THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

SEAWEED AS A SUSTAINABLE SOURCE OF FOOD PROTEIN

Maximizing seaweed protein content, protein recovery, and nutritional quality

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Department of Life Sciences

CHALMERS UNIVERSITY OF TECHNOLOGY

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Cover:
In the background, the ocean unites the fresh seaweeds - sugar kelp and sea lettuce - along with their protein ingredients as powders. *Painting made by my great and esteemed friend João Fernandes.*

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ABSTRACT

This thesis focuses on utilizing seaweed, such as *Saccharina latissima* and *Ulva fenestrata*, as sustainable food protein sources to complement terrestrial protein currently limited by land and water supply. While seaweed holds promise, its protein content is lower than pulses and antinutrients reduce protein nutritional quality. Additionally, *S. latissima* often contains excessive iodine, necessitating post-harvest blanching.

We aimed to produce protein-rich seaweed using food-process waters as nutrient sources; assess how blanching parameters impact downstream pH-shift-based protein extraction; create an efficient extraction method targeting aqueous-soluble and lipophilic proteins; and evaluate the influence of extraction on protein nutritional quality after *in vitro* digestion.

When food-process waters, mostly herring-derived, were added separately to the cultivation media of tank-cultivated *U. fenestrata*, protein content increased 2.4-fold compared to seawater media, reaching 24% per dry weight (dw). Growth rates generally remained unaffected and *S. latissima* was incompatible with this new nutrient loop.

Blanching sea-cultivated *S. latissima* at 45 or 80 °C for 2 minutes was equally effective at reducing iodine. However, biomass blanched at 45 °C provided higher protein extraction yields (23% vs. 14%). Iodine was still the limiting element for the daily adult consumption of extracts from blanched biomasses (0.5 g dw), though higher than extracts from crude biomass (0.1 g).

Employing 0.1-0.5% aqueous Triton X-114 during protein extraction from *U. fenestrata* followed by alkaline extraction provided a 3.3-fold increase in extraction yields (23%) compared to two alkaline extraction cycles. In both protocols, proteins were concentrated via isoelectric precipitation. It was confirmed that Triton disintegrated membranes, targeting also lipophilic proteins.

Digestibility of pH-shift extracts from *U. fenestrata* increased from 28% for crude biomass to 36%. Extraction also raised amino acid accessibility from 57% to 73%. When using the Caco-2 cell model, amino acids from *U. fenestrata* and extracts thereof were as bioavailable as casein.

Altogether, we raised seaweed protein content by recycling nutrients currently lost during food processing, improved protein extraction yields, and proved that extracts have higher digestibility than crude seaweed. Based on theoretical estimations, seaweed can offer a modest contribution to sustainable food systems, though this relies on scaling up seaweed production volumes.

Keywords: macroalgae, protein shift, cultivation, wastewater, protein isolation, elemental compositional, thermal water treatment, detergent, gastrointestinal digestion, intestinal permeability

LIST OF PUBLICATIONS

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

Paper I Stedt, K., **Trigo, J. P.**, Forghani, B., Nylund, G., Pavia, H., Undeland, I. Cultivation of seaweeds in food production process waters: evaluation of growth and protein content. Algal Research (2022), 63:102647.

Paper II **Trigo, J. P.**, Stedt, K., Schmidt, A. E. M., Kollander, B., Edlund, U., Nylund, G., Pavia, H., Abdollahi, M., Undeland, I. Mild blanching prior to pH-shift processing of *Saccharina latissima* retains protein extraction yields and amino acid levels of extracts while minimizing iodine content, Food Chemistry (2023), 404:134576.

Paper III **Trigo, J. P.**, Stedt, K., Steinhagen, S., Krona, A., Pavia, H., Abdollahi, M., Undeland, I. Harnessing the power of surfactants and alkaline aqueous solutions to efficiently solubilize and precipitate proteins from the seaweed *Ulva fenestrata.* Manuscript to be submitted.

Paper IV **Trigo, J. P.**, Engström, N., Steinhagen, S., Juul, L., Harrysson, H., Toth, G. B., Pavia, H., Scheers, N., Undeland, I. *In vitro* digestibility and Caco-2 cell bioavailability of sea lettuce (*Ulva fenestrata*) proteins extracted using pH-shift processing, Food Chemistry (2021), 356:129683.

OTHER SCIENTIFIC OUTPUT

Patent application based on Paper III duly filed with the Swedish Intellectual Property Office (PRV) on 31 August 2023:

Trigo, J. P., Undeland, I., Abdollahi, M. A process for extracting proteins from photosynthetic biomass.

Published papers during the PhD project not included in the thesis:

Paper 1 Jacobsen, M., Bianchi M., **Trigo, J. P.**, Undeland, I., Hallström E., Bryngelsson, S. Nutritional and toxicological characteristics of *Saccharina latissima*, *Ulva fenestrata*, *Ulva intestinalis*, and *Ulva rigida*: a review. International Journal of Food Properties (2023), 26, 2349-2378

Paper 2 **Trigo, J. P**., Bédard-Palmnäs, M., Juanola, M. V-L., Undeland, I. Effects of whole seaweed consumption on humans: current evidence from randomized controlled intervention trials, knowledge gaps, and limitations. Frontiers in Nutrition (2023), 10:1226168.

Paper 3 Stedt, K., Steinhagen, S., **Trigo, J. P.**, Kollander, B., Undeland, I., Toth, G. B., Wendin, K., Pavia, H. Post-harvest cultivation with seafood process waters improves protein levels of *Ulva fenestrata* while retaining important food sensory attributes. Frontiers in Marine Science (2022), 9:991359.

Paper 4 Sajib, M., **Trigo J. P.**, Abdollahi, M., Undeland, I. Pilot-Scale Ensilaging of Herring Filleting Co-Products and Subsequent Separation of Fish Oil and Protein Hydrolysates. Food and Bioprocess Technology (2022), 15, 2267–2281.

Paper 5 **Trigo, J. P.**, Alexandre, E. M. C., Oliveira, A., Saraiva, J. A., Pintado, M. Fortification of carrot juice with a high-pressure-obtained pomegranate peel extract: chemical, safety and sensorial aspects. International Journal of Food Science and Technology (2020), 55, 1599-1605.

CONTRIBUTION REPORT

Paper I João P. Trigo (JPT), as the second author, co-conceived the design of the study and performed the chemical characterization of the food process waters. Interpretation of data and writing parts of the manuscript corresponding to those analyses were under JPT's main responsibility. Further, JPT participated in the manuscript revision.

Paper II JPT, as the first author, co-conceived the design of the study and performed all the experimental work and analyses except for the seaweed cultivation as well as elemental and monosaccharide composition. Interpretation of results, manuscript drafting, and coordination of the editing of the manuscript were under the main responsibility of JPT.

Paper III JPT, as the first author, co-conceived the design of the study and performed all the experimental work and analyses except for the seaweed cultivation and microstructure analysis. Interpretation of results, manuscript drafting, and coordination of the editing of the manuscript were under the main responsibility of JPT.

Paper IV JPT, as the first author, co-conceived the design of the study and performed the protein determination and characterization methods, protein degree of hydrolysis, phenolic content, all calculations, and statistical analysis. Interpretation of results, manuscript drafting, and coordination of the editing of the manuscript were under the main responsibility of JPT.

ABBREVIATIONS

ATP	Adenosine 5′-triphosphate
CMC	Critical micelle concentration
DH	Degree of hydrolysis
dw	Dry weight
EFSA	European Food Safety Authority
EU	European Union
fw	Fresh weight
GHG	Greenhouse gas
GulA	Guluronic acid
HPLC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HPP	High pressure processing
HR-ICP-MS	High resolution inductively coupled plasma mass spectrometry
IMTA	Integrated multi-trophic aquaculture
ManA	Mannuronic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
OAT	Oat processing water
PEF	Pulsed electric field
PRI	Population reference intake
RAS	Recirculated aquaculture systems
RSW	Refrigerated seawater
SB-I	Salt brine I, i.e., from herring pre-salting
SB-II	Salt brine II, i.e., from ripening of herring in salt brine
SBW	Shrimp boiling water
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SGR	Specific growth rate
Sp.	Species
SPI	Spice brine
TAA	Total amino acids
TDI	Tolerable upper daily intake
TEAA	Total essential amino acids
TUB	Tub water, i.e., from pre-processing storage of whole herring in 3% salt

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Em memória de todo o orgulho que tinham no vosso neto, meus Queridos avós e eterna "abó" Adelaide

In memory of all the pride you had in your grandson, my dear grandparents and eternal grandmom Adelaide

1. INTRODUCTION

Global greenhouse gas (GHG) emissions from the livestock industry, including the required feed production represent 57% of all GHG emissions from the production of food and consume 8% of the global water supply (Schlink et al., 2010; X. Xu et al., 2021). Also, out of all agricultural land, the share taken up by grazing and feed production contributes to 77% (Gerber et al., 2013). Therefore, the expansion of terrestrial protein sources, especially livestock-based, to accommodate the diet demands of the ongoing demographic and socioeconomic growth is already constrained by pollution and access to arable land and fresh water. Hence, shifting towards alternative protein sources such as plants, insects, fungi, and algae, and to some extent, moderating the intake of protein-dense diets could serve as strategies to mitigate the environmental impact of food production (Ridoutt et al., 2021).

Proteins from marine organisms, such as seaweed, emerge as promising contributors to a more sustainable food system. Seaweed cultivation has a near-zero carbon footprint, does not require arable land, irrigation, or pesticides/insecticides, and can counteract eutrophication (Gephart et al., 2021). In addition, it is a key organism in seawater habitats ecosystems, which currently are threatened due to climate change (Cotas et al., 2023). Besides that, it can be a source of essential fatty acids, vitamins and minerals (Harrysson et al., 2018; Holdt & Kraan, 2011; Martínez–Hernández et al., 2018), while its characteristic umami- and ocean-like taste make it possible to create seafood diversification and develop food products with distinct sensorial properties such as fish analogues.

However, seaweed typically contains up to 11-35% protein on a dry weight basis (Holdt & Kraan, 2011). While this is a relatively good content, it is still lower than terrestrial protein crops e.g., soybean and far below fish and meat. Also, certain protein nutritional parameters such as digestibility have been reported as low due to polysaccharide-rich cell walls and the presence of phenolics (Fleurence et al., 2017; Tibbetts et al., 2016). Moreover, particularly brown seaweeds, can contain excessive amounts of iodine and non-essential elements, why post-harvest blanching is often required to reduce levels of some of these elements. Based on these challenges, the present PhD thesis, a core part of the Swedish CirkAlg project, was based on the premise that protein levels of seaweed can be improved through two approaches: (*i*) modifying media composition during its cultivation in tanks, and (*ii*) up-concentrating protein from crude and blanched seaweed using extraction methods. Moreover, such extraction methods can remove anti-nutrients, they were expected to improve protein digestibility and bioavailability. At the start of this PhD project, very little information was available on cultivation regimes to raise seaweed protein levels, and on protein extraction methods providing acceptable yields.

Given their economic significance in the emerging European seaweed aquaculture sector, the brown seaweed *Saccharina latissima* (common name "sugar kelp") and the green seaweed *Ulva fenestrata* (common name "sea lettuce") were identified as particularly important species to focus on from a protein perspective (Araújo et al., 2021). In this work, *U. fenestrata* refers to the northern hemisphere *Ulva lactuca*, which was reclassified with the former designation in 2019 (Hughey et al., 2019).

2. AIMS, RESEARCH GAPS & HYPOTHESES

This thesis aimed to evaluate the potential of seaweeds as food protein sources using *S. latissima* and *U. fenestrata* as the main model species. Specific experimental and theoretical aims were to:

- **a)** Screen different seaweed species and alternative nutrient sources for tank cultivation to identify the best combinations delivering high protein content and biomass specific growth (**Paper I**);
- **b)** Evaluate the effect of species and post-harvest treatments on biomass characterization in terms of ash, total monosaccharides, total amino acids, and elements (**Papers II-IV**);
- **c)** Assess the effect of different blanching temperatures and soaking on the extraction of proteins during pH-shift processing of *S. latissima* (**Paper II**);
- **d)** Invent a protein extraction method for *U. fenestrata* able to deliver higher yields than traditional methods (**Paper III**);
- **e)** Study the relationship between amino acid yield and total amino acid content of seaweed protein extracts, utilizing a combination of experimental data and relevant literature;
- **f)** Investigate potential deviations between high throughput protein determination methods and amino acid analysis when employed for the quantification of total protein extraction yields from seaweed, using experimental and literature data;
- **g)** Evaluate the effect of species, post-harvest treatments, and protein extraction method on protein extract characterization in terms of ash, total monosaccharides, total amino acids, elements, and protein/polypeptide size (**Papers II-IV**);
- **h)** Study how protein extraction from seaweed affects the limiting amino acids and amino acid chemical score (**Papers II-IV**);
- **i)** Examine the influence of pH-shift protein extraction in *in vitro* digestibility, amino acid accessibility, and amino acid bioavailability using the Caco-2 cell model (**Paper IV**);
- **j)** Calculate the potential global production and annual productivity of seaweed protein ingredients and compare it to plant-based protein sources, based on a combination of experimental and literature data.

Based on existing knowledge, identified main research gaps, and the above-stated experimental aims, a series of hypotheses were formulated which are presented in **Table 1**. The validity of each hypothesis is presented in Section 6.

Table 1 – Existing knowledge, research gaps, and formulated hypotheses for Papers I-IV.

Paper	Existing knowledge	Key references	Main research gap	Hypothesis
I	Cultivation of seaweeds with waters from fish aquaculture raised protein content of seaweed	Stedt, Pavia, et al. (2022)	There are no publications on process waters from the food industry as nutrient sources for seaweed	The protein content of seaweed would increase by adding food process waters rich in inorganic nutrients to the cultivation media (**H1**)
II	Thermal treatments are known to make microalgae less desirable for protein extraction	Wang et al. (2019)	The effect of blanching on seaweed protein extraction has yet to be addressed	Blanching of *S. latissima* would reduce protein extraction yields due to denaturation (**H2**)
III	Surfactants have been extensively used, especially in molecular biology, to extract membrane proteins	Smith (2017)	A complete extraction method for photosynthetic biomasses targeting soluble and insoluble proteins is yet to be reported	Sequential use of surfactant-containing and alkaline solutions would solubilize aqueous and lipophilic proteins from seaweed, thus increasing total extraction yields (**H3**)
IV	Protein digestibility of crude seaweed or seaweed protein extracts is dependent on the content of e.g., fiber, phenolics, and trypsin inhibitors	Fleurence et al. (2017); Tibbetts et al. (2016)	Crude seaweeds and respective protein extracts have not yet been compared in terms of protein digestibility and amino acid bioavailability	Structural disintegration and partial removal of antinutrient factors during protein extraction would improve digestibility and bioavailability (**H4**)

3. BACKGROUND

3.1. Seaweed and its taxonomy

Seaweed, also known as macroalgae, plays a key role in marine ecosystems by providing habitat and food for a variety of marine organisms. Despite converting carbon dioxide and water into organic matter through photosynthesis, they are not directly comparable to vascular plants. Seaweeds lack roots, stems, and leaves. Instead, they have thallus-like structures that vary in shape and size, ranging from small filamentous forms to large and complex structures. Moreover, although seaweed is often regarded as a multicellular organism it is important to highlight that it also encompasses unicellular representatives as seaweeds undergo unicellular stages at some stage in their life cycles, such as during the formation of spores, gametes, and zygotes (Hurd et al., 2014).

Traditionally, seaweeds have been categorized into three main groups based on their pigmentation: *Phaeophyta* (brown seaweed), *Rhodophyta* (red seaweed), and *Chlorophyta* (green seaweed). However, an examination of its taxonomy reveals that brown seaweed is phylogenetically very distant from red and green seaweeds as it belongs to the *Chromista* kingdom, while red and green seaweed to the *Plantae* kingdom. Therefore, red, and especially green seaweed, show a closer phylogenetic affinity to legumes, whereas brown seaweeds are more closely related to diatoms (Guiry & Guiry, 2022).

3.2. Seaweed aquaculture production and commercial applications in Europe

Global production of seaweed reached 36 million metric tons in 2020, of which less than 1% corresponded to seaweed produced in Europe. Moreover, in the same year, aquaculture was the predominant global source of seaweed with around 97% of the total amount being harvested with this strategy versus less than 2% in Europe (Araújo et al., 2021; FAO, 2022). Aquaculture-based seaweed production in Europe, which can prove a continuous and reliable biomass supply, is currently in its initial stages. However, the sector is witnessing a surge in interest and development, not the least due to EU policies and communications aiming to foster the development of an expanded seaweed aquaculture industry (European-Comission, 2018, 2022). As of 2020, around 46 companies were spread over Europe, with Norway being the country with the most companies producing aquaculture-based seaweed (13), followed by Spain (7), and Denmark (6) (Araújo et al., 2021). In the case of Sweden, only 1 company was compiled, however, at least 3-4 more have entered the market since then (Personal communication, Ingrid Undeland, 2023).

Around 90% of the total amount of seaweed harvested from aquaculture in Europe is brown seaweed, which can be explained by their relatively high productivity and simple life cycles (Hurd et al., 2014; Stévant, Rebours, et al., 2017). The remaining 10% includes *Ulva sp.,* while other species such as *Palmaria palmata* are cultivated by at least 6 companies each, without production volumes being available (**Table 2**). Interestingly, production volumes from harvesting of wild stocks are dominated by *Laminaria sp.* and *Ascophyllum nodosum* with around 292 000 tons of fresh weight harvested annually (Araújo et al., 2021).

Table 2 -Production amounts of aquaculture-based seaweed in Europe and number of companies that cultivate it. Adapted from Araújo et al. (2021).

Seaweed species	Production (tons fresh weight year^{-1})	Number of companies	Note
Saccharina latissima	376	26	-
Alaria esculenta	107	16	-
Ulva sp.	50	10	217 tons from wild stocks
Laminaria sp	n.a	8	209 772 tons from wild stocks
Palmaria palmata	n.a	6	455 tons from wild stocks
Others (*Chondrus crispus*, *Codium* sp., *Gracilariopsis longissima*, *Gracilaria* sp., *Porphyra* sp., *Undaria* sp.	n.a	26	-

n.a *not available*

Based on **Table 2** and the ongoing open-sea cultivation of seaweed by our industrial collaborators in the CirkAlg project, we opted for *S. latissima* and *U. fenestrata* as primary model species in this thesis.

Commercial applications of aquaculture-based seaweed in Europe have not yet been examined in detail, however, it is known that around half of seaweed producing companies direct their biomass to food or food-related uses such as hydrocolloid extraction and food supplements (Araújo et al., 2021). Moreover, in Europe, the market value of seaweed for human consumption is around 840 million euros and although the hydrocolloid market is saturated, a market study revealed there is plenty of room for other food applications to expand (Bergmans et al., 2021), such as production of seaweed protein ingredients.

3.3. Seaweed as a food protein source

3.3.1. Nutritional composition of whole seaweed as a protein source

Seaweed has a low to relatively high protein content depending on e.g., the species as shown in **Table 3** reporting the composition of the seaweed species listed in **Table 2** grouped by pigmentation.

Table 3 – Macronutrient composition (% of total dw) of seaweed species listed in Table 2 and comparison to terrestrial protein sources of plant and animal origin.

	Carbohydrates	Protein	Ash	Lipids	References
Brown seaweed	41-72	4-11[a]	20-48	1	(Albers et al., 2021; Harrysson et al., 2018; Schiener et al., 2015; Stévant, Marfaing, et al., 2017)
Green seaweed	26-48	5-20[a]	17-22	1-4	(Harrysson et al., 2018; Marsham et al., 2007; Morrissey et al., 2001; Steinhagen et al., 2021, 2022)
Red seaweed	42-75	8-35[a]	9-32	<1-3	(Galland-Irmouli et al., 1999; Harrysson et al., 2018; Marsham et al., 2007; Mouritsen et al., 2013; Rødde et al., 2004; Vasconcelos et al., 2022)
Soybean	33	40	5	22	(USDA, 2018a)
Beef	< 0.1	65	3	33	(USDA, 2018b)

Note that all data was retrieved from seaweed cultivated in Europe, regardless of whether it was harvested from aquaculture or wild stocks.
[a]Total crude protein (N×5)

Carbohydrates constitute the primary compositional fraction of seaweed and the lowest ranges have been reported for green species, which in this case only includes *Ulva sp.* (**Table 3**). The carbohydrate content of legumes such as soybean falls within the range for this species., however, the carbohydrate profile differs between the two: around 10 % of the total dw in soybean are soluble sugars and the remaining carbohydrates are dietary fiber i.e. polysaccharides, whereas, in *Ulva sp.*, most carbohydrates are dietary fiber (Berk, 1992; Holdt & Kraan, 2011). A similar trend is also found for brown and red seaweeds compared to soybeans (Holdt & Kraan, 2011). There is a high diversity in terms of polysaccharides between different colors of seaweeds: brown seaweeds primarily contain alginate, fucoidan, and laminarin; green seaweeds contain sulphuric acid polysaccharides such as ulvan, as well as sulfated galactans and xylans, and starch; red seaweeds contain agar, carrageenan, xylans, and

floridean starch. Most seaweeds contain cellulose, regardless of their color (Holdt & Kraan, 2011; Hurd et al., 2014; M. Prabhu et al., 2019).

Proteins are either the second or third most abundant fraction, alternating with ash. Compared to brown seaweeds, green and red species are closer in terms of protein content to soybeans. However, compared to raw beef, both soy and seaweed contain at least 2 times less protein on a dw basis (**Table 3**). Assuming the reference body weight for an adult of 63.3 kg (average of male and female) and the protein requirement of 0.75 g kg^{-1} day^{-1} (EFSA, 2019; WHO/FAO/UNU, 2007), one would need to consume at least 431, 238, and 148 g dw of brown, green, and red seaweeds, respectively, to fulfill this requirement, versus 119 g dw for soybean and 73 g dw (or 239 g fw) for beef. It should be noted that seaweed is often consumed in dry or wet form (Trigo et al. 2023). These consumption amounts suggest that for seaweed to become a protein source, its protein levels need to be improved during cultivation (Section 3.4), and/or proteins need to be up-concentrated via extraction methods (Section 3.5), the latter as already done with soybean proteins. It is also important to highlight that the protein requirement of 0.75 g kg^{-1} day^{-1} assumes that all dietary protein has the same digestibility as egg protein (WHO/FAO/UNU, 2007). However, seaweed has already been shown to contain relatively high amounts of dietary fiber, while eggs are fiber-free (Livsmedelsverket, 2023a). Therefore, the consumption estimations presented here are likely undercalculated since in Section 3.3.1 dietary fiber has been identified as a primary factor contributing to the moderate *in vitro* digestibility of proteins in whole seaweed.

Ash, which primarily consists of minerals, is higher in seaweed than soybean or beef (**Table 3**). The most abundant minerals in seaweed are sodium, potassium, calcium, and magnesium (Rupérez, 2002). Moreover, brown seaweed species such as *S. latissima* often contain high levels of iodine and non-essential elements, such as cadmium, lead, and inorganic arsenic (Jordbrekk Blikra et al., 2021; C. W. Nielsen et al., 2020; Stévant et al., 2018). Although iodine is an essential element, high levels of it can be toxic to humans.

Lipids are a minor fraction in the seaweed, with green and red seaweeds showcasing the highest maximum ranges, although still below what is found in soybean and raw beef (**Table 3**). Nonetheless, particularly red seaweeds can contain relatively high levels of the essential fatty acid eicosapentaenoic acid (EPA), with around 1% of the total dw (Harrysson et al., 2018; Mouritsen et al., 2013), while it is not found in soybean in detectable amounts (Livsmedelsverket, 2023b).

3.3.2. Protein quality

The protein quality is influenced by parameters such as amino acid profile, protein digestibility, and amino acid bioavailability (Conde et al. 2013).

Amino acid profile

According to **Table 4**, the total essential amino acid (TEAA) content of the seaweeds listed in **Table 2** is within what is reported for soybeans and beef. An examination of the individual amino acids reveals that seaweed seems a richer source of valine compared to soybean, while the latter is a relatively richer source of histidine. Among the three seaweed groups, red and brown seaweed have a slightly higher TEAA compared to green seaweed, probably due to the relatively higher content of methionine in the former two in comparison to green seaweed and also soybean. Based all these data, soybean and

seaweed proteins complement each other, making them a good combination for delivering all essential amino acids in proportions similar to those found in beef.

Table 4 – Essential amino acids (% of total amino acids) of seaweed species listed in Table 2 and comparison to terrestrial protein sources of plant and animal origin.

	Essential amino acids (% of total amino acids)								TEAA	References
	His	Ile	Leu	Lys	Met	Phe	Thr	Val		
Brown seaweed	1.5-2.6	4.8-5.5	6.8-10.1	4.9-8.9	2.0-3.0	4.5-6.1	4.7-6.8	5.8-7.7	36-49	(Abdollahi et al., 2019; Harrysson et al., 2018; Mai et al., 1994; Tibbetts et al., 2016)
Green seaweed	1.3-2.2	4.1-6.1	4.4-9.2	4.3-6.3	0.6-1.8	4.1-6.1	4.6-6.4	6.3-7.7	34-44	(Garcia-Vaquero et al., 2017; Harrysson et al., 2018; Mai et al., 1994)
Red seaweed	0.5-2.2	3.7-5.3	7.1-8.6	3.3-8.2	1.0-3.2	4.3-5.5	3.6-5.5	6.2-7.3	35-48	(Harrysson et al., 2018; Mouritsen et al., 2013; Tibbetts et al., 2016)
Soybean[a]	3.0	5.4	9.0	7.4	1.5	5.8	4.8	5.5	42	(USDA, 2018a)
Beef[a]	3.4	4.7	8.2	8.8	2.7	4.1	4.1	5.2	41	(USDA, 2018b)

TEAA *total essential amino acids*

[a]To allow a fair comparison between the reference proteins and seaweed data that often did not quantify cysteine and tryptophan, those amino acids were removed from the calculations for the reference proteins.

Protein digestibility

Protein digestibility is defined as the release of free amino acids and/or peptides from food during passage through the gastrointestinal tract. Typically, *in vitro* models are utilized as screening tools before conducting *in vivo* digestibility trials, aiming to reduce costs and bypass ethical constraints (Guerra et al., 2012). These models can be static, where they are conducted within a beaker or test tube, and have proved to be a good predictor of true digestibility (Bohn et al., 2018). They commonly encompass three sequential steps that replicate oral, gastric, and intestinal digestion by adding α-amylase, pepsin, and pancreatin with bile salts, respectively. Other parameters such as incubation time, temperature, pH, and enzymatic activity are also controlled to be as close as possible to physiological conditions. Limitations of these models include the lack of e.g., peristaltic movements, gradual addition of gastric fluid and gastric emptying, as well as treating the intestinal phase as one phase rather than as the sequential duodenal, jejunal, and ileal phases. Therefore, the static model is more suitable to evaluate digestion endpoints rather than kinetics (Brodkorb et al., 2019).

Evidence from the literature on protein digestibility of whole seaweeds is highly variable in terms of e.g., which *in vitro* digestion protocol was used. This makes comparisons across studies difficult. Nevertheless, available data suggests that protein digestibility is highly dependent on the species, regardless of its pigmentation (Bikker et al., 2020; Maehre et al., 2016; Tibbetts et al., 2016). Also, brown, green, and red seaweeds have lower protein digestibility than a soybean meal used for feed containing 46-52% protein dw (Bikker et al., 2016, 2020). Further, *P. palmata* showed an *in vitro* protein digestibility of 56-58% relative to that of casein (Galland-Irmouli et al., 1999; Marrion et al., 2003). **Table 5** enumerates potential reasons for the moderate protein digestibility of whole seaweed as well as the underlying mechanisms.

Table 5 – Possible reasons that influence protein digestibility of whole seaweeds and underlying mechanisms.

Possible reasons for moderate protein digestibility	Underlying mechanism(s)	References
Relatively high fiber content	- Protein is less likely to be accessible to the digestive enzymes - Fibers increase the viscosity of the chyme, slowing protein digestibility	(Mæhre et al., 2016; Marrion et al., 2003)
Intact cell walls	- Protein is less likely to be accessible to the digestive enzymes	(Marrion et al., 2003)
Proteins bound to or embedded in the cell wall	- Same mechanism as in "intact cell walls"	(Mæhre et al., 2016)
Presence of glycoproteins	- Glycosylation protects proteins against digestion by digestive enzymes	(Fleurence et al., 1999)
Presence of antinutrients such as phenolics, lectins, phytic acid, and protease inhibitors	- Protein is less likely to be accessible to the digestive enzymes due to phenol-mediated protein polymerization - Phenolics, phytic acid, and protease inhibitors (e.g., trypsin inhibitors) can reduce the efficiency of protein digestion, by inhibiting the action of digestive enzymes - Lectins can interact with intestinal epithelial cells, modifying their permeability (effect likely only captured with *in vivo* or cell models)	(Lund, 2021; Oliveira et al., 2009; Tibbetts et al., 2016; Vizcaíno et al., 2020)

Amino acid bioavailability

Following *in vitro* gastrointestinal digestion, assessing bioavailability alongside digestibility can provide a more accurate estimation of protein quality. This is due to the subsequent peptide degradation facilitated by cytoplasmatic and brush border peptidases produced by the intestinal absorptive cells i.e., enterocytes as illustrated in **Figure 1** (Wahbeh & Christie, 2011; Xu et al., 2019). Bioavailability is defined as the fraction of digested protein that is transported from the intestinal lumen to the bloodstream (Xu et al., 2019). In contrast to costly animal models characterized by restricted screening capacities, simple, reproducible, and relatively cheap intestinal cell models are extensively employed to study bioavailability. Over the years, several cell lines, such as Caco-2, HT-29, and Intestinal epithelial cell-6 have been used to simulate the human intestine *in vitro*. Among these, the Caco-2 cell model stands out as the most widely applied intestinal cell model as discussed in Section 4.8.4. However, intestinal cell models exhibit some limitations, such as they: (i) usually do not replicate peristaltic motions; (ii) lack representation of all intestinal cell types (especially monoculture models); (iii) do not produce the intestinal mucus layer (except models with HT-29 cells); and (iv) often do not capture complex host-microbiota interactions (Lea, 2015).

At the start of this PhD project, cell models or *in vivo* studies had not yet assessed the amino acid bioavailability of whole seaweed, protein extracts thereof, or even other photosynthetic biomasses e.g., leaves and grass. A previous study on protein isolates from chickpeas and lupin found that amino acids from the digested isolates were absorbed at a slower rate compared to lactalbumin and casein (Rubio & Clemente, 2009).

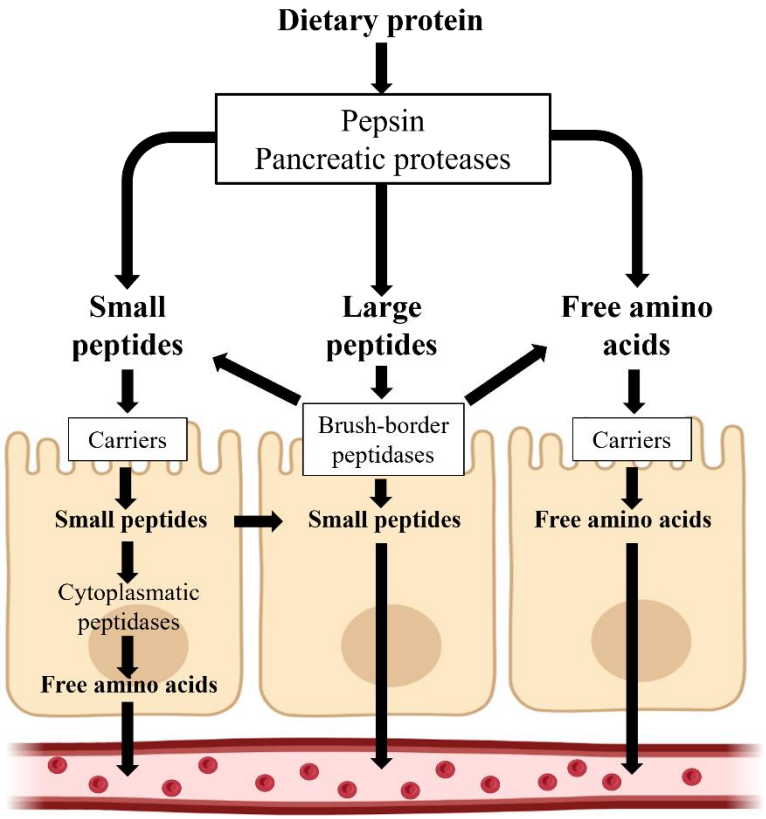

Figure 1 – Overview of digestion and absorption of dietary protein (Wahbeh & Christie, 2011; Xu et al., 2019).

3.4. Protein levels in seaweed and strategies to maximize it

As outlined in Section 3.3.1, protein levels of most seaweed species cultivated in Europe need to be raised to increase the ratio of protein to fiber and thereby improve protein digestibility. Also, to narrow the gap in protein content to terrestrial protein crops.

3.4.1. Abiotic factors affecting seaweed protein levels

By understanding which abiotic factors (i.e., non-living physical and chemical components) influence protein accumulation in seaweed one can manipulate cultivation aiming to maximize protein levels. It is known that different species can respond differently to a given abiotic factor (Coaten et al., 2023). **Table 6** lists multiple abiotic factors that have been proven to influence the protein content of the main model species included in this PhD project.

It has also been reported that some abiotic factors can interact with each other. For instance, Harrysson et al. (2018) reported for *U. fenestrata* that combining nitrate addition (5, 150, and 500 µM) with high addition levels of phosphate (50 µM) has a detrimental effect on protein content, compared to low phosphate levels (<1 µM). When it comes to non-abiotic factors, Steinhagen et al. (2021) found that gamete density at hatchery stages did not influence the protein content of open-sea farmed *U. fenestrata*. Other non-abiotic factors such as breeding and genetic techniques could however be used to obtain new seaweed strains (Khan et al., 2023) with e.g., high protein levels.

Table 6 – Abiotic factors influencing protein content in *U. fenestrata* and *S. latissima*.

Abiotic factor	Seaweed	Change leading to higher protein levels	Control group	Reference
Enviromental-related	*U. fenestrata*	↓ Irradiance (50 µmol m^{-2} s^{-1})	100 and 160 µmol m^{-2} s^{-1}	(Toth et al., 2020)
	S. latissima	↓ Water temperature (13 °C)	18 °C	
		↓ Water temperature	- (correlation)[1]	(Coaten et al., 2023)
		↑ Wind speed		
		↓ Relative humidity		
		↓ Atmospheric pressure		
		↑ August-September[2]	April-July	(Forbord et al., 2020)
Depth		↑ Depth (8-9 m)	1-2 m	
Media/seawater composition	*U. fenestrata*	↑ Nitrate (150, 500 µM)	< 5 µM	(Toth et al., 2020)
		= Phosphate (< 1 and 50 µM)	-	
		= pCO_2 (200, 400, and 2500 ppm)	-	
	S. latissima	↑ Nitrite	- (correlation)[1]	(Coaten et al., 2023)
		↑ Nitrite		
		↓ Phosphate		
		↑ Ammonium		
		↓ Total phosphorus		
		↓ pH		
Tide depth		↑ Tide depth		(Coaten et al., 2023)

[1]Relationship between abiotic factors and protein content was established through the correlation of data obtained from wild *S. latissima* harvested at different locations; [2]This result is likely related to the other listed environmental-related factors.

3.4.2. Use of underutilized nutrient sources as feedstock

Multiple studies have reported an increase in the protein or nitrogen content of seaweeds when aquaculture in association with fish cultivation wastewaters (Ashkenazi et al., 2019), recirculating aquaculture systems (RAS) and integrated multi-trophic aquaculture systems (IMTA) (Carneiro et al., 2021; Chatzoglou et al., 2020; Felaco et al., 2020). This beneficial effect is mainly attributed to changes in abiotic factors, such as increased availability of inorganic nitrogen due to its release from the fish/shellfish to the surrounding waters in the form of respiratory products and feces (Stedt, Pavia, et al., 2022).

Other water streams which are known to be rich in inorganic nitrogen are used to process waters from the food industry, not least the seafood industry (Forghani et al., 2022, 2023; Gringer et al., 2015; Osman et al., 2015). As water contacts with fish and shellfish, several forms of inorganic nitrogen and phosphorous leach out, resulting in outlets that could serve as feedstocks for protein accumulation in seaweed as has already been shown for microalgae (Forghani et al., 2022). Currently, these waters often undergo in-house pre-cleaning using non-food-grade chemicals before being released into public sewage systems, which entails substantial costs for the companies based on the large volumes of these waters being generated. For example, in a herring processing company handling 12 000 tons of fresh herring per year, costs for treating the used process waters (~35 000 m^3) reach around 5 euros per m^3 water (Personal communication, Ingrid Undeland, 2023). This is a strong incentive for finding ways to valorize these water streams or at the least reduce disposal costs as seaweed can act as a bioremediator. At the start of this PhD project, there were no studies on seaweed cultivation in outlet waters from the food processing industry, but the abundance of such waters provides plenty of stock for large-scale seaweed cultivation using hybrid (Stedt, Steinhagen, et al., 2022) or land-based systems. Exploring this new nutrient loop would ensure that valuable nutrients remain within the food chain, simultaneously transforming the costly disposal of these waters into a source of revenue by producing protein-enriched seaweed.

3.5. Up-concentration of seaweed proteins

As outlined in Sections 3.3.1 and 3.3.2, the up-concentration of seaweed proteins is necessary primarily to remove fibers and enhance the protein content of seaweed through the production of protein ingredients. The following subsection describes the qualitative aspects of seaweed proteins and their physical architecture in the seaweed biomass.

3.5.1. Challenges of seaweed protein extraction - including structural and biochemical differences compared to legumes

The commercialization of seaweed protein ingredients based on state-of-the-art knowledge is currently limited by factors such as relatively low extraction yields and highly complex and/or water-dense extraction protocols, hindering scalability (see further Section 5.3.4).

Over the years, multiple challenges have been proposed to explain why it is difficult to extract proteins from seaweed. These challenges include (*i*) the rigidity of the seaweed cell wall due to a strong polysaccharide network interwoven with proteins (Bjarnadóttir et al., 2018; Fleurence, Massiani, et al., 1995; Mæhre et al., 2016; Vásquez et al., 2019); (*ii*) the gel-forming properties of various seaweed polysaccharides that can reduce protein diffusion and hence its solubility (Fleurence, Le Coeur, et al., 1995; Jordan & Vilter, 1991); and (*iii*) interactions of proteins with charged polysaccharides and phenolic compounds which render protein solubilization difficult (Joubert & Fleurence, 2008; K. Wong & Cheung, 2001).

Protein extraction from whole seaweed, a photosynthetic active tissue, is often compared to protein extraction from dehulled seeds, particularly to soybean. This is due to soy protein being considered as a state-of-the-art product, given its vast production and application (Rajpurohit & Li, 2023). Seaweed has a more complex cell architecture and protein biochemistry than soybean (**Figure 2**). More specifically, in soybean most proteins are found on protein bodies that are abundant and easily accessible by the extraction solvent (Politiek et al., 2022). On the other hand, proteins in seaweed are scattered and highly compartmentalized within the cell, thus creating multiple physical barriers to the extraction solvent (Tenorio et al., 2018). Moreover, 50 to 90% of storage proteins in soy and pea are globulins, which are salt-soluble (Day, 2013). This facilitates protein extraction since most proteins share similar characteristics. Conversely, seaweed proteins are highly heterogeneous in terms of charge, solubility, and hydrophobicity. This heterogeneity arises from the unique characteristics of photosynthetic tissue, where proteins are instead classified depending on their function e.g., enzymatic or structural (Mendez & Kwon, 2021; Tenorio et al., 2018) or according to their solubility as aqueous-soluble (e.g. Rubisco) and lipophilic (e.g. thylakoid membrane proteins). In the latter classification, Rubisco has been shown to constitute 7-37% of the total soluble protein in seaweed (Iñiguez et al., 2019), while the exact contribution of lipophilic proteins to the total amount of proteins remains to be studied.

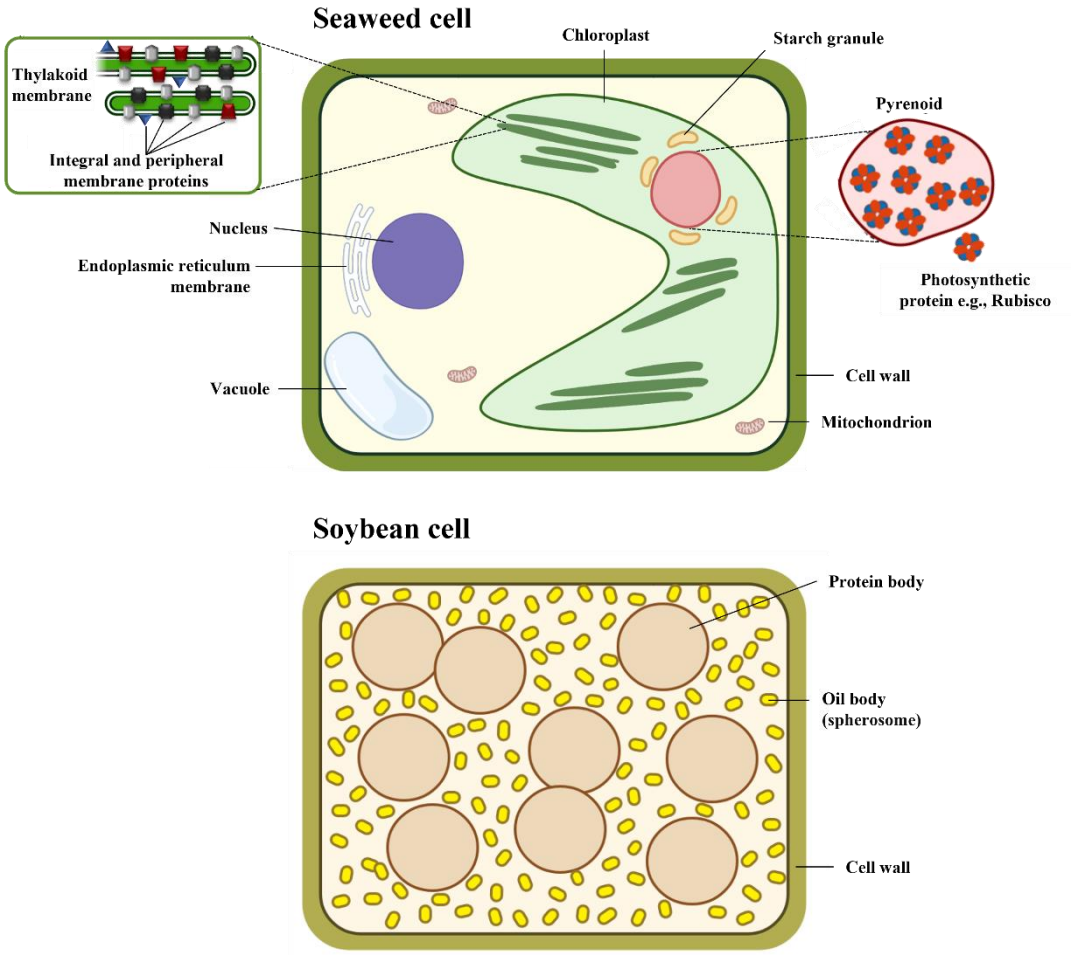

Figure 2 – Simplified schematic drawings of a general seaweed cell and soybean cotyledon cell. The schematic seaweed cell drawing is based on Hurd *et al.* (2014), Tenorio *et al.* (2018), and our own microscopic observations, while the soybean cotyledon cell is based on Politiek et al. (2022). Note that the drawings are not at scale.

Overall, these challenges, including the structural and biochemical differences to legumes, render seaweed biomass less suitable for protein extraction using traditional industrial methods, such as the pH-shift method also known as wet fractionation - characterized by alkaline solubilization followed by isoelectric precipitation, sometimes preceded by a solvent-based defatting step (Endres, 2001). Therefore, over the last ~30 years, seaweed-adapted versions of the pH-shift method have been developed, as well as seaweed protein extraction methods using other principles.

3.5.2. Overview of existing protein extraction methods for seaweed

Figure 3 depicts the typical steps of protein extraction methods reported for seaweed. These methods often start with cell disruption, followed by protein solubilization, and culminate with protein concentration. Each of these steps can be performed using a single principle, but it is more common to combine two or more principles.

Cell disruption can be applied directly to the biomass as pre-disintegration methods, such as traditional milling of dried biomass (Angell et al., 2017), ball/bead milling (Steinbruch et al., 2023), mincing (Juul, Steinhagen, et al., 2022), mechanical pressing (Juul, Danielsen, et al., 2021), and freeze-thawing (Wijers et al., 2020). Other cell disruption principles are usually applied to seaweed by mixing it with the extraction solvent such as high-speed homogenization (Postma et al., 2018), high-pressure processing

(HPP) (O' Connor et al., 2020), pulsed electric fields (PEF) (Robin et al., 2018), and ultrasound (Kadam et al., 2017), autoclaving (O' Connor et al., 2020), osmotic shock (Vilg & Undeland, 2017) and freeze-thawing (Wijers et al., 2020). Also within these principles, enzymatic hydrolysis often targets specific structural polysaccharides located in the cell walls, such as ulvan (Harrysson et al., 2019), carrageenan (Fleurence, Massiani, et al., 1995), and cellulose (Steinbruch et al., 2023). Many of these techniques can be combined aiming to maximize cell rupture, for example, mincing, followed by high-speed homogenization and then osmotic shock (Juul, Danielsen, et al., 2021).

The protein available for extraction and disruption of seaweed cells are closely interconnected since a high degree of cell disruption will often result in more protein being solubilized. Nevertheless, multiple studies have attempted to further increase protein solubility in water by (i) increasing temperature to 50 °C (Wijers et al., 2020), or by adding (ii) antioxidants to inhibit reactions between phenolics and proteins and also protein and lipid oxidation that can result in cross-linking (Juul, Møller, et al., 2021), (iii) bases as seaweed proteins are more soluble at alkaline pH values (Vilg & Undeland, 2017), (iv) organic solvents such as ethanol aid solubilization of hydrophobic proteins (Mendez & Kwon, 2021), (v) reducing agents to reduce S-S bonds to -SH (K. Wong & Cheung, 2001), (vi) polyether compounds plus inorganic salts to create a two-phase system where proteins are fractioned to the salt phase (Fleurence, Le Coeur, et al., 1995). A combination of techniques is also possible during this step, for example by combining ultrasound with the addition of base (Kadam et al., 2017). After the protein solubility step, centrifugation is often employed to remove insoluble components, such as non-disrupted cells and insoluble fibers. The accelerated solvent extraction sequence has also been applied to seaweed, where lipids, phenolics, and carbohydrates were removed before methanol-based protein solubilization (Harrysson et al., 2018).

Protein concentration in several studies consisted of freeze-drying the soluble-protein containing extract, as performed by e.g., Harnedy & FitzGerald (2013) and Jamshidi *et al.* (2018). Other principles involved protein precipitation via salting out using ammonium sulfate (Harrysson et al., 2018; K. Wong & Cheung, 2001), or via isoelectric precipitation preceded by freeze-thawing to maximize precipitation (Abdollahi et al., 2019) or without such freeze-thawing (Magnusson et al., 2019). The listed precipitation principles are often followed by centrifugation, which concentrates protein through dewatering/pelleting. In the field of protein hydrolysates from seaweed, membrane technology has been reported as a suitable concentration principle (Trigueros et al., 2022). However, we were unable to find works using the same principle in intact seaweed proteins.

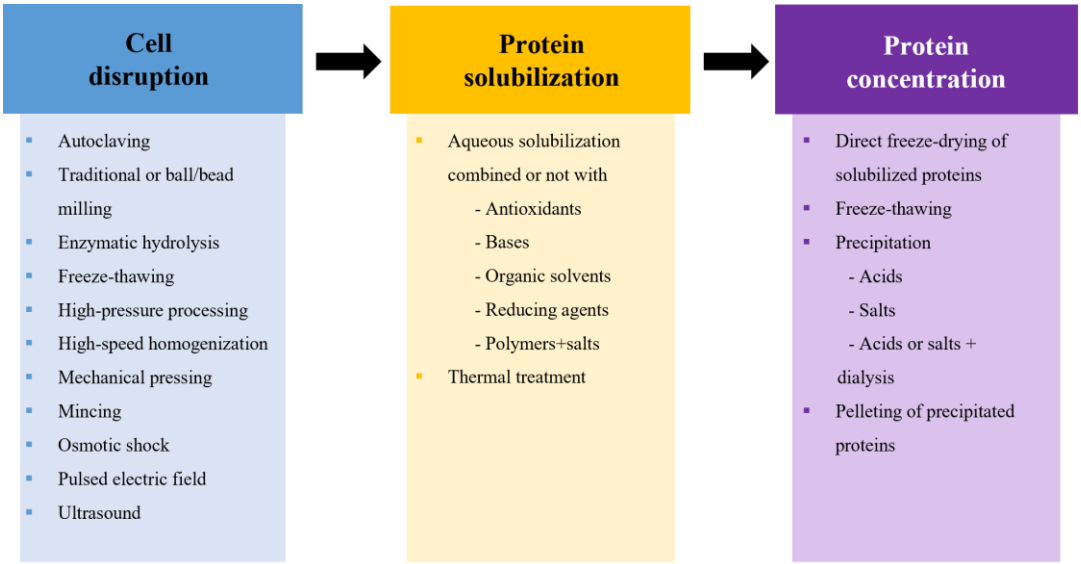

Figure 3 – Overview of steps included in reported protocols for protein extraction from seaweed.

3.5.3. Role of surfactants in membrane protein extraction

Seaweed protein solubilization techniques reported to date (**Figure 3**) primarily focus on aqueous soluble proteins, and they do not target the more lipophilic proteins within the thylakoid membranes, particularly integral proteins (**Figure 2**). Although the exact proportion of lipophilic to aqueous-soluble proteins remains to be addressed for photosynthetic biomasses, it is estimated that, for spinach, >70% of the total thylakoid membrane area is occupied by protein complexes (Kirchhoff et al., 2002). Therefore, finding strategies to solubilize lipophilic proteins in seaweed could lead to new extraction methods delivering higher protein yields.

Solubilizing integral membrane proteins requires the disruption of the lipid bilayer, a process commonly achieved through the use of surfactants within the field of molecular biology (Smith, 2017). Surfactants are amphipathic molecules containing hydrophobic and hydrophilic moieties. Thus, during protein solubilization, the hydrophobic moiety binds to the hydrophobic regions of the protein, while the hydrophilic one interacts with water (Orwick-Rydmark et al., 2016). A crucial aspect to consider when solubilizing proteins with surfactants is the surfactant's critical micelle concentration (CMC). The CMC represents a threshold where free surfactant molecules reorganize into micellar structures, which are characterized by having the hydrophobic moieties facing inwards (**Figure 4**). This structural transition is essential for the effective solubilization of lipophilic proteins as it facilitates their incorporation into the interior of these micelles (Smith, 2017). Therefore, at concentrations lower than the CMC, the surfactant monomers can perturb the membrane by partitioning into the lipid bilayer. At concentrations equal to or above the CMC, the membrane becomes saturated with surfactant monomers and breaks apart, generating protein-lipid-surfactant complexes (Figure 4). A further increase in surfactant concentration causes progressive de-lipidation of these complexes (Kalipatnapu & Chattopadhyay, 2005). Within molecular biology, common surfactants to use for solubilizing membrane proteins are e.g., Triton X-114 and Tween-20 (Orwick-Rydmark et al., 2016). The Tween-20 surfactant would be suitable for extraction of food grade proteins, calling for additional research.

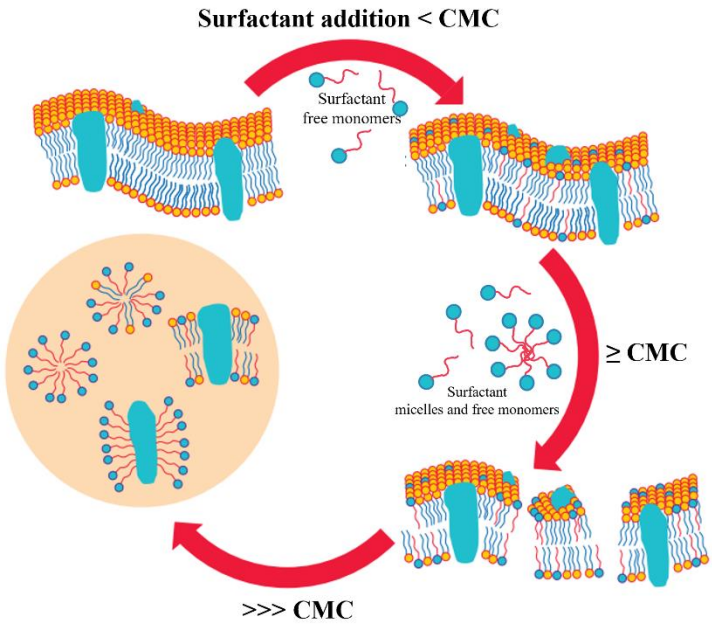

Figure 4 – Stages of biological membrane solubilization by surfactants as a function of surfactant concentration. Adapted from Kalipatnapu & Chattopadhyay (2005).

3.5.4. Effects of post-harvest biomass treatments on protein extraction

Seaweed is known to be highly perishable due to e.g., its high water activity (Perry et al., 2019; Pinheiro et al., 2019). Therefore, it is vital to stabilize and preserve the seaweed properly from the harvesting step until potential downstream protein extraction. Abdollahi et al. (2019) and Albers et al. (2021) evaluated how post-harvest biomass treatments, including freezing at -80 °C or -20 °C, oven-drying, sun-drying, freeze-drying and ensilaging influenced downstream pH-shift-based protein extraction of *S. latissima*. The authors found significant differences between treatments, with freeze-dried, oven-dried, and −20 °C frozen seaweeds resulting in significantly higher total protein yields compared to the remaining ones (Abdollahi et al., 2019). Another study focusing on fewer post-harvest treatments (freezing at −20 °C, freeze-drying, and air-drying at 40 and 70 °C compared to untreated control biomass) and more species (*C. crispus*, *Ascophyllum nodosum*, *S. latissima* and *U. lactuca*) found that for each of the four seaweed species, a different treatment resulted in the highest protein solubility yield following alkaline solubilization; air-drying for *C. crispus,* freeze-drying and freezing for *A. nodosum*, all treatments except air-drying at 70 °C for *S. latissima*, and fresh untreated for *U. lactuca* (Wijers et al., 2020). The effect of different drying types, specifically freeze-drying versus oven-drying, was also investigated by Wong & Cheung (2001) for three brown seaweed species. They reported that oven-drying resulted in higher total protein yields, after protein extraction involving aqueous solubilization with a reducing agent followed by protein salting out using ammonium sulfate (K. Wong & Cheung, 2001).

When it comes to biomass treatments having more purposes besides stabilization, such as rinsing and blanching, there is no available information regarding their impact on downstream protein extraction. Such information is relevant since blanching is widely adopted in industry to reduce the excessive iodine levels of *S. latissima* and because previous works on microalgae suggested that thermal treatments make the biomass less ideal for protein extraction due to protein denaturation (Wang et al., 2019). On the

other hand, rinsing is commonly applied to all types of seaweed to remove e.g., sand and epiphytes, and is expected to have a milder effect on protein denaturation.

Blanching and soaking can be conducted with fresh or saltwater (Krook et al., 2023; C. W. Nielsen et al., 2020; Stévant et al., 2018; Stévant, Marfaing, et al., 2017). While using saltwater minimizes cell osmotic breakage and likely co-leaching of important nutrients, its corrosive nature relative to freshwater can lead to increased maintenance costs for blanching equipment.

It is important to note that the term “blanching” often corresponds to high temperatures and short durations (80-95 °C, 1-2 min) when processing a food product. Nevertheless, within the seaweed industry, this term is applied even to processes involving temperatures as low as 45 °C. According to this convention, this thesis adopted a broader definition of blanching, extending it to encompass temperatures down to 45 °C. Alternatively, a fitting term could be “warm water treatment”.

4. METHODS AND METHODOLOGY CONSIDERATIONS

This section outlines and justifies the analytical methods used in this PhD project's experimental design. Also, it encompasses the methods' principles, and when deemed essential, assess their validity concerning the current state-of-the-art. For a comprehensive understanding of the specific methods' execution, the reader is referred to **Papers I-IV**.

4.1. Study design

The chosen study design addressed three main challenges related to seaweed as a potential food protein source: (*i*) its relatively low protein levels, (*ii*) the reported low protein extraction yields, (*iii*) and the complex composition of the seaweed matrix which may hamper protein digestibility. Concerning extraction, the design also included examining the impact of blanching, while regarding digestibility, the protein nutritional quality was followed through *in vitro* digestion trials and cell studies. **Figure 5** provides an overview of the different papers and the analysis performed.

Paper I comprised a screening test to maximize the natural protein levels of seaweed by cultivating it in media that contained nutrient-rich food process waters. The study monitored crude protein content and growth rate for different combinations of food process waters (in total eight different) and seaweed species (in total four different, including *S. latissima* and *U. fenestrata*).

Papers II and **III** focused on protein extraction from *S. latissima* and *U. fenestrata,* respectively. **Paper II** specifically investigated the effects of blanching or soaking on protein yield and characteristics of the protein extracts when using the pH-shift method. The protein extract characteristics included crude and elemental composition as well as protein/peptide size distribution.

Paper III served as a proof-of-concept study that explored a novel protein extraction method involving the use of surfactant and alkaline aqueous solutions for efficient protein solubilization and precipitation. The study also examined the polypeptide pattern of the different fractions produced, along with the crude composition of the final protein extracts. The biomass used was protein-enriched *U. fenestrata* cultivated based on the insights gained from **Paper I**.

Lastly, **Paper IV** provided a comprehensive assessment of the nutritional quality of protein extracts produced according to the method employed in **Paper II**. It focused on *in vitro* protein digestibility, amino acid accessibility, and amino acid cell bioavailability, offering a more complete understanding of the extraction method's nutritional implications.

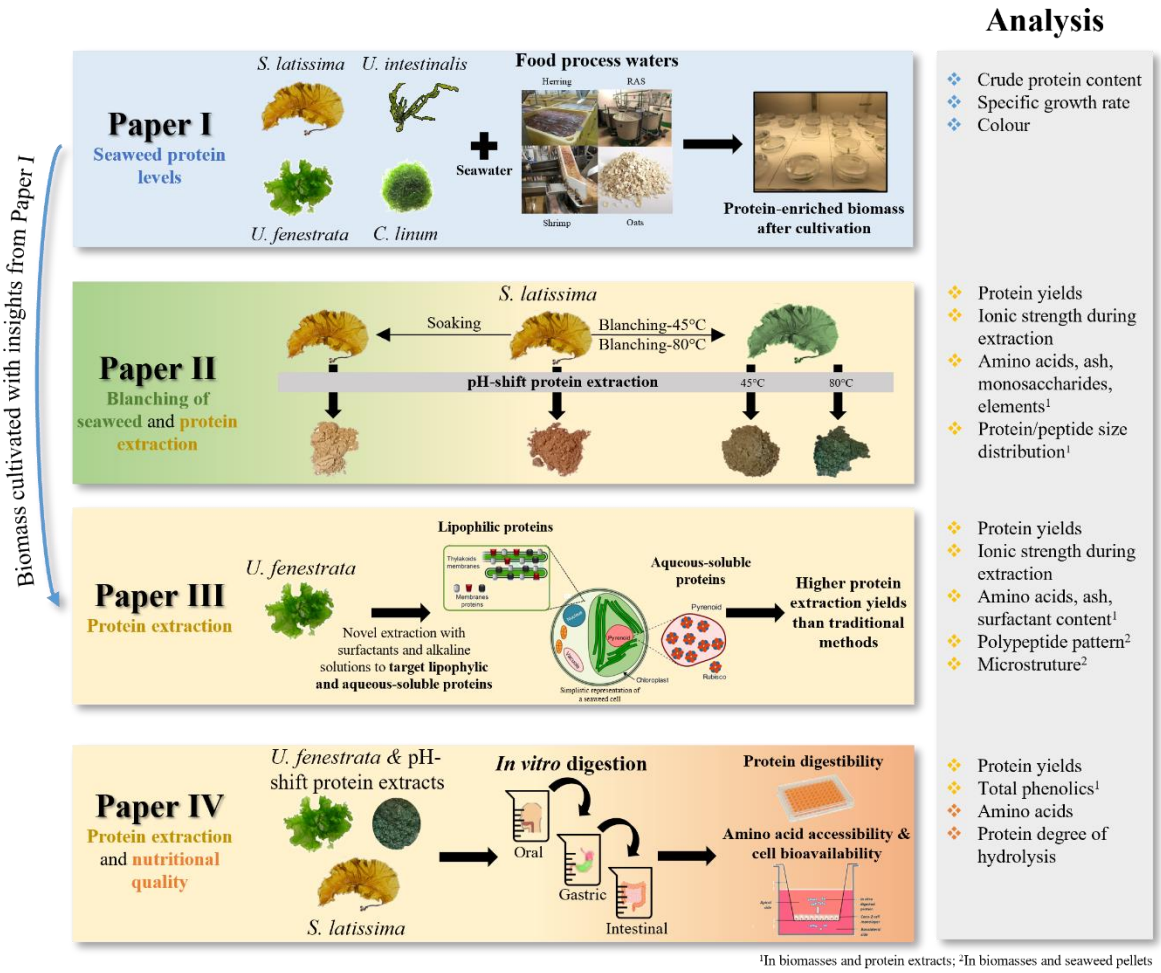

Figure 5 - Overview of Papers I-IV and the analysis performed.

4.2. Seaweed biomass and post-harvest handling

Table 7 provides details regarding the harvesting of the biomasses used in **Papers I-IV** as well as how they were handled post-harvest. All biomasses were collected either on the Swedish west coast (in the wild or from open sea farms) or in tank cultivation systems at Tjärnö Marine Station. Furthermore, in **Papers II-IV**, freshly harvested biomass was transported on the same day to Chalmers University of Technology as seaweed is known to be highly perishable (Perry et al., 2019; Pinheiro et al., 2019). After transportation, *S. latissima* was subjected to water treatments for 2 min, more specifically blanching at 45°C or 80°C or soaking with running tap water at 12 °C (**Paper II**). The choice of time-temperature binomials as well as the water-to-seaweed ratio was based on work reported elsewhere (Nielsen et al., 2020). All biomasses (**Papers II-IV**) were minced with a meat grinder (Model C-E22N, la Minerva) to ensure sample homogeneity before freezing at -80 °C. In **Paper III**, *U. fenestrata* had to be frozen before mincing since a preliminary trial revealed a substantial temperature build-up when mincing it while fresh. The minced biomass remained frozen, why all used biomasses ultimately were subjected only to a single freezing cycle at -80 °C

Table 7 – Overview of original seaweeds and their post-harvest handling.

	Paper I				**Paper II**	**Paper III**	**Paper IV**	
Species	*U. fenestrata*	*S. latissima*	*U. intestinalis*	*C. linum*	*S. latissima*	*U. fenestrata*	*U. fenestrata*	*S. latissima*
Harvest date	February 2020	January 2020 and October 2020	March 2020	August 2020	May 2020	March 2022	November 2019	June 2018
Harvest location in Sweden	Tjärnö Marine Station	Tjärnö Marine Station	Rossö Island	Ursholmen	Koster archipelago	Tjärnö Marine Station	Tjärnö Marine Station	Tjärnö Marine Station
Harvest coordinates	58°52′33.7″ N 11°08′44.9″ E	58°52′33.7″ N 11°08′44.9″ E	58°50′33.9″ N 11°09′06.6″ E	58°49′ 57.6″ N 10°59′19.2″ E	58°51′ 34.0″ N 11°04′06.2″ E	58°52′33.7″ N 11°08′44.9″ E	58°52′33.7″ N 11°08′44.9″ E	58°52′33.7″ N 11°08′44.9″ E
Cultivation type	Indoor tank	Indoor tank	Wild	Wild	Farm at open sea	Indoor tank	Indoor tank	Indoor tank
Generation	Gametophytes	Sporophytes	Not determined	Not determined	Sporophytes	Gametophytes	Gametophytes	Sporophytes
Handling after collection	Cultivation with media containing food process waters				Transported fresh to Chalmers	Transported fresh to Chalmers and frozen at -80 °C	Transported fresh to Chalmers	Transported fresh to Chalmers
Post-harvest treatment	None				Soaking or blanching, followed by mincing	Mincing while frozen	Mincing while fresh	Mincing while fresh
Storage prior experiments	*n.a*				Stored at -80 °C	Stored at -80 °C	Stored at -80 °C	Stored at -80 °C

n.a *not applicable*

4.3. Characterization and preparation of food process waters to be included in seaweed cultivation media

In **Paper I**, eight different food process waters derived from herring, shrimp, recirculated aquaculture systems (RAS), and oat processing industries were collected. The production flowchart of the five collected herring-derived process waters is depicted in **Figure 6**.

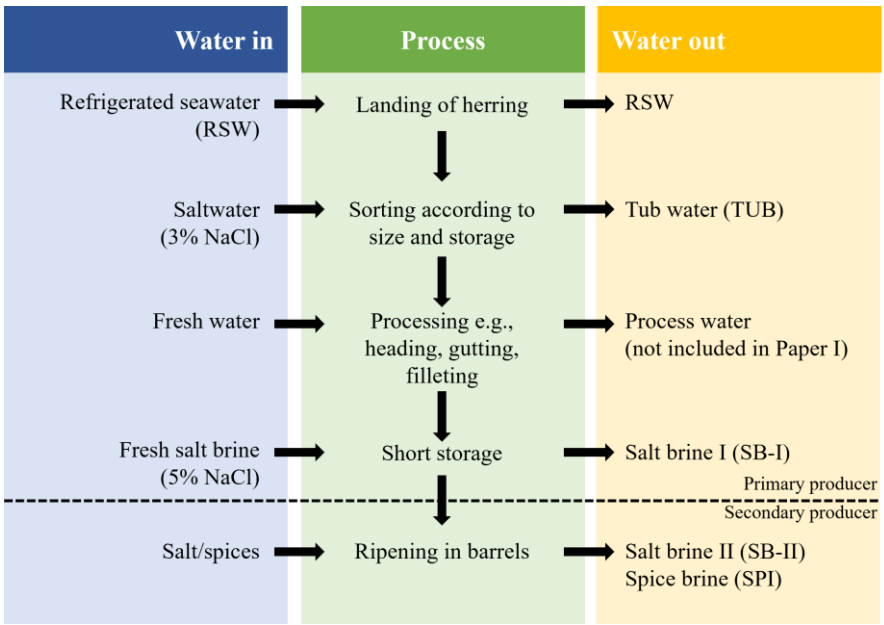

Figure 6 – Production flowchart with different herring-derived process waters and brines generated during marinated herring production.

All process waters were characterized in terms of total nitrogen and low-molecular-weight compounds, including ammonium, nitrate, nitrite, and inorganic phosphorous. Ammonium quantification relied on the reaction of α-ketoglutaric acid with ammonium and reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of L-glutamate dehydrogenase to form L-glutamate and oxidized nicotinamide adenine dinucleotide phosphate; the consumption of NADPH could be measured at 340 nm and was directly proportional to the ammonium concentration. Nitrate and nitrite quantification were based on the reaction of nitrate with nitrate reductase in the presence of β-nicotinamide adenine dinucleotide to yield nitrite which was then treated with sulphanilamide to form a diazocompound. This compound reacted with N-(1-naphthyl) ethylenediamine dihydrochloride to give an azo product that could be determined at 540 nm (Hooda et al., 2016). Inorganic phosphorous was quantified by reaction of phosphate ions with ammonium molybdate to form a phosphomolybdate complex that was measured at 700 nm (Sepulveda, 2013).

Seaweed cultivation media for tank-based systems is often sterilized by filtration or ultraviolet light. Therefore, before adding the food process waters to the cultivation media, it was important to sterilize them. Initially, we attempted sterilization through filtration. However, due to the presence of particulate material, this method proved to be unfeasible. Hence, we decided to employ a 60 µm filter to remove e.g., coagulated particulates and fish skin, followed by autoclaving sterilization. Since the addition of these waters was normalized based on their ammonium content, it was crucial to assess whether autoclaving sterilization affected the ammonium levels. The results demonstrated no significant changes in the ammonium content (148±13 mg/L before autoclaving vs. 159±10 mg/L after autoclaving).

4.4. Seaweed cultivation in food process waters and measurement of specific growth rate

All cultivation experiments in **Paper I** were performed in aerated Petri dishes as it was the viable strategy to test all combinations of the eight food process waters, four seaweed species, and respective six replicates. The cultivation parameters were standardized for all seaweeds – water temperature of 12 °C, light regime of 12:12 h (L:D), and irradiance of 70 µmol photons m^{-2} s^{-1} for 8 days. The water temperature corresponded to a realistic value along the Swedish west coast, while the irradiance to an average value experienced by intertidal seaweed in Sweden (Toth et al., 2020). The light exposure time corresponded with the average daylight hours throughout the year in the areas where the seaweeds were harvested.

Biomass growth, measured as specific growth rate (SGR), was calculated according to **Equation 1**.

$$SGR = \left(\frac{\ln(surface\ area\ or\ wet\ weight\ after\ cultivation) - \ln(surface\ area\ or\ wet\ weight\ before\ cultivation)}{Number\ of\ cultivation\ days} \times 100\right) \textbf{(Eq. 1)}$$

For *S. latissima* and *U. fenestrata,* SGR was calculated based on surface area (via photo-scanning), whereas for *U. intestinalis* and *C. linum,* it was based on wet weight. This distinction was necessary since the filamentous nature of the latter two species made it difficult to accurately estimate the surface area. Therefore, a reproducible method had to be developed to remove excess water before determining the biomass wet weight. Several preliminary methods were tested including: (1) pulling the seaweed against a beaker with forceps for 5 seconds, (2) shaking the seaweed for 5 seconds with forceps, and (3) placing the seaweed in a salad spinner for 20 seconds. After this, the wet weight was plotted against the respective dry weight, and methods 1 and 2 yielded the highest R-square for *U. intestinalis* and *C. linum,* 0.9415 and 0.9368, respectively.

4.5. Protein extraction

Protein extraction involved wet biomasses instead of oven-dried ones (**Papers II-IV**). This decision was made to avoid the need for drying the biomass and then rehydrating it for protein extraction, which would require unnecessary energy.

The pH-shift method with a freeze-thawing step was chosen for protein extraction in **Papers II** and **IV** as it previously achieved the highest protein yields and/or protein purities for *Ulva sp.* and *S. latissima* when compared to accelerated solvent extraction sequence and sonication in water followed by ammonium sulfate-induced protein precipitation (Harrysson et al., 2018). Although Angell et al. (2017) have shown that aqueous extraction followed by alkaline re-extraction of the obtained seaweed pellet and then protein recovery through isoelectric precipitation also has resulted in relatively good extraction yields for *Ulva ohnoi*, a direct comparison to the pH-shift method has not been done (Angell et al., 2017). The method reported by Angell et al. (2017) however comprised high water volumes - 19 times the seaweed weight in total - compared to only 6 in our pH-shift method optimized for seaweed. To limit the water footprint, the pH-shift method was therefore chosen in this thesis. In **Paper III**, a new extraction method was developed due to the yield and scalability limitations of existing methods, including the pH-shift method, when compared to yields obtained from terrestrial protein crops.

4.5.1. The pH-shift method

Figure 7 depicts a simplified scheme of the pH-shift method used in **Papers II** and **IV**, based on the protocol outlined by Harrysson et al. (2018). Briefly, the protocol involved homogenizing seaweed with distilled water, followed by an incubation for 1h to allow cell osmotic shock. Then, the pH of the homogenate was adjusted to the pH at which maximum protein solubility occurs and left to incubate for 20 min. Thereafter, the mixture was centrifuged to recover the solubilized proteins, which were present in the supernatant. Subsequently, the pH of the supernatant was lowered to the value inducing maximum protein precipitation and the supernatant was frozen at -20 °C. Finally, the thawed supernatant was subjected to centrifugation, which resulted in a second supernatant containing non-precipitated proteins and a protein extract, in the form of a pellet.

In **Papers II** and **IV**, slight modifications to the previously described protocol included the replacement of an Ultra-turrax T18 basic homogenizer for the more powerful Silverson LM5. A preliminary trial with *U. fenestrata* revealed similar total protein extraction yields between both homogenizers (3.5% versus 3.1%, respectively, N=1). The modification however allowed for the processing of larger quantities of seaweed, which was crucial for obtaining sufficient protein extract for the *in vitro* digestion experiments (**Paper IV**) and all chemical and elemental composition analyses (**Paper II**). In **Paper IV**, another modification was the addition of a pre-incubation step at pH 8.5 for 1h after the osmotic shock step. This adjustment was based on the findings reported by Harrysson et al. (2019) who observed a significant increase in total protein yield for *U. fenestrata* using this approach. However, in **Paper II**, this strategy was not implemented due to the lack of substantial yield improvements observed with

crude *S. latissima* – total yields were 20.5% and 20.1% with and without the pre-incubation step, respectively (N=1).

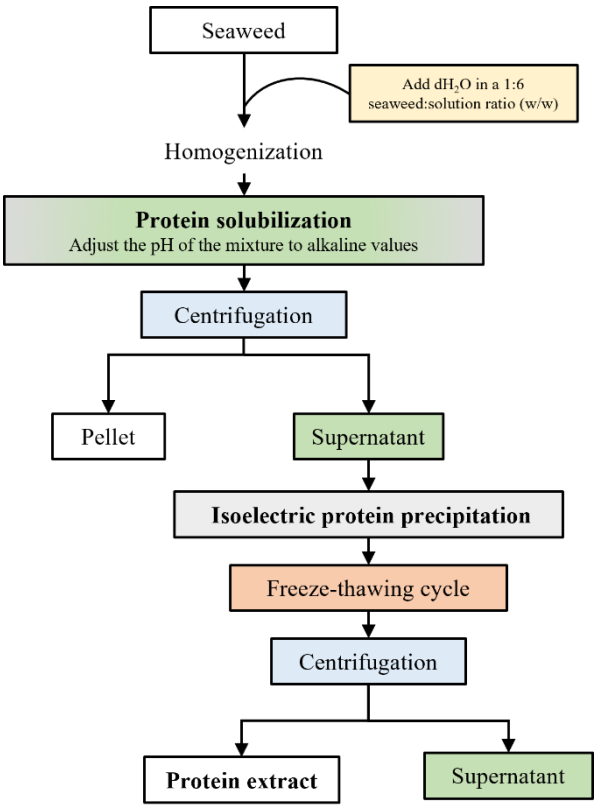

Figure 7 – Flowchart of the pH-shift method as adapted to seaweed. Green boxes indicate the fractions where protein content was measured to calculate protein solubilization, precipitation, and total yields.

The protein solubility and protein precipitation at specific pH values were determined using **Equations 2 and 3**, respectively, for every biomass in **Papers II and IV**.

$$\text{Protein solubility (\%)} = \frac{[\text{Protein in first supernatant}]\ (\frac{mg}{mL})}{[\text{Protein in homogenate}]\ (\frac{mg}{mL})} \times 100 \quad \textbf{(Eq. 2)}$$

$$\text{Protein precipitation (\%)} = 100 - \left(\frac{[Protein\ in\ second\ supernatant]\ (\frac{mg}{mL})}{[Protein\ in\ first\ supernatant]\ (\frac{mg}{mL})} \times 100\right) \quad \textbf{(Eq. 3)}$$

Furthermore, to determine the total extraction yield i.e., the percentage of proteins recovered in the protein extract relative to the input amount of proteins in the original biomass, **Equations 4-6** were used.

$$\text{Protein solubility yield (\%)} = \frac{\text{Protein in first supernatant } (mg)}{\text{Protein in homogenate } (mg)} \times 100 \quad \textbf{(Eq. 4)}$$

$$\text{Protein precipitation yield (\%)} = 100 - \left(\frac{Protein\ in\ second\ supernatant\ (mg)}{Protein\ in\ first\ supernatant\ (mg)} \times 100\right) \quad \textbf{(Eq. 5)}$$

$$\text{Total protein extraction yield (\%)} = \left(\frac{\text{Protein solubility yield}}{100}\right) \times \left(\frac{\text{Protein precipitation yield}}{100}\right) \times 100 \quad \textbf{(Eq. 6)}$$

It should be highlighted that several works dealing with protein extraction from seaweed have recovered the solubilized proteins directly via freeze-drying of the first supernatant (Kazir et al., 2019; O' Connor et al., 2020), while others have performed protein extraction without further recovery of the extracted proteins (Mendez & Kwon, 2021). In both cases, reported extraction yields can be directly comparable with either Equation 1 or Equation 3, assuming the cited works used the same protein determination method as ours.

4.5.2. The surfactant-based method

Figure 8 illustrates a simplified scheme of the surfactant-based method. The first processing cycle of this method involved homogenization of seaweed with either: (i) an aqueous solution of Triton X-114; or (ii) distilled water. In (ii) the pH of the homogenate containing seaweed and water was brought to pH 12. The homogenate was then incubated for 1h, followed by centrifugation to recover the solubilized proteins in the supernatant, whereas the resultant pellet was subjected to a second processing cycle. In this reprocessing, the pellet was homogenized with either one of the previous solutions. Thereafter, the homogenate was incubated and centrifuged, and the resulting supernatant was combined with the supernatant from the first processing cycle. The solubilized proteins were then precipitated at pH 2 and dewatered via centrifugation, thus resulting in a protein extract.

The water volumes chosen for the first and second processing cycles were 4- and 2-fold the weight of the used seaweed and pellet, respectively. Higher extraction volumes could have been chosen since it is reported they lead to higher protein extraction yields (Harrysson et al., 2018; Vilg & Undeland, 2017a). However, excessive water usage can be a decisive factor hindering the scalability of an extraction method due to cost and water footprint. Hence, the maximum total water volume decided upon was 6-fold the biomass weight, which aligned with the pH-shift method in **Papers II and IV**. Moreover, the change of water volumes between processing cycles one and two was made to maintain a consistent ratio between the ingoing amount of protein and the added water.

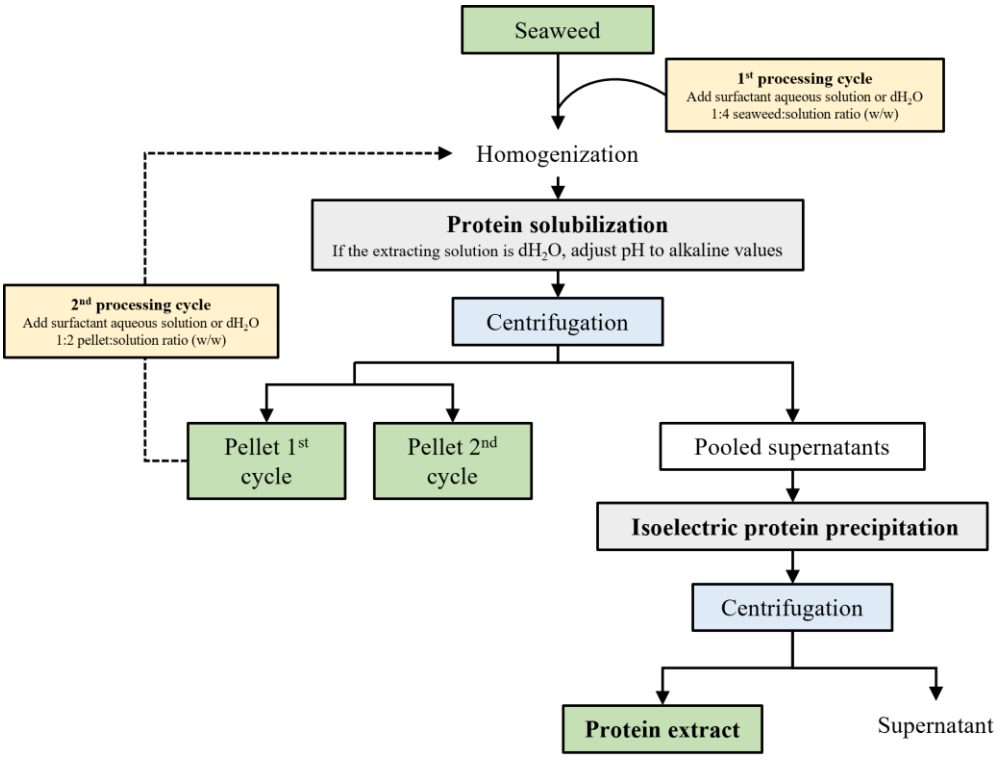

Figure 8 - Flowchart of the surfactant-based method created to extract seaweed protein. Green boxes indicate the fractions where the total nitrogen or amino acids were measured to calculate protein solubilization, precipitation, and total yields.

The N solubility, N precipitation, and total N yields of the surfactant-based method were calculated according to **Equations 7-10** by measuring the N content of the input biomass and the solid fractions

i.e., seaweed pellets and protein extracts. The reasons for changing from soluble protein analyses (**Papers II and IV**) to N-analyses (**Paper III**) are described in Section 4.6.1.

$$\text{N solubility yield of 1st cycle (\%)} = 100 - \left(\frac{\text{N in pellet 1st cycle [mg]}}{\text{N in original seaweed [mg]}} \times 100\right) \textbf{(Eq. 7)}$$

$$\text{N solubility yield of 2nd cycle (\%)} = \text{N solubility yield of 1st cycle} \times \left(100 - \left(\frac{\text{N in pellet 2nd cycle [mg]}}{\text{N in pellet 1st cycle [mg]}} \times 100\right)\right) \textbf{(Eq. 8)}$$

$$\text{N precipitation yield (\%)} = \left(\frac{\text{N in protein extract [mg]}}{(\text{N in original seaweed [mg]} - \text{N in pellet 1st cycle [mg]}) + (\text{N in pellet 1st cycle} - \text{N in pellet after 2nd cycle [}mg\text{]})} \times 100\right) \textbf{(Eq. 9)}$$

$$\text{Total N yield (\%)} = (\text{N solubility yield of 1st cycle} + \text{N solubility yield of 2nd cycle}) \times \text{N precipitation yield} \textbf{(Eq. 10)}$$

4.6. Analysis methods to determine the composition of original seaweeds and produced protein extracts

4.6.1. Protein determination

The principles, advantages, and limitations of each protein determination method used in **Papers I-IV** are presented in **Table 8**.

Table 8 – Protein determination methods used in this thesis as well as their principles, advantages, and limitations.

Method	Papers	Principle	Advantages	Limitations
Lowry method	II	Detection of cuprous ions produced by the reduction of cupric ions by peptides bounds under alkaline conditions	- Very high sample throughput - Does not measure nonprotein nitrogen	- Prone to interferences from e.g., reducing agents, surfactants - Only measures soluble protein
Total amino acids	II-IV	Hydrolysis of sample with HCl under hot conditions, followed by separation and detection of each amino acid in an e.g., LC-MS system	- Low sample interferences - Allows calculating amino acid profile	- Low recovery yields of Met, Cys, and Trp after acid hydrolysis - Low sample throughput
Total N using Dumas method	I-IV	Combustion of samples at > 1000 °C in the presence of oxygen. The formed NO_x is reduced to N_2 by copper and the nitrogen is analyzed by a thermal conductor	- High sample throughput - Low sample interferences	- Variations in nonprotein nitrogen content can reduce accuracy if a universal conversion factor is used - Regular maintenance of the equipment needed

LC-MS *liquid chromatography coupled with mass spectrometry*

In this PhD project, the Lowry method (Lowry et al., 1951) followed the modifications reported by Markwell et al. (1978), which consisted of adding sodium dodecyl sulfate (SDS) and an increased amount of copper tartrate. These modifications were ascribed to reduce interference from sucrose and chelating agents (Markwell et al., 1978). Additionally, to avoid interference from the chlorophyll present in *S. latissima* and *U. fenestrata*, the absorbance was read at 750 nm instead of 660 nm; both absorbances are within the region where the method's chromophore absorbs light (500-750 nm) (Olson & Markwell, 2007). Other potential sources of interference are the phlorotannins present in *S. latissima*. These phenolic compounds due to their reducing potential can lead to overestimations of the protein content. However, Harrysson et al. (2018) showed that the Lowry method remains suitable for the determination of protein yields when executing the pH-shift method, when compared to yields based on amino acid analysis (total protein yields of 25.1±0.9% and 22.1±0.8%, respectively). Therefore, the Lowry method was selected for **Paper II** due to its listed advantages (**Table 8**). Moreover, Harrysson et al. (2018) also reported that the Lowry method tends to overestimate protein content of *S. latissima* when compared to amino acid analysis, likely due to the presence of phlorotannins. Hence, in **Papers**

I-II, the protein content of the initial biomasses and respective protein extracts was reported based on amino acid analysis (**Paper II**) or total N (**Paper I**), the latter due to the large sample set. The presence of surfactants in the sample can also hinder accurate protein determination. In the case of the Lowry method, it is determined that it is only compatible with Triton X-114 at concentrations ≤0.03% (w/v) (Orwick-Rydmark et al., 2016). Therefore, for **Paper IV**, total N analysis was selected instead of the Lowry method, as the tested Triton X-114 concentrations exceeded 0.03%.

The conversion from total N content to crude protein content requires the use of a conversion factor. For seaweed, a meta-analysis conducted by Angell et al. (2016) determined an overall median factor of 5, which was adopted in **Papers I** and **IV**. The meta-analysis encompassed 103 seaweed species covering three taxonomic groups, multiple geographic regions, and diverse physiological stages (Angell et al., 2016). Since the authors reported slight fluctuations in this universal factor (Angell et al., 2016), we considered it important to complement, when possible, the N analysis with total amino acids - as performed in **Papers II-IV**. In **Paper I**, due to the low amount of seaweed recovered, it was only possible to conduct a total N analysis. However, the relative increase in protein content of seaweed cultivated with food process waters compared to the seaweeds cultivated in seawater and NH_4^+ could still be calculated to validate the findings. In a follow-up study that generated larger amounts of biomass cultivated in food process waters, we determined the N-to-protein conversion factors for *U. fenestrata* to be 6.1-7.3 (Stedt, Steinhagen, et al., 2022).

4.6.2. Other methods to determine composition

The composition of the original biomasses and produced protein extracts were also analyzed in terms of total ash, total monosaccharides, elements, and total phenolics. Their principles and the underlying motivations for their determination are described in **Table 9**. It should be noted that total phenolics were expressed as phloroglucinol equivalents since this compound is naturally present in brown seaweed, thus it can guarantee a more accurate quantification.

Table 9 – Principles and motivations for the compositional analysis methods other than protein determination methods.

Method		Paper(s)	Principle	Motivation(s)
Elemental composition	Inorganic arsenic	II	Hydrolysis with nitric acid and hydrogen peroxide followed by separation and detection with HPAEC-ICP-MS	- Knowledge about certain toxic arsenic species
	Other elements	II	Hydrolysis with nitric acid followed by separation and detection with HR-ICP-MS	- Seaweed can be a source of essential and non-essential elements
Total ash content		II, III	Combustion at 550 °C	- Seaweed is often abundant in minerals that can enhance the umami taste[1] - Major contributor to ionic strength, which in turn influences protein extraction - Low ash levels have been related to better N digestibility of seaweed protein extracts[2]
Total monosaccharide content		II	Two-step hydrolysis with sulfuric acid followed by separation and detection with HPAEC-PAD	- Polysaccharides are often the main macronutrients in seaweed[3] - Charged polysaccharides can be bound to elements e.g., divalent cations[4] - Potential co-extraction of carbohydrates during protein extraction that can affect protein precipitation and purity
Total phenolic content		IV	Phenolics react with the Folin-Ciocalteu reagent to produce molybdenum–tungsten blue that is measured at 750 nm[5]	- Amount of phenolics could explain differences in *in vitro* protein digestibility
Triton X-114 content		III	Ammonium cobaltothiocyanate reacts with poly (ethylene oxide) groups of Triton to produce a blue compound detectable at 625 nm[6]	- Protein extracts might contain this non food-grade surfactant

[1]Ikeda (2002), [2]Juul et al. (2022), [3]Holdt & Kraan (2011), [4]Makarova et al. (2023), [5]Malta & Liu (2014); [6]Garewal (1973)
HPAEC-PAD *High-performance anion-exchange chromatography with pulsed amperometric detection*; HR-ICP-MS *High resolution inductively coupled plasma mass spectrometry*

4.7. Polypeptide pattern and protein/peptide relative size distribution

Proteins can be characterized through e.g., sodium sulfate dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC). SDS-PAGE separates denatured proteins/polypeptides based on their molecular weight and delivers qualitative data. Size exclusion chromatography also separates proteins/peptides based on their molecular weight, using a chromatographic column packed with a gel containing porous beads, followed by UV detection for protein quantification. Although both techniques are used to separate protein based on molecular weight, they provide different types of data that can be relevant depending on the research question. In **Papers II** and **IV**, SEC was chosen since it delivers semi-quantitative data, which facilitated the visualization of protein/peptide size distribution as a function of *in vitro* digestion and extraction method. In **Paper III**, SDS-PAGE was primarily selected as it allowed us to study the effect of reducing conditions with β-mercaptoethanol. Running SEC under reducing conditions is not advisable as it can damage the chromatographic column.

4.8. Protein nutritional quality

Protein nutritional quality can be evaluated from various perspectives. In the case of **Papers II-IV**, one approach involved measuring the amino acid profile and comparing it to the amino acid requirements set by WHO/FAO/UNU (2007). Moreover, for **Paper IV**, a comprehensive assessment of protein nutritional quality was conducted, which encompassed not only the amino acid profile but also protein digestibility, amino acid accessibility, and Caco-2 cell amino acid bioavailability following *in vitro* gastrointestinal digestion.

4.8.1. Selection of the *in vitro* gastrointestinal digestion protocol

Over the years, different *in vitro* static digestion protocols have been adopted which can hamper comparisons between studies; this issue is especially relevant when it comes to the digestion of seaweed protein extracts (Fujiwara-Arasaki et al., 1984; Gajaria et al., 2017; Kazir et al., 2019; Maehre et al., 2016; K. H. Wong & Cheung, 2001). In an attempt to normalize the *in vitro* digestion field, a network of researchers recommended the adoption of an international harmonized digestion protocol named INFOGEST (Minekus et al., 2014), later updated to INFOGEST 2.0 (Brodkorb et al., 2019). However, in pre-trials, this protocol showed several limitations, two of them preventing the subsequent execution of cell bioavailability trials. The first issue was related to high viscosity of the very concentrated pancreatin suspension also containing undissolved particles. Both features made it challenging to pipette accurately, thus compromising reproducibility. Two publications have recently proposed different solutions to overcome this issue, but they were published after **Paper IV**. The first publication recommended preparing the pancreatin suspension using ultrasound treatment, followed by centrifugation and recovery of the supernatant, which was later adjusted to 100 U trypsin activity/mL (Sousa et al., 2023). The second publication recommended decreasing pancreatin activity by 10-fold as the authors did not observe statistically significant differences in terms of protein degree of hydrolysis when using this lower concentration versus the one described in the INFOGEST 2.0 protocol (Ariëns et al., 2021). The second issue was related to the protein content in the lysates obtained from cells treated with the INFOGEST 2.0 digests. These contents were 2 to 3 times lower for seaweed-containing digests and blank-digests, when compared to a digestion protocol that has been extensively used to study iron bioavailability, originally based on Glahn et al. (1996). This finding strongly suggested cell death, which was further supported by noticeable visual differences between the Caco-2 cells versus the Glahn et al. (1996) protocol. A reason for the potential incompatibility between the INFOGEST 2.0 protocol and Caco-2 cell transport studies may arise from the well-known toxicity of bile salts to Caco-2 cells, particularly at the relatively high concentrations used in the INFOGEST 2.0 protocol (Kondrashina et al., 2023). Other reasons such as pancreatic enzyme-induced degradation of Caco-2 cells were unlikely to occur as the final digests were heat-treated to stop enzymatic activity. Recent recommendations propose diluting the bile salt content from the original 10 mM to approximately 1 mM after digestion to enable compatibility of the INFOGEST protocol with *in vitro* epithelial absorption studies (Kondrashina et al., 2023). The recommended bile salt concentration closely aligns with the amount added to the apical compartment following the Glahn et al. (1996) protocol, which was 1.2 mM after 1:1 dilution with Hanks' balanced salt solution (**Table 10**).

Table 10 – Comparison of digestion parameters between the INFOGEST 2.0 protocol and the protocol selected for Paper IV.

***In vitro* digestion protocol**		**INFOGEST 2.0** Brodkorb et al. (2019)	**Paper IV** Based on Glahn et al. (1996)
Oral phase	Amylase activity (U/mL)*	75	75
	Incubation time (min)	2	2
	pH during incubation	7.0	7.0
Gastric phase	Pepsin activity (U/mL)*	2000	2000
	Incubation time (min)	120	60
	pH during incubation	3.0	2.0
Intestinal phase	Pancreatin activity (U trypsin activity/mL)*	100	0.6
	Bile salts content (mM)*	10	2.4
	Incubation time (min)	120	120
	pH during incubation	7.0	7.0

*Enzymatic activities and amount of bile salts correspond to the final volume at each digestion phase.

Taking into account the two discussed issues, we decided at the time of the experiments (2019) to select the digestion protocol based on Glahn et al. (1996). Modifications of the original Glahn protocol included: (i) the addition of an oral step similar to that of the INFOGEST 2.0 protocol (**Table 10**) since *Ulva sp.* contains starch plates surrounding the pyrenoids and starch granules among the thylakoid membranes (Farias et al., 2017); and (ii) an intestinal pH of 7.0 instead of the original pH 7.5 as the cells were grown at pH 7.0 until their differentiation. Both pH values are however within the physiological pH range of the small intestine (Fallingborg, 1999).

To prevent variations in the food protein to digestive enzymes ratio, all digestions were conducted on an equal protein basis. Specifically, the chosen amounts of protein were 34 and 67 mg, which were determined based on preliminary trials using casein as the model protein. During these trials, different protein amounts ranging from 34 to 750 mg were tested, and the amounts corresponding to 188, 375, and 750 mg were identified as cytotoxic.

4.8.2. Handling of final digests and measurement of amino acid accessibility

After *in vitro* digestion, the resulting digests were filtered with a 0.45 µm regenerated cellulose membrane. This was primarily done to ensure the removal of bacteria before cell culture work as later recommended by Kondrashina et al. (2023) as well as to remove particulate material that if added to the apical compartment would partially cover the cell monolayers. Such covering would hinder the recovery of the cell lysates and prevent potential transport of the soluble proteaceous material across the cell monolayers. After filtration, the filtrate was referred to as the *accessible fraction* since it corresponded to digestive material accessible for intestinal absorption (Rodrigues et al., 2022). The amino acid accessibility was calculated as the yield of amino acids in the filtrate compared to the digest. Before filtration, we stopped enzymatic activity by heat inactivation at 95 °C for 10 min. An alternative to filtration would be the use of enzymatic inhibitors, however, these can interfere with Caco-2 brush border enzyme digestion (Kondrashina et al., 2023).

4.8.3. *In vitro* protein digestibility

In **Paper IV**, *in vitro* protein digestibility was measured via protein degree of hydrolysis (DH%). This involved quantification of free N-terminal amino acids and comparison to a theoretical value of the total N-terminal amino acids, calculated by analyzing the amino acid profile of the respective sample. Two methods were considered to measure DH%: the OPA and TNBS methods as described by Nielsen et al. (2001) and Adler-Nissen (1979), respectively. Both methods involve a derivatizing step with o-phthaldialdehyde and trinitrobenzenesulphonic acid, respectively. These reagents react specifically with primary amino groups to form compounds with strong absorption at 340 nm (Adler-Nissen, 1979; Nielsen et al., 2001). Moreover, both methods have shown a strong correlation when applied to plant-based protein sources, such as soy (Nielsen et al., 2001). While the OPA method offers the advantage of higher sample throughput (Rutherfurd, 2010), it utilizes sodium tetraborate, a compound classified as a human reproductive toxicant. Therefore, based on the potential exposure risk, we opted to use the TNBS method; its protocol was scaled down to allow for microplate absorbance reading as described by Cavonius et al. (2015).

4.8.4. Caco-2 cell amino acid bioavailability

Caco-2 cells are a human cell line derived from a colon adenocarcinoma, initially isolated by Fogh et al. (1977) and provided by the American Type Culture Collection (HTB-37, ATCC). Although originating from the colon, Caco-2 cells undergo spontaneous differentiation into cells resembling mature duodenal enterocytes, both morphologically and functionally, when cultured for approximately five days after reaching confluence (Sambuy et al., 2005). Due to this characteristic, Caco-2 cells are extensively utilized as a model for studying e.g., the bioavailability of amino acids (Goulart et al., 2014; Mcgraw et al., 2014; Zhao et al., 2017), food contaminants (Faria et al., 2020), drugs (Birch et al., 2018), and minerals (Chang et al., 2023). Amino acid bioavailability was calculated according to **Equation 11**. For a comprehensive description of cell handling and amino acid transport studies, the reader is referred to **Paper IV**.

$$\text{Bioavailability (\%)} = \frac{\text{Amino acids in basolateral side after cell incubation [mg]}}{\text{Amino acid in apical side right before cell incubation [mg]}} \times 100 \quad \textbf{(Eq. 11)}$$

4.9. Microscopy

To visualize differences during protein precipitation of supernatants containing or not Triton X-114, light microscopy analysis was carried out in **Paper III** with a Axiostar Plus microscopy (Carl Zeiss Microscopy) using a 10x and 100x magnification. For the higher magnification, a drop of immersion oil was placed between the objective lens and the cover glass. This was to produce a brighter and sharper image since the oil had a refractive index equal to the lowest refractive index of glass components in between (McCrone et al., 1984). The reader is referred to **Paper III** to visualize the microscopic observations.

To visualize the effect of the surfactant and alkaline treatments on the seaweed biomass, the freeze-dried samples were rehydrated in distilled water for 1 hour before frozen in liquid nitrogen. Samples were then cut into 7 µm sections in a Leica CM3050S cryostat and visualized with an Olympus BX53 light microscope (Olympus Life Science).

4.10. Ionic strength

The ionic strength was measured during protein extractions in **Papers II** and **III** with a conductivity meter (CDM210, Meterlab). A sodium chloride calibration curve was used to convert the conductivity to NaCl equivalents. All measurements were conducted at 20 ± 1 °C.

4.11. Statistical analysis

Data in the thesis is reported as mean ± standard deviation. The t-test or one-way analysis of variance (ANOVA) was used, the latter was followed either by the Student-Newman-Keuls or Tukey's post hoc test for pairwise comparisons. Exceptions included when data was not normally distributed, for which the non-parametric Kruskal-Wallis test was used instead. The differences were considered statistically significant at a p-value less than 0.05. In **Paper I**, statistical analysis was carried out using RStudio (v.1.2.50001), whereas in **Papers II-IV** the SPSS Statistics software (version 26.0.0.0) was used.

5. RESULTS AND DISCUSSION

This section has a dual purpose: to present the main experimental findings of **Papers I-IV** and to provide an analysis across these papers. For a detailed description and analysis of the results, the reader is referred to the actual **Papers I-IV**. Moreover, this section occasionally formulates new research questions to enrich the discussion. It also includes a review of existing studies on protein extraction from seaweed. The latter allows the insights gathered on protein extraction from **Papers II-IV** to be compared and discussed within the broader context of this research field.

5.1. Characterization of the targeted food process waters and their effects on seaweed growth

Seaweeds primarily use inorganic nitrogen in the form of ammonium and nitrate as nutrient sources, which contributes to protein accumulation. Among these two sources, ammonium is reported as being the preferred one for uptake and assimilation (Hurd et al., 2014) and according to **Figure 9**, it emerged as the predominant source of inorganic nitrogen in most process waters subjected to analysis, ranging from 1.1±<0.1 mM to 8.8±0.1 mM. Therefore, when adding process waters to the seaweed cultivation media, the ammonium content was normalized to 0.02 and 0.2 mM. This was based on previous works that found the optimum ammonium concentration for growth of *U. lactuca* to be 0.02-0.04 mM, which followed the same behavior of a Michaelis-Menten equation (Nielsen et al., 2012; Waite & Mitchell, 1972). Additionally, within the tested ammonium range of 0.006 to 0.1 mM, Nielsen et al. (2012) observed a linear and positive correlation with biomass nitrogen content. Thus, an ammonium concentration of 0.2 mM could deliver biomass with maximal nitrogen content as the relationship between ammonium concentration and nitrogen content also follows the pattern of a Michaelis-Menten equation (Cohen & Neori, 1991).

Ammonium values were higher for herring salt brine I (SB-I), herring salt brine II (SB-II), and herring spice brine (SPI), compared to the remaining herring process waters. This can be explained by autolytic enzymatic protein degradation and microbial activity during the maturation/brining of herring fillets that produce ammonium (Tavares et al., 2021).

Nitrate was the main inorganic nitrogen source in oat- (OAT) and RAS-derived waters with 6.8±<0.1 and 3.1±<0.1 mM, respectively (**Figure 9**). The presence of nitrate in the RAS water is related to microbial nitrification of the ammonia previously excreted by fish (Schneider et al., 2007). However, in the case of OAT, we suspect that the presence of nitrate is due to a cleaning agent used during the batch cleaning process, as cereals themselves are a relatively poor source of nitrate (Zhong et al., 2022). OAT, together with RAS, were the only water streams that were not tapped off while still food grade. In most cases, nitrite was present below the method's detection limit (0.43 μM) and the highest recorded value was for OAT (0.3 mM).

Inorganic P, often a limiting nutrient for seaweed growth (Hurd et al., 2014), was found to be particularly abundant in SB-I, SB-II and SPI with values spanning from 21.2 to 33.6 mM (**Figure 9**). This is likely due to autolytic enzymatic reactions that degrade adenosine 5′-triphosphate (ATP) and its related products (Tavares et al., 2021). Refrigerated seawater (RSW) and tub water (TUB) had lower inorganic P levels ranging from 3.5±0.1 to 5.7±0.1 mM, while the remaining waters showed levels lower than 0.5 mM.

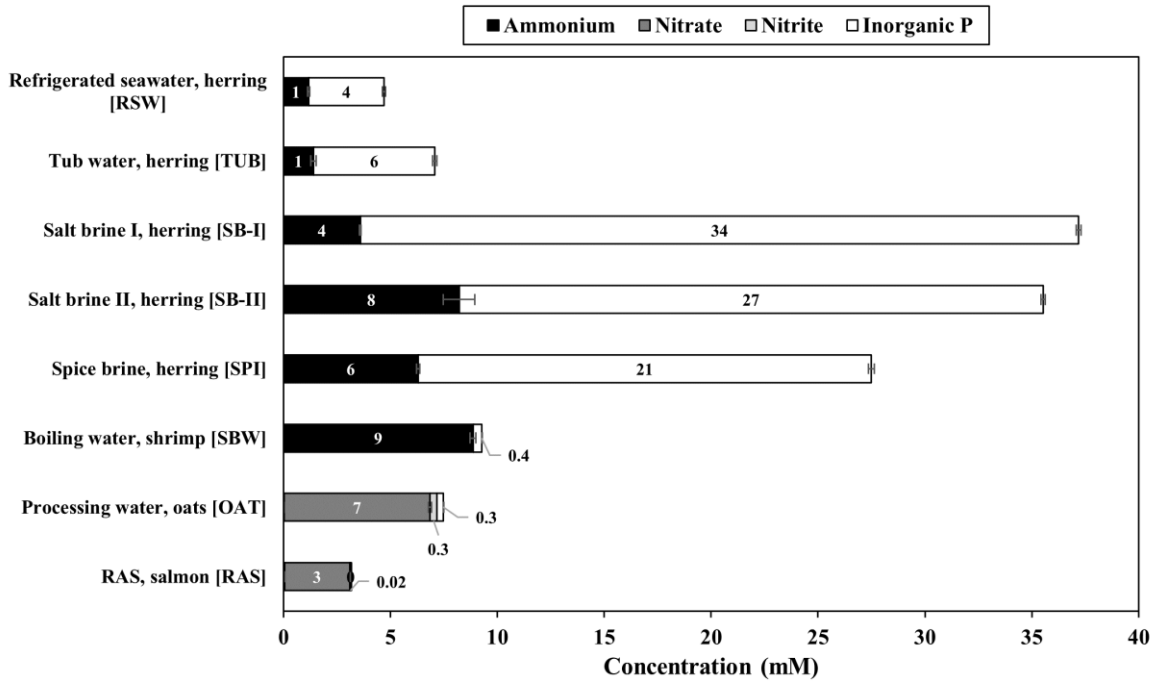

Figure 9 – Characterization of inorganic nutrients of undiluted food process waters to be included in seaweed cultivation media (N=3).

Generally, for *U. fenestrata*, *U. intestinalis*, and *C. linum*, the screening cultivation trials revealed similar SGRs between the ammonium-enriched seawaters and the media containing food process waters (**Figure 10**). Exceptions to this case, included higher SGRs for *C. linum* cultivated in RSW, TUB, and SPI diluted to 20 µM when compared to the respective ammonium-enriched seawater. Such increase was not significant when comparing to control seawater without added ammonium. Another exception was the negative SGR for *C. linum* cultivated in SPI diluted to 200 µM, which suggests the presence of inhibitory growth metabolites for this seaweed. Among the two ammonium concentrations tested, 20 µM generally yielded higher SGRs, which in certain cases exhibited statistical significance compared to 200 µM (**Figure 10**). This could also be the result of inhibitory growth metabolites being more diluted in the 20 µM dilution. Regarding *S. latissima*, it only showed positive SGRs in the seawater control (4.0-9.1 % d^{-1}) and ammonium-enriched seawater (3.3-3.9 % d^{-1}), whereas in the remaining treatments with process waters added, it died at the end of the cultivation period (data not shown).

Contrary to the green seaweeds tested, *S. latissima* is not an opportunistic species, so we posit that its inability to cope with media containing process waters can be attributed to the relatively high levels of nitrate in the diluted OAT- and RAS-waters (> 1000 µM), as well as the elevated inorganic P levels found in the diluted herring- and shrimp-derived process waters (> 50 µM). For comparison, typical concentrations of nitrate and inorganic P in surface coastal sea waters range from undetectable to approximately 30 µM and 2 µM, respectively (Hurd et al., 2014). To test this hypothesis, future

experiments could for instance normalize the nitrate concentration to levels of up to 150 µM, which *S. latissima* have been shown to tolerate (Boderskov et al., 2022).

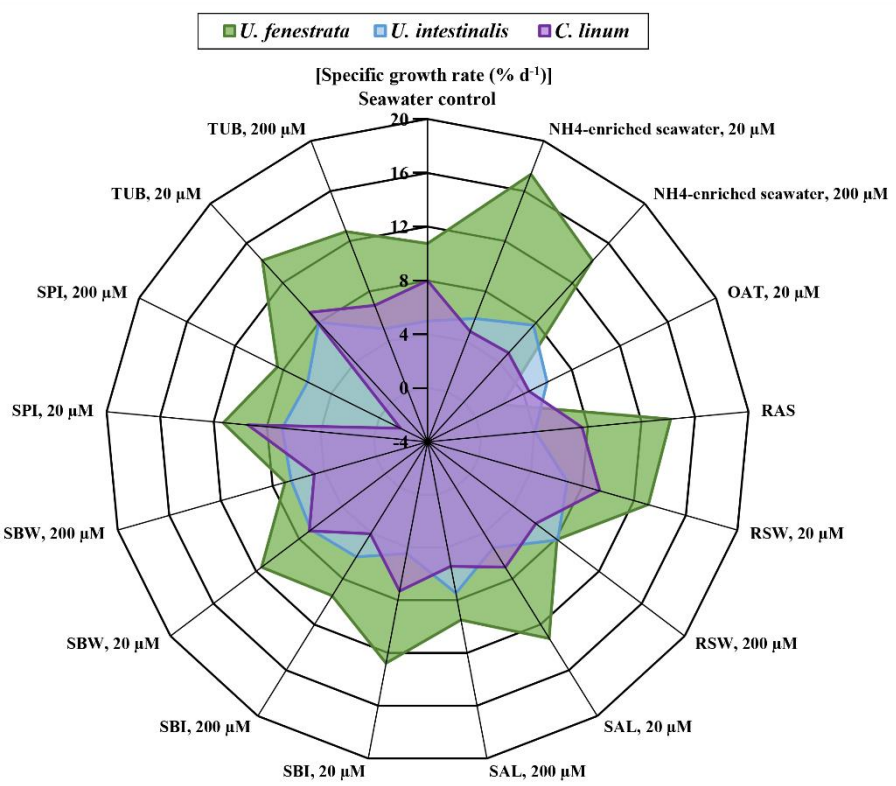

Figure 10 – Specific growth rate (SGR, % d^{-1}) at the end of the cultivation period of *U. fenestrata*, *U. intestinalis*, and *C. linum* cultivated in seawater control, NH_4^+-enriched seawater and food process waters (N=6).

5.2. Composition of seaweed biomasses and its relation to cultivation media, species, and post-harvest treatments

The chemical composition of seaweed has an impact on downstream protein extraction as well as on its use as a future food commodity. **Table 11** presents the composition of the seaweed biomasses used in this thesis and how it was affected by cultivation in food process waters species, species, and post-harvest treatments.

Table 11 – Composition of seaweed biomasses used in this thesis in terms of N content (% dw), total amino acids, ash, monosaccharides, and phenolics (N≥3, unless stated otherwise).

Paper	Biomass	Nitrogen content (% total dw)	Amino acid analysis			Total ash (% total dw)	Total monosaccharides (% total dw)	Total phenolics (% total dw)[b]
			Total AA (% total dw)	TEAA (% total AA)	N-to-protein factor			
I	*C. linum*		n.d	n.d	n.d	n.d	n.d	n.d
	Seawater control	1.3						
	NH_4^+ enriched seawater	1.6-2.0						
	Process waters,20 µM	1.9-3.9						
	Process waters,200 µM	2.0-5.0						
	U. fenestrata							
	Seawater control	2.0						
	NH_4^+ enriched seawater	1.7-3.9						
	Process waters,20 µM	3.6-4.7						
	Process waters,200 µM	3.8-4.6						
	U. intestinalis							
	Seawater control	1.2						
	NH_4^+ enriched seawater	1.3-1.7						
	Process waters,20 µM	1.8-4.4						
	Process waters,200 µM	3.0-4.2						
	S. latissima	n.a						
II	*S. latissima*	1.7±<0.1[a]	4.9±0.3	44.3	2.9	29.9±0.9	64.9±0.8	n.d
	S. latissima, soaked	1.7±<0.1[a]	5.4±0.2	41.4	3.2	22.4±1.8	77.6±2.1	
	S. latissima, blanched 45 °C	2.3±<0.1[a]	10.1±0.1	41.0	4.4	15.3±1.4	66.2±0.1	
	S. latissima, blanched 80 °C	2.5±<0.1[a]	11.1±0.4	42.4	4.5	15.4±2.5	71.1±4.5	
III	*U. fenestrata*	5.4±0.1	23.6±0.4	33.2	4.4	26.2±0.4	n.d	n.d
IV	*U. fenestrata*	4.3±0.1	16.5±2.5[a]	37.6[a]	3.9	n.d	n.d	0.09±<0.01[a]
	S. latissima	1.7±<0.1	7.4±0.1	44.7	4.5			0.85±0.04

[a]N=2; [b]expressed as grams of phloroglucinol equivalents per 100 g dw sample
dw *dry weight*; AA *amino acids*; TEAA *total essential amino acids*; n.d *not determined*

5.2.1. Nitrogen and amino acid content

According to **Table 11**, all three variables; cultivation media, species and post-harvest treatment, had an impact on the nitrogen content as well as on total and essential amino acids plus the N-to-protein conversion factor.

Effect of food process waters as nutrient sources (Paper I)

In general, cultivation of seaweeds in seawater containing food process waters as nutrient sources raised the total nitrogen content of the seaweed biomass up to almost 4-fold, when compared to cultivation in only seawater or ammonium-enriched seawater. Moreover, a dilution to 200 µM ammonium proved more effective at raising biomass nitrogen content, particularly in *C. linum*. Among the different process waters evaluated, most resulted in seaweeds with similar nitrogen content as the corresponding seawater control, apart from SPI and OAT which were responsible for the lower end of the nitrogen intervals presented in **Table 11**. Nitrogen content of *S. latissima* was not determined since the seaweed died at the end of the cultivation. Overall, the higher biomass nitrogen levels obtained following cultivation with process waters as well as the better adaption of green seaweeds versus *S. latissima* to process water additions align with the conclusions from a meta-analysis conducted by Stedt, Pavia, et al. (2022). Their analysis examined the relation between media nitrogen levels and biomass nitrogen when cultivating seaweed with wastewaters, predominantly from fish aquaculture (Stedt, Pavia, et al., 2022).

Effect of species (Papers I-IV)

The nitrogen content and total amino acid content (TAA) varied between species, with the highest values being recorded for *U. fenestrata,* followed by *S. latissima*, *U. intestinalis* and lastly *C. linum* (**Table 11**). Regarding unprocessed *S. latissima*, the nitrogen and amino acid levels correlated well between **Papers II** and **IV** while for *Ulva* they spanned from 2.0 to 5.4% on a dw basis. In literature, values for unprocessed cultivated *U. fenestrata*, *S. latissima*, *C. linum*, and *U. intestinalis* have been reported be situated between 2.4-5.5% (Steinhagen et al., 2021; Wheeler & Bjornsater, 1992), 0.8-2.2% (Schiener et al., 2015), 2.8-4.1% (Ansari & Ghanem, 2017), and 2.0-5.1% (Wheeler & Bjornsater, 1992), respectively, on a dw basis. Thus, nitrogen content for *U. fenestrata* and *S. latissima* in this thesis are close to, or within, those ranges, whereas for *C. linum* and *U. intestinalis,* they are lower than what is reported. While TAA were higher in *U. fenestrata* than in *S. latissima*, an opposite trend was found for TEAA with average values of 35.4% and 44.5% of TAA, respectively, corresponding to 6.7-7.9% and 2.2-3.3% of the total dw, in the same order. Another work that analyzed *U. fenestrata* and *S. latissima* found a similar trend for TEAA although less pronounced - 38.7 and 41.8% of TAA, respectively (Harrysson et al., 2018). The reader is referred to **Table 17** where the analysis of the amino acid chemical score and limiting amino acids of these biomasses is described.

The conversion factors for N-to-protein calculated in **Papers II-IV** were found to be lower than the universal factor of 5 proposed for seaweed (Angell et al., 2016); from 2.9 to 4.5. Furthermore, we observed fluctuations in these factors between and within species. These variations are likely due to differences in the non-protein nitrogen fraction, which includes chlorophyll, nucleic acids, free amino acids, and inorganic nitrogen (Angell et al., 2016). It should be noted though that the calculated factors might be slightly underestimated due to the complete degradation of tryptophan during the acid hydrolysis preceding amino acid analysis. Unpublished data from us revealed that *S. latissima* had a tryptophan content of 0.3% of TAA (0.014% total dw), while reported values for *Ulva sp.* are 0.3-0.7% of TAA (Fleurence, 1999; Peña-Rodríguez et al., 2011). Nevertheless, we recommend that nitrogen analysis be accompanied, whenever possible, by amino acid analysis.

Effect of post-harvest treatments (Paper II)

Blanching of *S. latissima* led to an increase in the total nitrogen content (**Table 11**). This was likely due to the observed lower ash content in these biomasses, which led to an up concentration of nitrogen. A similar explanation can also account for the observed increase in TAA. Interestingly, when compared to unprocessed *S. latissima*, the N-to-protein conversion factor was higher after blanching, but similar after soaking. These variations in conversion factors further support the importance of study-specific determinations of N-to-protein conversion factors and suggest that leaching of non-protein nitrogen occurred to a greater extent during blanching than during soaking. Regarding TEAA, both blanching and soaking statistically retained the same value as before treatment, which is consistent with data from Nielsen et al. (2020).

5.2.2. Total ash

Effect of species (Papers II-III)

The total ash content represented more than 25% of the total dw of *S. latissima* and *U. fenestrata* and was slightly higher for *S. latissima* (**Table 11**). Seaweed is well-known for having a high ash content due to its strong bioadsorptive and bioaccumulative capacities (Circuncisão et al., 2018).

Effect of post-harvest treatments (Paper II)

Blanching and soaking with freshwater resulted in biomasses with lower ash content, with the lowest values being recorded for blanched *S. latissima,* regardless of the blanching temperature. This aligned with previous studies that performed blanching and/or soaking of *S. latissima* (Krook et al., 2023; C. W. Nielsen et al., 2020). In their cases, blanching for 30-120 seconds resulted in biomasses with an ash content of 9.1-15.4% dw (Krook et al., 2023; Nielsen et al., 2020) and when instead soaking, in 18.4% dw (C. W. Nielsen et al., 2020), thus placing our data in the upper range.

5.2.3. Total and individual monosaccharide content

Total and individual monosaccharide content were only measured in **Paper II.** The incentive was primarily to correlate the increase in certain non-essential elements after blanching with changes in the alginate content. Nevertheless, analyzing content of total and/or individual monosaccharides can provide new insights into ideal protocols for downstream protein extraction.

Soaking *S. latissima* in fresh water significantly increased total monosaccharide content on a dw basis relative to unprocessed *S. latissima*, while blanching retained it (**Table 11**). **Figure 11** presents the content of each monosaccharide. Relative to unprocessed *S. latissima*, blanching resulted in a significant increase in alginate levels (i.e., guluronic acid+mannuronic acid) and a decrease in mannitol levels. These results are consistent with those from Stévant et al. (2018), where they soaked *S. latissima* for 22 h in warm freshwater at 32 °C.

Analysis of the monosaccharide profile (data not shown) revealed no significant changes induced by soaking compared to unprocessed *S. latissima*. Conversely, blanching resulted in biomasses with a higher relative content of alginate (58-64% of the total monosaccharides) compared to unprocessed *S. latissima* (44%). This was likely due to the leaching of mannitol to the blanching waters since its content was only 4-8% of the total monosaccharides in blanched biomasses versus 44% in the unprocessed *S. latissima*.

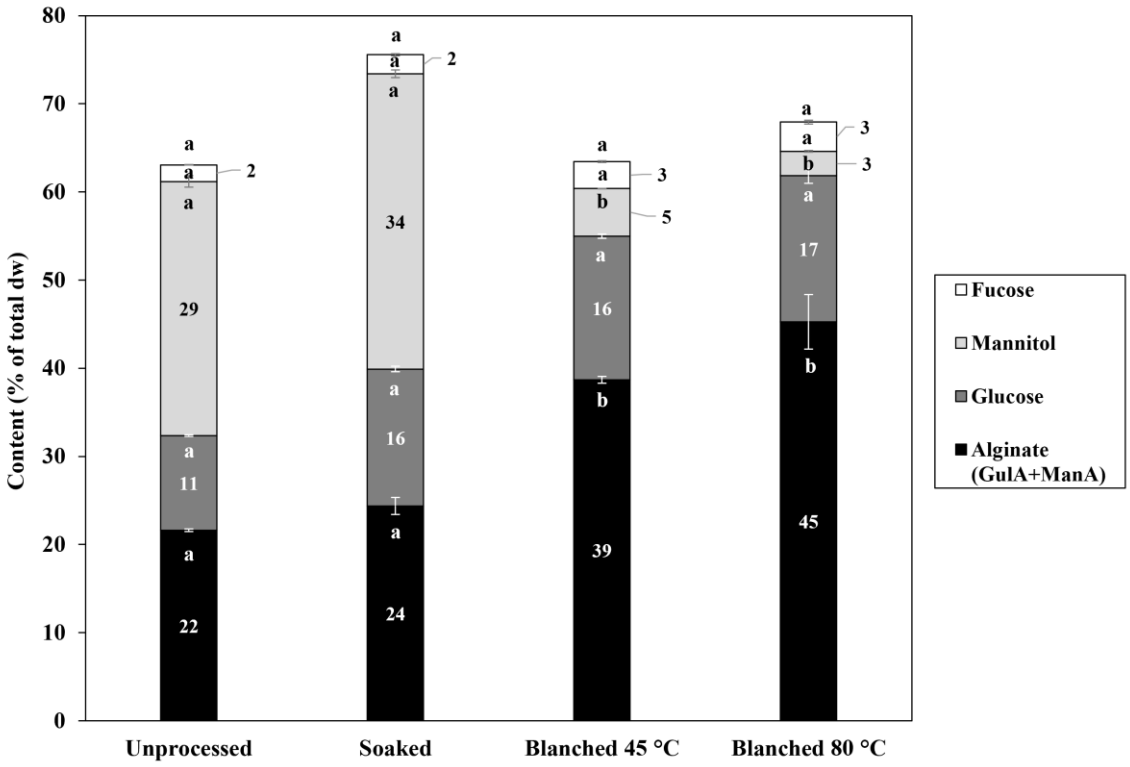

Figure 11 – Monosaccharide content (% dw) of unprocessed, soaked, and blanched *S. latissima* (N=2). Only monosaccharides representing more than 2% of the total dw were included in the figure to facilitate readability. Different letters (a-b) for each monosaccharide mean statistical differences ($p<0.05$). GulA means guluronic acid and ManA means mannuronic acid.

Overall, these results indicate that blanching, a common practice in the industry to reduce the iodine content of *S. latissima*, also impacts the carbohydrate fraction. Such compositional differences can influence downstream protein extraction due to, for example, selective polysaccharide co-extraction (Section 5.4.3) and viscosity differences that affect mass transfer rates (Tenorio et al., 2018).

5.2.4. Total phenolic content

In **Paper IV**, total phenolic content of *S. latissima* was around 9.4 times higher than *U. fenestrata*. Other studies reported a content ranging from 0.4 to 1.2 % total dw for *S. latissima* (Vilg et al., 2015), which aligns with the results in **Table 11**. Moreover, others have reported that brown seaweed usually contains more phenolics than *Ulva sp*. (Holdt & Kraan, 2011).

5.2.5. Non-essential elements and selected essential elements

The species *S. latissima* often contains high levels of iodine and non-essential elements, such as cadmium, inorganic arsenic, and lead. Blanching is a simple strategy that has been studied in several previous works (Jordbrekk Blikra et al., 2021; Krook et al., 2023; C. W. Nielsen et al., 2020; Stévant et al., 2018) to partially reduce the levels of some of these elements. Despite existing literature on this topic, it was reported in **Paper II** to allow for subsequent assessments of blanching-induced effects on protein extraction. Moreover, eight essential elements were also examined, of which iron and zinc are of significant relevance as they are both low in vegetarian diets (Craig, 2010).

Blanching significantly reduced iodine levels by around 85% compared to the unprocessed biomass (**Figure 12**). It should be noted that in **Paper II** a non-parametric statistical test should have been used for iodine and lead, which then would have shown statistical differences between blanched and

unprocessed biomasses (Kruskall-Wallis test, p-value=0.006 for blanched 80 °C vs. unprocessed and p-value=0.024 for blanched 45 °C vs. unprocessed). Relative to the unprocessed biomass, cadmium and lead levels were higher after blanching, especially at 80 °C, which could be related to the larger relative amount of alginate after blanching. Moreover, blanching retained levels of iron and zinc (**Figure 12**) as well as inorganic arsenic (data not shown). Based on the tolerable upper daily intakes (TDI) for cadmium, iodine, inorganic arsenic, and lead (EFSA, 2006, 2009b, 2009a, 2013) and the population reference intake (PRI) for iron and zinc (EFSA, 2019), iodine was the limiting factor for the daily consumption of blanched *S. latissima*, which should be kept at 1.3-1.5 g dw versus only 0.2 g dw for unprocessed seaweed. All these amounts were at least 20 times lower than the ones needed to reach the TDIs or PRIs of the remaining elements. Our unpublished data on unprocessed *U. fenestrata* indicates that iodine remains the limiting element also for this species, however, the seaweed could be consumed up to 16.7 g dw before reaching the TDI for iodine. Therefore, *U. fenestrata* might hold a higher potential as a whole food source, although randomized controlled human trials shall be conducted to determine how much of the iodine is bioavailable. According to our recent review (Trigo et al., 2023), no randomized controlled human trials have been conducted assessing the iodine bioavailability of whole *Ulva sp*. or *S. latissima*.

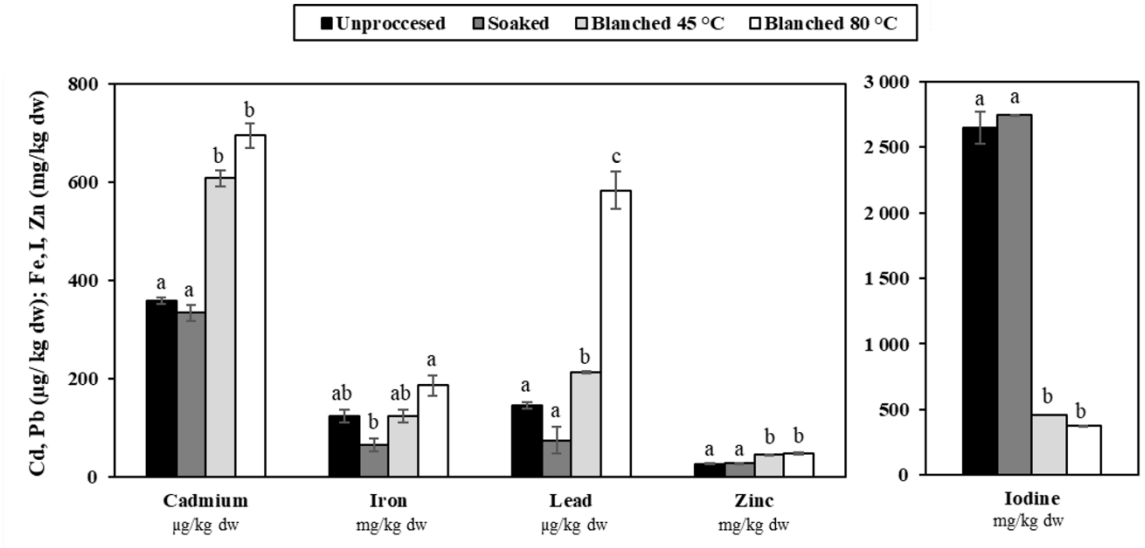

Figure 12 – Non-essential elements and selected essential elements of unprocessed, soaked, and blanched *S. latissima* (N=3). Different letters (a-c) within each element mean statistical differences ($p<0.05$).

5.3. Protein up-concentration and impact of species, post-harvest treatments, and extraction method

5.3.1. Protein yields based on the Lowry protein analysis method

Table 12 presents the protein yields (solubility yield, precipitation yield and total yield) based on protein measurements done with the Lowry analysis method (**Papers II** and **IV)** as well as the maximum yields reported in literature.

Effect of species (Papers II and IV)

In **Papers II** and **IV**, alkaline protein solubilization resulted in a solubility yield of around 40% for unprocessed *S. latissima* and *U. fenestrata*. As shown in **Table 12**, this value falls within the previously

reported ranges for *S. latissima* (21-91%) and *Ulva sp. (*3-63%) (Abdollahi et al., 2019; Harrysson et al., 2018, 2019; Juul, Danielsen, et al., 2021; Juul, Steinhagen, et al., 2022; Postma et al., 2018; Prabhu et al., 2019; Robin et al., 2018; Vilg & Undeland, 2017), but is lower than that reported for the red seaweed *Porphyra umbilicalis* (68%) (Harrysson et al., 2018). Unfortunately, other studies focusing on red seaweeds expressed protein solubilization as g protein per 100 g dw seaweed without providing the corresponding protein content of the seaweed, something which hinders unit conversion (Harnedy & FitzGerald, 2013; Jamshidi et al., 2018; Mendez & Kwon, 2021; Pimentel et al., 2020). It should be noted that the highest previously reported yield values for *S. latissima* corresponded to protein extraction from dried and milled biomass (Abdollahi et al., 2019) while in **Paper II**, wet-minced biomass was used. Since milling often results in larger sample surface area towards the extraction solvent, potentially enhancing protein solubility, these results are not directly comparable with ours. However, we opted for wet-minced biomass as it saves costs and energy associated with biomass de- and re-hydration. Others who have used a fresh-minced *S. latissima* reported a protein solubility yield ranging from 21 to 53% (Harrysson et al., 2018; Vilg & Undeland, 2017), thus placing our data in the upper range.

After protein solubilization in **Papers II** and **IV**, proteins were recovered via isoelectric precipitation. The average protein precipitation yield for *S. latissima* (47%) was higher than for *U. fenestrata* (28%), albeit no significant differences were found (t-test, p=0.084). A similar trend was also detected in literature, with protein precipitation yields being slightly higher for *S. latissima* (30-67%) compared to *Ulva sp*. (29-51%) (Abdollahi et al., 2019; Harrysson et al., 2018, 2019; Juul et al., 2021, 2022; Veide Vilg & Undeland, 2017). The only found data point for red seaweed, corresponding to *P. umbilicalis,* was within the previous ranges (41%) (Harrysson et al., 2018).

Total protein yields were higher with *S. latissima* (**Paper II)** (19%) compared to *U. fenestrata* (**Paper IV)** (11%), although differences were not statistically significant (t-test, p=0.093). Analysis of other works using isoelectric precipitation (**Table 12**), revealed that: (i) total protein yields from *S. latissima* spanned over a narrower range compared to *U. fenestrata* - 11-26% versus 4-29%, respectively, and (ii) our data is in the upper range for *S. latissima* and in the mid-range for *U. fenestrata*. Regarding *U. fenestrata*, two studies using a similar extraction method as in **Paper IV**, reported total protein yields of 8% (Juul, Danielsen, et al., 2021) and 29% (Harrysson et al., 2019). The *U. fenestrata* biomass in those two studies and in **Paper IV** originated from the same type of long-term cultivation that was kept by parthenogenetic proliferation of a gametophytic strain. Factors explaining such contrasting differences in total yield are thus likely due to the use of different post-harvest preservations; dry-milled seaweed in the Harrysson et al. (2019) study and wet-minced seaweed in ours and Juul, Danielsen, et al. (2021) studies. Another factor could be related to the stage of maturation, more specifically if *U. fenestrata* was on its vegetative or fertile stage. Thus, distinct stages of maturation between the studies might have resulted in the different profiles of protein expression, which in turn influenced protein extraction yields. Studies on red and brown seaweed have demonstrated that RuBisCo is more expressed in gametophytes than sporophytes, while the effect of vegetative or fertile stage was not addressed (Wang et al., 2011).

Effect of post-harvest treatment (Paper II)

The pH-shift method coupled with a freeze-thawing step was conducted on unprocessed, soaked, and blanched *S. latissima* biomasses. Protein extraction from treated biomasses, especially the blanched ones, resulted in a significantly lower protein solubility yield compared to unprocessed biomass (**Table 12**). We attributed this outcome to the reduced proportion of small molecular weight protein/peptides induced by blanching (Section 5.3.7) and/or increased proportion of water-insoluble protein aggregates (Sashikala et al., 2015). Although blanched biomass gave rise to a reduced amount of solubilized proteins compared to unprocessed biomass, it significantly increased protein precipitation yields, particularly after blanching at 45 °C. The precipitation yield obtained from this biomass (83%) was in fact the highest value reported in literature for seaweed and it was similar to that reported from pea protein extraction (Sajib et al., 2023) (**Table 12**). We posit this finding to be partially related to the reduced ionic strength induced by blanching, as confirmed later through a dialysis trial. In that trial, we dialyzed the supernatant containing the solubilized proteins from the unprocessed biomass, reducing its ionic strength from 88 mM to 44 mM, equivalent to the supernatant from the blanched 45°C biomass. Such change resulted in a significant 18 percentage point increase in protein precipitation yield (**Table 12**). A similar effect of salt removal via dialysis of the supernatant was demonstrated by Juul et al. (2022) for *U. fenestrata*. They observed that lowering the ionic strength from 140 mM to 31 or 5 mM increased protein precipitation yield by 28 or 50 percentage points, respectively (Juul, Steinhagen, et al., 2022). Another reason potentially explaining the higher protein precipitation yields with the blanched biomasses is related to the co-extraction of charged polysaccharides as analyzed in Section 5.4.3. As some proteins are known to be embedded with cell wall fibers such as alginate (Domozych, 2016; Shao & Duan, 2022) it is plausible that the elevated precipitation yields can be attributed to this co-extraction phenomenon.

Based on the high precipitation yield that offset the relatively low protein solubility yield, total protein yields were the highest for the blanched 45 °C biomass (23%). This total yield was similar to that obtained with unprocessed biomass and was significantly higher than the ones from the soaked and blanched 80 °C biomasses.

Effect of precipitation conditions (Paper II)

Adding a freeze-thawing cycle to improve protein precipitation can jeopardize the economic sustainability of the pH-shift process. Thus, we evaluated the effect of dialysis as a potential replacer of this strategy since membrane processes are widely implemented in the dairy and beverage industries (Charcosset, 2021). Overall, dialysis against a buffer with an ionic strength of 44 mM achieved similar precipitation yields to a freeze-thawing cycle (**Table 12**). Dialysis could likely achieve higher precipitation yields by dialyzing against a lower ionic strength (Juul, Steinhagen, et al., 2022). Interestingly, a freeze-thawing cycle following dialysis resulted in a synergetic effect, surpassing the outcomes of both individual strategies.

Table 12 – Protein solubility yields, protein precipitation yields, and total protein yields based on Lowry measurements obtained during protein extraction from brown, green and red seaweeds. Reported yields correspond to Papers II and IV (N=2) and data available in literature. In all cases, "pH-shift" refers to alkaline solubilization followed by isoelectric precipitation at low pH.

Reference	Biomass	Protein extraction method	Protein solubility yield (%)	Protein precipitation yield (%)	Total protein yield (%)
Paper II	*S. latissima*	pH-shift	**36.4**±2.1	**21.0**±3.7[a]	**7.7**±1.8[a]
		pH-shift + freeze-thawing		**42.4**±0.6[a]	**14.5**±0.2[a]
		pH-shift + dialysis during isoelectric precipitation		**40.3**±2.5[a]	**14.7**±1.7[a]
		pH-shift + dialysis during isoelectric precipitation + freeze-thawing		**60.1**±0.1[a]	**20.6**±<0.1[a]
		pH-shift + freeze-thawing	**41.5**±0.8	**46.7**±0.3	**19.4**±0.8
	S. latissima, soaked	pH-shift + freeze-thawing	**34.6**±0.4	**44.0**±2.4	**15.2**±0.7
	S. latissima, blanched 45 °C	pH-shift + freeze-thawing	**28.3**±0.3	**82.5**±0.8	**23.3**±1.4
	S. latissima, blanched 80 °C	pH-shift + freeze-thawing	**20.1**±0.3	**67.9**±0.9	**13.9**±0.4
Abdollahi et al. (2019)		pH-shift + freeze-thawing	**91**	**43**	**26**
		pH-shift	**91**	**30**	**11**
Vilg & Undeland (2017)		pH-shift	**21**	**33**	n.a
Harrysson et al. (2018)		pH-shift + freeze-thawing	**53**	**67**	**25**
		Ultrasound-assisted water solubilization + protein salting out + dialysis	-	-	**3**
		Accelerated solvent extraction sequence	-	-	**1**
Paper IV	*U. fenestrata*	pH-shift + freeze-thawing	**38.8**±2.1	**28.4**±8.0	**11.1**±3.7
Juul et al. (2021a)		pH-shift + freeze-thawing	**25**	**33**	**8**
		Mechanical pressing + isoelectric precipitation + freeze-thawing	**12**	**32**	**4**
Juul et al. (2021b)		Mechanical pressing + antioxidant + isoelectric precipitation + freeze-thawing	**13**	**46**	**5**
Juul et al. (2022)		pH-shift + freeze-thawing	**24**	**29**	**7**
		Mechanical pressing + isoelectric precipitation + freeze-thawing	**14**	**35**	**5**
		Mechanical pressing + pH-shift on pulp + freeze-thawing	**24** (pressing) + **23** (pH-shift on pulp)	**35** (pressing) and **82** (pH-shift on pulp)	**24**
Harrysson et al. (2019)		pH-shift + freeze-thawing	**63**	**48**	**29**
		pH-shift + enzyme treatment + freeze-thawing	**56**	**51**	**25**
Harrysson et al. (2018)		pH-shift + freeze-thawing	**68**	**12**	**6**
		Ultrasound-assisted water solubilization + protein salting out + dialysis	-	-	**20**
		Accelerated solvent extraction sequence	-	-	**< 1**
Postma et al. (2018)	*Ulva lactuca*	Aqueous solubilization (with osmotic shock)	**20**	-	-
		Aqueous solubilization (with high-speed homogenization)	**39**		
		Enzyme-assisted solubilization in water	**26**	-	-
		Pulsed electric field-assisted solubilization in water	**15**	-	-
Prabhu et al. (2019)	*Ulva ohnoi*	Pulsed electric field-assisted solubilization in water	**15**	-	-
Robin et al. (2018)	*Ulva sp.*	Pulsed electric field-assisted solubilization in water + dialysis	**3**	-	-
Harrysson et al. (2018)	*Porphyra umbilicalis*	pH-shift + freeze-thawing	**68**	**41**	**23**
		Ultrasound-assisted water solubilization + protein salting out + dialysis	-	-	**5**
		Accelerated solvent extraction sequence	-	-	**4**
Harnedy & FitzGerald (2013)	*Palmaria palmata*	Aqueous solubilization + alkaline solubilization on seaweed pellet	n.a	-	-
		Enzyme-assisted solubilization	n.a		
Mendez & Kwon (2021)	*Devaleraea mollis*	Alkaline solubilization + enzyme treatment	n.a	-	-
Mendez & Kwon (2023)		Alkaline solubilization + enzyme treatment + acid precipitation	n.a	n.a	n.a
		Osborne protein fractionation	n.a	-	-
Pimentel et al. (2020)	*Porphyra dioica*	pH-shift	n.a	n.a	n.a
Jamshidi et al. (2018)	*Gelidiella acerosa*	Enzyme-assisted solubilization in acetate buffer	n.a	-	-

[a]Precipitation yields at pH 2.5, so not directly comparable with the remaining unmarked precipitation yields of Paper II that were conducted at pH 2.0; n.a *not avaiable*

5.3.2. Protein yields based on the total N analysis method

In **Paper III**, protein solubility yield, protein precipitation yield, and total protein yields were based on N measurements since the presence of Triton X-114 could interfere with the Lowry method (Section 4.6.1). Total protein yields based on N were not reported for **Papers II** and **IV**, but were calculated for this thesis. By calculating yields based on N-data, the effect of species and post-harvest treatment followed similar trends to the previous section. An exception was the effect of soaking in **Paper III** that delivered a total N yield similar to the blanched 45 °C biomass.

In **Paper III** a new protein extraction method was developed, and results showing the optimized yields are presented in **Table 13**. The optimization process involved processing of wet-minced *U. fenestrata* with alkaline solution at pH 12 or with 2% Triton X-114 and reprocessing the resulting pellet with one of these solutions. The highest total N solubility yield was observed when the biomass was processed with Triton X-114, followed by reprocessing the pellet with alkaline solution. The second optimization trial involved reducing Triton X-114 levels to 0.5 and 0.1%, which besides retaining N solubility yields enabled protein precipitation to occur.

Effect of extraction method (Papers II-IV)

The total N solubility yield in **Paper III** (sum of yields from first and second processing cycles) spanned from 49 to 63%, the upper range corresponding to 0.5 or 0.1% Triton X-114, which gave significantly higher yields than the control comprising two cycles of alkaline extraction. We later confirmed that the superior N yield was likely due to (i) Triton X-114 targeting thylakoid membrane proteins and disrupting the chloroplast membranes (Section 5.3.6) and/or (ii) significant ash removal during surfactant treatment that likely promoted a salting-in during the second processing cycle with water at pH 12.

Among other works in the literature reporting N solubility yields (Angell et al., 2017; Fleurence et al., 1995; Kadam et al., 2017; Kandasamy et al., 2012; O' Connor et al., 2020; Wijers et al., 2020), only Kadam et al. (2017) and Wijers et al. (2020) reported yields up to 60% (**Table 13**). In their case, the biomass was however dried-milled, which could provide a solubilization advantage relative to our extraction protocol. Nonetheless, Kadam et al. (2017) followed a multi-step approach for protein extraction from *Ascophyllum nodosum*. The first step involved aqueous solubilization, followed by processing the seaweed pellet with 0.4 M HCl and then reprocessing of the second seaweed pellet with 0.4 M NaOH (Kadam et al., 2017). On the other hand, Wijers et al. (2020) performed protein solubilization with 0.1 M NaOH on the same species, followed by high-speed homogenization and one-hour incubation at 50 °C. In both studies, the pH values after the addition of acid or base were not disclosed and protein recovery via e.g., isoelectric precipitation was not attempted (Kadam et al., 2017; Wijers et al., 2020). Thus, their final product was a protein-containing solution.

Protein extraction with Triton X-114 increased by more than 3 times the N precipitation yield compared to the control treatment (**Table 13**). We hypothesized that such significant differences were due to the precipitation of proteins bound to charged lipid membranes containing a phosphate or sulfonic group with a pKa close to pH 2 (Buchanan et al., 2015; Schaller et al., 2011; Simidjiev & Barzda, 1997). In comparison to the literature, Angell et al. (2017) observed precipitation yields around 2 times higher than those we obtained using Triton X-114. In their study, wet-blended *Ulva ohnoi* was subjected to an

aqueous extraction with a seaweed:water ratio of 1:5. After centrifugation, the resulting pellet was reprocessed with water at pH 12 following a pellet:water ratio of 1:20. Therefore, the higher precipitation yields reported by Angell et al. (2017) might be the outcome not only of a different species but also of the extensive dilution of salts caused by the high water ratios they used. This conjecture is supported by the dialysis trials conducted in **Paper II** and in Juul et al. (2022) study. In our extraction method (**Paper III**), we selected water ratios up to 1:4 as this option is more practical and realistic when aiming for industrial scalability. For instance, processing 1 metric ton of *U. fenestrata* with our method would require about 5.4 tons of water (already considering the water usage of the second processing cycle), whereas, for the method developed by Angell et al. (2017), the same amount of *U. ohnoi* would require 19.0 tons of water (assuming the mass balances provided by the authors).

Among **Papers II-IV**, total N yields were the highest for the surfactant-based method (17-20%), followed by the pH-shift method on unprocessed, soaked, and blanched 45 °C *S. latisima* (9-13%), and lastly the control treatment of the surfactant-based method and the pH-shift method applied to *U. fenestrata* and blanched 80°C *S.latissima* (5-6%). In the existing literature, Angell et al. (2017) was the only study reporting total N yields, and their results fell within the range obtained by the surfactant-based method.

Table 13 - Protein solubility yield, protein precipitation yield, and total protein yields based on N measurements done during protein extraction from brown, green and red seaweed. Yields correspond to Paper III (N=2) and data available in literature. In all cases, "pH-shift" refers to alkaline solubilization followed by isoelectric precipitation at low pH.

Reference	Biomass	Protein extraction method		N solubility yield (%)	N precipitation yield (%)	N total yield (%)
Paper II	*S. latissima*	pH-shift + freeze-thawing		n.d	n.d	**8.9**[a,c]
	S. latissima, soaked					**12.3**[a,c]
	S. latissima, blanched 45 °C					**12.8**[a,c]
	S. latissima, blanched 80 °C					**5.8**[a,c]
Wijers et al. (2020)[b]	*S. latissima*	Alkaline solubilization		**44**	-	-
	Ascophyllum nodosum			**60**		
O' Connor et al. (2020)	*Fucus vesiculosus* (*Alaria esculenta*)	Ultrasound-assisted solubilization + protein salting out + dialysis		**35 (18)**	-	-
		High-pressure processing-assisted solubilization		**24 (15)**		
		Autoclave-assisted solubilization		**24 (17)**		
Kadam et al. (2017)	*Ascophyllum nodosum*	Aqueous solubilization + alkaline solubilization on pellet (or acid solubilization of pellet)		**56 (17)**	-	-
		Aqueous solubilization + alkaline solubilization on pellet (or acid solubilization of pellet) + ultrasound		**57 (43)**		
		Aqueous solubilization + alkaline solubilization on pellet + acid solubilization of 2nd pellet		**60**		
		Aqueous solubilization + acid solubilization on pellet + alkaline solubilization of 2nd pellet		**51**		
Wong & Cheung (2001)	*Sargassum patens* (*S. henslowianum, S. hemiphyllum*)	Aqueous solubilization + alkaline solubilization with reducing agent of pellet + protein salting out + dialysis		**48 (10, 33)**[d]	-	-
Paper III	*U. fenestrata*	Surfactant-based solubilization + alkaline solubilization of pellet + isoelectric precipitation	Alkaline (control)	**48.4±1.7**	**9.2**±1.2	**4.6**±0.6
			0.1% Triton	**60.2±1.0**	**29.2**±3.8	**17.0**±1.6
			0.5% Triton	**62.6±2.6**	**33.0**±1.3	**20.2**±0.6
Paper IV		pH-shift + freeze-thawing		n.d	n.d	**5.1**±0.8[a]
Angell et al. (2017)	*Ulva ohnoi*	Aqueous solubilization + alkaline solubilization of pellet + isoelectric precipitation		**43**	**62**	**19**
Wijers et al. (2020)[b]	*Ulva lactuca*	Alkaline solubilization		**33**	-	-
Fleurence et al. (1995)[b]	*Ulva rotundata* (*Ulva rigida*)	Aqueous solubilization		**14 (10)**	-	-
		Solubilization with Tris-HCl buffer		**14 (9)**		
		Solubilization with Tris-HCl buffer + ultrasound		**16 (10)**		
		Solubilization with Tris-HCl buffer + alkaline solubilization of pellet		**25 (18)**		
		Aqueous solubilization + alkaline solubilization of pellet		**36 (27)**		
		Solubilization with polyether polymer and salt in an aqueous two-phase system		**32 (19)**		
		Enzyme-assisted solubilization in phosphate buffer		**22 (19)**		
Wong & Cheung (2001)	*Ulva lactuca*	Aqueous solubilization + alkaline solubilization with reducing agent of pellet + protein salting out + dialysis		**36**[d]	-	-
Kandasamy et al. (2012)[b]	*Ulva linza* (*Ulva compressa, Ulva tubulosa*)			**46 (37, 32)**[d]	-	-
O' Connor et al. (2020)	*Palmaria palmata* (*Chondrus crispus)*	Ultrasound-assisted solubilization + protein salting out + dialysis		**13 (35)**	-	-
		High-pressure processing-assisted solubilization		**15 (16)**		
		Autoclave-assisted solubilization		**22 (22)**		
Wijers et al. (2020)[b]	*Chondrus crispus*	Alkaline solubilization		**42**	-	-
Kumar et al. (2014)[b]	*Kappaphycus alvarezii*	Aqueous solubilization + alkaline solubilization with reducing agent of pellet + protein salting out + dialysis		**43**[d]	-	-
Wong & Cheung (2001)	*Hypnea charoides*	Aqueous solubilization + alkaline solubilization with reducing agent of pellet + protein salting out + dialysis		**46**[d]	-	-
	Hypnea japonica			**45**[d]		

[a]Total yield calculated as follows: [mg N of input material/mg N of obtained protein extract]×100; [b]Protein was determined via the Kjeldahl method, while for unmarked studies the Dumas method was used; [c]N=1, since the recovered protein extract was only weighed in one replica; [d]Assuming that 100% of the solubilized protein was precipitated with ammonium sulphate; n.d *not determined*

5.3.3. Total amino acid yields of pH-shift and surfactant-based methods

Table 14 presents the biomasses that underwent protein extraction in **Papers II-IV** and prior literature. In **Figure 13**, the corresponding total amino acid yields are plotted against the purity of the obtained protein extracts. This correlation is essential for evaluating the efficiency and resource utilization of a protein extraction method.

Table 14 – List of works, including Papers II-IV and data available from literature, that measured total amino acid yields (N=2, for papers of this thesis, unless stated otherwise) and amino acid purity of the produced seaweed protein extracts. The plot illustrating the relationship between these two measurements can be found in Figure 13. In all cases, "pH-shift" refers to alkaline solubilization followed by isoelectric precipitation at low pH.

Reference	Biomass	Protein extraction method		No.
Paper II	*S. latissima*	pH-shift + freeze-thawing		1[a, b]
	S. latissima, soaked			2[a, b]
	S. latissima, blanched 45 °C			3[a, b]
	S. latissima, blanched 80 °C			4[a, b]
Harrysson et al. (2018)	*S. latissima*	pH-shift + freeze-thawing		5
		Ultrasound-assisted solubilization with water + protein salting out + dialysis		6
		Accelerated solvent extraction sequence		7
Paper III	*U. fenestrata*	Surfactant-based solubilization + alkaline solubilization of pellet + isoelectric precipitation	Alkaline (control)	8
			0.1% Triton	9
			0.5% Triton	10
Paper IV		pH-shift + freeze-thawing		11[a]
Harrysson et al. (2018)		pH-shift + freeze-thawing		12
		Ultrasound-assisted solubilization + protein salting out + dialysis		13
		Accelerated solvent extraction		14
Magnusson et al. (2019)	*Ulva ohnoi*, soaked	Alkaline solubilization + isoelectric precipitation		15
Robin et al. (2018)	*Ulva sp.*	Pulsed electric field-assisted solubilization in water + dialysis		16[c]
Harrysson et al. (2018)	*Porphyra umbilicalis*	pH-shift + freeze-thawing		16
		Ultrasound-assisted solubilization in water + protein salting out + dialysis		17
		Accelerated solvent extraction sequence		18

[a]Total yield calculated as follows: [total amino acids of input material/ total amino acids of obtained protein extract]×100; [b] N=1; [c]Yield corresponds to amino acid solubilization yield as solubilized protein were recovered via freeze-drying

Based on **Figure 13**, TAA yields were the highest for the surfactant-based method (22-23%), which were also higher when compared to other works (Harrysson et al., 2018; Magnusson et al., 2019; Robin et al., 2018). This was followed by the pH-shift method as applied to unprocessed, soaked, and blanched 45 °C *S. latissima* (13-15%), while the control treatment to the surfactant-based method as well as the pH-shift method as applied to *U. fenestrata* and blanched 80°C *S. latissima* showed the lowest yields (6-8%). Moreover, the surfactant-based method, particularly the one using 0.1% Triton X-114, delivered one of the best yield-purity balances, both when compared to **Papers II-IV** and to prior literature (Harrysson et al., 2018; Magnusson et al., 2019; Robin et al., 2018). It is important to highlight that this was achieved without introducing a freeze-thawing step as used in **Papers II, IV** and by Harrysson et al. (2018) or by applying a very high water-to- seaweed ratio such as that used by Magnusson et al. (2019) (ratio of 1:30).

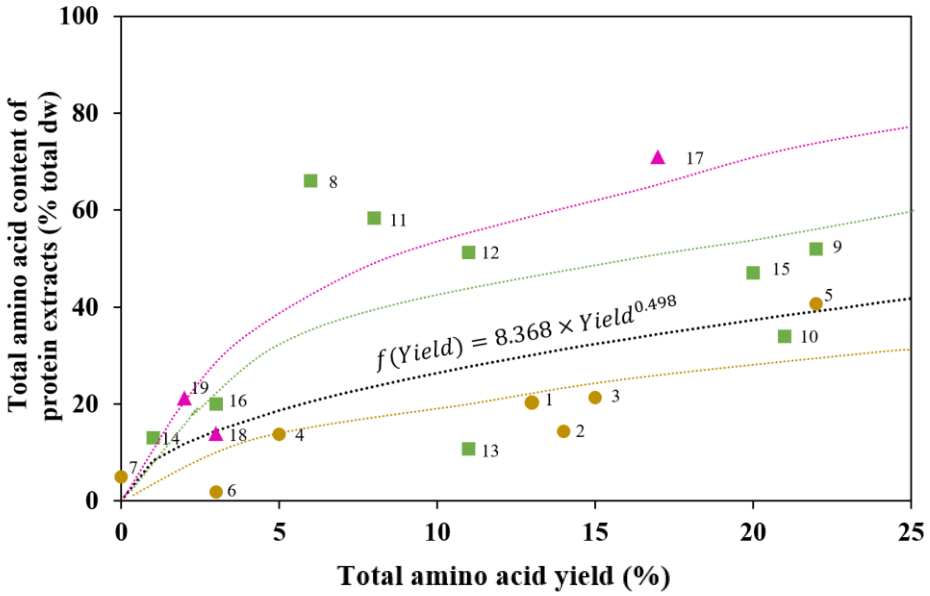

Figure 13 – Correlation between total amino acid yield and amino acid purity of the produced seaweed protein extracts from Papers II-IV and from data available in literature. The data labels correspond to the list found in Table 14.

Among the various regression models tested to correlate amino acid yields with purity, such as linear, logarithmic, inverse, quadratic, cubic, compound, power law, S, growth, exponential, and logistic, the power law was the one showing the highest R-square (0.370) and level of significance, $F(1, 17)=10.00$, $p=0.006$ for an average among all seaweed colors (**Figure 13**). A similar trend was observed when correlating amino acid yields with amino acid up-concentration relative to original biomass (data not shown). In **Figure 13**, we also sketched a possible power law regression as a function of the seaweed color, although more data points would be needed to perform the actual regression. For more robust models, additional data points would be needed to balance and account for the various effects plotted, such as species, post-harvest treatments, and specific extraction methods.

To date, reported protein extraction from seaweed has not surpassed yields of 30% (**Figure 13**), why we consider it important that efficient and scalable methods such as the one developed in **Paper III** are integrated in a cascade biorefining aiming to maximize resource efficiency and reduce waste streams (Magnusson et al., 2019; Wahlström et al., 2018). Regarding the purity of amino acids, the highest recorded levels stand at 71% (**Figure 13**), which categorizes the seaweed extract as a "concentrate" (≥ 65% purity dw) using the same terminology in industries like soy (Endres, 2001). To obtain a protein "isolate" (≥ 90% purity dw), demands additional purification steps. However, this would raise costs and environmental impact. Such pure extracts have not been the goal of the CirkAlg project, but rather on developing multifunctional protein ingredients. These ingredients encompass various nutrients and compounds that enhance sensory and textural qualities, including salts and fibers.

5.3.4. Protein yield comparison between seaweed and legumes

As mentioned earlier, total protein yields obtained from seaweed are consistently below 30%, regardless of the used protein determination method. In contrast, legumes, such as lupin have been found to achieve yields as high as 66% (based on N measurements) when using the pH-shift method without a freeze-thawing step (Bähr et al., 2014). Although freeze-thawing has been widely used for protein extraction from seaweed (Abdollahi et al., 2019; Harrysson et al., 2018, 2019; Juul, Danielsen, et al., 2021; Juul, Møller, et al., 2021; Juul, Steinhagen, et al., 2022) to maximize total yields e.g., from 8 to 15% in *S. latissima* (**Table 12**), its addition is costly and may pose challenges when attempting to scale up production. The yield disparity between legumes and seaweed can be attributed to the function and type of proteins present in each biomass, as discussed in detail in Section 3.5.1.

5.3.5. Impact of analysis method on protein yield determination from seaweed

Protein yields in **Papers II-IV** were calculated using two or more protein determination methods. **Figure 14** presents a correlation between total protein yields based on total amino acids, with yields based on Lowry and total N analyses. Additionally, data from Harrysson et al. (2018) were incorporated into this correlation to establish a more robust linear model. The model hereby comprises data not only from *U. fenestrata* and *S. latissima,* but also from *P. umbilicalis*. Yields based on Lowry (N=14, whereof 5 belonging to this thesis) or total N (N=8, all belonging to this thesis) exhibited a significant correlation with yields based on total amino acids ($p<0.001$). However, total N measurements showed a slightly higher R-square (0.9369 vs. 0.8715) and lower root mean squared error (1.08% vs. 1.26%). Both methods appear suitable for quick estimations of protein yields, although Lowry tends to slightly overestimate protein yields, while total N tends to provide lower estimates. Therefore, we recommend performing amino acid analysis whenever possible for more accurate results.

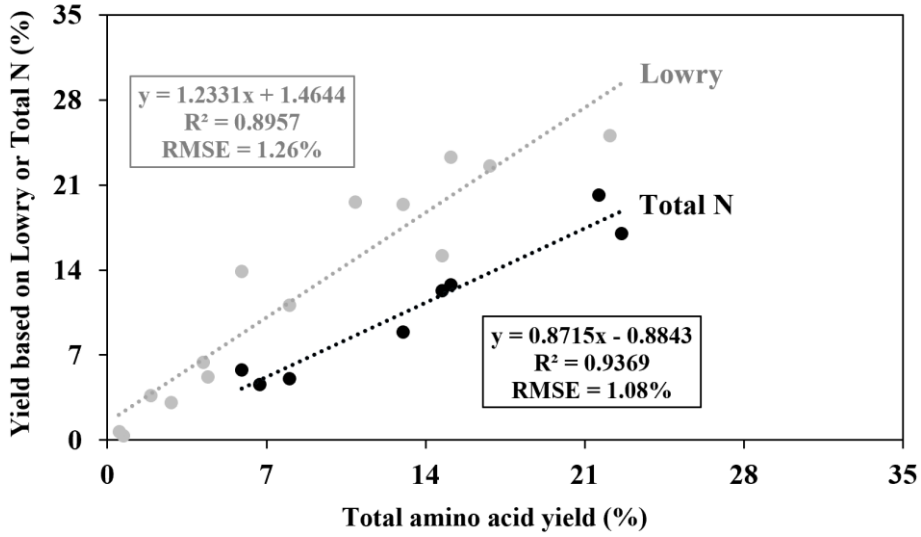

Figure 14 – Correlation between total yields based on amino acid analysis, which provide the most accurate estimation, with those from Lowry and total N.

5.3.6. Seaweed microstructure and polypeptide pattern as influenced by surfactant-based extraction method

The polypeptide sizes of biomasses, intermediate fractions and extracts in **Paper III** were analyzed through SDS-PAGE as it allows to study the effect of reducing conditions. The main finding was that solubilization with 2% Triton X-114 resulted in bands appearing at the top of the stacked gel and at >250 kDa. Both bands were absent in the original biomass and after alkaline solubilization at pH 12 (**Figure 15**). Elsewhere, Tamayo et al. (2017) reported similar observations after extraction of leaf membrane proteins using the Triton X-114 phase-partition method. These bands probably correspond to aggregated light harvest protein complexes, which in their disaggregated state, are part of the thylakoid membranes. This assumption was supported by: (*i*) the strong green coloration of the supernatant recovered following Triton solubilization when compared to the nearly colorless supernatant obtained after extraction at pH 12 (refer to **Paper III**) and (*ii*), the observed microstructure differences induced by Triton, but not by alkaline conditions, indicating disruption of chloroplasts and loss of chlorophyll (**Figure 16**). Co-extraction of chlorophyll has previously been utilized as an indicator for the concentration of thylakoid membranes in thylakoid extracts from spinach (Emek et al., 2010). Hence, solubilization with Triton likely targets more proteins, explaining the higher N solubility yields obtained, when compared to alkaline solubilization (**Table 13**). Another observation on **Figure 15** was that the lane corresponding to the first supernatant from control treatment showed less intense bands than the remaining lanes. This could reflect the presence of many small peptides running off the gel.

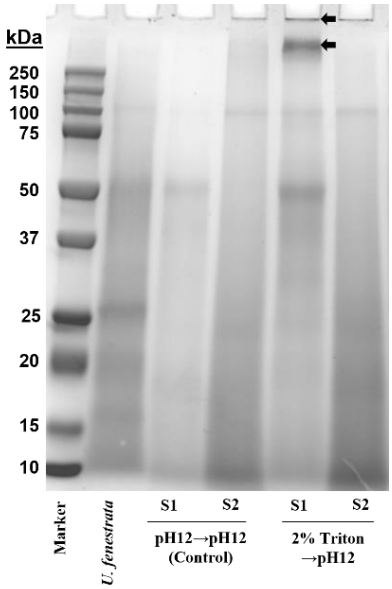

Figure 15 - Polypeptide patterns of *U. fenestrata* biomass and supernatants from first and second processing cycles (S1 and S2) analyzed through SDS-PAGE under reducing conditions. Twenty micrograms of protein were loaded in each lane.

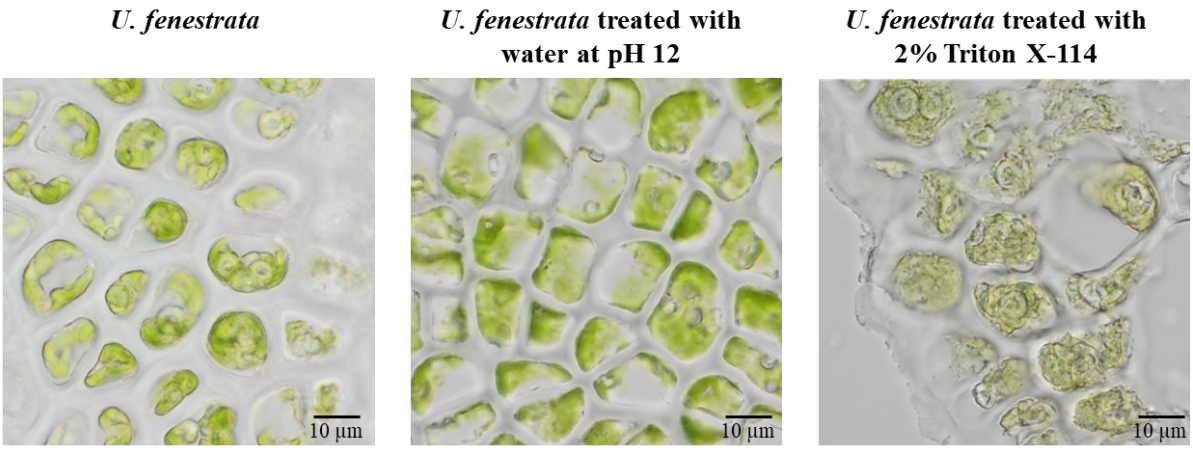

Figure 16 – Light microscopic pictures of cryosectioned unprocessed *U. fenestrata* and resulting seaweed pellets after treatment with alkaline and surfactant aqueous solutions.

5.3.7. Protein/peptide relative size distribution - effects of species, post-harvest treatments and protein extraction

Table 15 shows the relative distribution of proteins and peptides in seaweed biomasses as a function of species, post-harvest treatments and subjection to pH-shift protein extraction. It should be noted that only molecules soluble in phosphate buffer (0.1 M, pH 7.5) were captured. To facilitate data interpretation, protein/peptides were grouped in fractions of increasing molecular sizes.

Effect of species (Papers II and IV)

Crude *S. latissima* and *U. fenestrata* shared a comparable protein/peptide size profile, with one notable distinction in the fraction 5-10 kDa. In *S. latissima* this fraction was absent, whereas in *U. fenestrata* it constituted 10.4% of the total SEC peak area and corresponded to peptides/protein with a molecular weight of 5.8 and 8.3 kDa. Differences in the remaining fractions were relatively minor, being less than 6 percentage points (**Table 15**).

Effect of post-harvest treatments (Paper II)

Comparing crude *S. latissima* to blanched samples, we observed a significant increase in the relative proportion of the >670 kDa fraction (7% versus 14-32%, respectively), particularly when blanching was performed at 80°C. We attribute this phenomenon to the formation of soluble protein aggregates, as reported in previous studies (Mession et al., 2013; W. Wang et al., 2010). Moreover, the relative abundance of the fraction 1-5 kDa decreased after blanching, likely due to leaching of small soluble peptides into the blanching waters and/or due to 1-5 kDa peptides also contributing to >670 kDa aggregates.

Effect of protein extraction (Papers II and IV)

Protein extraction of crude *S. latissima* and *U. fenestrata* increased the relative proportion of the >670 kDa fraction from 1-7% in the biomass to 32-55% in the protein extracts. Extraction also decreased the relative proportion of the 1-5 kDa fraction from 79-83% to 15-22%. Similar patterns were found for protein extracts from soaked and blanched *S. latissima*, although changes were less pronounced (**Table 15**). It is possible that pH-shift processing promotes protein aggregation by exposing hydrophobic regions that are normally buried within the protein's interior, as found during herring pH-shift-based protein extraction (Marmon et al., 2012). The same authors also demonstrated that hydrogen bonds and S-S bonds were formed during protein extraction (Marmon et al., 2012). Another study reported that alkali-soluble proteins recovered from a previously acid-treated seaweed pellet resulted in a large peak at $>10^6$ kDa, which was not observed when extracting alkali-soluble proteins from the original biomass (Kadam et al., 2017).

Table 15 – Protein/peptide relative size distribution (%) in a form of a heat-map of seaweed biomasses and respective pH-shift protein extracts (N=3).

Paper			Relative size distribution (%)					
			< 1 kDa	1 - 5 kDa	>5-10 kDa	>10-100 kDa	>100-670 kDa	>670 kDa
II	*S. latissima*	Biomass						
		Protein extract						
IV	*U. fenestrata*	Biomass						
		Protein extract						
II	*S. latissima*, soaked	Biomass						
		Protein extract						
	S. latissima, blanched 45 °C	Biomass						
		Protein extract						
	S. latissima, blanched 80 °C	Biomass						
		Protein extract						

5.4. Composition of protein extracts and effects of extraction method, species, and post-harvest treatments

Analyzing the chemical composition of protein extracts from seaweed is a crucial step that facilitates the assessment of their nutritional value, potential food safety concerns, and their viability as multifunctional protein ingredients. **Table 16** presents the composition of produced protein extracts in terms of TAA, total ash, monosaccharides, and phenolics as well as the influence of species, post-harvest treatments, and extraction methods on these compounds.

Table 16 - Composition of protein extracts in terms of total nitrogen, amino acids, ash, monosaccharides, and phenolics (N=3, unless stated otherwise).

Paper	Biomass	Protein extraction method	Amino acid analysis			Total ash (% total dw)	Total monosaccharides (% total dw)	Total phenolics (% total dw)[b]
			Total AA (% total dw)	TEAA (% total AA)	N-to-protein factor			
II	*S. latissima*	pH-shift + freeze-thawing	20.2±0.2▲	45.5▲	4.18▲	18.3±0.3▼	34.4±0.8[a]▼	n.d
	S. latissima, soaked		14.3±0.7▲	45.0▲	3.80▲	22.7±0.5▼	49.5±4.1[a]▼	
	S. latissima, blanched 45 °C		21.3±0.6▲	43.1▲	5.20▲	10.4±0.9▼	52.1±0.1[a]▼	
	S. latissima, blanched 80 °C		13.8±0.1▲	42.0	4.56	7.4±0.6▼	44.7±1.2[a]▼	
III	*U. fenestrata*	Surfactant-based method Control	66.2±2.8▲	36.8▲	6.14▲	7.6[b]▼	n.d	n.d
		0.1%	52.1±1.9▲	37.4▲	5.76▲	5.9[b]▼		
		0.5%	34.1±2.2▲	36.5▲	5.37▲	3.5[b]▼		
IV		pH-shift + freeze-thawing	58.4±5.7[a]▲	40.7▲	6.06▲	n.d	n.d	0.13±0.02[c]▲

[a]N=2; [b]N=1 due to limitation on sample amounts; [c]expressed as grams of phloroglucinol equivalents per 100 g dw sample; [d]Solubilization with 0.1 or 0.5% Triton X-114 or water at pH 12 (control) + alkaline solubilization of pellet + isoelectric precipitation
▲ and ▼ indicate an increase and decrease, respectively, compared to the respective biomass, while the absence of these symbols means the values were similar; dw *dry weight*; AA *amino acids*; TEAA *total essential amino acids*; n.d *not determined*

5.4.1. Amino acid analysis

According to **Table 16**, protein extraction generally increased TAA, TEAA, and N-to-protein conversion factors.

Effect of species (Papers II-IV)

The TAA content of the protein extracts from crude *S. latissima* was 1.7 to 3.3 times lower compared to those from *U. fenestrata*, regardless of the extraction method (**Table 16**). However, the amino acid up-concentration factor relative to the original biomass was slightly higher for *S. latissima* (4.1) than for *U. fenestrata* (1.4-3.5). A similar trend was observed for TEAA - 46% for protein extracts from crude *S. latissima* and 37-41% for *U. fenestrata*. A previous work also storing the wet *S. latissima* at -80 °C before pH-shift extraction (Abdollahi et al., 2019), found slightly higher TAA (26% dw) and TEAA (49%) as well as a lower up-concentration factor (3.4 times) when compared to our findings for protein extracts from *S. latissima*. A study on oven-dried *U. fenestrata* revealed that pH-shift-based extraction resulted in a protein extract with 51% dw of TAA, a TEAA of 42%, and an up-concentration of 2.6 (Harrysson et al., 2018). These values fall within or close to the range observed in our data comprising wet *U. fenestrata* frozen at – 80 °C.

The N-to-protein conversion factors were 4.2 for protein extracts from crude *S. latissima* and between 5.4 to 6.1 for protein extracts from *U. fenestrata* (**Table 16**). Harrysson et al. (2018) reported a conversion factor of 7.7 for a pH-shift protein extract from unprocessed *S. latissima*. Therefore, it is likely that compositional variations within the same species, particularly in terms of non-protein nitrogen, influence the composition of the final protein extracts, thus explaining the different conversion factor between **Paper II** and Harrysson et al. (2018). For protein extracts from *Ulva sp*., earlier reported conversion factors vary from 5.3 to 5.8 regardless of the extraction method (Juul, Stødkilde, et al., 2022; Magnusson et al., 2019), which aligns with the range observed in our data.

Overall, *U. fenestrata* delivered purer protein extracts compared to *S. latissima*, although the ratio of essential amino acids and amino acid up-concentration was higher in the latter. Moreover, similary to seaweed biomasses, we recommend that nitrogen analysis shall be accompanied by amino acid analysis, especially for protein extracts from *S. latissim*a.

Effect of post-harvest treatment (Paper II)

Blanching *S. latissima* at 45 °C followed by pH-shift extraction resulted in a protein extract with similar purity as the protein extract from the crude biomass (> 20% dw). On the other hand, soaking and blanching at 80 °C yielded protein extracts with a significantly lower purity (< 15% dw). Amino acid up-concentration of protein extracts relative to the respective biomass decreased in protein extracts from soaked and blanched biomasses (2.1 and 1.5-1.9 times, respectively) compared to the protein extract obtained from crude *S. latissima* (**Table 16**). Magnusson et al. (2019) also reported an amino acid up-concentration of 2 times after extracting protein from soaked *U. ohnoi* in water at 40 °C, although the crude biomass was not subjected to protein extraction. In **Paper II**, the TEAA only significantly increased for protein extracts from crude, soaked, and blanched 45 °C biomasses, which also presented the highest TEAA values among all analyzed samples. The extracts also had higher N-to-protein conversion factors compared to their respective biomasses and the highest factors were found for protein extracts derived from the blanched biomasses.

Effect of protein extraction method (Papers II-IV)

Protein extraction from *U. fenestrata* with the pH-shift method comprising a freeze-thawing step (**Paper IV**) and the surfactant-based method using 0.1% Triton X-114 (**Paper III**) resulted in protein extracts with similar total amino acid content (> 52% dw) (**Table 16**). However, the amino acid up-concentration was higher for the pH-shift method either with *U. fenestrata* or *S. latissima*: 3.5-4.1 times for pH-shift vs. 2.2 times for surfactant-based method. However, we anticipate that further refinement of the surfactant-based method by lowering surfactant levels and/or washing the protein extract may help to narrow the difference between the surfactant-based method and the pH-shift method. Furthermore, the higher TEAA in pH-shift extracts from *U. fenestrata* likely resulted from the higher TEAA found in the original biomass compared to the one used in the surfactant-based method, including the control treatment (**Table 11**). Thus, for more accurate comparisons, it would be preferable to perform the assessment using the same batch of *U. fenestrata* for both methods.

Other extraction methods such as (i) ultrasound-assisted solubilization in water, followed by protein precipitation with ammonium sulphate and then dialysis (Harrysson et al., 2018), (ii) accelerated solvent extraction sequence (Harrysson et al., 2018), and (iii) mechanical pressing, followed by isoelectric precipitation (Juul, Stødkilde, et al., 2022) generally resulted in protein extracts with lower TAA (2-21% dw) and lower up-concentration factors (0.2-2.6 times). Regarding the TEAA, Wong & Cheung (2001) and Fleurence et al. (1995) reported ratios between 37 to 39% for protein extracts from *Ulva sp.,* which aligns with our findings. The protein extraction method employed by Wong & Cheung (2001) was aqueous solubilization, followed by alkaline solubilization with β-mercaptoethanol of the seaweed pellet, and then protein precipitation with ammonium sulphate and finally dialysis (Wong & Cheung, 2001), while Fleurence et al. (1995) solubilized proteins with a similar method as theirs but without the protein precipitation step.

Overall, based on our data and data available from others, the pH-shift and surfactant-based methods yielded the highest amino acid purities and up-concentrations. Also, the TEAA appears to be more dependent on the original biomass rather than the type of extraction method.

5.4.2. Total ash

Protein extraction resulted in extracts with lower ash content compared to the respective biomass (**Table 16**). Possible explanations for this are due to the water added to start protein extraction that dilutes the minerals and to some minerals remaining in solution after protein isoelectric precipitation.

Effect of post-harvest treatment (Paper II)

Protein extracts from the blanched biomasses showed the lowest ash content, which was significantly different from the other protein extracts (**Table 16**). This is likely attributed to that ash is significantly diluted already during the blanching (**Table 11**). Magnusson et al. (2019) reported an ash content of 5% dw for a protein extract from soaked *U. ohnoi*, which is similar to protein extracts obtained from our blanched *S. latissima*. However, the effect of soaking versus blanching or even no pre-treatment on the ash levels in extracts was not evaluated (Magnusson et al., 2019).

Effect of protein extraction method (Papers II-III)

Papers II and **III** measured the ash content of protein extracts from different species, which unfortunately hinders accurate comparisons (**Table 16**). However, when comparing the surfactant-derived extracts from *U. fenestrata* with protein extract data available from literature, the former method yielded relatively lower ash contents (4-6%) compared to those obtained from the pH-shift method (14%), ultrasound-assisted solubilization in water, followed by protein precipitation with ammonium sulphate and dialysis (19%), accelerated solvent extraction sequence (39%), and mechanical pressing, followed by isoelectric precipitation (39-51%) (Harrysson et al., 2018; Juul, Stødkilde, et al., 2022). This difference could be because the original biomasses had varying ash levels across the studies and/or because more ash was dissolved due to the two-step solubilization of the surfactant-based method, and most of it stayed in solution after isoelectric precipitation.

5.4.3. Total and individual monosaccharide content

Data showed that total monosaccharide content decreased after protein extraction (**Table 16**) and analysis of the individual monosaccharide content revealed several side findings (**Figure 17**). Firstly, we found a positive correlation (N=4; R-square 0.9929) between protein precipitation yield and content of alginate in the protein extracts (Precipitation yield (%)=1.285×[Alginate%]+38.43), although more data points are needed to further strengthen this correlation. Secondly, high levels of fucose were present in the protein extracts versus only 2-3% total dw in the initial biomasses. This sugar is the main monomer of fucoidan, thus there is a strong likelihood that fucoidan was co-extracted during the pH-shift method. Fucoidan contains sulfate ester groups (Zayed et al., 2020), and it has been determined that these groups have very low pKa values, often lower than 1.0 (Guthrie, 1978). Therefore, protein precipitation at pH 2.0 may have induced neutralization of some sulfate ester groups, subsequently promoting their hydrophobic interactions with proteins.

Overall, these findings indicate that charged polysaccharides (e.g., fucoidan, alginate, and likely ulvan in Ulva sp.), beyond salt, likely contribute to pushing downwards the isoelectric point of seaweed proteins. As a result, when performing protein extraction, it is commonly observed that maximum precipitation yields are achieved at pH levels lower than pH 3, as determined in **Papers II** and **IV** and elsewhere (Angell et al., 2017; Harrysson et al., 2018; Juul, Møller, et al., 2021; Vilg & Undeland, 2017). Thus, we suspect that this phenomenon is partially attributed to the binding of charged polysaccharides to proteins at pH < 3, which enhances protein aggregation and subsequent precipitation. Future studies shall test this hypothesis by minimizing the interference from charged polysaccharides through e.g., fractionation (Schwenzfeier et al., 2014), or studying the behavior of a pure protein solution in the presence of these polysaccharides. In both potential studies, it would be important to

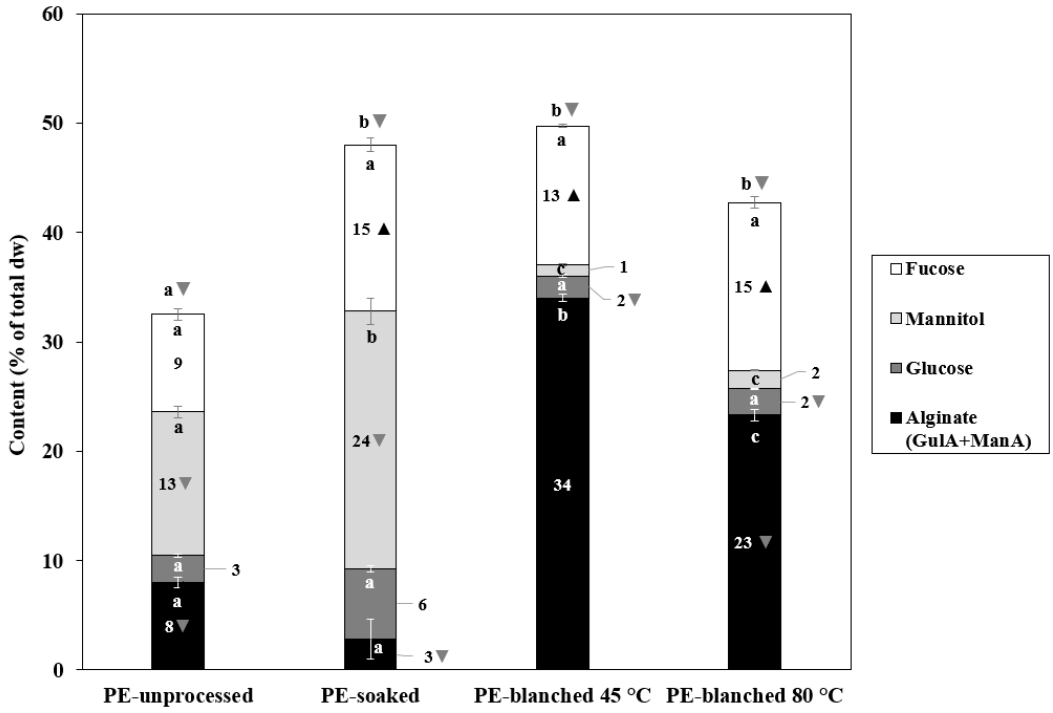

Figure 17 – Monosaccharide content (% dw) of unprocessed, soaked, and blanched *S. latissima* (N=2). Only monosaccharides representing more than 2% of the total dw were included in the figure. Different letters (a-c) for each monosaccharide mean statistical differences ($p<0.05$). ▲ and ▼ indicate an increase and decrease, respectively, compared to the respective biomass, while the absence of these symbols means the values were similar.

normalize ionic strength since we already demonstrated in **Paper II** that this factor strongly influences protein precipitation yields.

5.4.4. Total phenolic content

The pH-shift protein extract of *U. fenestrata* in **Paper IV** exhibited 1.4 times higher total phenolic content compared to the crude seaweed biomass (**Table 16**). In contrast to other works that reported phenolic content of seaweed protein extracts as gallic acid equivalents (Wong & Cheung, 2001), we opted for phloroglucinol as the standard, which hinders direct comparisons. This choice was supported by the fact that phloroglucinol is the monomer of phlorotannins, commonly found in brown seaweed (Holdt & Kraan, 2011). The concentration of phenolic compounds alongside proteins could reflect the binding of polyphenols to the proteins as well as affect the e.g., color and taste of the extracts.

5.4.5. Non-essential elements and selected essential elements

In **Paper II**, the elemental composition of pH-shift protein extracts from *S. latissima* was investigated. **Figure 18** presents the analysis of non-essential elements along with two essential elements - iron and zinc.

The key findings revealed that blanching the seaweed at 45 °C led to protein extracts with significantly higher levels of cadmium and zinc, while iodine and iron levels were lower compared to protein extracts from crude *S. latissima* (**Figure 18A**). To understand the impact of protein extraction from a nutritional/potential toxicological perspective, a comparison was made between the elemental content of the protein extracts and their respective biomasses (**Figure 18B**). This comparison demonstrated that iron and lead were significantly concentrated, especially in protein extracts obtained from unprocessed and soaked biomasses. In contrast, iodine values did not differ between blanched biomasses and their corresponding protein extracts (Kruskall-Wallis test, p-value=0.166 for protein extract from blanched 80 °C vs. blanched 80 °C biomass and p-value=0.419 for protein extract from blanched 45 °C vs. blanched 45 °C biomass). A similar pattern was observed for inorganic arsenic (data not shown).

Based on the TDI for cadmium, iodine, inorganic arsenic, and lead (EFSA, 2006, 2009b, 2009a, 2013) and the PRI for iron and zinc (EFSA, 2019), it was found that iodine was the limiting factor for the daily consumption of protein extracts, similar to the limitation observed for the biomasses. To not exceed TDI for iodine, the daily consumption of protein extracts from blanched *S. latissima* should be kept below 0.51 g dw, whereas protein extracts from unprocessed seaweed should be limited to 0.25 g dw. These amounts are at least 50 times lower than the quantities required to reach the TDIs or PRIs of the other elements. Interestingly, if one disregards the TDI for iodine, then a 5 g dw portion of protein

extracts would provide 12% to 21% of the PRI for iron. The same portion would though provide negligible amounts of zinc (≤ 2% of PRI).

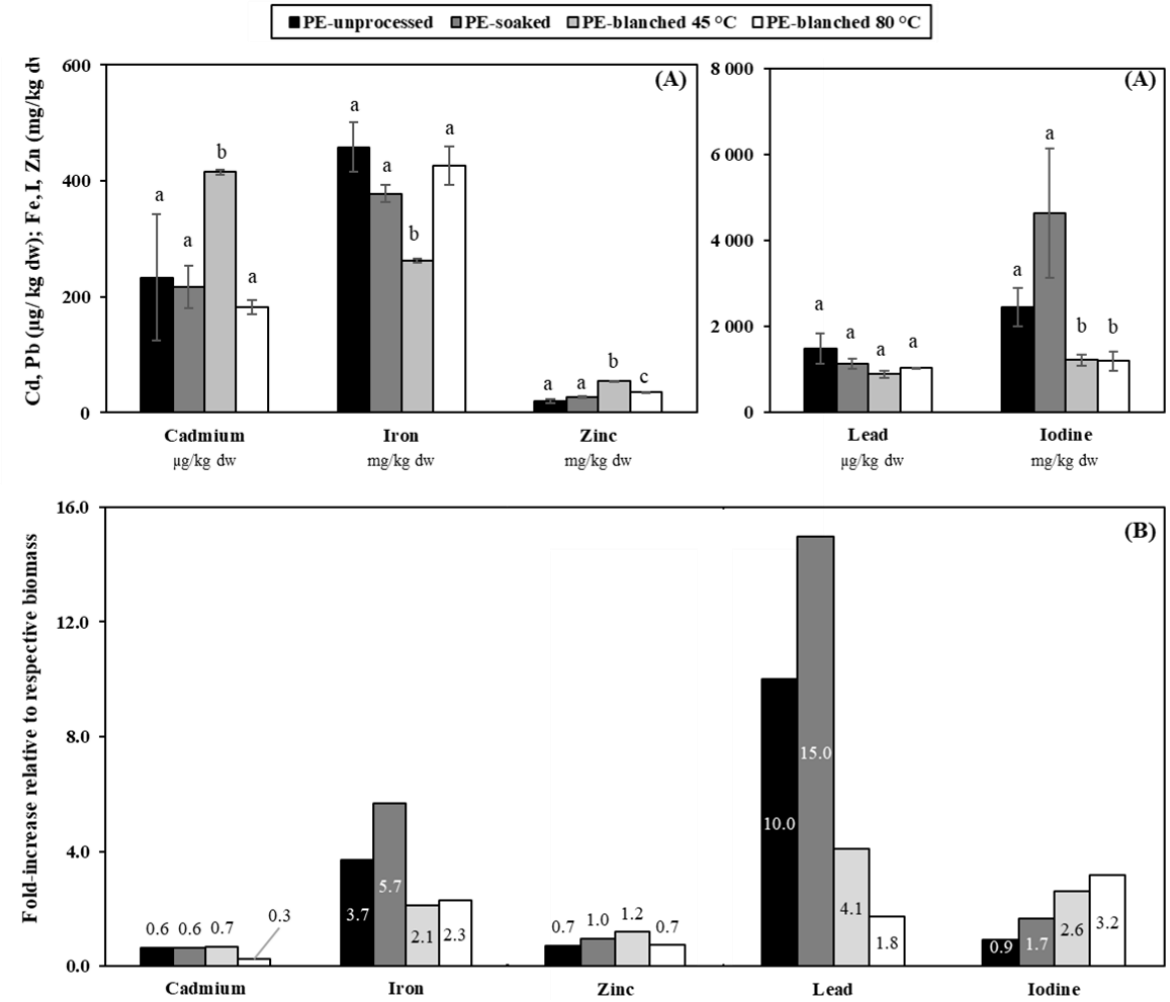

Figure 18 – Content (A) and fold-increase relative to respective biomass (B) of non-essential elements and selected essential elements of dry protein extracts from unprocessed, soaked, and blanched *S. latissima* (N=2). Different letters (a-c) for each element mean statistical differences ($p<0.05$).

5.5. Protein nutritional quality as affected by species, post-harvest treatment, and protein extraction method

In this thesis, the protein nutritional quality of seaweed biomasses and protein extracts thereof was evaluated via the essential amino acid profile, *in vitro* protein digestibility, amino acid accessibility, and Caco-2 cell amino acid bioavailability.

5.5.1. Limiting amino acids and amino acid chemical score

Data from the amino acid profile along with the amino acid requirements established by WHO/FAO/UNU (2007) can be used to determine the limiting amino acids and to calculate the amino acid chemical score as proposed by FAO/WHO (1991).

Effect of species (Papers II-IV)

The limiting amino acids of unprocessed *S. latissima* were histidine or methionine, whereas for *U. fenestrata* they were lysine, methionine, and to a lesser extent histidine. The amino acid chemical scores varied especially within species, whereas they were similar between species (55-88% for *U. fenestrata* and 66-93% for *S. latissima*) (**Table 17**). Prior literature reported similar patterns of limiting amino acids and found scores of 51-106% for *S. latissima* (Bak et al., 2019) and 22-100% for *U. fenestrata* (Harrysson et al., 2018; Juul, Stødkilde, et al., 2022), thus placing our data within those ranges.

Effect of post-harvest treatment (Paper II)

Blanched *S. latissima* did not show any limiting amino acid, which contrasts with the soaked biomass, where histidine was identified as the limiting amino acid. Additionally, the amino chemical score of blanched biomasses was found to be nearly two times higher when compared to crude *S. latissima* (**Table 17**). This suggests that the blanching process had a positive impact on the amino acid profile, resulting in a more balanced and nutritionally favorable composition.

Effect of protein extraction method (Papers II-IV)

Both the pH-shift and surfactant-based methods (except 0.5% Triton X-114) showed improvements in the amino acid chemical score and generally reduced the number of limiting amino acids compared to the initial *U. fenestrata* biomass (**Paper III**). This can be explained by the reduction of non-essential amino acids during protein extraction, particularly glutamate (data not shown). In the case of the surfactant-based method, the resulting protein extracts had an amino acid chemical score around 65%. This lower score when compared to the pH-shift method was likely due to the already low score of the initial biomass that was 55% (**Table 17**). Mechanical pressing followed by isoelectric protein precipitation on *U. fenestrata* was reported to yield a protein extract with a score of 114% versus 100% in the initial biomass (Juul, Stødkilde, et al., 2022). Thus, the relative difference between extract and biomass is similar to our surfactant-based method.

Interestingly, the score for protein extracts from unprocessed, soaked, and blanched 45 °C *S. latissima* (**Paper II**) is close to those of casein isolate (**Paper IV**) and soy protein isolate (**Table 17**).

Table 17 – Limiting amino acid(s) and amino acid chemical score of seaweed biomasses and respective protein extracts.

Reference	Sample	Limiting amino acid(s)[a]		Amino acid chemical score[b]	
		Biomass	Protein extract	Biomass	Protein extract
Paper II	*S. latissima*	**His**	None	66	145
	S. latissima, soaked	**His**	None	64	140
	S. latissima, blanched 45 °C	None	None	112	145
	S. latissima, blanched 80 °C	None	None	115	110
Paper III	*U. fenestrata*	Lys, **Met**, His	Lys, **Met**, His (Control) **Met**, His (0.1% Triton) Lys, **Met**, His (0.5% Triton)	55	64 (Control) 67 (0.1% Triton) 49 (0.5% Triton)
Paper IV	*U. fenestrata*	**Lys**, Met	None	88	104
	S. latissima	**Met**	-	93	-
	Casein isolate	-	None	-	124
Abdollahi et al. (2018)	Soy protein isolate	-	None	-	141

[a]Determined according to the WHO/FAO/UNU requirements for an adult (WHO/FAO/UNU, 2007); [b]Calculated according to FAO/WHO (1991) as it follows: Score (%) = (mg of limiting essential amino acid(s) per g of protein)/(amino acid requirement for each limiting amino acid(s) in mg amino acid per g protein))×100; limiting amino acids in bold were the ones yielding the lowest amino acid chemical score.

5.5.2. *In vitro* protein digestibility, amino acid accessibility, and Caco-2 cell amino acid bioavailability

Figure 19 summarizes the *in vitro* protein digestibility (%DH), amino acid accessibility, and Caco-2 cell amino acid bioavailability measured in **Paper IV**. The main findings were that protein digestibility and amino acid accessibility were significantly higher after pH-shift protein extraction from *U. fenestrata*. Furthermore, the *Ulva* protein extract showed a comparable digestibility to casein, while unprocessed *S. latissima* were significantly less digestible and accessible than crude *U. fenestrata*. Regarding amino acid bioavailability, no differences were detected across all samples when excluding the digestion blank.

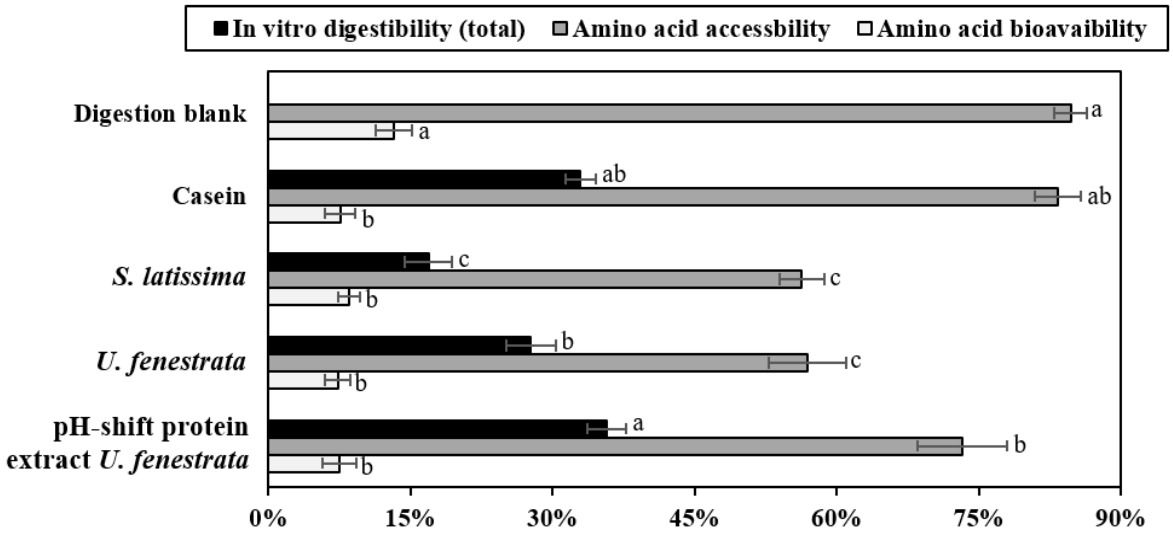

Figure 19 – *In vitro* digestibility measured as protein degree of hydrolysis (%), amino acid accessibility (%), and amino acid Caco-2 cell bioavailability of seaweed biomasses and pH-shift protein extract from *U. fenestrata* (N=3). Different letters (a-c) for each parameter mean statistical differences ($p<0.05$).

In **Paper IV**, we hypothesized the increase in protein digestibility following protein extraction of *U. fenestrata* was partially attributed to the removal of fiber. We demonstrated in **Paper II** - although with a different species - that pH-shift extraction indeed resulted in protein extracts with a lower carbohydrate content than the respective biomass (**Table 16**). Moreover, Tibbetts et al. (2016) reported an inverse correlation between *in vitro* protein digestibility and phenolic content of seaweed biomasses. However, phenolics were concentrated during protein extraction of *U. fenestrata* (**Table 16**), so we were unable to relate their correlation with our data. Therefore, it is more likely that the removal of fiber, other anti-nutrients (e.g., lectins, phytic acid, protease inhibitors), and/or mechanical disintegration of seaweed cells during protein extraction played a pivotal role in improving protein digestibility following pH-shift-based protein extraction. Regarding bioavailability, it is likely that extraction-induced modifications such as tentative protein-phenolic crosslinking, protein aggregation (**Table 15**), and amino acid racemization (Juul, Danielsen, et al., 2021) did not influence bioavailability.

In vitro test tube and cell models only serve as screening tools, and an important subsequent step involves comparing or confirming their findings with *in vivo* tests. After the publication of **Paper IV,** Juul, Stødkilde, et al. (2022) studied the effect of protein extraction of *U. fenestrata* on N digestibility in rats. In their study, N digestibility was calculated by determining the difference between N intake from seaweed or seaweed extracts and N excreted in feces, divided by the N intake from seaweed or extract. The authors reported a significant increase in the N digestibility of protein extracts relative to the crude *U. fenestrata* (Juul, Stødkilde, et al., 2022). Our findings from **Paper IV** align with these data, since the higher amino acid accessibility of the protein extract provides more amino acids, in absolute terms, to the basolateral side (75±23 µg versus 46±8 µg in crude *U. fenestrata*, $p>0.05$), despite the relative Caco-2 cell bioavailability being the same.

5.6. Potential global production and annual productivity of seaweed protein ingredients compared to other plant-based protein sources

This section presents a comprehensive analysis of the potential global production of seaweed protein ingredients, along with assessments of their respective weight yield and annual productivity. The seaweed-based data was benchmarked against lupin, pea and soybean. The former two legumes were chosen since they can be cultivated in moderate climate areas like Northern Europe (Berghout et al., 2015; Sandberg, 2011), while soybean is one of the most produced plant-based protein sources worldwide (Rajpurohit & Li, 2023). Hence, these comparisons offer insights into the present and potential future viability of seaweed as a protein source.

According to **Table 18**, the current global production of seaweed leads to potential protein extraction amounts that are comparable to those that can be extracted from lupin. Additionally, seaweed protein extracts have the potential to deliver up to 86% and 5% of the amount of protein potentially extracted from peas and soybeans, respectively. The potential contribution of seaweed to the ongoing protein shift seems relatively modest based on current production amounts. However, unlike protein ingredients from soybean, seaweed protein extracts can be regarded as multifunctional, as they contain potential bioactive polysaccharides, such as fucoidan (**Paper II**), omega-3 fatty acids like eicosapentaenoic acid and α-linolenic acid (Harrysson et al., 2018), and essential elements such as iodine and iron (**Paper II**).

Besides that, our ongoing research is evaluating if protein extracts from *U. fenestrata* can be a rich source of biologically active vitamin B12.

Table 18 – Global production of cultivated and wild-harvested seaweed (brown, green, and red species) as well as selected plant-based protein sources in 2019 (in million metric tons) given as wet weight, dry weight, total protein, and potential protein extracted.

Global production in 2019 (in million metric tons)	Protein source				Assumptions	References
	Seaweed	Lupin	Pea[a]	Soybean		
Wet weight biomass	35	1.3	19.7	69.8	The 69.8 million tons is equal to 20% of the global production of soy that is allocated for human food consumption	Celente et al. (2023), FAO (2023), Ritchie & Roser (2021)
Dry weight biomass	3.5-7	1.2	4.1	62.8	Dry matter of 10-20% (seaweed, raw), ~90% (mature lupin and soybean seeds, raw), 21% (green pea, raw)	**Papers II-IV**, (USDA, 2018c, 2018d, 2018a)
Total protein	0.4-1.8	0.5	1.1	25.1	Protein contents of 10-25% dw (seaweed, raw), 40% dw (mature soybean and lupin, raw), 26 % dw (green pea, raw)	**Papers II-IV**, (USDA, 2018c, 2018d, 2018a)
Potential protein extracted	0.1-0.6	0.3	0.7	12.6	Protein extraction yields of 20-35% (seaweed)[b], 66% (lupin), 60-67% (pea), 50% (soybean) using wet-fractionation extraction methods	**Paper III**, Berghout et al., (2015), Schmidt et al. (2022), Verfaillie et al. (2023)

[a]The FAO source does not disclose which pea cultivar it corresponds to; [b]Based on Paper III, which reported the highest yield compared to literature. Since the extraction method needs further refinement, we estimated a total yield ranging from 20% to 35%.
dw *dry weight*, ww *wet weight*

In **Table 19**, a second exercise was conducted, which is especially relevant assuming the seaweed aquaculture industry will grow in the future. The findings indicated that the extraction method employing 0.1% Triton X-114 (**Paper III**) has the potential to deliver 3.6 to 4.1 times more annual protein productivity yield compared to the control pH-shift treatment (**Paper III**) and the pH-shift method with a freeze-thawing step (**Paper IV**). Furthermore, when comparing protein extraction yields between seaweed and legumes such as lupin and soybean, the surfactant-based method applied to seaweed demonstrated significantly superior productivity yields, being 8-17 times higher than lupin treated with the pH-shift method (Berghout et al., 2015) and 3-6 times higher than soybean subjected to enzymatic-assisted solubilization in water, followed by recovering a protein-enriched pellet via centrifugation.

Table 19 – Weight yield (% of dw biomass) and potential annual productivity (in terms of biomass or protein ingredient and protein amounts) of *Ulva sp.* or selected plant-based protein sources, including two commercially available food protein ingredients.

Product	Weight yield (% of dw biomass)	Potential annual productivity yield		Assumptions	References
		Tons dw ha^{-1} $year^{-1}$	Tons of protein ha^{-1} $year^{-1}$		
Ulva sp. (raw)	-	45-100[a]	6.8-20	Protein content of 15-20% dw	Bruhn et al. (2011), Magnusson et al. (2019), Reznik & Israel (2012), Papers III-IV
U. fenestrata protein extract				-	
- 0.1% Triton X-114	10.5±0.9	4.7-10.5	2.5-5.3		**Paper III**
- Control pH-shift	2.3±0.3	1.0-2.3	0.7-1.5		
- pH-shift + freeze-thawing	2.3±0.3	1.0-2.3	0.6-1.3		**Paper IV**
Lupin (mature seed, raw)	-	1.2	0.5	Dry matter of ~90% and protein content of 40% dw	FAO (2023), USDA (2018a)
Lupin protein isolate (≥90% protein)	27	0.3	~0.3	-	Berghout et al. (2015)
Soybean (mature seed, raw)	-	2.5	1.0-1.1	Dry matter of ~90% and protein content of 40% dw	FAO (2023), USDA (2018b)
Soy protein concentrate (70% protein)	44	1.1	0.8	-	Loman et al. (2016)

[a]Range corresponds to estimates of *Ulva sp.* cultivation based in Denmark (Bruhn et al., 2011), Israel (Reznik & Israel, 2012), and Australia (Magnusson et al., 2019). The first and second studies corresponded to land-based cultivation (45 and 70 tons dried seaweed per year per hectare, respectively) whereas in the latter the type of cultivation is not disclosed.

Based on these two exercises, while an efficient protein extraction method was developed in **Paper III**, its potential impact on the protein market remains constrained by the relatively low production levels of seaweed (**Table 18**). Therefore, to unlock the full potential of seaweed as a protein source (**Table 19**), we call for efforts to scale up seaweed cultivation. We estimate that at least 147 million metric tons of dried seaweed would need to be produced annually to reach the same amount of protein extracted as that globally from soybean (**Table 18**). This amount would require an ocean area of around 3.3 million hectares (slightly smaller than Belgium) for an annual productivity of 45 tons of dried seaweed per year and hectare (Bruhn et al., 2011). Furthermore, open-sea cultivation of *U. fenestrata* in Sweden is expected to yield ≥4 tons of dried seaweed per year per hectare, although optimized cultivation protocols are yet to be developed (Personal communication, Göran Nylund, 2023). Using this productivity yield, the same 3.3 million hectares would produce an amount of extracted seaweed protein equal to ≤10% of the amount of protein globally extracted from soybean (**Table 18**), which we consider a reasonable percentage for an alternative protein source.

6. CONCLUSION AND RECOMMENDATIONS

This PhD thesis aimed to evaluate the potential of seaweed as food protein sources using *S. latissima* and *U. fenestrata* as the main model species. A strategy to raise the biomass protein levels with food process waters was addressed as well as extraction methods to up-concentrate the proteins into ingredients. Finally, nutritional properties of biomasses and extracts were in focus. The main conclusions and recommendations are listed below.

The primary source of inorganic nitrogen in most of the studied food process waters was ammonium, why its level was normalized to 20 and 200 µM before starting the cultivation screening experiments with *U. fenestrata*, *U. intestinalis*, *C. linum*, and *S. latissima*. Generally, cultivation of the three green seaweed species in media containing food process waters raised the nitrogen content, when compared to cultivation with only seawater or ammonium-enriched seawater hence **confirming hypothesis 1**. *S. latissima* was found to be incompatible with additions of food process waters.

Based on compositional analyses of the initial seaweed biomasses, the N-to-protein conversion factors were found to be lower than the universal factor of 5 proposed by Angell et al. (2016) for seaweed. Thus, we recommend that **N analysis be accompanied, whenever possible, by amino acid analysis** when seaweed proteins are in focus**.** Moreover, blanching of *S. latissima* resulted in a relative increase in alginate, which was likely linked to the higher cadmium and lead levels after blanching, especially when done at 80 °C for 2 min.

More than 25 studies were found in literature dealing with protein extraction from seaweed. Our data, combined with the data from these studies, revealed that protein extraction from ***S. latissima*** **generally yielded higher protein precipitation and total yields compared to** ***U. fenestrata***. Regarding our data on blanching, it reduced protein solubilization yield, however, protein precipitation yield was significantly higher, particularly after blanching at 45 °C for 2 min. We further explored the mechanism behind this phenomenon through: (i) a dialysis model (ii) correlation analyses between precipitation yield and content of alginate in the protein extracts. Dialysis revealed that **blanching induced a reduction in ionic strength, which positively influenced protein precipitation**. Since the higher precipitation yields counterbalanced the lower solubility yield, blanching at 45 °C was able to retain the same total protein yields as was found for unprocessed *S. latissima*. Hence **hypothesis 2 was partially rejected** and we recommended **mild blanching temperatures if *S. latissima* is to be used for protein extraction purposes.**

Total protein yields calculated based on N and amino acid data were higher for the surfactant-based method when compared to the pH-shift method with a freeze-thawing step and other methods in literature. We attributed this positive outcome to the superior N solubility and precipitation yields provided by Triton X-114. The former is likely because Triton: (i) targeted a broader range of polypeptides and appeared to disrupt chloroplasts, thus **likely confirming hypothesis 3** and (ii) **removed significant amounts of ash,** thereby likely promoting a salting-in scenario during the second processing cycle. The superior protein precipitation with Triton X-114 was probably derived from co-precipitation of charged lipid membranes.

The **power law equation was the one best describing the relationship between amino acid yields and amino acid purity**. Moreover, **yield estimations based on the Lowry protein analysis method or N analyses leads to deviations**, more specifically, slight over- and underestimations, respectively.

Protein extracts from *U. fenestrata* exhibited higher total amino acid content compared to those from *S. latissima*, although the ratio of essential amino acids and amino acid up-concentration was higher in the latter. Furthermore, based on our data and elsewhere, the **pH-shift and surfactant-based methods yielded the highest amino acid purities** and up-concentration factors. Regarding monosaccharide content, the pH-shift method increased the relative content of fucoidan and alginate, which can partially explain why the pI of seaweed proteins is pushed to very low pH values. Moreover, **iodine was the limiting factor for the daily adult consumption of protein extracts**, which should be kept below 0.51 g dw for protein extracts from blanched *S. latissima* versus below 0.25 g dw for protein extracts from unprocessed *S. latissima* biomass.

Both the pH-shift and surfactant-based methods reduced the number of limiting amino acids compared to the initial biomass and improved the amino acid chemical score. *In vitro* protein digestibility and amino acid accessibility were significantly higher after pH-shift protein extraction from *U. fenestrata*, while no differences were found for amino acid bioavailability using the Caco-2 cell model. Thus, **hypothesis 4 was partially confirmed**.

Based on theoretical estimations, **seaweed proteins have an annual productivity at least 3-times higher than soy proteins**. However, extracted proteins from seaweed will only make minor contributions to the ongoing protein shift unless the seaweed aquaculture industry ramps up significantly.

Overall, this thesis introduced a novel nutrient loop that can increase seaweed's natural protein levels, provided recommendations for post-harvest treatments that optimize downstream protein recovery, developed an efficient and scalable protein extraction method, and showcased improvements in the nutritional quality of the extracted protein. By highlighting the potential of seaweed as an alternative protein source, this thesis serves as a useful building block to promote a more environmentally friendly and resilient food system.

7. FUTURE PERSPECTIVES

Future studies shall aim to find the **best ammonium levels that guarantee optimal protein content and biomass growth** when using food process waters as nutrient sources.

As demonstrated in Paper II, iodine is a major limiting factor for the consumption of unprocessed and blanched *S. latissima* as well as protein extracts thereof. Although randomized controlled trials are warranted to determine **iodine bioavailability** of seaweed (Trigo et al., 2023), the high iodine content can potentially impair the interest of *S. latissima* as a food protein source. Therefore, one could focus future cultivation efforts on other kelp species, such as *Alaria esculenta* that have lower iodine levels and are also cultivated in Europe (Araújo et al., 2021; Roleda et al., 2018). Alternatively, the surfactant Triton-X-114 solubilized more ash than the alkali-based method in Paper III, implying that **surfactant treatment should be further tested to reduce iodine levels** in *S. latissima* coupled or not with blanching.

As demonstrated in this thesis, protein extraction yields in seaweed do not yet surpass 30%. To enhance these yields, we consider that new research questions need to be formulated aiming to gain fundamental knowledge on the **role of ionic strength in protein precipitation**. Seaweed proteins, unlike other food proteins, such as those from legumes, cereals, and animals, are likely adapted to function at relatively high ionic strengths – a significant feature that has been mostly overlooked within the scientific community (Celente et al., 2023). Further, our data suggest that co-precipitation of charged polysaccharides plays a role in protein precipitation yields. Thus, future works shall further investigate this by **minimizing the interference from charged polysaccharides** and/or by studying the behavior of a pure protein solution in the presence/absence of these polysaccharides to achieve a controlled contribution from these molecules.

In Paper III, a proof-of-concept protein extraction method was developed, which proved to be efficient and potentially more desirable to scale up than current alternatives. Further research shall focus on testing **food-grade alternatives to Triton X-114**, such as Tween 20 (European-Comission, 2011). Additionally, optimization studies are required to find a balance between maximizing total protein yield and protein purity, while **minimizing surfactant presence** in the final product. Furthermore, it would be relevant to assess the **organic P** and **fatty acid profile of the protein extracts** since we expect co-extraction of membrane lipids with membrane proteins. The co-extraction of polyunsaturated fatty acids (Harrysson et al., 2018), provides the basis for a multifunctional protein ingredient with added market value. Another relevant nutrient to be analyzed is vitamin B12 since it is known to be protein-bound (Bito et al., 2018) and since our ongoing research suggests that *U. fenestrata* is a rich source of **biologically active vitamin B12**. Quantification of **folic acid** could also be relevant as a recent study suggested that seaweed could be a source of this vitamin often lacking in vegetarian diets (Koseki et al., 2023).

Beyond method refinement and further compositional analysis, technological challenges to be addressed relate to the **oxidative stability** and **color** of the produced protein extracts. The first challenge can be enhanced from the co-extraction of unsaturated fatty acids that are highly prone to oxidation (Harrysson et al., 2021), which can lead to **off-flavor formation** during storage. A strategy to mitigate

this could be the **addition of antioxidants** during protein extraction (Zhang et al., 2022) and storage of extracts in darkness (Harrysson et al., 2021). The second challenge relates to the co-extraction of chlorophyll with the proteins. Although it adds cost and climate footprint to the protein extraction that could be assessed through a **life cycle analysis**, industry often seeks protein ingredients with a neutral color as it widens the range of food products it can be added to. Previous research has shown that chlorophyll can be removed during protein extraction by ultracentrifugation (Juul, Møller, et al., 2021), while heat treatment can be used to fractionate chlorophyl-binding proteins from the colorless RuBisCO (Tamayo Tenorio et al., 2016). To develop milder and more scalable strategies for chlorophyll removal, a closer examination of the existing knowledge of **chlorophyll binding to photosystems**, as reviewed by e.g., Murray et al. (2006), is essential.

Although we expect protein extraction for seaweed to become even more efficient in the short to medium term perspective, likely, at least half of the proteins would still be lost into side streams. Therefore, to offset potential economic losses and reduce waste, one should create a **cascade biorefining** targeting e.g., ulvan and flavour-providing molecules. In the case of Paper III, the strong ability of Triton to remove ash could be an important feature for downstream extraction of ulvan since low-salt biomass enhances its extraction efficiency (Kidgell et al., 2019).

A major incentive to extract proteins from seaweed is the improvement of its digestibility and bioavailability. On this matter, Paper IV and recent works elsewhere achieved promising findings using *in vitro* and rat models. However, to establish robust evidence, **digestibility shall be tested using more complex monogastric animals**, such as pigs, and eventually proceed to randomized controlled clinical trials involving human subjects.

Besides protein nutritional quality, the **sensorial profile** of the protein extracts is a key parameter for its successful commercialization. Our ongoing research revealed no major changes in most sensory descriptors when compared to crude seaweed, which opens the door for the development of food products requiring e.g., umami taste and “ocean” taste.

Seaweed protein ingredients are considered **novel food under EU regulations**. Therefore, before their commercialization it is necessary to demonstrate the safety of the novel food to EFSA. Safety aspects evaluated include for instance anticipated intake of novel food, its absorption, excretion, and allergenicity (Turck et al., 2021). There is an ongoing assessment of the allergenicity risk of protein from the red seaweed *Palmaria palmata* (Garciarena et al., 2022).

Lastly, based on theoretical estimations, for seaweed proteins to make a significant contribution to the ongoing dietary protein shift, the **seaweed aquaculture industry needs to expand**. Thus, we call upon policymakers, academics, and industry stakeholders to collaborate and take action to facilitate this transformation.

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