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Aquaculture of green seaweed Ulva intestinalis in brackish water and its potential as food and feed ingredient

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The global population growth predicts soaring demands on food production. Ecological constraints necessitate a thorough examination of alternative protein sources to animal derived products. Recently, seaweeds have been gaining attention as a sustainable source of nutrition that could contribute to the rapidly developing global food production demands. However, seaweeds react differently to environmental stimuli such as salinity and temperature. The purpose of this study was to study how temperature and nutrient density in low salinity affect the growth, protein content and amino acid profile of a green seaweed, *Ulva intestinalis*. Additionally, cultivation potential in the low salinity of the Baltic sea was evaluated. Samples of *U. intestinalis* were cultivated in orthogonal combinations of two temperatures and two nutrient levels for four weeks, 10 °C and 25 °C, F/2 (high) and F/20 (low) Medium, respectively. Ground and freeze-dried samples were quantified for total chlorophyll and protein content using absorbance in dimethylformamide and the Lowry Protein Assay, respectively. The amino acid profile was determined using HPLC-FLD and a Zorbax Eclipse AAA column to detect the derivatized amino acids. Samples grown in 10 °C maintained a relatively consistent growth rate of 38 % and 26 % (w.b.) on average, respectively, while samples grown in 25 °C plateaued in biomass growth between the 2nd and 3rd week. The chlorophyll a, chlorophyll b, and carotenoid contents of both F/2 25 °C and F/2 10 °C samples were about 2.2, 8.3, and 2.0-fold compared to both F/20 25 °C and F/20 10 °C samples, respectively. Temperature significantly affected the total protein content as well as serine, histidine, and lysine concentrations, while nutrient level affected significantly only lysine.

Keywords: seaweed, *Ulva*, *Ulva intestinalis*, cultivation, protein, amino acids, HPLC, Lowry

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1 Introduction

Reports by The Food and Agriculture Organization of the UN (FAO) forecast the demand for seafood to be doubled in the 21st century, with the majority of the increased demand to become the burden of aquaculture (Bjerregaard et al. 2016; Cook, Gachon, and Badis 2016; FAO 2018; Rosegrant et al. 2002; Tacon and Barg 2001). The majority of the future demand for aquaculture products is expected to target protein-rich fish and/or shrimps that currently are grown almost entirely on manufactured aquafeeds containing fishmeal. The current practise of feeding protein-rich fish and shrimps, which are predicted to occupy most of the future demand for aquaculture products (Tacon and Metian 2008; Pallaoro, Vieira, and Hayashi 2016) is considered environmentally, ecologically and socially unsustainable praxis (Rosegrant et al. 2002; Martinez 1998; Naylor et al. 2000).

Throughout history, seaweed has been cultivated using a traditional long-line technique in coastal sea areas as a cost-effective edible commodity (Bjerregaard et al. 2016; Gjedrem, Robinson, and Rye 2012). Today, about 221 macroalgae species are considered of commercial value, ten of which are extensively cultured, including brown macroalgae (*Sargassum fusiforme, Undaria pinnatifid and Saccharina japonica*), red macroalgae (*Gracilaria* spp., *Eucheuma* spp., *Porphyra* spp. and *Kappaphycus alvarezii*) and green macroalgae (*Caulerpa* spp., *Monostroma nitidum* and *Enteromorpha clathrate*). The Japanese kelp, *Saccharina japonica*, comprises more than 33 % of the global macroalgae production, followed by *Eucheuma* spp., 17 %. The global macroalgae production has doubled from 14.7 to 30.4 million tonnes between 2005 and 2015. The largest producers of cultivated species were China, Indonesia, the Republic of Korea and the Philippines totalling in 29.4 million tonnes, while China, Chile and Norway produced the largest harvest for wild species totalling in 1.1 million tonnes in 2015. The nutritional composition of macroalgae is high in magnesium, sodium, potassium, calcium, sulphur, chlorine, phosphorus, and micronutrients, such as zinc, selenium, iron, iodine, fluoride, molybdenum, manganese, nickel, boron and cobalt, as well as vitamins, like B12, A and K. A small amount of macroalgae can provide, for instance, the daily requirement of iodine for an adult, 150 µg/day, which is especially true for brown algae like kelp, which has an iodine concentration of 1.5 to 8.0 mg/g. Generally, macroalgae are regarded as a decent source of protein, fiber and minerals for humans. (Ferdouse et al. 2018).

FAO (Cai et al. 2021) estimated global seaweed production, cultivation and wild collection combined, shares of 47.65 % and 51.48 % for brown and red seaweeds, respectively, leaving only a meager 0.1 % for green seaweeds, of which *Ulva* comprises about 10 % in 2019. Unsurprisingly, microalgae's global production remains tiny compared to seaweeds', 56 500 and 35 800 000 tonnes, respectively, which is still more than the green seaweeds' global production, 33 000 tonnes.

Spirulina is by far the most consumed microalgae thanks to its rich protein content and healtht promoting, among others lowering blood pressure, sugar and lipids levels, and even renal protection (Spolaore et al. 2006). Spirulina's lipid profile consists of high degree of hypocholesterolemic γ-linoleic acid (GLA), free-radical neutralizing phycobiliproteins, and some B-vitamins (Sajilata, Singhal, and Kamat 2008). Perhaps the greatest recognition Spirulina has achieved after being labelled "super food" by the WHO is being sent to the Internation Space Station by by the National Aeronautics and Space Administration (NASA) thanks to its rich nutrient profile (Khan, Bhadouria, and Bisen 2005). To mention a few comparisons, Spirulina has 7.7 times the protein of tofu, 2.8 times the calcium of milk, 32 times the β-carotene of carrots, and 52 times the iron of spinach (Capelli and Cysewski 2010).

Globally, the greatest producer of Spirulina is Hainan Simai Enterprising Ltd. located in China's Hainan province. This farming behemoth produces 200 000 kg of dried biomass each year, constituting 25 % of the national production and an appreciable 10 % of the world's production. Meanwhile, the largest Spirulina production plant by area belongs to the Earthrise Company, located in California, USA, covering an area of 440 000 m^2 (Spolaore et al. 2006).

Chlorella is also an extensively consumed microalgae, whose worldwide retail is over US $38 billion (Yamaguchi 1996). The primary substance found in Chlorella that is beneficial for human health is beta-glucan, which acts as an immunostimulant, scavenges free-radicals, and decreases blood lipids (Yaakob et al. 2014). *Chlorella* also contains plenty of protein at 48 %, PUFAs at 39 % of total lipids, and phosphorus at 1.8 g/100 g (dw)(Tokuşoglu and Ünal 2003).

The most used plant materials in fish feed are corn, wheat and soy. Soy meal has the highest protein content at approximately 240 g/ kg. Since methionine is the limiting amino acid in soy and its concentration in macroalgae can be up to three times higher the potential of macroalgae as a supplement to soy in fish feed should be considered (Mæhre et al. 2014).

1.1 Taxonomy and life history of *Ulva intestinalis*

Marine macroalgae, more commonly known as seaweed, are generally found in coastal areas. Since the mid-19th century, they have been empirically categorised based on the colour of their thallus, the undifferentiated vegetative tissue, into three groups: brown, red and green algae. *Ulva* are a genus of edible marine green algae, which is more commonly known as 'Sea Lettuce'. Currently, *Ulva* is comprised of 130 taxonomically approved species (Guiry 2019a; 2019b). *Ulva* belong to a family of green algae, *Ulvaceae*, which, in turn, belong in the order of *Ulvales* along with *Ulvellaceae* and *Kornmanniaceae*. *Ulvales* belong to the *Ulvophyceae* class of green algae (**Fig. 7**)(Zhu et al. 2015).

Ulvophyceae differ from the other two core chlorophyte classes, *Trebouxiophyceae* and *Chlorophyceae*, in that they are primarily distributed along coastal regions, preferring rocky coastlines and tropical lagoons. In general, *Ulvophyceae* reside mainly in marine benthic environments, the ecological zone at the lowest level of a body of water, where they are thought to have emerged from during the mid-Neoprotezoic to establish a key role in several coastal regions to this day. While brown and red algae inhabit almost exclusively marine environments, green algae, including *Ulva*, are also common in freshwater, but can be found even in terrestrial areas, such as rocks, walls, houses, and tree bark when provided adequate moisture. Along with the "paraphyletic assemblage of free-living unicells with a wide variety of cell shapes" classes of the prasinophytes and a relatively recently suggested deep water class, palmophyllales, core chlorophytes form the Chlorophyta taxon of green algae (**Fig. 8**). (Leliaert, Verbruggen, and Zechman 2011).

Macroalgae and seagrasses maintain complex marine ecosystems and vivid coastal environments that have drawn the attention of hunter-foragers for more than 100 000 years (Ainis et al. 2014). In fact, some archeologists have postulated that edible macroalgae along with fish and marine mammals available in littoral and intertidal areas, like mangrove forests and estuaries, have played a significant part in expansion from Africa via the Southern Dispersal Route around 100 000 years ago and later from Northeast Asia to the American (Erlandson and Braje 2015). Additionally, archeologists have recovered remains of macroalgae from hearths and other human structures in an occupational layer in the Monte Verde in the tip of South America dating back 14 000 years (Dillehay et al. 2008).

Nowadays, macroalgae is frequently used as *nori* sheets for sushi wrapping, as a soup stock additive in *kombu*, and as *wakame* in soups and salads mostly in Japan, China and South

Korea. Red macroalgae, like *Pterocladis*, *Gracilaria* and *Gelidium*, have a significant role in human nutrition and other uses, for example, as a binding agent in food stuff and as a bacterial substrate in the form of agar gel. Brown macroalgae, such as *Saccharina japonica*, *Undaria pinnatifid*, and *Sargassum fusiforme*, as well as green macroalgae, like *Ulva* sp., *Monostroma* sp., *Enteromorpha* sp., *Codium* sp., and *Caulerpa* sp., are essential staple foods in Asia. They are mostly consumed raw, dried or boiled in soups and stews. A particular extract, carrageenan, branded after the red macroalgae, Irish moss or carrageen moss, discovered in Carraigin, Ireland, has been utilized as a gelatin substitute and for traditional healing for over 1 000 years. Other important algal extracts include agar, or agar agar, which has maintained a robust demand in Asia as a foodstuff for, at least, 300 years, and alginates, which textile printing industry has utilized for almost 1000 years. Agar is primarily produced from the polysaccharides of a red macroalgae, *Gracilaria*, China being the largest producer (2.7 million tonnes) in 2015. These days, carrageenan and alginates are utilized in many food applications as thickening components in bakery products, ice creams, confectionaries, salad dressings, puddings, jellies, jams, chewing gum, dairy products like chocolate milk, beverage mixtures and in processed meats, sausages and fish, as well as clarifying of wines and beers. Carrageenan and alginates also have applications in pharmaceuticals as emulsifiers, binders, stabilizers and for dental moulds. Carrageenan occurs as three main types: kappa, lambda and iota. They are produced primarily from *E. cotonii*, *Chondrus crispus* and *E. spinosum*, respectively. Kappa carrageenan is broadly utilized in food additives for its ability to form rigid gels, lambda carrageenan constitutes the creamy sensation in dairy products, and iota carrageenan is softer and more clastic. There are three main processing methods for production of refined carrageenans: alcohol, potassium chloride and semi-refined processing. (Ferdouse et al. 2018).

Several species of seaweed are exceptionally high in aspartic acid and glutamic acid, which are amino acids exhibiting a particular flavour that led to finding and establishing the taste sensation known as umami (MacArtain et al. 2007). A little known fact is that, monosodium glutamate itself, one of the world's most popular umami flavour enhancer, was first detected in the brown seaweed *Laminaria japonica* also known as kombu (Marcus 2007).

U. intestinalis was first described by Carl Linnaeus in his work ‘Species plantarum’ in 1753 but was assigned its current heterotypic name again in 2007 after a series of taxonomical reclassifications, of which the second most recent was *Enteromorpha intestinalis*.

The application of *U. intestinalis* as a protein source for humans has been assessed. *U. intestinalis* along with *P. palmata* and *V. lanosa* have been recognised to have equivalent or higher content of all the essential amino acids by dry weight in comparison to the three most commonly consumed cereals, rice, corn and wheat (Mæhre et al. 2014). Another study suggests that *U. intestinalis* (as *Enteromorpha*) has the highest amount of total omega-3 polyunsaturated fatty acids and the lowest omega-6 to omega 3 ratio compared to the other five studied macroalgae: *Ulva lactuca*, *Sargassum ilicifolium*, *Colpomenia sinuosa*, *Hypnea valentiae* and *Gracilaria corticate (Rohani-Ghadikolaei, Abdulalian, and Ng 2012)*.

1.2 Reproduction and habitat

Species of the genus *Ulva* have been described as “rapidly growing opportunists” for their remarkable frequency and speed of reproduction. Correspondingly, the algae have a relatively short lifespan and reach maturity at a specific point of development, instead of depending on environmental triggers. *U. intestinalis* remains actively reproductive throughout all seasons, but reaches the peak development and reproduction in the course of the summer, which is especially the case for the populations in the north. (Burrows 1991).

U. intestinalis is capable of disseminating across notable distance, for example, spores and gametes, *swarmers*, have been shown to have reached exposed artificial substrata on a submarine plateau 35 km away (Amsler and Searles 1980). The alga can often be found in abundance on high shores, where the principal factor affecting algae distribution is desiccation stress, and, in some cases, can even reside above the tidal limits of the shore (Guiry 2019a). *U. intestinalis* generally binds permanently to suitable substrata, however, under some circumstances, the alga may detach from the substratum, and float to the surface by being buoyed up by gas, while it continues to grow in mats. In this case the thalli tend to lose their tubular form, spread, and create unattached monochromatic sheets. (Back, Lehvo, and Blomster 2000)

The distribution of *U. intestinalis* is considered cosmopolitan throughout coastal regions (**Fig. 6**)(OBIS 2019). This holds true for its distribution in the Baltic Sea as well, except for environments with salinities less than 2 ng/kg (**Fig. 9**) (Leskinen, Alström-Rapaport, and Pamilo 2004).

The Baltic Sea is a harsh environment with low light intensities, low temperature, low salinity and ice cover during winter. Despite of this, both gametophytes and sporophytes of *U.*

intestinalis seem to survive in this demanding environment. Considering that both gametophytes and sporophytes can reproduce asexually in *U. intestinalis* they could, in theory, form two reproductively isolated populations. The low genotypic diversity along with a high allelic diversity also implies that over-wintering happens predominantly as diploid spores from sporophytes rather than as diploid zygotes created by two fusing gametes. (Leskinen, Alström-Rapaport, and Pamilo 2004).

1.3 Physical and chemical sensitivity

U. intestinalis like many other algae can be exposed to extreme environmental variations. *U. intestinalis* can tolerate several weeks of staying in entirely dried up rock pools, while turning totally bleached on the uppermost layers, however staying damp under the bleached fronds. Additionally, it is an excellent shelter for copepods in supralittoral rockpools thanks to its ability to persist in dehydrated environments makes it (McAllen 1999). Moreover, *U. intestinalis* has been reported to be tolerant against increase in temperature, e.g. its brown seaweed cousins of the genus *Ascorphyllum* and *Fucus* were eradicated from a rocky coast heated to 27-30 °C by a power station in Maine, meanwhile the abundance of the opportunist alga increased significantly near the outfall (Vadas, Keser, and Rusanowski 1976). Its tolerance against decrease in temperature is prominent as well, being able to tolerate temperatures as low as -20 °C (Kylin 1917).

U. intestinalis has been assessed to be intermediately intolerant to synthetic chemical pollution, which has been underlined by evidence for detrimental effects upon the species viability and lethal damage. For example, although herbicides generally are not used directly in the marine environment, they may contaminate estuarine areas via river discharge and runoff. Herbicides, Paraquat and 3AT, have been experimented on the alga for their impacts on the settlement, germination and growth of *U. intestinalis*. Zygotes can mature into filaments in the presence of Paraquat at 7 mg/L, but at higher concentrations the germination is deferred. Between the two herbicides, *U. intestinalis* has been found to be more tolerant to Paraquat than to 3AT (Moss and Woodhead 1975).

Some synthetic chemicals utilized as antifouling agents may be used directly in the marine environment. The presence of a particular s-triazine herbicide, Irgarol 1051, which is utilized as a component in antifouling paints on pleasure boats and ships, has been reported to have adverse effects on the alga. Concentrations above of 120 ng/L can inhibit considerably the growth of *U. intestinalis* spores under laboratory conditions, whereas concentration of 22 ng/L has no observable effect. Additionally, Irgarol 1051 can inhibit photosynthetic efficiency in the adult frond of *U. intestinalis* under laboratory conditions with a half maximal effective concentration (EC_{50}) of 2.5 µg/L after 72 h, which would suggest a weak detrimental effect on *U. intestinalis* reproduction inside harbours, where the concentrations of Irgarol are found to be the highest, is therefore probable (Scarlett et al. 1997).

The metal toxicity of different algal species has been studied extensively. Generally, the order of metals from the most toxic to the least toxic is thought to be mercury, copper, cadmium, silver, lead and zinc based on inactivation of enzyme systems at different exposure levels (Rai, Gaur, and Kumar 1981; Rice, Leighty, and McLeod 1973). Later studies have calculated EC50 values from the growth curves of several seaweed species cultivated under increasing heavy metal – added as non-organic salts – exposure levels, and found the following mean based toxicity hierarchy: Cd> Co> Cr> Cu> Pb> Ni> Zn> Al> Fe (Lukavský and Cepák 2003). Species of the genus *Ulva* are promising for the surveillance of heavy metals in littoral areas and estuaries since they appear frequently in both environments. In addition, laboratory trials have demonstrated that accretion of copper, zinc, cadmium and lead by four different species of *Ulva* is adequately analogous to legitimize pooling samples of the genus for field surveillance (Say, Burrows, and Whitton 1990). However, the interactions of salinity and temperature with metal toxicity are indistinct. For example, the accumulations of zinc, manganese and cobalt in *U. intestinalis* can be amplified by reducing the salinity (Munda 2009).

U. intestinalis is probable to show intolerance to hydrocarbon contamination. The general effects include inhibition of respiration and photosynthesis, smothering, interference with reproduction and bleaching, which can result in the extermination of the contaminated populations. On the contrary, the species is prone to recuperate quickly from oil pollution accidents. For example, the *Torrey Canyon* tanker oil incident in 1967 resulted in the death of grazing species in the area, while a dense flush of ephemeral green algae of the genus *Ulva* and *Blidingia* emerged on the rocky coast after only a couple weeks and endured for up to one year (Hayward, Shields, and Nelson-Smith 1996).

Interestingly enough, the species of *Ulva* have been found to be unaffected by accumulated radionuclides, such as zirconium, niobium, cerium and even plutonium-239 (Clark 1997).

U. intestinalis is relatively tolerant to nutrient changes in nutrient concentrations. For example, nitrogen enrichment can increase its growth, while also thriving in un-enriched conditions. In fact, high levels of nutrient concentrations have been found to alleviate the adverse effects of the decreased salinity can have on the growth of the alga (Kamer and Fong 2001). On the contrary, excessive growth of green seaweeds in response to nutrients derived from anthropogenic river influxes is becoming an increasingly common phenomenon, especially in the Baltic Sea. Climate change is expected to escalate the ongoing algal blooms, eutrophication, in the Baltic Sea, and favour the green seaweeds with increasing temperature and nutrient levels and decreasing salinity levels. (Takolander, Cabeza, and Leskinen 2017; Rinne et al. 2018).

The consequences of extensive proliferation of green algae are severe, since decomposing algae deplete oxygen, resulting in drastic reduction in faunal abundance (Berezina et al. 2017). Moreover, only a few specific invertebrates, such as amphipods and gastropods can effectively graze these macroalgae, of which the consumption of *U. intestinalis* has been found to be the lowest (Golubkov et al. 2018).

1.4 Nutritional composition

The nutritional composition of macroalgae is high in magnesium, sodium, potassium, calcium, sulphur, chlorine, phosphorus, and micronutrients, such as zinc, selenium, iron, iodine, fluoride, molybdenum, manganese, nickel, boron and cobalt, as well as vitamins like B12, A and K. A small amount of macroalgae can provide, for instance, the daily requirement of iodine for an adult, 150 µg/day, which is especially true for brown algae like kelp, which has an iodine concentration of 1 500 to 8 000 µg/g. Generally, macroalgae are regarded as a decent source of protein, fiber and minerals for humans. (Ferdouse et al. 2018).

The protein content can drastically vary between different macroalgae species. In red macroalgae, the protein content of dried matter ranges from 1 % to 45 %, in brown macroalgae from 5 % to 20 %, and in green macroalgae from 3 % to 30 %. Thus, the protein and amino acid contents in macroalgae correspond to those of beef. However, concerns have been raised regarding possible overestimation of the protein content by the Kjeldahl method, which uses a conversion factor of 6.25 to determine the protein content in macroalgae. Considering the non-protein sources of nitrogen in macroalgae, species-specific conversion factors ranging from 3.57 to 5.72 have been suggested for macroalgae. (Cherry et al. 2019).

In recent years, the sulphated polysaccharides, ulvan, a major component of the cell wall of *Ulva* have attracted plenty of attention from researchers. Ulvan is comprised of D-glucuronic acid, and its C5-epimer l-iduronic acid, sulphated L-rhamnose, and a minor fraction of D-xylose. In ulvan, α-l-rhamnose-3-sulfate-1,4-β-d-glucuronic acid, α-l-rhamnose-3-sulfate-1,4-α-d-iduronic, and α-l-rhamnose-3-sulfate-1,4-β-d-xylose repeat as the main units. (Robic et al. 2009).

A study compared the prebiotic effect of three macroalgae polysaccharides, laminaran, porphyran, and ulvan using an individual intestinal bacteria fermentability test as well as an *in vitro* human fecal fermentation system. All three polysaccharides were found to be fermented by species of *Lactobacilli*, Bacteroides, and Bifidobacteria in the individual culture test. Ulvan and laminaran also stimulated the production of SCFAs in the *in vitro* fecal fermentation test. (Seong et al. 2019).

The nutritional composition of *U. intestinalis* and its seasonal variation is shown in **Table 1** (Jannat-Alipour et al. 2019; Escobido, Orbita, and Orbita 2016; Rohani-Ghadikolaei, Abdulalian, and Ng 2012; Mæhre et al. 2014; Metin and Baygar 2018; Benjama and Masniyom 2011), which illustrates the seasonal variation in the nutritional composition of the macroalgae. Metin & Baygar (2018) also measured the variation in chlorophyll-*a* content from August to November, January and April: 4.1; 4.9; 5.0; 5.9 (g/100g), respectively. The data on the amino acid profile of *U. intestinalis* is shown in **Table 2**. The findings of previous studies are compared to the essential amino acid (EAA) requirements suggested by the WHO (Joint Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition, 2007). This comparison demonstrates a moderate lack of histidine and lysine *in U. intestinalis*. The EAA requirements of different sized salmons have been investigated as well (Hua and Bureau 2019).

Table 1. Nutritional composition of U. Intestinalis harvested from different regions at different seasons (Jannat-Alipour et al. 2019; Escobido, Orbita, and Orbita 2016; Rohani-Ghadikolaei, Abdulalian, and Ng 2012; Mæhre et al. 2014; Metin and Baygar 2018; Benjama and Masniyom 2011).

Reference	Country	Harvest date	Protein (g / 100 g)	Lipids (g / 100 g)	Carbohydrates (g/100 g)	Ash (g / 100 g)	Moisture (g / 100 g)
Jannat-Alipour *et al*., 2019.	Iran	–	13.6	2.7	57.0	19.0	8.4
Escobido *et al*., 2016.	Philippines	Jan – Apr	5.6	0.4	37.3	–	–
Rohani-Ghadikolaei *et al*., 2012.	Iran	–	10.5	2.9	35.5	–	–
Maehre *et al*., 2014.	Norway	May 2010	11.3	1.03	–	55.3	–
Metin & Baygar, 2018.	Turkey	Aug 2013	13.4	1.3	58.0	14.8	12.1
		Nov 2013	4.7	0.2	70.6	11.8	12.2
		Jan 2014	6.3	0.2	69.0	12.5	11.2
		Apr 2014	12.3	0.02	59.8	17.4	10.7
Benjama & Masniyom, 2011.	Thailand	Dec 2007	16.4	8.7	62.2[1]	28.4	5.4
		Apr 2008	19.5	7.3	51.3[1]	26.9	7.2

[1] Carbohydrates expressed as total dietary fiber.

[2] Rows missing moisture value express nutritional values as dry weight.

Table 2. Amino acid composition of U. intestinalis, WHO EAA requirements for adults and EAA requirements for Atlantic Salmon.

	Jannat-Alipour et al., 2019	Benjama and Masniyom, 2011	Indispensable AA requirements of adults, WHO (2007)		Hua & Bureau, 2019
Essential AAs	g / 100 g protein		g/ 100 kg per day	g/ 100 g protein[c]	% diet d.m.[d]
Arginine	6.83	4.71	ND	ND	1.67
Histidine	1.22	0.76	1.0	1.5	ND
Isoleucine	3.66	3.2	2.0	3.0	1.03
Leucine	8.27	6.22	3.9	5.9	1.35
Lysine	3.9	2.73	3.0	4.5	2.45
Methionine	2.9[a]	ND	1.5	2.2[a]	2.50
Phenylalanine	8.41[b]	4.3	2.5	3.8[b]	1.00
Threonine	5.61	5.17	1.5	2.3	1.55
Tryptophan	ND	ND	0.4	0.6	1.36
Valine	7.25	5.06	2.6	3.9	0.29
Tyrosine	3.95	1.86	ND	ND	1.73
Non-essential AAs					
Alanine	7.03	8.6	ND	ND	
Aspartic acid	9.28	10.5	ND	ND	
Glutamic acid	13.14	10.9	ND	ND	
Glycine	7.16	5.29	ND	ND	
Proline	2.17	3.95	ND	ND	
Serine	5.45	5.58	ND	ND	
Total AAs	92.28	78.9			
Total EAAs	46.83	31.4	18.4	27.7	
Total NEAAs	45.45	47.5			
EAA/Total AA	0.51	0.4			

[a] Methionine + cysteine

[b] Phenylalanine + tyrosine

[c] Mean nitrogen requirement of 105 mg nitrogen/kg per day (0.66 g protein/kg per day).

[d] EAA requirements for 100 – 500 g weight salmon

[e] ND: not defined

1.5 Protein isolation for food applications

Processing seaweed biomass into a protein rich isolate involves two primary steps, extraction and concentration. Traditional extraction methods include acid-alkaline, freeze-thawing, pressing, and high shear homogenization, while novel extraction methods include high-pressure processing (HPP), microwave-assisted extraction (MAE), and sub- and supercritical water extraction (SWE).

These are usually assisted with at least one of the following procedures: sonication, pulsed electric field (PEF), osmotic shock (OS), and enzymatic hydrolysis. The second step, protein concentration, methods include isoelectric precipitation that is also known as pH-shift, salting-out, ion-exchange chromatography, and membrane separation. Most of these methods have been in regular use in biochemistry for decades, but seaweed proteins and their isolation began gaining attention only in the 90's. (de Souza Celente, Sui, and Acharya 2023).

The first quarter of the 21st century has produced significant improvements since the experiments of the early 90's that struggled to recover even 1 % of algal protein using enzyme-assisted extraction (Fleurence et al. 1995).

Alkaline treatment yields a wide range of protein solubilization, at least, from 15 to 90 % even in modern studies. It is often combined with assisting treatments such as enzymatic hydrolysis, osmotic shock, or different drying methods to maximize protein solubilization. (Mateus et al. 2024; Vilg and Undeland 2017; Harrysson et al. 2018; Abdollahi et al. 2019).

A study on protein extraction of *Saccharina latissima* compared the effects of length, temperature and volume of osmotic shock, and its contribution to the total solubilized protein after a subsequent alkaline extraction. The osmotic shock solubilized between 50 % and 75 % at most of the total solubilized protein. Moreover, a 58 % increase in protein solubilization with increased OS extraction volume, 1:20 to 1:60 (dw biomass to water at 2 h, 4 °C), was also reported. (Vilg and Undeland 2017).

Sometimes sonication is more effective than alkaline extraction. A study comparing three seaweeds, *S. latissima*, *U. lactuca*, and *Porphyra umbilicalis*, found that the alkaline extraction at pH 12 solubilizes about 60 % of the protein for all three seaweeds, but the highest recovered protein for *U. lactuca*, 19.6 %, was achieved with sonication and the subsequent ammonium-sulfate concentration. (Harrysson et al. 2018).

Different drying methods and freeze/thawing in strong acidity can also affect solubilization and total extraction yield. A study compared freeze, oven, and sun-drying on solubilizing proteins from a brown seaweed, *S. latissima*, in subsequent alkaline extraction (pH 12) and pH-shift precipitation (pH 2) with and without a freeze/thaw (F/T) between the precipitation and the final centrifugation. The freeze-drying produced by far the highest protein solubilization and total recovery at 90.9 % and 26.4 % with F/T, respectively, followed by oven-drying at 77.2 % and 26.1 % with F/T, respectively, and sun-drying at 64.9 % and 18.6 % & with F/T, respectively. Indeed, across all samples freeze/thawing consistently doubled total protein recovery. Interestingly, oven-drying with F/T produced the highest final protein concentration at 40.5 %. (Abdollahi et al. 2019).

Another study compared three physical processing based protein extraction methods, sonication (1h, 42 Hz), HPP (600 MPa, 4 min), and autoclaving (124 °C, 101 kPa, two 15 min cycles) after osmotic shock assistance (1:50 w/w). A 100 µ muslin bag was used for insoluble-soluble separation for the latter two. Studied seaweeds included *Fucus vesiculosis*, *Alaria esculenta*, *Palmaria palmata* and *Chondrus crispus*. Sonication and the subsequent ammonium-sulphate concentrating on *C. crispus* yielded the highest total protein recovery at 35.2 %. (O' Connor et al. 2020).

Only a handful studies have evaluated the effect of subcritical water extraction (SWE) on seaweed fractionation. A kinetic study subjected post-agar extraction industrial solid residue from a red seaweed, *Gelidium sesquipedale*, to 185 °C at different SW residence times using two semi-continuous fix-bed reactors, 127 cm^3 and 27 cm^3. Perhaps surprisingly, higher protein solubilization was achieved by increasing the flow rate, i.e. decreasing the residence time (RS). In fact, protein solubilization plateaued at 96 % and 100 min for the 3 min RS, and at 80 % and 150 min for both 4.2 min RS and 6 min RS in the smaller reactor. A small amount (~6 %) of protein was hydrolyzed into free amino acids in all treatments. A follow-up study explored the scale-up prospects of SW treatment using two discontinuous reactors, a 0.5 L lab reactor and a 5 L pilot reactor at 175 °C and 130 °C. The protein solubility was about 37 % at 175 °C, and less than 20 % at 130 °C in both reactors. At most, 1.8 % of the protein was hydrolyzed into free amino acids. (E. Trigueros et al. 2021; E. Trigueros et al. 2023).

The pH-shift also known as isoelectric precipitation (IEP) is probably the most studied and the most popular algal protein isolation method. It requires careful optimization to account for species specific differences in protein isoelectric point (pI). Using a pH-shift of 2 to 12 on concentrating alkaline extracted *S. latissima* proteins appears to be capped at 40 % (dw) when protein contents of the raw seaweed biomass are 8-10 % (dw) yielding protein concentration factors (PCF) between 4.0 and 5.0 (Abdollahi et al. 2019; Harrysson et al. 2018). Using the regular pH-shift water extracted *U. lactuca* proteins have been concentrated to 51 % from an original content of 20 % (dw), while alkaline extracted *P. umbilicalis* proteins have been concentrated to 71 % from an original content of 32 % (dw) (Harrysson et al. 2018).

Studies on salting out seaweed proteins using ammounium sulphate are scarce and the results inconsistent. Salting out *U. lactuca* proteins has yielded total protein recoveries comparable to F/T-assisted pH-shift methods after extraction at around 20 %, although with very poor PCF values of <1 (Harrysson et al. 2018). O' Connor et al. (2020) recovered 35 % and 18 % of total protein with PCF values of 1.6 and 1.5 from sonicated and freeze-dried *C. crispus* and *A. esculenta*, respectively. Proteins of alkaline-sonicated *U. rigida* and *U. fasciata* have been concentrated to 30 % with a PCF of 3.3 using 85 % ammonium sulphate saturation at the cost of low total protein recovery, 10.5 ± 0.5 % (Kazir et al. 2019).

Concentrating algal proteins using ultra- or nanofiltration (UF) has been poorly studied. Using 22.8 µm and 68.4 µm filters on sonicated and alkaline treated samples of *Ascophyllum nodosum* has resulted in a PCFs of 4.1 with a 9.0 % loss and 3.9 with a 11.6 % loss, respectively, after only 0.5 h, 3 500 g centrifugation (Kadam et al. 2017).

A comparison of two hydrophilized PTFE membranes, 0.45 µm and and 1.0 µm, on filtration yield (protein recovery in the permeate) from pressed and heated (45 °C) *Ulva* sp. pulp produced comparatively meek yields of 19.4 ± 0.7 % and 22.7 ± 1.2 %, respectively, which was attributed to the inability of sizeable protein complexes over 250 kDa to pass through the filter (Juel et al. 2024). Kazir et al., (2019) performed two cycles of sonication and centrifugation in NaOH (10 % w/v) on *Ulva* sp. and *G. conferta* before filtering (0.45 µm) the combined supernatants and purifying the protein with an alkaline ion exchange chromatography producing highly concentrated protein isolates with concentrations of, 70 % and 86 %, respectively, from the original raw seaweed protein with concentrations of 9.0 % and 25 % (dw), respectively, although the total protein recovery yield was merely 10–11 %, for both *Ulva* sp. and *G. conferta*.

Studies on protein extraction and isolation methods and their efficacies are compiled in **Table 3** (E. Trigueros et al. 2021; E. Trigueros et al. 2023; Mateus et al. 2024; Vilg and Undeland 2017; Trigo et al. 2023; Abdollahi et al. 2019; Harrysson et al. 2018; O' Connor et al. 2020; Kazir et al. 2019; Kadam et al. 2017).

The problem in the extraction is that seaweed proteins are often part of the cell membrane (Lourenço et al. 2002). Much to researchers' dismay, some proteins are trapped by the cell wall and extracellular matrix (ECM) polysaccharides leading to low yield. Cermeño et al. (2020) state that the seaweed proteins are "cross-linked covalently to polysaccharides via disulfide bridges within the cell wall assembly" and conclude that these ”anionic or neutral polysaccharides and polyphenol-rich seaweed cell walls inhibit protein solubilization under the high dispersion viscosity.”

Table 3: Protein solubilization and total recovery yields, protein content (dw; PC) in the isolate and the raw seaweed biomass, and protein concentration factor (PCF) for several seaweeds and treatments.

Author	Species	Extraction	Concentration	Sol %	Rec %	PC$_{ISO}$%	PC$_{RB}$%	PCF
Trigueros et al., 2021	G. sesqui pedale	SW treatment at 185 ºC	None	96 ± 1	-	-	21 ± 1	-
Trigueros et al., 2023		SW treatment at 175 ºC	None	38	-	-	-	-
Mateus et al., 2024	G. corneum	Enzymatic x2 + Alkaline x2	Consecutive pH-shifts	52 ± 1	34 ± .0	-	15 ± .2	-
			Salting		-			
		Water x2 + Alkaline x2	Consecutive pH-shifts	15	6 ± .0			
			Salting		7 ± .0			
Undeland & Vilg, 2016	S. latissima	OS + Alkaline	pH-shift	60	16	18	9	2.0
Trigo et al., 2023	S. latissima	Soaked and minced	pH-shift with Freeze/ Thaw	-	21 ± .1	20 ± .2	5 ± .3	4.1
		Blanched 45 °C + minced		-	21 ± .1	21 ± .6	5 ± .3	4.3
Abdollahi et al., 2019	S. latissima	Freeze-dried + Alkaline		91 ± .3	26 ± .1	38 ± .7	8 ± .3	4.6
		Oven dried + Alkaline		77 ± 7	20 ± 1	41 ± .5	8 ± .1	5.1
Harryson et al., 2018	S. latissima	Alkaline solubilization		-	25 ± 1	41 ± .5	10 ± .2	4.0
	P. umbilicalis			54 ± 5	23 ± 7	71 ± 4	32 ± 2	2.2
	U. lactuca			62 ± 5	8 ± .1	51 ± 2	20 ± 1	2.6
		OS	Salting	-	20 ± 1	10 ± .1	20 ± 1	0.5
O' Connor et al., 2020	C. crispus	OS + Sonication + Freeze drying		-	35 ± 4	23	14	1.6
	A. esculenta			-	18 ± 5	9	6	1.5
Kazir et al., 2019	U. rigida & U. fasciata	Sonication in alkaline conditions x2		-	10 ± 1	30 ± .1	9	3.3
			Alkaline ion exchange	-		70 ± 4	9	7.8
	G. conferta			-		86 ± 2	25	3.4
Kadam et al., 2017	A. nodosum	Sonication + Alkaline	UF 22,8 µm	57 ± 2	52 ± 4	29	7 ± .0	4.1
			UF 68,4 µm		51 ± 5	28	7 ± .0	3.9

1.6 Seaweed cultivation potential in the Baltic Sea

As mentioned before, the tolerance of *U. intestinalis* for low salinity allows it to grow practically across the entire Baltic Sea, with the exception of innermost areas, where salinity drops under 2 µg/kg (Leskinen, Alström-Rapaport, and Pamilo 2004), which could be explained by the tolerance of *U. intestinalis* for low salinities under high levels of nitrogen and phosphorus (Kamer and Fong 2001), which may occur in polluted estuaries connected to wastewater sources or fish farms. There is a handful of studies on the cultivation potential of macroalgae in the Baltic Sea.

Macroalgae and macrofauna and integrated systems thereof have been cultivated in the Baltic Sea for different research purposes. Some macroalgae absorb riverine nitrogen from estuaries sufficiently enough to replace portable direct measurements of nitrogen in ammonium and nitrate when precise quantification is not required. *Furcellaria lumbricalis* has been cultivated in open waters to determine the potential of its biotechnologically applicable compounds, of which furcellaran, lutein and phenolic content could be relevant to food applications. A pond cultivation experiment on *Gracilaria tenuisipitata* and *U. intestinalis* in brackish seawater (6–7 ‰) in the Gryt archipelago on the east coast of Sweden recorded a growth rate in biomass of 4 ‰ per day and a complete inhibition, respectively. Nutrient absorption and growth rate increased with the temperature, and the researchers determined a feasible cultivation period of 5 to 6 months using wastewater e.g. from trout cultivation, in southern Sweden. (Kersen et al. 2017; Haglund and Pedersén 1993).

In another experiment *Ceramium rubrum*, *Polysiphonia* sp. and *Fucus vesiculosus* were cultivated in gauze bags in unpolluted brackish water for 10 days and at 12 stations in a polluted estuary for 14 days according to salinity gradient to measure the contribution of anthropogenic nitrogen sources. Salinity, nitrate $\delta^{15}N - NO_3^-$ -values (^{15}N in NO_3^- relative to atmospheric N_2 per thousand) were measured along the estuary that stretched for 15 km from the dam separating the estuary from the river. A significant decrease in nitrate concentration in the immediate vicinity of the dam was observed from 250 µM to 122.5 µM after the 14-day cultivation period despite the low salinity, 1.4 ‰. (Deutsch and Voss 2006).

In a Finnish study non-seeded polyethylene substrata consisting of insect nets and braided ropes were installed in two non-tidal locations in Naantali and Hanko with strong seasonal variation in temperature and irradiation, low salinity, and ice cover in winter. The first setup in Naantali consisted of substrata placed at three different distances, 2 m, 5 m, and 150 m from nearby fish cages cared by the Natural Resource Institute Finland. The other two commercial fish farms were located 150 m and 300 m further. The second setup was in a nature reserve. The total nitrogen and phosphorus in Naantali ranged 300 - 380 and 15 - 30 µg/L., respectively. Corresponding values for Hanko were 280 to 400 µg/L and 14 to 28 µg/L. During the yearlong study period, *Cladophora glomerata* and *Ulva* spp. comprised the main macroalgal fractions on the substrata, however most of the biomass was accumulated in the invertebrates. After 5-month incubation, the total biomass on the substrata was comprised of 21 g/m^2 of nitrogen and 3 g/m^2 of phosphorus on average, which was doubled after a total of 14.5 months. (Suutari et al. 2017).

Samples of *U. intestinalis* from the southern Baltic Sea, Gulf of Gdansk, and Vistula Lagoon have shown a relatively average abundance of microelements and heavy metals in the following descending order: K, Na, Mg, Ca, & Mn, Zn, Cu, Pb, Ni, Cd. The highest mean Zn, Cu, Pb, Cd levels in biomass were found in samples from the Gulf of Gdansk, 64.1 ± 40.5; 4.92 ± 2.33; 3.77 ± 2.17; and 0.44 ± 0.24 µg/g d.w., respectively, and the corresponding water concentrations were 1.62; 0.80, 0.088, and 0.014 µg/L. The highest mean Ni and Mn levels (µg/g d.w.) 8.20 ± 1.6 and 361.5 ± 111, in biomass were found in samples from the Vistula Lagoon, and the Ni water concentration (µg/L) in the location was 5.0. (Żbikowski, Szefer, and Latała 2006).

A case study on the metal content of different algal species from the eastern Gulf of Finland found similar relative average abundances of heavy metals. Sediment and algal samples were harvested from Neva bay, the outer and inner estuary, where hydrological conditions and landscape features vary noticeably. The inner estuary is a transition zone, where the condition changes from freshwater to brackish water. Metal concentrations of Mn, Zn, Pb, Cu and Cd (µg/g) in the surface sediments of these parts varied significantly, 57.7 – 405, 7.1 – 77.6, 6.2 – 240, 1.4 – 55.7, 0.03 – 0.5, respectively. The measured metal concentrations of Zn, Cu, Pb, and Cd, (µg/g) in biomass of *U. intestinalis* were 17.5 ± 2.6, 5.4 ± 0.5, 3.1 ± 0.6 and 0.12 ± 0.03. (Gubelit et al. 2020).

The Panel on Contaminants in the Food Chain (CONTAM Panel) defined a threshold dose lower bound associated with 1 % extra risk for neurodevelopmental effects in children of 12 µg/L of lead in blood, corresponding to 0.50 µg/kg b.w. per day, and a margin of exposure (MOE) of 10 was deemed adequate that is an exposure equal to or less than 0.05 µg /kg b.w. /day. Therefore, a 0.33 g and a 0.4 g portion of dry *U. intestinalis* sample with a lead concentration of 3.77 µg/g d.w. from the Gulf of Gdansk and of 3.14 µg/g d.w. from the Gulf of Finland, respectively, would meet the daily MOE of lead for a 25 kg child. However, the mean dietary exposure to lead in European adults ranges from 0.36 to 1.24 µg/kg b.w. /week and 0.80 to 3.10 µg/kg b.w. /week in children – both surpassing the adequate MOE – cereals remaining as the primary source of dietary exposure. To achieve the lower or higher end of the weekly lead exposure of adults a 100 kg adult would have to consume a portion of dry *U. intestinalis* sample of 9.55 g or 33 g from the Gulf of Gdansk (Pb 3.77 µg/g), and of 11.5 g or 39.5 g from the Gulf of Finland (Pb 3.14 µg/g), respectively. EFSA has set the tolerable weekly intake (TWI) of cadmium, which is mainly toxic to kidneys and bones, at 2.5 µg/ kg b.w. /week. Diet is the main source of cadmium for non-smokers. A 100 kg adult would meet the TWI by consuming a 568 g and a 2 kg portion dry *U. intestinalis* from the Gulf of Gdansk (Cd 0.44 µg/g) and Gulf of Finland (Cd 0.12 µg/g). Using unprocessed seaweed biomass in exposure assessment, in all likelihood, results in an overestimation since the effects of cooking and processing on the final concentrations in the algal biomass as well as bioavailability are not considered. Washing and cooking may substantially decrease the levels of several metals like iodine. In the absence of a more robust health risk assessment of metal contamination in seaweeds maximum or recommended consumption levels are yet to be officially defined in Europe. (Monteiro et al. 2019; Żbikowski, Szefer, and Latała 2006; Gubelit et al. 2020).

The overall regulation and research on the dioxin, polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyl (PCB), and radionuclides contamination in algae in the Baltic Sea is scarce. Species of red algae are often used to measure such contaminants in sea or fresh water. Samples of *Ceramium tenuicorne* collected near Askö Island in the Stockholm archipelago contained total hydroxylated polybrominated diphenyl ethers, total methoxylated polybrominated diphenyl ethers (MeO-BDE), and total PCBs at average concentrations and ranges (ng/g d.w.) of 140 (110 - 220), 4.6 (4.1 - 5.5), and 1.7 (1.2 - 2.7), respectively, most of which are suspected to be metabolized to a degree by the alga (Malmvärn et al. 2008). Samples of *U. intestinalis* harvested from coastal regions in the northern Baltic contained total MeO-BDEs and total bromoanisoles at average concentrations of 45 and 726 pg/g wet weight, respectively (Bidleman et al. 2019). Samples of *Ulva* and *Cladophora* species found in the Gulf of Gdansk contained a total average concentration of 15 different PAHs of 64 ng/g d.w. (Filipkowska et al. 2008). Compared to other macroalgae, including red and brown, species of *Ulva* have shown the lowest ^{137}Cs activity, below 5 Bq/ kg d.w., and *Polysiphonia* *fucoides* has ranked the highest at 22.5 Bq/ kg d.w., while the average concentration of ^{137}Cs in the southern Baltic Sea was 35 Bq/m^3 in 2010 (Zalewska and Suplińska 2013).

Five years and even almost instantly after the Chernobyl nuclear accident and the plutonium fallout that quickly ensued, the increase in radioactivity concentration of the brown macroalgae, *Fucus vesiculosus*, in the Baltic Sea was not found statistically significant (Holm 1995). Unfortunately, EFSA's declared maximum levels (ML) of total dioxins and PAHs for seafood exclude seaweed, and include muscle meat of fish and crustaceans, whose ML values are 8.0 and 5.0 mg/kg wet weight, respectively (Barroso 2011).

Considering the physical and chemical sensitivity as well as its adaptability to the harsh environment of cold climate, low salinity, and short growing season of the Baltic Sea, *U. intestinalis* shows great potential in cultivation for food and feed purposes. The purpose of this study was to examine the effect of temperature and nutrient level in low salinity mimicking conditions in the Baltic Sea on the biomass growth, protein content, and amino acid profile on *U. intestinalis*. This study expects to find higher nutrient levels to ameliorate the effect of low salinity on *U. intestinalis* growth, or, in other words, to find *U. intestinalis* to thrive in higher nutrient levels. Knowledge gained from this study could further expand our understanding of seaweed growth and health in low salinity environments.

2 Materials and Methods

2.1 Cultivation

The thalli of *U. intestinalis* were earlier collected in coastal region of Naantali, Finland on 4.11.2019 and in Uittamo, Finland on 12.11.2019 and washed with distilled water and moved into a nutrient solution, where it acclimated for two weeks before the experiment. The experiment was commenced by pre-weighing four (2.0 - 2.5 g) *U. intestinalis* thalli, which were submerged in 800 mL of two standard nutrient F/2 solutions and of two low nutrient F/20 solutions prepared in 5.0 PSU tap water (**Table 4**)(Guillard and Ryther 1962).

Table 4. Nutritional composition of the standard F/2 and low F/20 nutrient solutions.

	F/2	*F/20*	
$NaNO_3$	882	88.2	µM
NaH_2PO_4	36.2	3.62	µM
Na_2CO_3	106	10.6	µM
$FeCl_3 \cdot 6\ H_2O$	11.7	1.17	µM
$Na_2(EDTA) \cdot 2\ H_2O$	11.7	1.17	µM
$CuSO_3 \cdot 5\ H_2O$	39.3	3.93	nM
$Na_2MoO_4 \cdot 2\ H_2O$	26.0	2.60	nM
$ZnSO_4 \cdot 7\ H_2O$	76.5	7.65	nM
$CoCl_2 \cdot 6\ H_2O$	42.0	4.20	nM
$MnCl_2 \cdot 4\ H_2O$	91.0	9.10	nM
Thiamine HCL (vitamin B1)	296	29.6	nM
Biotin	2.05	0.205	nM
Cyanocobalamin (vitamin B12)	0.369	0.0369	nM

Pairs of the thalli in standard nutrient and low nutrient solutions were cultivated in 25 °C and in 10 °C. The pump was switched on to initiate the recirculation of the nutrient solution at a plastic valve-set rate flow rate. Both thalli pairs were provided as near as was possible a uniform vertical illuminance with a LED light (400 µmol/fotons/s) in a light-tight carton container. The LED lights were controlled by a timer to provide a 12:12 hr. light/dark cycle. The cultivation period was four weeks, while both the nutrient solution was replaced and the thalli weighed weekly. Four additional thalli samples collected from Saaremaa and Viinistu (Estonia) as well as from Seili and Uittamo (Finland) were included for comparison.

2.2 Sample pretreatment

The thalli were freeze dried in dark, -29 °C, 140 µbar, 96 h (VirTis Wizard 2.0, SP Scientific). Freeze dried samples were ground into powder with liquid nitrogen and a mortar.

2.3 Chlorophyll and carotenoid concentration

Powdered thalli samples (10 - 15 mg) were dissolved into 1 mL of N,N-dimethylformamide (EMPARTA®, Merck KGaA, Germany) in triplicates, shaken, and vortexed. Extraction was continued by centrifugation, 12 000 g, RT, 5 min, after which 0.9 mL of supernatant was collected. The absorbance of the extraction solution was measured at four wavelengths: 480, 646.8, 663.8 and 750 nm. Concentrations (µg/mL) of chlorophylls *a* and *b* and total carotenoids were derived from three equations. (Wellburn 1994).

$C_a = 12A_{663.8} - 3.11A_{646.8}$,

$C_b = 20.78A_{646.8} - 4.88A_{663.8}$, and

$C \text{ (carotenoids)} = (1000{*}A_{480} - 1.12{*}C_a - 34.07{*}C_b)/245$.

2.4 Total protein content and amino acid profile

Approximately 50 mg of powdered thalli samples were added to 4 mL of ultrapure water (Milli-Q®), 20 h. These samples were centrifuged 4 500 g, 20 min, RT, and supernatants collected. Exactly 1 mL of protein extraction buffer (0.1 M NaOH, 0.5 % (w/V) SDS) was added to the pellet, left in shaker for 1 h, RT, and centrifuged 4 500 g, 20 min, RT, and supernatants collected. The two supernatants were combined resulting it total volume of 5 mL. The combined supernatant samples were diluted to 1:3 with ultrapure water, triplicate samples prepared for the protein assay, and kept in 4 °C.

The reagents of the Lowry Protein Assay were added to each protein sample and the BSA (Sigma, A9647, Loc: 4217) standard samples (0, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 mg/mL) as instructed. Lowry's Solution was added to each sample in 5:1, vortexed, and incubated 15 min. The 1.0 N Folin Phenol Reagent was added to each sample in 1:12, vortexed, and incubated 30 min. The final concentrations were 14.8 mg/mL; Na_2CO_3; 3.06 mg/mL NaOH; 1.21 mg/mL Na-K-tartrate; 0.62 mg/mL $CuSO_4$; 0.15 mg/mL Na-tartrate, and 0.08 N Folin Reagent. Absorbances were measured at 750 nm. The absorbances of the protein samples were compared against the BSA standard curve. (Lowry et al. 1951).

The hydrolysis of the proteins was initiated by dissolving approximately 20 to 25 mg of powdered thalli into 2.5 mL of 6 M HCl in duplicates. The airspace of the sample tubes was flushed with nitrogen gas before placing the samples in an oven preheated to 110 °C, 22 h. Each hydrolysate was diluted to 20 mL with ultrapure water.

Derivatization of the hydrolyzed protein samples and the amino acid standard mixtures (AASM) was completed by pipetting borate buffer (0.5 %-V 3-MPA, pH adjusted to 10.2 with NaOH), protein hydrolysate or AASM, *o*-phthalaldehyde (OPA, in borate buffer), 9-fluorenylmethyl chloroformate (FMOC-Cl, in ACN) and ultrapure water into autosampler vials in this exact order before the injection. The AASMs were prepared by diluting an AA standard stock solution containing 17 AAs (PN: 5061-3331, Sigma-Aldrich) and dissolving four AAs in powder form, Gln, Asn, Trp, and Hyp, to 12.5, 25, 50, 100, 200 µM for each AA, except for Cys, 6.25, 12.5, 25, 50, 100 µM with ultrapure water for constructing the calibration curve. The final pre-injection concentrations of the borate buffer, 3-MPA, protein hydrolysate, AASM, OPA, and FMOC-Cl were 28.1 mM, 0.035 V-%, 1.4 V-%, 1.4 V-%, 7.0 nM, 7.0 nM, 0.14 mg/mL, and 0.07 mg/mL, respectively.

The HPLC-FLD equipment included the following: Shimadzu LC-20AD coupled with a SIL-20AC autosampler (4 °C), a column oven CTO-20AC (40 °C), a communication module (CBM-20A), a RF-20Axs fluorescence detector (Channel 1: ex 340 nm, em 450 nm; Channel 2: ex 266 nm, em 305 nm), a column (Zorbax Eclipse AAA: 4.6 x 150 mm, 3.5 µm) with a pre-column. The mobile phases consisted of solvent A (40 mM Na_2HPO_4 adjusted to 7.80 ± 0.01) and solvent B (45:45:10 (V/V/V), ACN : MeOH : H2O). Flow rate was set to 2 mL/min, and stop time to 26 min. Auxiliary pump settings included 100 mL/min^2 maximum flow ramp, 50×10^{-6} compressibility A, 20 µL minimal stroke, 115×10^{-6} compressibility B, auto minimal stroke B. The autosampler settings included 200 µL/min draw speed, 600 µL/min eject speed, and 0.0 mm draw position. The gradient was set as following: 0 min – 0 % B, 1.9 min – 0 % B, 18.1 min – 57 % B, 18.6 min – 100 % B, 22.3 min – 100 % B, 23.2 min – 0 % B, 26 min – 0 % B. The injection volume was set to 5 µL. The AAs were quantified by comparing the areas of chromatographic responses of *U. intestinalis* protein samples to the constructed AASM standard curve.

All correlations and standard curves were calculated using standard linear regression tools. Statistical significance was quantified with analysis of variance (ANOVA) and student’s t-test.

3 Results

The average weekly wet biomass (w.b.) productivity varied greatly between the cultivated *U. intestinalis* samples. After two weeks only the F/2 10 °C cultivated sample maintained a clearly consistent and the highest weekly relative growth rate (RGR) in the range of 30 – 47 %, averaging at 38 % (w.b.) until the end of the four week experiment. The total biomass growth of F/2 10 °C cultivated *U. intestinalis* was 260 % after four weeks (**Fig. 1**).

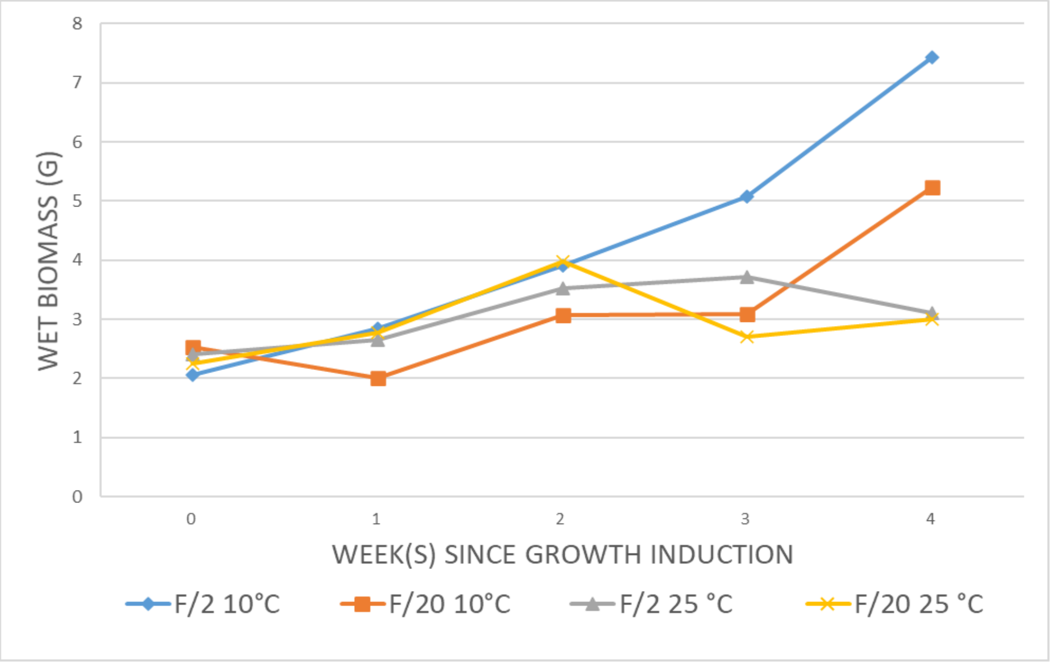

Figure 1: *U. intestinalis* was cultivated in orthogonal combinations of two nutrition concentrations, F/2 (high) and F/20 (low), and two temperatures, 25 °C and 10 °C.

Chlorophyll content was used as an indicator of the *U. intestinalis* well-being. Concentrations (µg/mL) of chlorophylls *a* and *b* and total carotenoids were calculated using the absorbances of the extraction solutions measured at four wavelengths: 480, 646.8, 663.8 and 750 nm. Interestingly, chlorophyll *b* contents of the thalli grown in F/2 were clearly higher than chlorophyll *a*, whereas the exact opposite was true for the thalli grown in F/20. Thalli grown in F/2 10 °C showed a relatively high chlorophyll content, despite its poor biomass yield. Concentrations of chlorophyll *a* and *b* and total carotenoids are shown in Figure 2. Additionally, references of chlorophyll contents of *U. intestinalis* are included for comparison (Bianchi, Kautsky, and Argyrou 1997; Shafique et al. 2010).

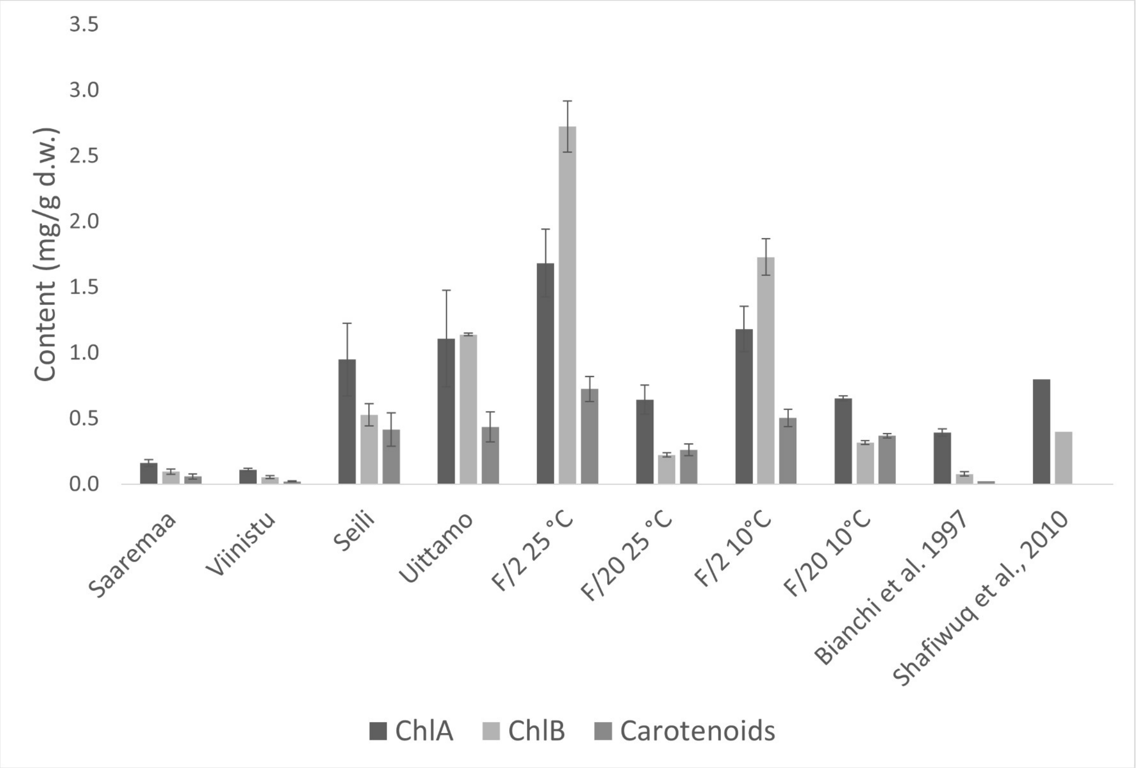

Figure 2: Chlorophyll *a* & *b* and carotenoid contents of cultivated, collected, and reference *U. intestinalis* samples (N = 4) (Bianchi, Kautsky, and Argyrou 1997; Shafique et al. 2010).

The total protein content was determined using the Lowry Protein Assay (LPA). The method is based on colorization of protein, which is mediated by two specific reactions, and measuring the spectrophotometric absorbance of the end product. In the first reaction, proteins react non-catalytically with copper ions in an alkaline solution, where approximately 1 copper ion binds to 4 amino acid residues. In the second reaction, Folin Reagent containing phosphomolybdatephosphotungstic acid is added, which is reduced by the aromatic acids present in the copper ion treated sample. This reaction yields an end product with a blue colour. Finally, the quantity of proteins was estimated by measuring the absorbance at 750 nm of the end product in comparison with the BSA standard curve (R^2=0.9804). (Lowry et al. 1951).

The protein content (% d.w.) results from the Lowry Protein Assay (LPA) is compared to the compiled research data in **Figure 3.**

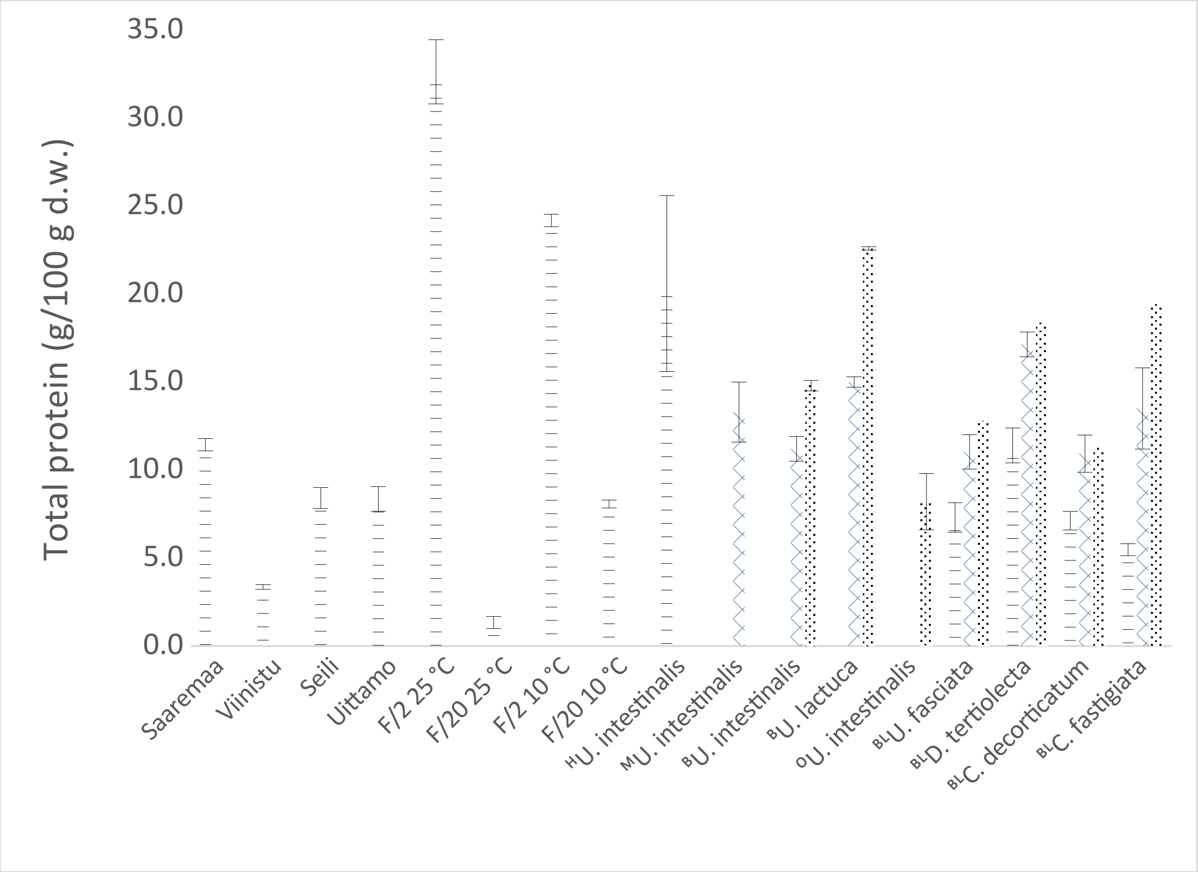

Figure 3: Protein content of *U. intestinalis* cultivated in orthogonal combinations of two nutrition concentrations, F/2 (high) and F/20 (low), and two temperatures, 25 °C and 10 °C and reference seaweeds determined by LPA (dashed), total AA residues (crossed), and nitrogen conversion (dotted) methods. (Haroon et al., 2000[H]; Mæhre et al., 2014[M]; Biancarosa et al., 2017[B]; Olsson et al., 2020[O]; Barbarino & Lourenco, 2013[BL]).

The amino acid profile (AAP) was determined using a semi-quantitative HPLC analysis. Agilent 1100 HPLC enables a pre-column, automated, online derivatization using *o*-phthalaldehyde (OPA) for primary AAs and 9- fluorenylmethyl chloroformate (FMOC-Cl) for secondary AAs. The acid hydrolyzed protein/peptide samples are derivatized in an alkaline buffer solution. The primary AAs react first with OPA using 3-mercaptopropionic acid (3-MPA), whereas the secondary AAs are derivatized using FMOC. The addition of 3-MPA into the indoles decreases their hydrophobicity, which results in the OPA derivatives eluting chromatographically before the FMOC derivatives. (Henderson et al. 2000).

Results of the AAP analysis are included and compared to WHO's (2007) recommended EAA intakes for adults in **Table 5**. Examples of HPLC chromatograms of 10 °C F/2 cultivated *U. intestinalis* are shown in **Figure 4** and **Figure 5**.

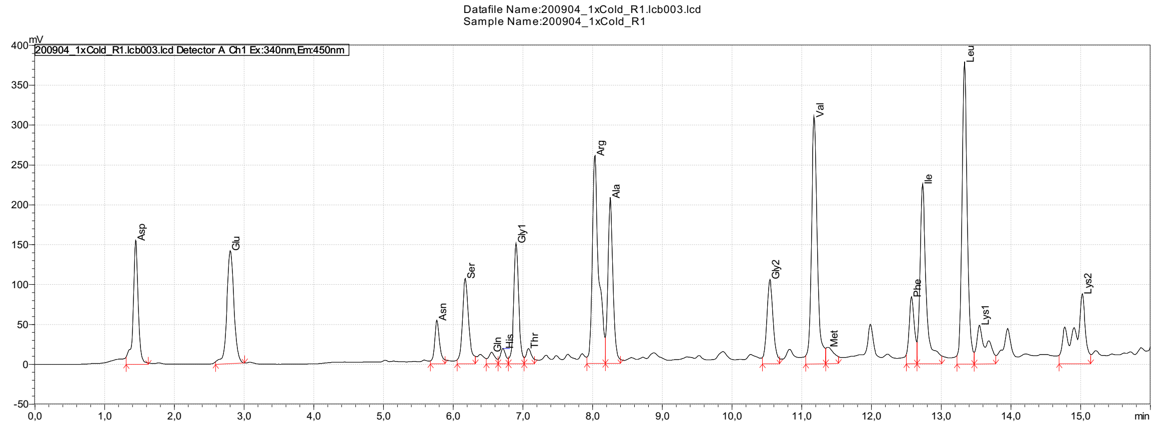

Figure 4: HPLC chromatogram (λ_{Ex}=340 nm, λ_{Em}=450 nm) of 10 °C F/2 cultivated *U. intestinalis* amino acid analysis.

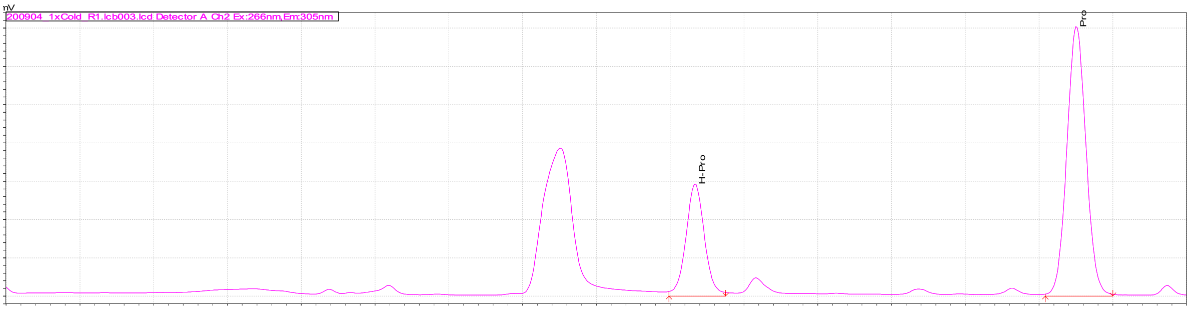

Figure 5: HPLC chromatogram (λ_{Ex}=266 nm, λ_{Em}=305 nm) of 10 °C F/2 cultivated *U. intestinalis* amino acid analysis.

Table 5: The amino acid profile (AAP) was determined using a derivatization based HPLC analysis. The sample results are compared to WHO's (2007) recommended EAA intakes for adults (mean nitrogen requirement of 105 mg nitrogen/kg per day (0.66 g protein/kg per day))[A]*. Met+Cys*[B]*, Tyr+Phe*[C]

	Saare maa	Viini stu	Seili	Uit tamo	F/2 25 °C	F/20 25 °C	F/2 10 °C	F/20 10 °C	g/100g prot[A]
Asp+ Asn	3.4	3.6	3.8	5.0	5.8 ± 2.5	4.0 ± 1.5	3.9 ± 0.1	4.8 ± 0.3	ND
Glu+ Gln	3.7	4.0	4.1	5.7	7.0 ± 7.2	11.2 ± 13	10.2 ± 6	4.7 ± 0.0	ND
Cys	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	See Met
Ser	18.7	20.0	18.8	14.6	12.5 ± 10	13.6 ± 3.1	21.9 ± 1.8	21.8 ± 1.5	ND
His	8.9	9.5	9.2	6.8	5.1 ± 3.3	5.7 ± 1.1	8.6 ± 1.6	8.6 ± 0.4	1.5
Thr	0.9	0.0	0.0	0.3	0.6 ± 1.2	0.7 ± 0.6	1.6 ± 0.3	1.6 ± 0.1	2.3
Arg	5.3	4.4	5.0	10.4	15.3 ± 5.9	10.5 ± 2.9	9.5 ± 17	10.6 ± 1.7	ND
Ala	3.0	2.9	3.2	5.2	7.0 ± 1.3	5.3 ± 2.0	4.4 ± 0.7	5.4 ± 0.6	ND
Tyr	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.4	See Phe
Val	2.2	2.2	2.4	2.3	2.2 ± 0.3	1.8 ± 0.3	1.5 ± 1.3	1.7 ± 0.1	3.9
Met	0.0	0.0	0.0	0.0	0.0 ± 0.3	0.6 ± 0.1	1.0 ± 0.9	1.0 ± 0.0	2.2[B]
Phe	1.8	1.9	2.1	1.8	1.8 ± 0.0	1.8 ± 0.1	1.7 ± 0.0	1.7 ± 0.4	3.8[C]
Ile	3.1	3.3	3.4	3.5	3.7 ± 0.6	3.2 ± 0.5	2.1 ± 0.6	2.5 ± 0.2	3.0
Leu	3.7	4.0	3.4	5.0	6.1 ± 0.7	5.0 ± 0.9	3.4 ± 1.5	3.4 ± 0.1	5.9
Hyp	8.8	9.4	9.8	8.5	6.8 ± 5.4	8.1 ± 0.0	6.0 ± 2.8	6.7 ± 1.0	ND
Pro	10.0	10.7	7.5	10.3	12.0 ± 7.4	12.3 ± 0.0	8.2 ± 0.9	5.6 ± 0.7	ND
Gly sum	3.9	3.8	4.2	4.7	5.1 ± 0.8	4.2 ± 1.0	3.8 ± 0.2	4.2 ± 0.2	ND
Lys sum	22.6	20.3	23.2	16.0	9.0 ± 4.6	12.1 ± 0.8	11.9 ± 6.0	15.1 ± 3.6	ND
EAA sum	43.2	41.2	43.6	35.5	28.6	30.8	31.7	35.7	27.7
	100	**100**	**100**	**100**	**100**	**100**	**100**	**100**	

4 Discussion

4.1 Biomass growth and health

While the tolerance of *U. intestinalis* to a wide range of salinity levels has been well documented (Kamer and Fong 2001), a fact that is reflected in studies as not finding salinity impactful to its growth rate (Bews et al. 2021; Ruangchuay et al. 2012; Kim, Zhao, and Kim 2021). However, the impact of temperature, not to mention the optimal temperature, seems to be far from settled.

In this study the samples grown in 10 °C, F/2 and F/20, maintained a consistent growth rate of 38 % and 26 % (w.b.) on average, respectively, while the samples grown in 25 °C, F/2 and F/20, plateaued in growth only after 2 to 3 weeks and actually lost some biomass at the end of the 4 week cultivation. Consequently, the total biomass growths for samples grown in 10 °C, F/2 and F/20, were 260 % and 107 % (w.b.), and for samples grown in 25 °C, F/2 and F/20, were 29 % and 33 %, respectively. Variation in the growth rate, however, was statistically significant only between the F/2 samples, 10 and 25 °C (paired t-test: $p < 0{,}05$; **Fig. 1**).

Ruangchuay et al., (2012) cultured *U. intestinalis* under 20, 25, 30 °C, in 20 ppt salinity, 60 μmol photon $m^{-2}s^{-1}$, Modified von Stosch (Grund) medium and a 12:12-hr light:dark period for 8 weeks, and observed the greatest wet weight at 25 °C, which peaked after 4 weeks of cultivation and actually decreased by 22 % until the end. This decrease is comparable to this study's growth rates of the samples grown in 25 °C, which lost about 18 % in wet weight during the latter half of the experiment.

Conversely, Kim, Zhao, and Kim (2021) cultivating *U. intestinalis* germlings under 15, 20, 25 °C, in a seawater medium (824 μM DIN, 33 μM PO_4^{3-}, 30 PSU), 60 μmol photon $m^{-2}s^{-1}$, and a 12:12 h L:D period for 27 days found that samples grown in 15 °C produced the longest thalli by far. Finally, Brito (2018) cultured *U. intestinalis* under 12, 16, 20 °C, 1.28 μM NO_3^-, 0.16 μM PO_4^{3-}, 35 PSU, 100 μmol photon $m^{-2}s^{-1}$, and a 12:12 h L:H period for 5 weeks only to find no significant effect on growth by temperature.

Bews et al. (2021) cultivated *U. intestinalis* in pilot-scale tanks in orthogonal combinations of three nutrient concentrations by adding 190, 95, 0 mg / L of phosphorus, nitrogen, and kalium to seawater, and of two salinities, 35 and 15 PSU, in 15–17 °C, and in 70–90 μmol photons $m^{-2}s^{-1}$ at a period of 12:12 L:D to observe that samples grown in the medium nutrient level (95 mg / L) produced the highest total biomass, 1.3 and 1.7-fold, compared to high (190 mg /

L) and low (only seawater) levels, respectively, while also maintaining a consistent growth during the 35 day cultivation regardless of salinity. In a smaller laboratory setup *U. intestinalis* was cultured in four different nutrient concentrations, 70, 175, 350, 1 050 µg/L of nitrogen and 46, 115, 230, 690 µg/L of phosphorus, in 20 °C, 75 µmol photons $m^{-2}s^{-1}$ at a period of 16:8 L:D to find that the average growth rate was highest at the lowest nutrient concentration, while decreasing drastically with increased nutrient concentration, which was attributed to a too strong aeration that increased salinity through evaporation and contributed to cell plasmolysis (Balina et al. 2017).

U. intestinalis germlings grown under four different nutrient levels, 1.54, 12.8, 32.0, 102 µM NH_4^+ and 0.2, 2.0, 6.5, 10.0 µM PO_4^{3-}, in 15 °C, 30 PSU seawater medium, 90 µmol photon $m^{-2}s^{-1}$, with a 12:12 h L:D period for 27 days revealed that samples grown in the 12.8 µM NH_4^+ produced the greatest average thalli length, while samples grown in 32.0 µM NH_4^+ were not far behind (Kim, Zhao, and Kim 2021).

Finally, Kim, Zhao, and Kim (2021) suggest that “Compared to growth under the different temperature and light conditions, variation in nutrient availability appeared less stimulating for *U. intestinalis* germling growth than the other factors.” which would be in agreement with the findings of this study, where temperature was a significant factor for biomass growth.

The chlorophyll contents of the cultivated *U. intestinalis* samples do not seem to correspond to their growth rates or total biomass. The chlorophyll a, chlorophyll b, and carotenoid contents of both F/2 25 °C and F/2 10 °C samples were about 2.2, 8.3, and 2.0-fold compared to both F/20 25 °C and F/20 10 °C samples, respectively (**Fig. 2**). Interestingly, this difference seems to appear between their protein contents as well, as suggested by a modest correlation between the chlorophyll b and protein contents (R^2=0.842)(**Fig. 2, 3**)

The F/2 25 °C cultivated sample suffered from an unrecognized competing seaweed that had a visibly darker pigment. The intruding seaweed persisted even after removing all visible thalli. This could explain the overall poor health of the F/2 25 °C cultivated sample.

4.2 Protein content and amino acid profile

The Lowry Protein Assay yielded a wide range of protein content between the samples, 1.3 - 32.6 g/ 100 g (d.w.), demonstrating significant variation between the temperatures (ANOVA: F = 19.256, p = 0.0482) (**Fig. 3**). Such results are not unheard of including within *Ulva*. Major factors in determining the protein content of *Ulva* include seasonal changes and geographical location, even within the Baltic sea. An investigation in the Gulf of Gdansk using the BSA standard and the LPA method reported the highest protein contents in spring and in autumn, at the start and at the end of the growing season, and the lowest protein content in the summer, 20.6 ± 5.0 to 9.4 ± 4.6 % d.w. for protein in *Enteromorpha* spp., respectively (Haroon et al. 2000). Another factor that demands paying attention to is the quantification method itself. A Norwegian research team determined a specific nitrogen-to-protein (NtP) factor of 4.7 ± 1.0 for *U. intestinalis* using the total nitrogen and total AA data, 2.4 ± 0.3 % and 11.2 ± 0.7 % (d.w.), respectively (Biancarosa et al. 2017). A Swedish research team used this exact factor, 4.73, to calculate the protein content of *U. intestinalis* along the Swedish coast, where highest content was reported in samples near Helsingborg, 8.2 ± 1.6 % (d.w.) (Olsson et al. 2020).

A pair of Brazilian researchers evaluated four different methods to quantify algal protein content. The comparison of the NtP conversion, the LPA method, the Bradford method, and total AA yielded several interesting discrepancies such as 30 % lesser protein content (% d.w.) on average when comparing the Lowry method to the total AA, and a calculated specific NtP factor of 4.7 ± 0.4 using the total AA data for the three out of four included green seaweeds, *U. fasciata*, *D. tertiolecta*, and *C. decorticatum*, leaving *C. fastigiate* as the outlier with a factor of 3.13. (Barbarino and Lourenço 2013).

However, Harrysson et al., (2018) found almost exactly an opposite tendency, where the Lowry method consistently resulted in notably greater total protein yield estimates compared to the total amino acids based protein yield analysis. On average, the Lowry method resulted in 34 % higher estimates of total protein yield. Interestingly, Mæhre et al., (2014) found a similar tendency, when estimating the protein contents of cod, salmon, shrimp, wheat, and a red seaweed, *P. palmata*.

The amino acid profile analysis revealed that three amino acids, serine, histidine, and lysine varied significantly between the temperatures (paired t-test: $p < 0.05$, **Table 5**), among which lysine varied significantly between the nutrient levels as well (paired t-test: $p < 0.05$, **Table 5**). However, this might be explained by the relatively high lysine content deviation in the F/2 25 °C and 10 °C samples, 9.0 ± 4.6 and 11.9 ± 6.0, respectively. All samples of collected and cultivated *U. intestinalis* cleared WHO's (2007) EAA intake recommendations based on a mean nitrogen requirement of 105 mg nitrogen/kg per day (0.66 g protein/kg per day) for histidine, isoleucine, lysine, while only F/2 25 °C cleared the recommendations for leucine. All samples fell short of the recommendations for threonine, valine, methionine, and phenylalanine for about 50 % on average (**Table 5**). This does not correspond to previous studies, where *U. intestinalis* samples fell short of the WHO (2007) recommendations only for histidine (Benjama and Masniyom 2011; Jannat-Alipour et al. 2019). Finally, the total EAA varied significantly between the collected and cultivated samples (paired t-test: $p < 0.05$, **Table 5**), but not between temperatures or nutrient levels.

The EAA recommendations (% of diet) for a medium sized salmon were met practically by all *U. intestinalis* samples for all EAAs except for tyrosine and methionine (**Table 5**) (Hua and Bureau 2019).

However, comparing cultivated and collected samples reveals a potential advantage of U. intestinalis. The protein contents of the F/2 25 °C and F/2 10 °C samples werer about 4-fold and 3-fold compared to the collected samples. This suggests an advantageous protein conversion ratet in a high nutrient environment.

4.3 Applications

The processing potential of *U. intestinalis* into human nutrition, animal feed, and fertilizer has been assessed.

Physical processing seems to contribute significantly to protein recovery yield which can be isolated and measured with protein solubilization after extraction, since significant losses occur during concentration as well. Purely alkaline solubilization seems extract between 34 and 62 % of the total seaweed protein (Vilg and Undeland 2017; Harrysson et al. 2018), and when assisted by oven or freeze drying can extract up to 77 and 91 % of the total protein, respectively (Abdollahi et al. 2019), which has only been surpassed by a subcritical water treatment using a semi-continuous reactor by extracting 96 % of the total protein (E. Trigueros et al. 2021).

Ammonium-sulphate salting seems to produce protein isolates between 10 and 30 %, and when combined with physical processing like osmotic shock, sonication, and freeze drying seems to yield total protein recoveries between 7 and 35 % (**Table 3**).

The total protein recovery after a pH-shift concentration does not seem to correlate with any particular extraction method or the protein content in the original raw seaweed biomass ($R^2 = 0.04$) or even the solubilized (%) protein ($R^2 = 0.3$) and seems to yield protein recoveries of 20 ± 6 % (**Table 3**). Instead, the protein content of the crude seaweed alone would appear to predict the protein content of the pH-shift derived final isolate ($R^2 = 0.81$), which varied between 18 and 71 % (**Table 3**).

Overall, both the total protein recovery (ANOVA: F = 14.489, p = 0.00027; **Table 3**) and protein concentration (ANOVA: F = 7.324, p = 0.0048; **Table 3**) seem to vary significantly between the concentration methods, ultrafiltration, pH-shift, salting, and ion exchange. More importantly, perhaps, extraction efficiency, if defined simply as recovery% / solubilized% protein, varied significantly between ultrafiltration and pH-shift methods (paired t-test: $p < 0.05$; **Table 3**) suggesting that a significant amount of protein is lost during the pH-shift precipitation unlike the during ultrafiltration, which was reported to be about 10 % (Kadam et al., 2017). Finally, considering the highest results (**Table 3**) combining freeze-drying assisted alkaline extraction or subcritical water treatment with ultrafiltration or ion exchange chromatography could yield very promising results. Additionally, since the protein content in the isolates varied significantly with the seaweed species (ANOVA: F = 7.00, p = 0.036; **Table 3**), the pH-shift method could be optimized for specific seaweed species.

Charitably speaking, combinations of protein extraction and concentration methods that recover and isolate less than 50 % of seaweed protein cannot compete with microalgae-derived products, which have already achieved prominence and a firm spot on the global food market for their nutritional advantages. Moreover, a truly efficient method would deliver high recovery and isolation regardless of the seaweed type or nutrition profile.

Spirulina sp. is regularly reported to contain between 50 % and 70 % protein (dw) (Spolaore et al. 2006). Moreover, *Chlorella* and *Spirulina* proteins can be extracted and concentrated to 80 % (dw) with a 15.1 % recovery, and to 80–90 % (dw) with a 37–61 % recovery, respectively, using the alkaline solubilization and pH-shift methods (Ladjal-Ettoumi et al. 2024; eParimi et al. 2015).

Finally, *U. intestinalis'* biofertilizing effect on the growth rate of tomato plants has been assessed. Several seaweeds including *U. intestinalis* collected from the Gulf of Gdańsk were dried and added to gardening soil. The growth rates (heigth, cm) of both seaweed and commercial tomato plant fertilizer suplemented tomato plants were significantly higher to the unsuplemented control, but not between each other (Filipkowska et al. 2008).

Arguably, microalgae displays superior qualities compared to seaweed from the perspective of protein recovery and isolation. This does not mean that seaweeds, including *Ulva* despite its extremely marginal share in the global seaweed market, do not have a place on our plates.

Retailers specializing in Asian cuisine provide a wide variety of seaweed products, which also includes green laver that is also known as aonori (アオノリ) in Japan, as parae (파래) in Korean, and as hutai (滸苔) in China. It is made from two species, *Ulva* and *Monostroma*, and is utilized in miso soups and stir-fried dishes sometimes also in powder form. Dried and powdered *Ulva* could also be used in fish meal and in fertilizers.

Conclusions

Temperature was found to significantly affect *U. intestinalis* biomass growth rate and contents of histidine and serine. Lysine content was significantly affected by both temperature and nutrient level. Comparison of cultivated and collected samples suggests that *U. intestinalis* could considerably benefit from both a high nutrient and warm temperature environment from the perspective of all markers of health: biomass, chlorophyll, carotenoid and protein content.

Further studies could examine where the respective accuracies of Lowry, total AA, nitrogen conversion methods are acceptable, and whether chlorophyll content is a reliable indicator of protein content.

Follow-up experiments could further study how different nutrient levels affect *U. intestinalis* growth to determine locations in the Baltic Sea offering opportune growth conditions for a potential pilot farming study. Additional commercially promising species could be included to determine if some can thrive under conditions with extreme temperature variation, while others under conditions with extreme nutrient level variation. Locations downstream of water treatment plants could provide a stable nutrient flow all year, while littoral areas with riverine water enriched by different farming operations upstream could potentially provide a variable nutrient flow depending on seasonal or even daily weather factors. Additional factors potentially affecting the biomass productivity would be the local invertebrates feeding on the seaweeds.

Current protein extraction and concentration methods do not allow seaweeds to compete with microalgae-derived products on the global market. Where seaweeds, including *U. intestinalis*, could have an advantage is in their simpler cultivation installment and maintenance requirements, since microalgae must be cultivated in isolated and protected pools with an artificially maintained constant fluid flow. In addition to the green seaweeds’ well established culinary utilization in the Asian cuisine *U. intestinalis* could be used as a viable biofertilizer to commercial fertilizers.

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Appendices

U. intestinalis' location within the Ulvophyceae class.

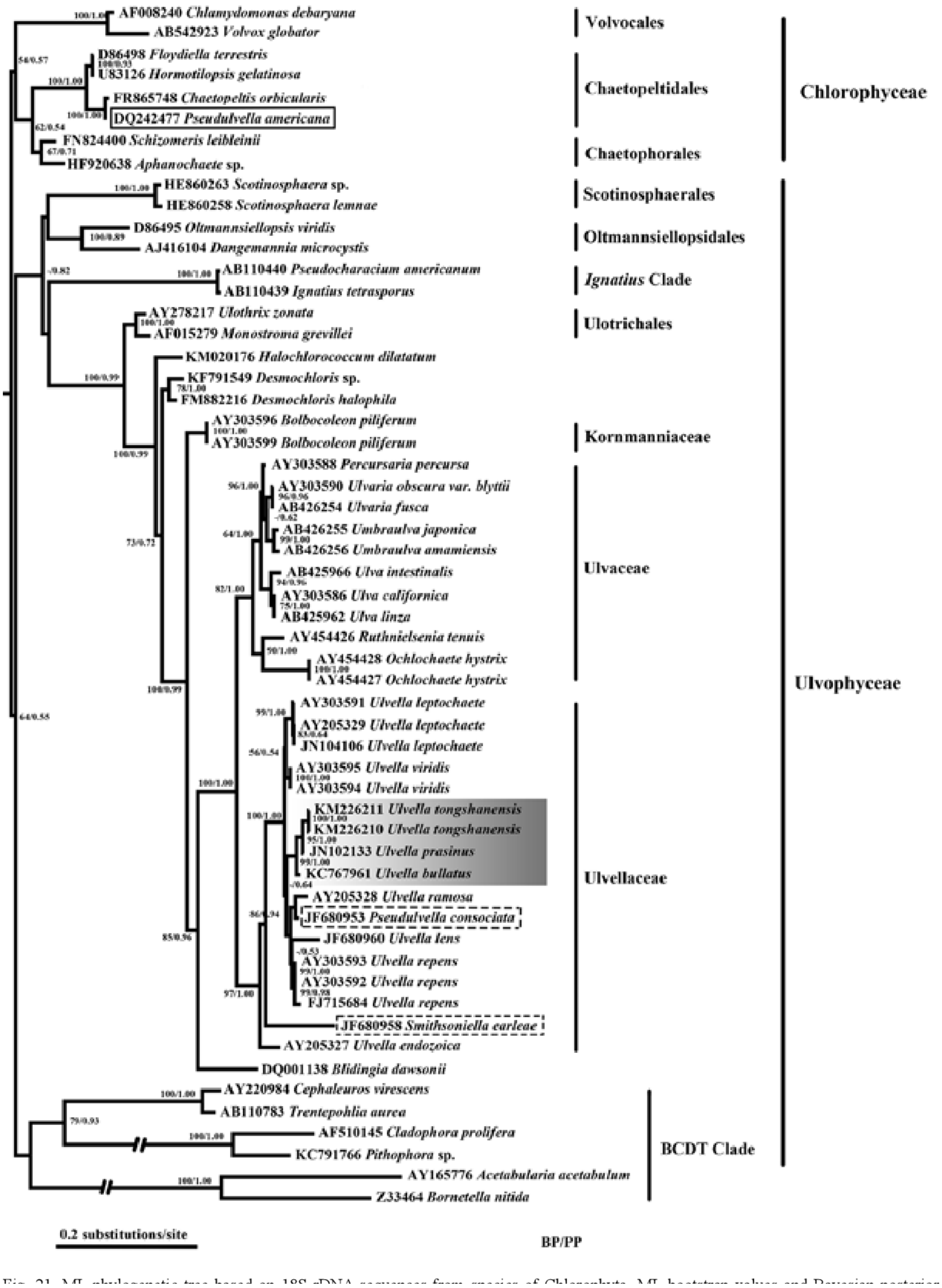

Figure 6: *U. intestinalis*' location within the *Ulvophyceae* class.

U. Intestinalis' global occurrence

Figure 7: *U. Intestinalis*' global occurrence (OBIS, 2019).

Ulvophyceae's location within the *Chlorophyta* division

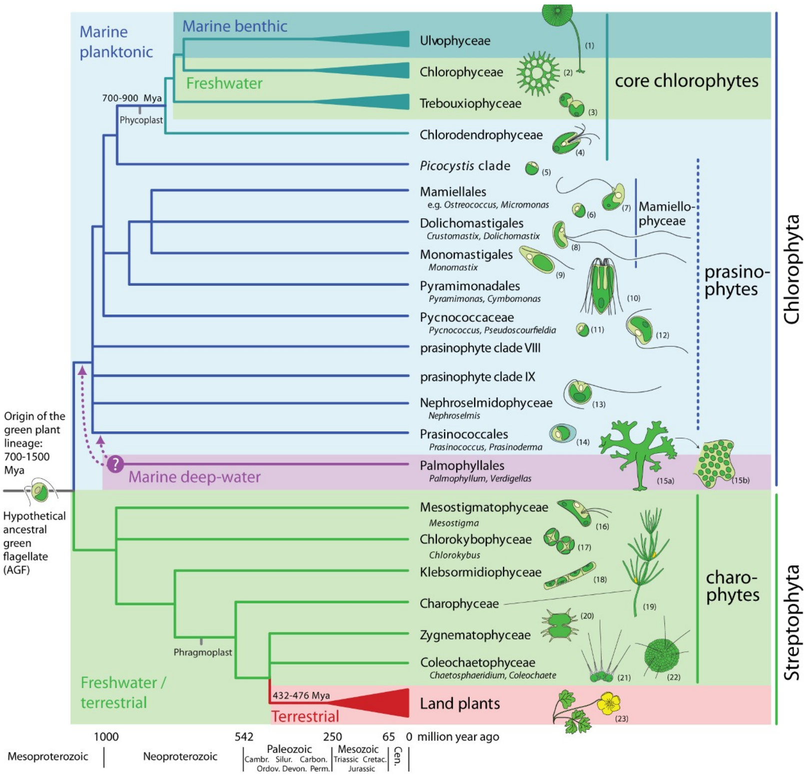

Figure 8: *Ulvophyceae*'s location within the *Chlorophyta* division.

U. intestinalis’ distribution in the Baltic Sea

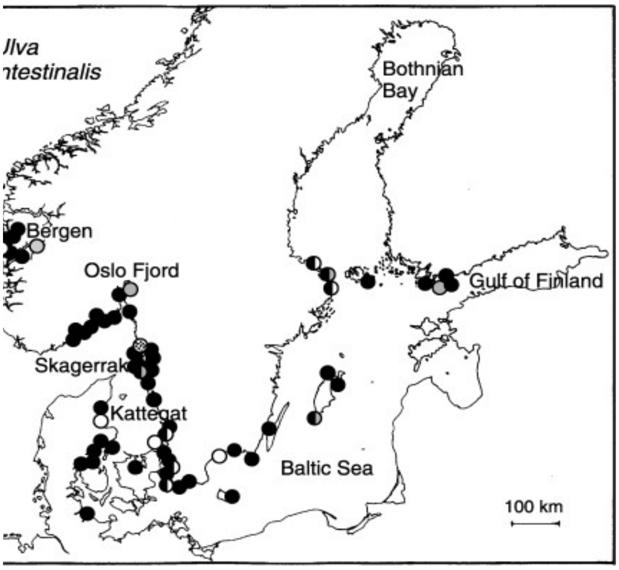

Figure 9: *U. intestinalis’* distribution in the Baltic Sea (Leskinen, Alström-Rapaport, and Pamilo 2004).