessible amino acids, enzymatic degradation being 9.6-fold more effective than Potter homogenisation.

Comparing the three different processing methods (table 4), presented as relative change in accessible amino acids compared to their corresponding raw materials, it was seen that heat treatment was 3.5-fold more effective than Potter homogenisation. Enzymatic degradation of cell wall polysaccharides showed to be the most effective method for increasing the amount of accessible amino acids, being 2.6-fold more effective than heat treatment.

Table 4: Relative changes in accessible total amino acids in dulse (*Palmaria palmata*) subjected to three processing methods compared to their raw counterparts. The processing methods were boiling in water for 30 minutes (paper II), Potter homogenization (paper III) or enzymatic treatment (paper III). Values are presented as % change relative to the raw alga.

	Heat_30min	Potter	Enzyme
Essential amino acids (EAA)			
Threonine	110.5	45.8	284.2
Valine	109.0	40.7	306.3
Methionine	120.6	65.2	373.8
Isoleucine	112.4	48.3	319.8
Leucine	116.7	40.5	307.2
Phenylalanine	119.5	52.0	317.2
Lysine	117.6	72.3	285.4
Histidine	158.7	40.4	275.2
Tryptophan	n.a.	n.a.	n.a.
Non-essential amino acids (NEAA)			
Aspartic acid*	77.7	-14.6	201.0
Serine	114.8	41.3	292.0
Glutamic acid*	62.0	-25.0	197.0
Proline	21.7	-37.5	130.0
Glycine	102.8	26.6	238.5
Alanine	93.4	54.0	246.5
Cysteine	320.9	752.7	2135.3
Tyrosine	144.1	193.6	858.7
Arginine	130.8	86.1	375.3
Sum TAA	99.6	27.4	263.4

n.a.: not analysed

* Aspartic acid and glutamic acid represent the sums of aspartic acid + asparagine and glutamic acid + glutamine, respectively, as asparagine and glutamine are present in their acidic forms after acidic hydrolysis.

As mentioned in sections 2.2.1 and 2.3.2, some of the essential amino acids, in particular lysine, may be negatively affected by processing. The effect of processing on the content of essential amino acids was thus examined in both of these papers. Neither relative amount of essential amino acids nor relative content of lysine were significantly affected by the treatments (figure 4 in **paper II** and figure 3 in **paper III**). Boiling in water is considered a relatively gentle processing method, not exposing the food item to direct heat or very high temperatures. Thus, the negative effects on proteins are limited. The loss of free amino acids as a result of boiling was, however, significant. For *P. palmata*, the free amino acid content was 75 % lower after boiling compared to raw material (table 2 in **paper II**), while the corresponding loss for *A. esculenta* was around 60 %. The purpose of the enzymatic treatment applied in this study was to degrade the polysaccharides in the cell wall and the enzymes used were

polysaccharidases, whose targets are glycosidic bonds and not peptide bonds. The effects on the proteins was thus limited.

5.2.2 *In vitro* gastrointestinal digestion

Intact proteins are not absorbed through the gastrointestinal system, but they have to be degraded into free amino acids. Thus not only a high protein content, but also the digestibility of proteins is a measure of a food item's dietary value. The digestibility of plant proteins is as previously mentioned lower than that of animal protein, but several *in vitro* studies have shown that processing improves the digestibility of plant proteins [144-146]. Also for seaweeds *in vitro* models of digestibility have been performed, giving highly variable results, ranging from 2% to 90 % digestibility [83, 147-149]. However, there are large variations in choice of enzymes, reaction times and evaluation of digestibility between these studies, so direct comparison is difficult. In addition, none of these studies have included effects of processing.

The *in vitro* gastrointestinal digestion method used in this project was chosen due to its similarity to *in vivo* protein digestion, as types and concentrations of enzymes, along with reaction times are physically relevant. In both **paper II** and **paper III**, the results showed that the amount of liberated amino acids available for absorption increases throughout the digestion process for both raw and treated samples (figure 2a in **paper II** and figure 4 in **paper III**).

Figure 5: Total amino acids liberated after mouth, stomach and intestinal phases in boiled and enzyme treated *Palmaria palmata* related to the corresponding non-treated sample. Values are presented as % change related to raw alga.

In figure 5, the relative differences between raw and processed samples are shown for *P. palmata* boiled for 30 minutes and enzymatically treated *P. palmata*, respectively. As can be seen, the liberation of amino acids was higher in enzymatically treated samples than in the boiled samples at all sampling points during the digestion process. As stated in section 5.2.1 the amount of accessible amino acids increased by 99 % in the boiled samples and 263 % in the enzyme-treated samples. At the end of the digestion process the relative difference in liberated amino acids between raw and treated samples were 70 % for the boiled samples and 266 % for the enzyme-treated sample. This means that if defining the protein digestion as the amount of liberated amino acids in the gastrointestinal model relative to the amount of accessible amino acid in the undigested samples (table 3 in **paper II** and table 2 in **paper III**), it is seen that the relative digestibility is slightly decreased as a result of boiling whereas it remains the same in enzyme-treated samples. However, due to the increased accessibility as a result of treatment, the amount of utilizable protein increases.

5.2.3 Protein extraction

Protein extraction and purification have become frequently used techniques for optimal utilization of proteins from different sources. For instance in fish feed, where there has been a shift in protein sources from marine to terrestrial during the recent decades, the plant proteins are added as soy hydrolysates or wheat gluten and not as complete plants. This is due to the removal of indigestible fibres in the extraction process. In addition, in most extraction protocols the proteins are partially hydrolysed, making them more easily accessible for the fish.

Extraction of proteins from seaweeds have been presented in several papers, using different extraction protocols [83, 93, 119, 120]. As for the digestibility studies, the protocols are very different, making direct comparisons difficult. Most studies conclude that polysaccharidases are efficient in degrading the cell walls, but due to a generally low extraction yield, there are discrepancies in whether or not the use of enzymes is economically feasible in an industrial scale.

The purpose of **paper III** was to further develop and optimise existing protein extraction protocols in order to increase protein extraction yield. A range of extraction solutions, times and temperatures, along with mechanical or enzymatic disruption of cell walls were tested. The results showed that combining several principles was the most efficient approach and that the method giving the highest extraction yield was enzymatic pre-treatment by a combination of polysaccharidases, followed by alkaline extraction. This procedure resulted in a protein extraction yield of 38.3 %, a result that is higher than most other studies on the topic and shows that this procedure is promising as a means to increase

the utilization potential of seaweeds. Further optimisation of the method is nevertheless necessary in order to obtain maximal cost-benefit.

6 Conclusions and further work

The nutritional values of the seaweeds analysed in this project were mainly in accordance with previous findings as lipid contents were generally low, while mineral contents were high. Protein contents in red seaweeds were generally higher than in the other classes. However, the variation in protein contents within the brown and green seaweeds were large. The protein contents were generally lower than presented in many other studies, most likely due to methodological differences. However, also seasonal and geographical variations may have influenced these differences.

Containing sufficient amounts of essential amino acids in order to cover the human requirements, several of the seaweed proteins were recognized as complete proteins. Both protein content and quality of *P. palmata* were higher than that of wheat, corn and rice. A simulated gastrointestinal digestion model indicated that the amount of amino acids available for absorption after equivalent intakes were higher for *P. palmata* than for the three cereals, making this alga a better protein source in food and feed compared to the cereals.

In *P. palmata*, heat treatment increased the amount of accessible amino acids and the amount of amino acids liberated during a simulated gastrointestinal digestion model. The contents of essential amino acids were not negatively affected as a result of the process. For *A. esculenta*, no equivalent changes were observed, indicating that there are species differences.

Also enzymatic treatment of *P. palmata* increased the amount of accessible amino acids and the amount of amino acids liberated during a simulated gastrointestinal digestion model. The same treatment also increased the protein extractability.

A major limitation of this study was that only *in vitro* experiments were performed. Such basal studies may provide valuable indications in a range of fields, but confirming the results in pre-clinical and eventually clinical trials is warranted. At present, no data on true digestibility of seaweed proteins exist. Prior to increasing the use of seaweeds in food and feed, it is important to gain this knowledge through pre-clinical studies and an intervention study focussing on this is currently being planned.

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