Seaweeds as a future protein source: innovative cultivation methods for protein production

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“*Känner du lukten från havet och skogen*
Det är grannens septitank
Hör du vågorna som slår mot stranden
Det är grannens vattenklosett
Tänk vad naturen är underbar
Tänk vad naturen är fin”

- Philemon Arthur & The Dung

ABSTRACT

As the global population is projected to reach approximately 10 billion people by 2050, it is estimated that we will need to produce up to 60% more food compared to 2010. Although the current food production system contributes to 25% of greenhouse gas emissions worldwide, accounts for 70-80% of eutrophication and freshwater usage, and occupies half of all ice- and desert-free land, it fails to meet the global nutritional needs. Furthermore, with extreme weather events and heat waves affecting terrestrial food production systems, it is evident that we need to look elsewhere to produce sustainable, protein-rich, and nutritious food. Recently, seaweeds have emerged as a promising part of this solution. Cultivating seaweeds requires no arable land, freshwater supply, or high nutrient input. Furthermore, seaweeds have high productivity that outperforms many terrestrial crops such as wheat, seeds, and soybeans. The protein often contains all the essential amino acids, making seaweeds a favorable protein source for human consumption. However, even though seaweeds often have protein contents in the range of some beans and pulses, it is lower than in soybeans. Therefore, their protein content needs to be increased if seaweeds are to become a competitive protein source in the future.

This thesis aims to explore the potential of seaweeds as a sustainable future protein source. It specifically focuses on optimizing seaweed cultivation to boost both growth rates and protein content. To achieve this, the effects of different cultivation conditions and the potential of one kelp and three green seaweed species are investigated. A novel nutrient loop is explored, wherein industrial food production process waters (FPPWs) are used as seaweed growth media. By conducting a meta-analysis, as well as land-based experiments that combine physiological, biochemical, chemical, and sensory analyses, the thesis aims to establish the potential for seaweed cultivation in nutrient-rich process waters.

The findings from this thesis show that seaweeds can become a promising alternative food source in the ongoing dietary protein shift. The results show that all groups of seaweeds (brown, green, and red) can be cultivated in various nutrient-rich process waters; but green seaweeds have the highest potential. After identifying the green seaweed species *Ulva fenestrata*, which usually has a crude protein content of 10-20% dry weight, as a promising candidate, its cultivation in FPPWs yielded protein content of up to 37% dry weight. Furthermore, the biomass yield was up to six times higher compared to when grown in seawater. The safety aspects of consuming the biomass were confirmed by showing that large quantities of the biomass can be consumed every day without exceeding health-based reference points for heavy metals. Also, no sensory attributes regarded as negative were found after cultivation in the FPPWs. In conclusion, this thesis illustrates a novel nutrient loop, where the disposal of industrial food production process waters can be turned into nutrient-rich and valuable biomass through seaweed cultivation.

Keywords: Macroalgae, *Saccharina latissima*, *Ulva fenestrata, Ulva intestinalis*, *Chaetomorpha linum*, cultivation conditions, nitrogen content, amino acids, heavy metals, food safety, wastewater, process water, circularity, blue economy

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vi beräknas vara cirka 10 miljarder människor på jorden år 2050. Uppskattningsvis kommer vi behöva producera upp till 60% mer mat än vad vi gjorde år 2010. Trots att livsmedelsproduktionen idag står för 25% av de globala växthusgasutsläppen, 70-80% av den globala övergödningen och vattenanvändningen, samtidigt som den upptar hälften av all is- och ökenfri mark, är den otillräcklig för att tillgodose världens näringsbehov. Dessutom kommer ökade extremväder och värmeböljor negativt påverka produktionen av livsmedel på land. Det är tydligt att vi behöver hitta nya odlingssystem för att producera hållbar, proteinrik och näringsrik mat. Makroalger (tång) har uppmärksammats som en lovande gröda i detta skifte. Odling av makroalger kräver ingen odlingsbar mark, färskvattenanvändning eller gödsling. Dessutom har makroalger en hög produktivitet, som ofta överträffar landbaserade grödor såsom vete, baljväxter och sojabönor. Proteiner i makroalger innehåller därtill alla de essentiella aminosyrorna som människor behöver. Men även om proteininnehållet i makroalger är jämförbar med t.ex. vissa bönor och andra baljväxter, är det lägre än i sojabönor. För att makroalger ska bli en konkurrenskraftig proteinkälla i framtiden är det därför viktigt att försöka öka dess proteininnehåll.

Syftet med denna avhandling är att utforska makroalgers potential som en hållbar framtida proteinkälla. Avhandlingens fokus är på att optimera odling av makroalger för att öka makroalgernas tillväxt och proteininnehåll. För att uppnå detta undersöktes effekterna av olika odlingsförhållanden samt potentialen hos en kelpart (brunalg) och tre grönalger. En ny ”näringsloop” utforskades, där industriellt livsmedelsproduktionsvatten (FPPWs) användes som tillväxtmedium för makroalger. Genom en meta-analys samt landbaserade experiment kombinerades fysiologiska, biokemiska, kemiska och sensoriska analyser, med syftet att undersöka potentialen för makroalgsodling i näringsrika produktionsvatten.

Resultaten från denna avhandling visar att makroalger har en lovande potential att bli en framtida proteinkälla i det pågående skiftet mot nya proteinkällor. Resultaten visar att alla grupper av makroalger (bruna, gröna och röda) kan odlas i näringsrikt produktionsvatten, men att gröna makroalger har högst potential. Den gröna algarten *Ulva fenestrata* identifierades som en speciellt lovande kandidat, och genom odling i FPPWs lyckades proteinhalten höjas till 37% torrvikt från dess genomsnittliga 10-20%. Dessutom producerades upp till sex gånger mer biomassa när *U. fenestrata* odlades i FPPWs jämfört med i vanligt havsvatten. Resultaten visar dessutom att biomassan kan konsumeras i stora mängder varje dag utan några problem för att överskrida referensvärden för tungmetaller, och efter en sensorisk analys av biomassan påträffades inga egenskaper som kunde uppfattas som negativa för konsumenten. Sammanfattningsvis presenterar denna avhandling en effektiv näringsloop där outnyttjat produktionsvatten från livsmedelsindustrin omvandlas till näringsrik och värdefull biomassa genom odling av makroalger.

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

Paper I:	**Stedt, K.,** Pavia, H., & Toth, G.B. (2022). Cultivation in wastewater increases growth and nitrogen content of seaweeds: a meta-analysis. *Algal Research* 61, 102573. https://doi.org/10.1016/j.algal.2021.102573
Paper II:	**Stedt, K.,** Trigo, J.P., Steinhagen, S., Nylund, G.M., Forghani, B., Pavia, H., & Undeland, I. (2022). Cultivation of seaweeds in food production process waters: evaluation of growth and crude protein content. *Algal Research* 63, 102647. https://doi.org/10.1016/j.algal.2022.102647
Paper III:	**Stedt, K.,** Gustavsson, O., Kollander, B., Undeland, I., Toth, G.B., & Pavia, H. (2022). Cultivation of *Ulva fenestrata* using herring production process waters increases biomass yield and protein content. *Frontiers in Marine Science* 9, 988523. https://doi.org/10.3389/fmars.2022.988523
Paper IV:	**Stedt, K.,** Steinhagen, S., Trigo, J.P., Kollander, B., Undeland, I., Toth, G.B., Wendin, K., & Pavia, H. (2022). Post-harvest cultivation with seafood process waters improves protein levels of *Ulva fenestrata* while retaining important food sensory attributes. *Frontiers in Marine Science* 9, 991359. https://doi.org/10.3389/fmars.2022.991359
Paper V:	Steinhagen, S., **Stedt, K.,** Undeland, I., & Pavia, H. A step towards closing the food-waste gap in novel protein sources: post-harvest protein boost of the crop *Ulva* by food process water. (Manuscript).
Paper VI:	**Stedt, K.,** Toth, G.B., Davegård, J., Pavia, H., & Steinhagen, S. (2022). Determination of nitrogen content in *Ulva fenestrata* by color image analysis – a rapid and cost-efficient method to estimate nitrogen content in seaweeds. *Frontiers in Marine Science* 9, 1081870. https://doi.org/10.3389/fmars.2022.1081870

Other papers not included in the thesis:

Trigo, J.P., **Stedt, K.,** Schmidt, A.E.M., Kollander, B., Edlund, U., Nylund, G.M., Pavia, H., Abdollahi, M., & Undeland, I. (2023). 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* 404, 134576. https://doi.org/10.1016/j.foodchem.2022.134576

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).

Wendin, K., **Stedt, K.,** Steinhagen, S., Pavia, H., & Undeland, I. Sensory aspects and consumer attitudes and preferences of *Ulva fenestrata* –

impact of protein level, time for harvest and cultivation conditions. (Submitted).

Toth, G.B., Hargrave, M., **Stedt, K.,** Steinhagen, S., Visch, W., & Pavia, H. Advances in Swedish seaweed aquaculture: production and biomass quality. (Submitted).

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ABBREVIATIONS

TML	Tjärnö Marine Laboratory
FAO	Food and Agricultural Organization of the United Nations
FPPW(s)	food production process water(s)
HPPW(s)	herring production process water(s) - a type of FPPW
TUB	tub water from in-house pre-processing storage of whole herring at a primary processor - a type of HPPW
SAL	salt brine from the maturation of herring fillets at a secondary processor - a type of HPPW
S. latissima	*Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders, 2006 (sugar kelp)
U. fenestrata	*Ulva fenestrata* Postels & Ruprecht, 1840 (sea lettuce)
U. intestinalis	*Ulva intestinalis* Linnaeus, 1753 (gutweed)
C. linum	*Chaetomorpha linum* (O.F. Müller) Kützing, 1845
NH_4^+	ammonium
NO_3^-	nitrate
NO_2^-	nitrite
P	inorganic phosphorus/ortophosphate
AA/TAA/EAA/TEAA	amino acid/total amino acids/essential amino acids/total essential amino acids
Heavy metals	arsenic, cadmium, lead, and mercury are referred to as "heavy metals" in this thesis, even though arsenic is strictly speaking a metalloid
US EPA	United States Environmental Protection Agency
EFSA	European Food Safety Authority
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MLs	maximum allowed levels of heavy metals in foodstuffs set by the European Union's Commission Regulation (EC) No. 1881/2006 (version 03/05/2022)
RfD	reference doses for heavy metals set by the US EPA

BACKGROUND

THE PROTEIN SHIFT

As the global population continues to grow, so does the demand for protein-rich and nutritious food. Current food production practices fail to meet global nutritional needs while degrading terrestrial and aquatic ecosystems (Poore and Nemecek, 2018). Global food production practices contribute to a quarter of the total global greenhouse gas emissions, occupy almost half of all ice-free and desert-free land, and are responsible for 80% of eutrophication and 70% of global water use (Poore and Nemecek, 2018; Gephart et al., 2021). Furthermore, agriculture systems are being severely impacted by extreme weather events and heat waves, resulting in reduced yields and increased pests and diseases (Skendžić et al., 2021). Still, it is projected that global food production will need to increase by 60-70% by 2050 to feed the growing population (Alexandratos and Bruisnma, 2012; van Dijk et al., 2021). Strikingly, even though the world's surface is 70% water, aquaculture production is only 1% of the agriculture production (around 100 million and 10 billion tonnes year^{-1}, respectively) (FAO, 2021a, 2022).

Growing competition for land, water, and energy will affect our ability to produce food, as will the urgent need to reduce the environmental impacts of current food systems, such as red meat production and overexploitation of fish stocks (Godfray et al., 2010; Crona et al., 2023). As a result, the impact on, and resilience of natural resources, ecosystems, and climate could be significantly reduced by addressing how we produce food (FOLU, 2019). It is evident that we need to produce more sustainable, protein-rich, and nutritious food with a lower environmental footprint. In Europe, the plant-based food industry grew by almost 50% between 2018 and 2020, underlining the shift towards more sustainable food choices (Smart Protein Project, 2021). By including 10% of seaweeds in our food, it is possible to save 110 million hectares of cropland and grassland currently used for growing food (Spillias et al., 2023), an area roughly equivalent to the total arable land in China.

Food from aquatic environments is often nutrient-rich (Golden et al., 2021) while having lower emissions and impacts on land and water than their terrestrial counterparts (Gephart et al., 2021) (**Figure 1**). In particular, non-fed species produce the lowest emissions during production. For example, seaweeds extract more nitrogen (N) and phosphorus (P) during their growth than what is emitted during their production (**Figure 1**). Furthermore, many seaweeds have high productivity compared to terrestrial crops (Mata et al., 2016), while also having proteins with favorable amino acid profiles for human consumption (Fleurence, 2004; Mæhre et al., 2014). This has led to an interest in cultivating seaweeds as a way of producing protein-rich and nutritious food with low environmental impact (Duarte et al., 2022).

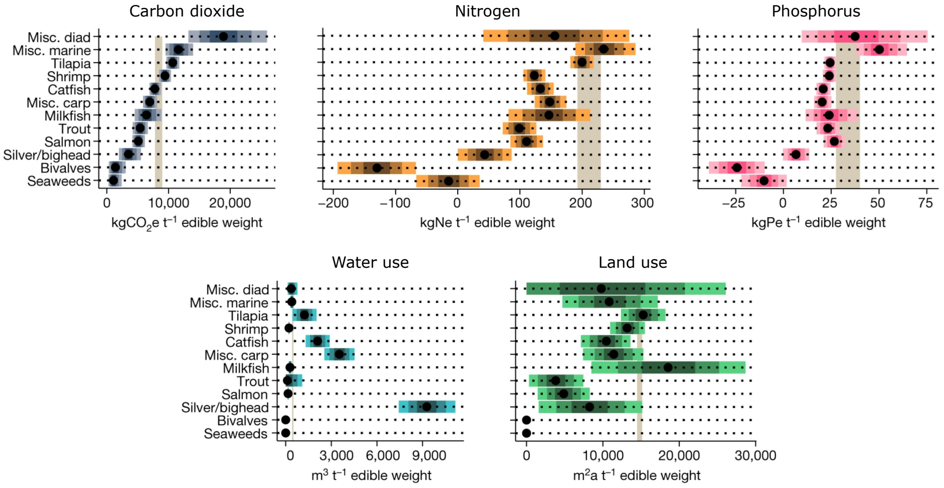

Figure 1. Impact of farmed aquatic food. Chicken production which is often considered the most efficient terrestrial animal-source food production system is represented by the beige band. *Reproduced and adapted with permission from Springer Nature: License number 5544801478146* from Gephart et al., 2021.

SEAWEED AQUACULTURE

Controlled, large-scale seaweed cultivation has been practiced in Asia for decades but has only recently gained interest in Europe and the Western world (FAO, 2018; Araújo et al., 2021). Today, countries in Asia produce 99.9% of the global seaweed production (**Figure 2**). Global seaweed production is increasing at a rate of 6-8% year^{-1}, and the annual production is currently estimated at 35 million tonnes with a market value of around USD 15 billion (Cai et al., 2021; Duarte et al., 2022; FAO, 2022). Compared to Asian production, where the majority of seaweeds are cultivated, harvesting from wild stocks still dominates production in Europe (FAO, 2018; Araújo et al., 2021). If seaweed production in Europe is to increase from 270,000 tonnes in 2019 to 8 million tonnes in 2030 (European Commission, 2022b), Europe needs to develop environmentally friendly seaweed aquaculture, rather than overexploiting wild stocks (Araújo et al., 2021). In addition to being environmentally friendly and more sustainable than wild harvest, seaweed aquaculture can help meet the processing industry's need for traceable, high-quality, and predictable biomass yields.

Despite seaweeds constituting over 12,000 described species worldwide, five species account for nearly 90% of the commercial production (Hafting et al., 2015; FAO, 2022) (**Figure 2**). In terms of biomass, red seaweeds constitute the major part of cultivated seaweeds (61%), followed by brown seaweeds (39%). Green seaweeds, on the other hand, only account for a very small proportion (<0.2%) of the worldwide cultivation (FAO, 2020). Brown seaweeds are produced primarily for human food, while red seaweeds are also extracted for hydrocolloids like carrageenan and agar, which are used for cosmetics, pharmaceuticals, animal feed, fertilizers, and as vegan thickener and stabilizer to substitute gelatin (Bixler and Porse, 2011; Buschmann et al., 2017).

Each seaweed species has specific environmental requirements to grow, but in general, they all need enough nutrients and light, as well as appropriate salinity and temperature conditions (Campbell et al., 2019). The cultivation usually occurs in sea farms in coastal areas close to the shore or offshore (Araújo et al., 2021). Although these systems are practical and cost-effective for large-scale farming, they are limited by seasonal changes (Roleda and Hurd, 2019; Araújo et al., 2021; Forbord et al., 2021; Steinhagen et al., 2022). Land-based cultivation is a promising alternative for cultivating seaweeds that are not suitable for sea-based cultivation. With land-based cultivation, it is also possible to control the production cycle and biomass composition of the seaweeds independent of the season, allowing traceable, high-quality, and predictable biomass yields (Hafting et al., 2012; Mata et al., 2016). However, the commercial interest in land-based cultivation is slowed down by high costs due to sophisticated infrastructure and maintenance requirements (Hafting et al., 2012; Mata et al., 2016).

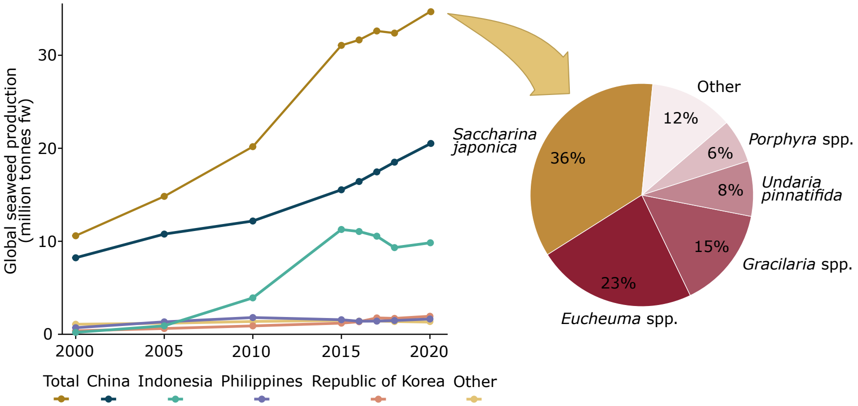

Figure 2. Global seaweed production between 2000 and 2020 for the main producers and cultivated species (reproduced data from FAO (2022)).

SEAWEEDS AS A SOURCE OF PROTEIN

Despite covering 70% of the Earth's surface, only about 15% of human protein intake comes from the ocean (Costello et al., 2020). Seaweeds have been consumed as food all over the world for thousands of years, however, outside of Asia, it is not commonly included in people's diets nowadays (Peñalver et al., 2020). However, seaweeds are becoming increasingly recognized as an interesting food source worldwide, largely due to the rising popularity of Asian cuisine, and the nutritional benefits often associated with seaweed consumption (Mouritsen et al., 2018). Furthermore, the European Union has recognized seaweeds as an essential source of alternative proteins to help establish sustainable food systems and global food security (European Commission, 2020).

Seaweeds are generally classified into three major groups by their pigmentation of brown (Phaeophyta), green (Chlorophyta), and red (Rhodophyta), however, it is important to recognize that, just like terrestrial crops, seaweeds are a highly diverse group of organisms. Consequently, they also differ in their biochemical composition, growth, and environmental requirements, both within and between groups (**Table 1**). In general, seaweeds have many nutritional properties interesting for consumers, such as low fat content, while being rich in high-quality proteins, carbohydrates, minerals, antioxidants, vitamins, and other essential micronutrients (Holdt and Kraan, 2011; Fleurence et al., 2012; Mæhre et al., 2014; Bjerregaard et al., 2016; Machado et al., 2020). Furthermore, they generally have high productivity compared to terrestrial crops (Mata et al., 2016). Their protein content depends on both environmental conditions and taxonomy, and is generally highest in red, followed by green, and brown species (Černá, 2011; Fleurence, 2016). Even though some red seaweed species are often cited at protein contents up to 47% of dry weight (dw), more commonly reported levels for seaweeds fall within 5-25% protein dw (Fleurence, 2004; Černá, 2011). Hence, seaweed protein content is relatively high compared to most vegetable staple foods, but still not as high as in, for example, soybean biomass (33-45% dw; Grieshop and Fahey, 2001; Thakur and Hurburgh, 2007; Henchion et al., 2017).

Table 1. Biochemical composition of brown, green, and red seaweeds, as well as pulses (lentils, chickpeas, and beans), and soybeans.

% dw	**Brown seaweeds**	**Green seaweeds**	**Red seaweeds**	**Pulses**	**Soybeans**
Protein	3-19* [1-3, 5-8, 13]	3-23* [2, 3, 5-10, 12]	3-38* [1-8]	17-35 [14, 15]	33-45 [6, 16-19]
Lipids	0.5-9 [1, 3, 5, 6, 8, 11]	0.8-5 [3, 5, 6, 8-11]	0.1-13 [1, 3-6, 11]	0.8-7 [15]	15-22 [17, 18]
Ash	14-51 [1, 3, 5-8, 13]	14-78 [3, 5-10]	7-84 [1, 3-8, 11]	1-5 [15]	3-6 [17, 18]
Carbohydrates	20-62 [1, 5, 8, 13]	33-63 [5, 7-9]	31-73 [1, 5, 7, 8]	55-65 [15]	9-39 [18, 19]

*Seaweed protein content originally estimated using the 6.25 conversion factor from nitrogen has been recalculated with the more conservative conversion factor of five for more accurate comparisons.

[1]Tibbetts et al. (2016), [2]Fujiwara-Arasaki et al. (1984), [3]Véliz et al. (2023), [4]Cian et al. (2014), [5]Rodrigues et al. (2015), [6]Mæhre et al. (2014), [7]Nunes et al. (2017), [8]Fleurence (2016), [9]Steinhagen et al. (2022), [10]Toth et al. (2020), [11]Fleurence et al. (1994), [12]Shuuluka et al. (2013), [13]Vilg et al. (2015), [14]Ahuja et al. (2017), [15]Boye et al. (2010, and references therein), [16]Thakur and Hurburgh (2007), [17]Grieshop and Fahey (2001), [18]Redondo-Cuenca et al. (2007), [19]Karr-Lilienthal et al. (2005)

To assess the quality of protein in food, it is essential to determine the amino acid (AA) profile (Machado et al., 2020). Since humans cannot produce all AAs on their own, it is necessary to consume the essential amino acids (EAA) through food. The nine EAA include phenylalanine, histidine, isoleucine, lysine, leucine, methionine, threonine, valine, and tryptophan (WHO/FAO/UNU, 2007). Interestingly, the protein in most seaweed species contains all the EAA, and many species have a high content of methionine, which is often lacking in plant-based proteins like soybeans and other pulses (Fleurence, 2004; Mæhre et al., 2014; Henchion et al., 2017). Seaweed protein is therefore often considered a high-quality protein (Fleurence, 2004; Henchion et al., 2017). However, despite seaweeds' relatively high protein content, the protein's digestibility in its unprocessed form is often poor due to tough polysaccharide-rich cell walls of the seaweeds (Tibbetts et al., 2016; Trigo et al., 2021; Thiviya et al., 2022). To increase the digestibility and to market seaweeds as an interesting protein ingredient, the proteins could be concentrated and extracted, similar to what is done with soybean (Preece et al., 2017; Trigo et al., 2021).

CULTIVATION IN PROCESS WATERS

Seaweeds are known to grow faster and accumulate nitrogen when cultivated in nutrient-rich waters (Ryther et al., 1981; Habig et al., 1984). Integrating seaweed cultivation with industries that produce nutrient-rich process waters could therefore enhance the protein content in seaweeds while creating a circular system that recycles nutrients. Although the idea of using seaweeds as biofilters for water treatment has been around for some time (Huguenin, 1976), it has received newfound interest since the introduction of integrated multitrophic aquaculture (IMTA) (Neori et al., 2004). In IMTA systems seaweeds are usually cultivated in integration with fed aquaculture to utilize the considerable amounts of nutrients leaking from aquaculture farms (Chopin et al., 2001; Neori et al., 2004; Ridler et al., 2007; Troell et al., 2009; Abreu et al., 2011; Handå et al., 2013; Fossberg et al., 2018; Nardelli et al., 2019; Nederlof et al., 2022). These studies have shown the positive effects of cultivating seaweeds close to fish farms through the remediation of nutrients from the water, and increased growth and protein content of the seaweed biomass.

However, many industries produce process water that cannot be used as cultivation media in the ocean. These waters can be the result of many different processes and activities, such as land-based aquaculture farming, industrial food processing, and municipal wastewater management (Sode et al., 2013; Neveux et al., 2016; Arumugam et al., 2018; Forghani et al., 2020). Thanks to their high nutrient concentration, there is a great potential to integrate many of these activities with land-based seaweed cultivation, which could create circular systems where nutrients are not wasted but used to produce sustainable protein-rich biomass of seaweeds.

For many food companies, disposing of process water constitutes a significant cost. However, by integrating land-based seaweed cultivation with these waters, they could possibly be utilized instead of being treated as waste. Land-based cultivation also creates an opportunity to cultivate high-value species with morphologies unsuitable for sea-based cultivation (Hafting et al., 2012). Furthermore, by integrating seaweed cultivation with industries that generate large quantities of nutrient-rich process waters, some major challenges for land-based seaweed cultivation, such as seawater intake and costly infrastructure, are addressed. Until the studies included in this thesis, there were, to my knowledge, no reports on seaweed cultivation in outlet waters from the food processing industry, even though these provide a wide range of nutrients in a food-grade state.

CONSUMING SEAWEEDS CULTIVATED IN PROCESS WATERS

Seaweeds may accumulate toxic elements (commonly grouped as "heavy metals") from their surrounding environment (Gaudry et al., 2007; Ortiz-Calderon et al., 2017). The bioaccumulation of heavy metals in seaweeds is largely attributed to polysaccharides in their cell walls, which have metal-binding characteristics (Duinker et al., 2016; Ortiz-Calderon et al., 2017; Roleda et al., 2019). Different species have different types of polysaccharides, which bind heavy metals to various degrees (Ortiz-Calderon et al., 2017; Roleda et al., 2019). Furthermore, the levels of heavy metals in seaweed biomass can be affected by several factors, including the age and shape of the seaweeds, as well as the environmental conditions in which they are grown (Ortiz-Calderon et al., 2017; Roleda et al., 2019; Véliz et al., 2023). This results in highly variable accumulation of heavy metals both between and within species. To date, there are no general regulations from the European Commission regarding maximum levels of heavy metals in seaweeds intended as food (European Commission, 2018). However, with the increasing interest in consuming seaweeds, it is important to determine possible toxic elements in the seaweed biomass to help estimate potential health risks associated with its consumption.

Another important factor in successfully introducing seaweeds to the market is the sensory quality of the biomass. The appearance, odor, flavor, taste, and texture, play a significant role in the public acceptance of novel food (Lawless and Heymann, 2010; Moerdijk-Poortvliet et al., 2022; Young et al., 2022). Consumer attitudes toward seaweeds as food are predominantly positive (Wendin and Undeland, 2020; Young et al., 2022). However, to increase the understanding and recognition of seaweeds as a protein source and their gastronomic potential, their sensory attributes need to be further investigated. This is especially important when cultivating seaweeds in media that might impact the sensory qualities of the seaweed.

AIMS OF THE THESIS

The overall aim of this thesis was to explore seaweeds as a future protein source using industrial food production process waters (FPPWs) as growth media. The main goals were to identify seaweed species suitable for cultivation in FPPWs, as well as to improve their growth and increase their protein content. More specifically, I investigated how different FPPWs could be used as growth media for the green seaweed sea lettuce (*Ulva fenestrata*), and to a lesser extent for two other green seaweeds (*Ulva intestinalis,* and *Chaetomorpha linum*), and for the brown seaweed sugar kelp (*Saccharina latissima*). I did this by conducting a meta-analysis, as well as land- and sea-based experiments combining physiological, biochemical, heavy metal, and sensory analyses.

The specific aims of each study completed, or in progress, are detailed below:

Paper I: Meta-analysis: The aim of this study was to synthesize the current literature to assess the effect that different types of process water have on seaweed growth and nitrogen content. The objective was to determine which types of process water and seaweed can be used for seaweed cultivation.

Paper II: Screening of FPPWs and seaweed species: The aim of this study was to investigate growth and crude protein content in *S. latissima, U. fenestrata, U. intestinalis,* and *C. linum* when cultivated in dilutions of eight different FPPWs.

Paper III: Upscaling of selected herring production process waters (HPPWs): The aim of this study was to investigate how upscaling of the system, using two promising HPPWs, affects the growth and crude protein content of *U. fenestrata.* The amino acid composition and heavy metal content of the *U. fenestrata* were measured to determine if biomass grown in the two HPPWs is a nutritious and safe source of protein.

Paper IV: Post-harvest cultivation in HPPWs: The aim of this study was to investigate if the HPPWs can be used as a short-term, post-harvest treatment to boost the protein content of seafarm cultivated *U. fenestrata*, harvested at a suboptimal condition (based on protein content). Growth, crude protein content, amino acid composition, and heavy metal content were measured. In addition, a sensory analysis of the biomass was carried out to assess if its sensory attributes were affected by the cultivation in the HPPWs.

Paper V: Boost of high protein content further: The aim of this study was to investigate if HPPW can be used as a short-term, post-harvest treatment to further boost the protein content of seafarm cultivated *U. fenestrata,* harvested at its natural peak protein content. In addition to growth, crude protein content, and amino acid composition, the biomass was also analyzed for fish allergens.

Paper VI: Using color to estimate protein content: The aim of this study was to develop models that estimate the nitrogen content (crude protein content) in *U. fenestrata* based on color image analysis. From the models, the aim was to develop a web-based application that reports nitrogen content based on uploaded images, and to develop a printed color guide for quick, in-field estimations of nitrogen content.

METHODS

This section provides an overview of the main methods used in the thesis. It starts with a brief explanation of the meta-analysis, followed by descriptions of the different methods used in the experimental part of the thesis. More detailed descriptions can be found in the specific papers.

META-ANALYSIS

In **paper I**, the effect of different process waters (referred to as wastewaters in **paper I**) on seaweed growth and nitrogen content was quantitatively explored using a systematic review. In the systematic review, a literature search was conducted based on specific criteria to ensure reproducibility. Web of Science, Google Scholar, and Scopus were searched using a defined search term, which resulted in 1649 relevant papers. After screening the titles and abstracts, as well as applying a set of well-defined inclusion criteria, 40 papers remained (see **paper I**). The systematic review was then followed by two meta-analyses (effect on growth, and nitrogen content) to statistically analyze and compare the results from the primary studies (**Figure 3**). To compare the different studies, their outcome was summarized using a metric that puts all studies on the same scale. This measure is called the effect size, and the magnitude and direction of the effect size are weighted by the study sample size and variance. The effect size from the individual studies can then be combined to calculate a single numerical value of the overall treatment effect across all studies. In this study, data from the 40 different previously published papers were normalized by calculating their effect sizes using the Hedges' *d* method. Furthermore, moderators (seaweed group, experimental method, and types of process water) were included to categorize data into different subgroups and compare their group means.

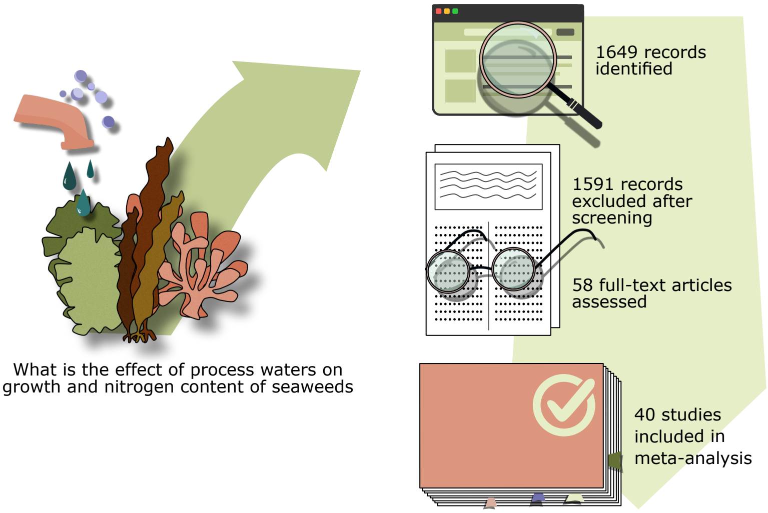

Figure 3. Schematic image of the process of the meta-analysis in **paper I**.

SEAWEEDS FOR EXPERIMENTS

Sporophytes of *S. latissima* (**paper II**) and clonal foliose-shaped gametophytes of *U. fenestrata* (**papers II-III**) were collected from indoor tank cultivation systems at Tjärnö Marine Laboratory (TML, 58°52′33.7″ N, 11° 08′44.9″ E). The *U. intestinalis* (**paper II**) was collected at Rossö, located on the Swedish west coast (58°50′33.9″ N, 11°09′06.6″ E), while *C. linum* (**paper II**) was collected in intertidal rock ponds at Ursholmen (58°49′57.6″ N, 10°59′19.2″

E), also located on the Swedish west coast. Taxonomic identification of *Ulva* strains was based on molecular identification of the *tufA* marker gene (**papers II-VI**), while *S. latissima* and *C. linum* were identified by their morphological characters (**paper II**). The seaweeds were kept in tank cultivations at TML until used in the experiments. The reason these species were chosen for this thesis was because they are economically important, have been grown successfully in similar settings, or are known as opportunistic species that grow quickly and easily incorporate nitrogen.

In **papers IV-VI**, clonal foliose-shaped gametophytes of *U. fenestrata* were collected from sea-based seaweed farms (2 ha, 100 x 200 m) located in the Koster archipelago (Skagerrak), Sweden (58°51′34.0″ N, 11°04′06.2″ E), and the Bohuslän coastline (Skagerrak), Sweden (58°38′34.0″ N, 11°12′59.0″ E). The gametophytes, which had grown between six and nine months at the sea-based farms, originated from the offspring of gametophytes from the long-term indoor tank cultivation at TML and were out-planted after hatchery.

As the main focus of this thesis is on the crop *U. fenestrata,* its life cycle is shortly described (**Figure 4**). *Ulva* has an isomorphic biphasic life cycle, meaning that the two phases of haploid gametophytes and diploid sporophytes are morphologically identical. While the sporophytes originate from gametes from gametophytes, the gametophytes can originate from either haploid zoids from sporophytes or from clonal unmated gametes (parthenogenetic gamete propagation). In **papers II-VI**, gametophytes of *U. fenestrata* from clonal unmated gametes are used. This is mainly because long-term tank cultivations through parthenogenetic gamete propagation are easier to maintain, but also because differences observed between clonal gametophytes in the experiments will likely be due to the treatment effect rather than genetic differences between individuals.

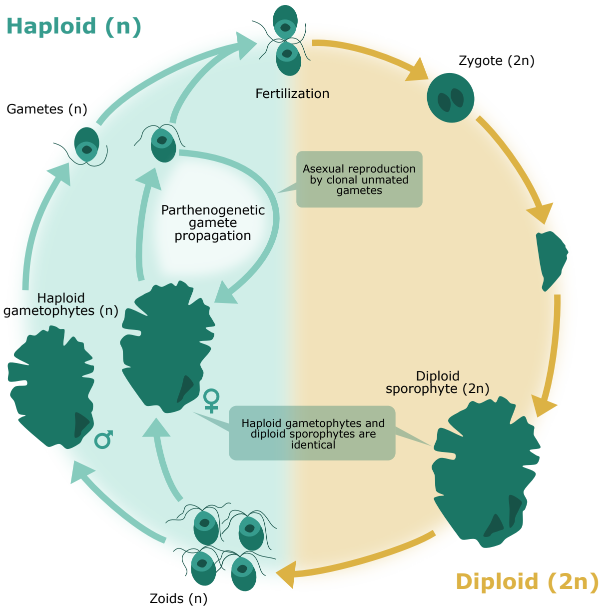

Figure 4. The isomorphic biphasic life cycle of *Ulva fenestrata*.

PROCESS WATERS AND EXPERIMENTAL SETUPS

In **paper II**, eight different FPPWs from seafood industries and an oat processing industry were tested as cultivation media (**Table 2**). For **papers III-V,** two types of water from herring production (HPPW), namely TUB and SAL, were identified as promising waters to proceed with. TUB refers to water emerging from the industrial storage of herring in plastic tubs with saltwater (3% NaCl) for up to four days, prior to the herring being filleted. SAL refers to water emerging from the maturation of herring fillets stored in barrels with saturated salt brine for up to two years.

The FPPWs were characterized for pH, total nitrogen (TotN), ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and inorganic phosphorus/orthophosphate (P) at Chalmers University of Technology. The pH measurements were performed with a pH meter (PHM 210, Meterlab, Hach, USA). Total nitrogen was analyzed with a LECO Nitrogen Analyzer (TruMac N, LECO Corporation, USA) using EDTA 9.56 as standard. Inorganic nitrogen was quantified using commercial enzymatic kits (AA0100, Sigma, USA, and Cat. No. 11746081001, Roche Diagnostics, Germany), while P was measured with a standard curve made with monopotassium phosphate, as reported by Qvirist et al. (2015).

After collection of the FPPWs, the waters were filtrated to remove coarse particles (>300 µm) and then stored at -60°C. For **paper II**, the waters were sterilized by autoclaving for 20 min at 1200 kPa. The waters were analyzed before and after this thermal treatment to ensure no influence on the NH_4^+ concentration. Autoclaving was not performed on the waters for **papers III-V** as it was not considered a realistic scenario for upscaling. When used in an experiment, the FPPWs were thawed, and diluted with filtered (0.2 µm+UV-light treated) deep-sea (40 m) seawater to reach the desired concentration of NH_4^+ for the specific experiment (see below). In all experiments, the FPPWs were renewed every second day to avoid nutrient depletion.

Since NH_4^+ is the preferred nitrogen source for most seaweeds, and the main nitrogen found in the FPPWs, the FPPWs were diluted based on their NH_4^+ concentration. The NH_4^+ concentration was normalized to 20 and 200 µM NH_4^+ in **paper II**, and 25 µM NH_4^+ in **papers III-V**. These final concentrations were chosen to include optimal growth and nitrogen content of seaweeds based on previous work (Waite and Mitchell, 1972; Cohen and Neori, 1991; Nielsen et al., 2012; Rugiu et al., 2020), as well as personal experience and insight from preparing **paper I**. Examples of the different experimental setups used in this thesis are shown in **Figure 5**.

Table 2. Description of the different process waters used in this thesis (**papers II-V**), and their characterization of inorganic nutrients. Different batches of TUB and SAL were used in the different papers, and the min-max values from these batches are shown.

Type of water	**Acronym**	**Origin**	**Provider**	**Water description**	**NH_4^+ (µM)**	**NO_3^- (µM)**	**NO_2^- (µM)**	**P (µM)**
Refrigerated seawater	RSW	Herring	Sweden Pelagic AB	From on board refrigerated seawater tanks	1160	6	n.d	3500
Tub water	TUB	Herring	Sweden Pelagic AB	From storage tubs with herring in 3% NaCl	1400-2300	9-20	3	5700-22100
Salt brine I	SBI	Herring	Sweden Pelagic AB	From pre-salting of headed/gutted herring in 5% NaCl	3600	16	n.d	33600
Salt brine II	SAL	Herring	Klädesholmen Seafood AB	From maturation of herring fillets in saturated salt brine	8000-21800	n.d	n.d	15400-27300
Spice brine	SPI	Herring	Klädesholmen Seafood AB	From maturation of herring fillets in spice brine	6300	12	n.d	21200
Shrimp boiling water	SBW	Shrimp	Bua Shellfish AB	From steaming of shrimps	8900	11	n.d	410
Oat processing water	OAT	Oat	Oatly AB	From processing of oat to oat milk	30	6800	330	300
Recirculated aquaculture system (RAS) water	RAS	Salmon	Nordic Aquafarms AS	Salmon RAS water after biofiltration-nitrification process	40	3100	17	40

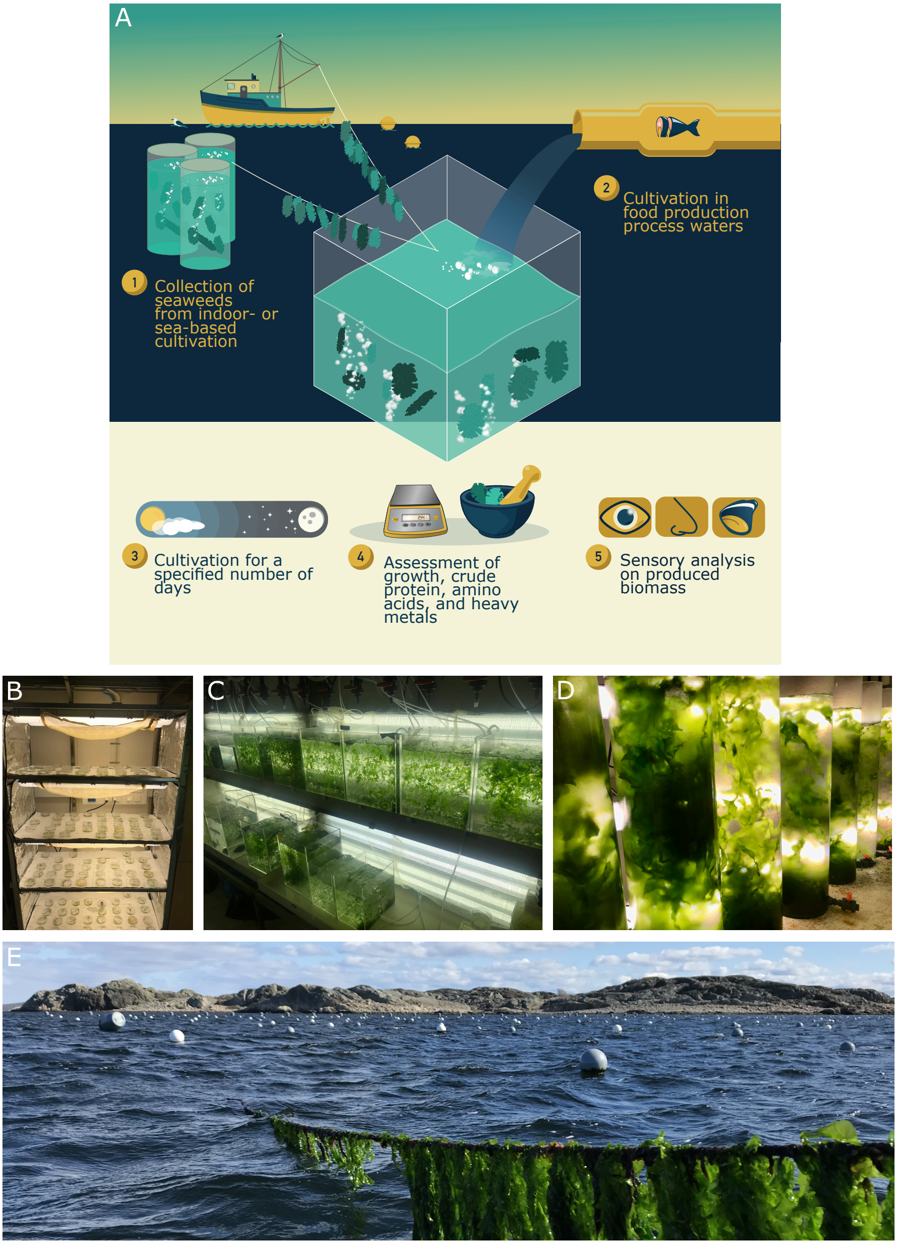

Figure 5. (**A**) Example of the experimental setups scaled up from (**B**) 100 mL Petri dishes in **paper II**, (**C**) 14 L tanks in **papers III-IV**, and (**D**) 45 L tanks in **paper V**, as well as (**E**) the sea-based farm in **papers IV-VI**.

PHYSIOLOGICAL AND BIOCHEMICAL MEASUREMENTS

Growth

Growth was measured either by weight or area in this thesis. In **paper II**, the growth was measured by the specific growth rate (SGR) for area for *S. latissima* and *U. fenestrata*, by analyzing images taken at the start and end of the experiments in ImageJ (ImageJ V. 2.0.0-rc-69/1.52p). Due to the filamentous morphological characteristics of *U. intestinalis* and *C. linum*, the SGR for these species was measured by fresh weight (fw). The SGR was calculated using the formula $SGR=((Ln(A_t)–Ln(A_0))/t)x100$, where A_t is the area/weight after t days and A_0 is the initial area/weight. In **papers III-V**, the biomass yield was measured by the increase in fw. When using fw, standardized methods were used to remove excess water.

Color (RGB)

In **papers II** and **VI,** images of the seaweeds were analyzed for the three band colors red (R), green (G), and blue (B), (RGB), using either ImageJ (**paper II**) or a web-based image color summarizer **(paper VI**). The RGB values were used in **paper II** to indicate the physiological status of the seaweeds, while in **paper VI** they were correlated to nitrogen tissue content and used to estimate the nitrogen tissue content of *U. fenestrata*.

Nitrogen and crude protein content

In this thesis, nitrogen content was used to estimate crude protein content. This approach was chosen over direct protein extraction methods, as it is simple, reproducible, and a widely preferred method in experimental studies. Direct protein extraction methods on the other hand are not standardized and prone to inaccuracies in both extraction and quantification of the protein, leading to underestimations in protein content and making comparisons between studies more difficult (Angell et al., 2016; Biancarosa et al., 2017).

For **papers II-VI**, the seaweed tissues were freeze-dried and homogenized to a fine powder before performing the desired biochemical analyses. The total nitrogen content was analyzed using an elemental analyzer in **paper II**. Due to technical issues with this machine, *C. linum* was analyzed using a LECO Nitrogen Analyzer. In **papers III-VI**, the nitrogen content was analyzed using an elemental analyzer coupled to an isotope-ratio mass spectrometer. The nitrogen was converted to crude protein using a conversion factor of five, as opposed to the traditionally used factor of 6.25. The reason being that the latter inaccurately assumes that the total protein in seaweeds constitutes 16% nitrogen and that all nitrogen is in the form of protein (Angell et al., 2016).

Amino acid composition

The quality of the proteins was assessed by the AA profile, with respect to the content and composition of EAA. In **papers III-IV**, high-performance liquid chromatography coupled with mass spectrometry detector (HPLC-MS) was used to analyze the total amino acids (TAA) at Chalmers University of Technology. In **paper V**, the TAA were analyzed at Eurofins Food & Feed Testing Sweden AB, using ion chromatography coupled with ultraviolet detector (IC-UV). These methods are not able to recover tryptophan, and the HPLC-MS method is not able to recover cysteine. For both methods, glutamine and asparagine are co-determined with glutamic and aspartic acid, respectively.

Heavy metal content

The toxic elements arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) were analyzed in **papers III-IV,** as they are considered elements of major health concern. These elements are referred to as "heavy metals", even though arsenic is strictly speaking a metalloid. The heavy metals were analyzed at the Swedish Food Agency, using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x) following standard procedures of NMKL no. 186 and EN 15763:2009, while inorganic arsenic (iAs) was analyzed using HPLC-ICP-MS (Agilent 1260 Infinity Quaternary LC and Agilent 7700x) according to the EN 16802:2016.

There are no regulations from the European Commission on the maximum levels of these heavy metals in seaweeds for food purposes, other than for food supplements. Therefore, the heavy metal contents were compared to the maximum allowed levels (MLs) in general foodstuffs set by the European Union's Commission Regulation (EC) No. 1881/2006 (version 03/05/2022). Furthermore, simple exposure assessments were performed to indicate if consuming the cultivated biomass posed risks concerning heavy metal exposure.

MAIN RESULTS AND DISCUSSION

OVERVIEW OF KEY FINDINGS

The main results from the different papers in relation to their defined aims are summarized below:

Paper I: The meta-analyses show that all groups of seaweed (brown, green, and red) can be cultivated in a wide range of process waters, in both ocean- and land-based settings, to produce biomass with enhanced growth and nitrogen content. It also identifies a gap in the literature, showing that very few studies have explored the cultivation of seaweeds in process waters from sources outside the aquaculture industry.

Paper II: The results show that there is a high potential for all the tested FPPWs as growth media for the green seaweeds, but not for the kelp. In general, 20 μM NH_4^+ treatments performed better or similar to the 200 μM NH_4^+ treatments. In the most promising cases, growth rates were up to 64% higher, and crude protein content was almost four-fold higher in FPPWs compared to seawater controls.

Paper III: The key finding from this study is that after only 14 days, the crude protein content of *U. fenestrata* was three times higher in the HPPWs compared to the seawater control. Reaching protein content over 30% dw, the protein content is comparable to soybeans and pulses. Additionally, the potential health risks (related to heavy metal content) of consuming the biomass were very low.

Paper IV: The results show that it is possible to extend the sea-based cultivation season of *U. fenestrata* when aiming for high protein levels, by post-harvest treatment in HPPWs. By cultivating seaweeds harvested late in the season (high biomass yield but low protein content) in HPPWs, it was possible to rapidly increase the protein content to similar levels as early in the season when the protein content is at its peak. The biomass cultivated in HPPWs could be consumed as food based on the documented levels of heavy metals, and its sensory attributes were not regarded as negative.

Paper V: The results show that the natural peak protein content of *U. fenestrata* can be boosted through short-term post-harvest treatment with HPPW. The crude protein content was increased by over 70% and reached an average of 37% dw when cultivated in the HPPW. Additionally, no fish-related allergenic effects were detected in the biomass.

Paper VI: The results show that the color of *U. fenestrata* can be used to accurately estimate the nitrogen content of the biomass. Based on the produced models, a web-based application was developed that automatically analyzes the nitrogen content from uploaded images. Furthermore, a color guide was produced that can easily be brought to the field to estimate the nitrogen content of *U. fenestrata*.

META-ANALYSIS

When synthesizing the literature in **paper I**, it was found that both the growth and nitrogen content of seaweeds are increased when cultivated in process waters. Green seaweeds benefit more in terms of growth and nitrogen content compared to brown and red seaweeds (**Figure 6**). This is not surprising as green seaweed species are often characterized as opportunistic species that grow fast (Taylor et al., 2001). Being able to tolerate large fluctuations in environmental parameters (Bäck et al., 2000; Ye et al., 2011), they are ideal candidates for cultivation in process waters. In the case of brown seaweeds, almost all studies were from IMTA settings with the kelp *S. latissima*. The effect of process water is, therefore, harder to assess since this group is not well represented by different species of seaweed or process waters.

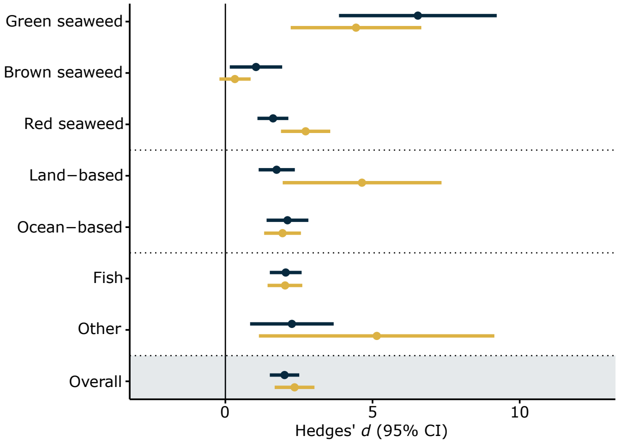

Figure 6. The effect of process water on growth (blue) and nitrogen content (yellow). A significant positive effect is identified by a Hedges' *d* value greater than zero.

The results further show that the benefits of cultivating seaweeds in process waters are applied to both land-based and ocean-based cultivation. There is a strong tendency for nitrogen content to be more positively affected when cultivated in land-based settings, which can possibly be explained by seaweeds grown in ocean-based settings often being at some distance from the nutrient source. Cultivation in process waters has the potential to advance land-based cultivation, which has thus far been slowed down by costly infrastructure and maintenance (Hafting et al., 2012; Mata et al., 2016). Land-based cultivation also makes it possible to cultivate various seaweed species with morphologies unsuitable for ocean-based cultivation in a range of unexplored process waters, such as from industrial food processing industries.

The meta-analysis also clearly reveals a gap in the literature, where the focus is on a few selected seaweed species and types of process waters. However, the studies included beyond traditional fish aquaculture process waters indicate that using alternative process waters has the potential to yield fast-growing and nitrogen-rich seaweed biomass. This concept was further explored throughout this thesis.

GROWTH AND CRUDE PROTEIN CONTENT

It has long been known that seaweeds grow faster and accumulate nitrogen when cultivated in nutrient-rich waters (Ryther et al., 1981; Habig et al., 1984). Accordingly, this thesis clearly shows the potential of using FPPWs to increase both the growth and protein content of seaweeds. After cultivation in eight different FPPWs emerging from recirculated salmon aquaculture systems, as well as from herring, shrimp, and oat processing, the green seaweed species (*U. fenestrata*, *U. intestinalis*, and *C. linum*) increased in both growth and crude protein content, as compared to when cultivated in seawater controls (**paper II, Figure 7**). Growth rates of the green seaweeds were up to 64% higher, and crude protein content was almost up to four times higher when cultivated in the FPPWs, compared to seawater controls.

The brown species, *S. latissima*, had negative growth rates in all the tested FPPWs, which resulted in no biomass to perform crude protein content analysis on. The negative growth may be attributed to the inherent characteristics of kelps, which are not opportunistic species and usually grow in much lower inorganic nitrogen and phosphorus concentrations (Forbord et al., 2012; Bruhn et al., 2016; Roleda and Hurd, 2019). It is important to note that the dilution in the experiment is based on NH_4^+, but other species of nitrogen and phosphorus are also present. It is possible that the total nitrogen and/or phosphorus concentrations in the waters were a shock for *S. latissima*, or alternatively, there were some other unidentified compounds in the water that inhibited the growth (Tegner et al., 1995; Coelho et al., 2000). Regardless, *S. latissima* was not considered a promising species for cultivation in these settings.

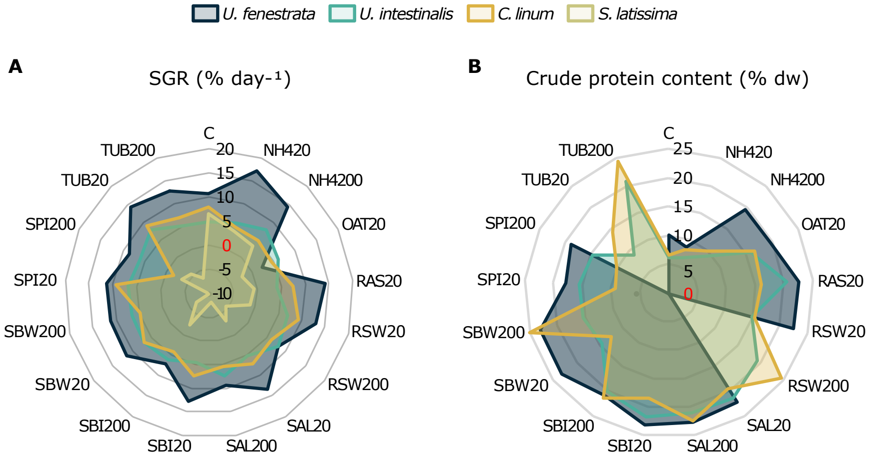

Figure 7. **(A)** Specific growth rate (SGR, % day^{-1}), and **(B)** crude protein content (% dw) of *Ulva fenestrata*, *Ulva intestinalis*, *Chaetomorpha linum*, and *Saccharina latissima* in the respective FPPWs. Values are mean. Details about measures of variability and statistics can be found in **paper II.** Samples for crude protein content of *U. fenestrata* in TUB20, TUB200, and RSW200 were destroyed during analysis and could therefore not be included.

For the green seaweed species, growth rates were generally higher in FPPWs diluted to 20 µM compared to 200 µM NH_4^+, while crude protein content was similar in the two dilutions. For some seaweed species, a high NH_4^+ concentration can have an inhibitory effect on growth (Roleda and Hurd, 2019). Even if *Ulva* species can tolerate high NH_4^+ concentrations, their growth rate and nitrogen content often stagnate at around 20-60 µM (Waite and Mitchell, 1972; Cohen and Neori, 1991; Taylor et al., 2001; Nielsen et al., 2012). This indicates that FPPWs with high NH_4^+ concentration possess a high dilution capacity, which is promising for industries producing limited quantities of FPPW or those with seasonal production.

Even though the results from **paper II** were promising, the experiments were small-scale (100 mL Petri dishes). In the next step, two promising FPPWs from the herring processing industry (HPPWs), namely SAL and TUB, were selected. These waters were selected based on the results from **paper II**, additional unpublished results, and considerations of the FPPWs' characterization and availability. Furthermore, *U. fenestrata* was chosen as the most promising candidate for cultivation in these settings.

In **paper III,** it is shown that the positive results of increased growth and crude protein content are maintained when the cultivation setting is scaled up. The results show that *U. fenestrata* cultivated in the HPPWs had four to six times higher biomass yields and three times higher crude protein content compared to when cultivated in seawater (**Figure 8**). After only 14 days, the crude protein content was over 30% dw. This crude protein content is comparable to soybeans (35-40% dw) (Grieshop and Fahey, 2001; Thakur and Hurburgh, 2007), and pulses such as lentils, chickpeas, and beans, in which crude protein makes up 17-30% dw (Boye et al., 2010). Extrapolating these results indicates a production of 14-19 t dw ha^{-1} year^{-1}. By assuming a protein content of 30% dw this results in 4-6 t protein ha^{-1} year^{-1}. This is over three times more productive than the average soybean protein production of 1.4 t protein ha^{-1} year^{-1} (assuming production of 3 t ha^{-1} year^{-1} of soybean containing a protein content of 45% dw; Grieshop and Fahey, 2001; Ainsworth et al., 2012; FAO, 2021b). Although these extrapolations are rough and assume a stable year-round production, they are necessary to provide possible productivity scenarios. Furthermore, this system was not optimized for optimal biomass yields, and the start density used in the experiment was very low compared to possible cultivation densities.

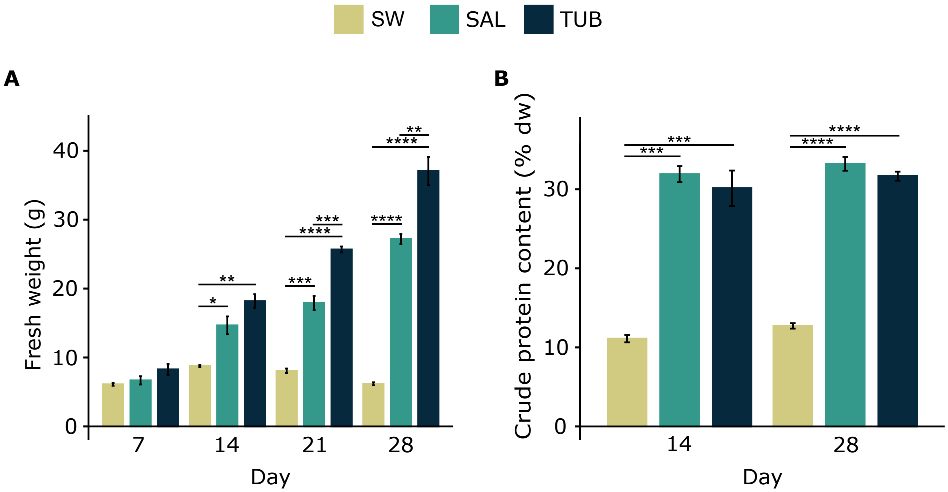

Figure 8. (A) Fresh weight (g) and **(B)** crude protein content (% dw) of *Ulva fenestrata* cultivated in seawater control (SW), and the two herring production process waters salt brine (SAL), and tub water (TUB). Error bars show standard error, and significant differences are denoted by asterisks.

In **paper IV**, the HPPWs (SAL and TUB) were used to extend the sea-based cultivation seasons of *U. fenestrata* when aiming for high protein levels. Generally, harvesting *U. fenestrata* late in the season will allow for higher biomass yields. However, there is a trade-off as the protein content drastically decreases during this period (Steinhagen et al., 2022). **Paper IV** demonstrates that it is possible to extend the sea-based cultivation season by post-harvest cultivation in the HPPWs for 7-14 days to reach similar levels of crude protein as those observed early in the season when the crude protein content is at its peak (March/April; 21% dw) (Steinhagen et al., 2022). This method can help sea-based farms maximize their output of proteins.

In **paper V**, the HPPW (TUB) was instead used to further boost the protein content of the sea-based cultivated *U. fenestrata* when harvested at its natural peak protein content (March/April; 21% dw). By cultivating the *U. fenestrata* for 14 days in the TUB, the crude protein content was increased by over 70% reaching an average of 37% dw (**Figure 9**). Such high protein yields are important for the subsequent downstream application of biomass in the food value chain. To my knowledge, this is also the first time that such high crude protein contents have been achieved in green seaweed biomass when using the conversion factor of five. For example, the frequently cited protein content of 47% dw for red seaweeds (Fleurence, 2004), is calculated from a nitrogen content of 7.6% dw using the conversion factor of 6.25 (Fujiwara-Arasaki et al., 1984). However, by applying the conversion factor of five, as used throughout this thesis, it instead results in a crude protein content of 38% dw.

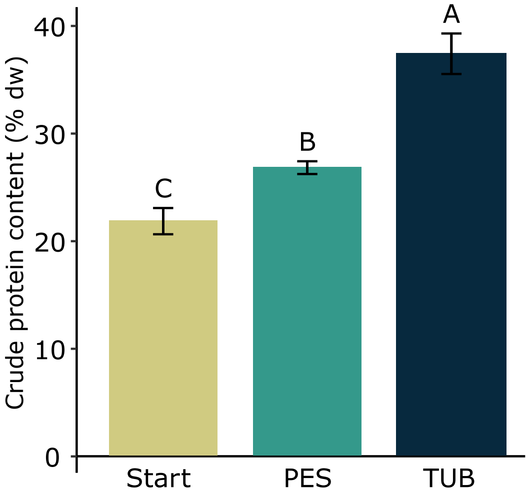

Figure 9. Crude protein content (% dw) of *Ulva fenestrata* at the start of the experiment and after 14 days post-harvest treatment in Provasoli Enriched Seawater (PES) and the herring production process water (TUB). Error bars show standard deviation, and significant differences are denoted by capital letters above bars.

AMINO ACID COMPOSITION AND PROTEIN QUALITY

The results of this thesis (**papers III-V**) show that cultivation of *U. fenestrata* in HPPWs strongly influences the AA composition of the biomass, resulting in high-quality protein biomass. All amino acids were present at 2-8 times higher levels in *U. fenestrata* cultivated in the HPPWs compared to seawater. As a result of the high levels of some non-essential AAs, the TEAA were generally lower in seaweed biomass from SAL (33.8-36.4%), and TUB (33.2-37.2%) compared to from seawater (36.3-40.6%). Nevertheless, these TEAA values are higher than in many terrestrial plants, while being comparable to protein products such as eggs and soybeans (Fleurence, 2004; Mæhre et al., 2014; Henchion et al., 2017). Furthermore, apart from tryptophan, which could not be recovered using this method, all the EAA were found in the biomass (**Table 3**).

Table 3. Summarized profiles of essential amino acids (EAA) and total essential amino acids (TEAA) in *Ulva fenestrata* cultivated in seawater (SW), salt brine (SAL), and tub water (TUB) from **papers III-V**, as well as the amino acid (AA) profiles recommended by WHO/FAO/UNU (2007).

EAA	**SW (% of TAA)**	**SAL (% of TAA)**	**TUB (% of TAA)**	**Recommended AA profile (% of protein)**
Valine	5.8-7.6	5.9-6.6	5.7-6.4	3.9
Threonine	5.1-7.1	5.6-6.0	5.4-6.6	2.3
Isoleucine	3.8-4.6	3.7-3.8	3.5-4.2	3.0
Leucine	7.4-8.5	6.1-6.7	6.0-6.4	5.9
Lysine	3.2-5.3	4.9-5.8	4.7-5.0	4.5
Methionine	0.5-2.6	1.5-1.9	1.3-1.8	1.6
Histidine	1.4-2.4	1.2-1.3	1.2-2.0	1.5
Phenylalanine	5.0-5.4	4.6-5.0	4.6-5.1	3.8*
TEAA	36.3-40.6	33.8-36.4	33.2-37.2	

*sum of phenylalanine and tyrosine.

To achieve the recommended daily intake of all EAA from only consuming *U. fenestrata*, an adult needs to consume about 1200-1500g dw of *U. fenestrata* cultivated in SW and about 140-300g dw of *U. fenestrata* cultivated in the HPPWs. These values can seem high but are comparable to the required amount for soybeans (170 g dw; Grieshop and Fahey, 2001). Furthermore, the seaweed proteins can be concentrated by different extraction processes, which also improves the protein's digestibility (Harrysson et al., 2019; Trigo et al., 2021; Juul et al., 2022a, b). Developing such extraction methods will be important if seaweeds are to play a significant role in transforming the food systems.

The average nitrogen-to-protein conversion factor from **papers III-V** is 5.12, which corresponds well to the conversion factor of five that was adapted throughout this thesis (Angell et al., 2016). However, it should be noted that there was a variability in the conversion factor between the different studies and treatments. This is because not all of the nitrogen in seaweeds is in the form of protein, and some proteins are made up of more nitrogen-rich AAs than others (Shuuluka et al., 2013; Mæhre et al., 2014; Angell et al., 2016). A protein with high levels of nitrogen-rich AAs (such as arginine, histidine, and lysine), therefore, generally has a lower nitrogen-to-protein conversion factor (Sosulski and Imafidon, 1990; Shuuluka et al., 2013). However, it is also important to note that the applied method for AA analysis may result in an underestimation of the conversion factor, as some AAs are not recovered while others may be destroyed during chemical analysis. Altogether, the results from this thesis support using nitrogen content as a reliable method to estimate crude protein content, facilitating easy comparisons between studies, when amino acid analysis is not possible.

HEAVY METAL CONTENT AND FOOD SAFETY ASPECTS

There is a concern that seaweeds may accumulate toxic elements, specifically the inorganic form of arsenic (iAs), which is known to be carcinogenic (Duinker et al., 2020; Blikra et al., 2021). The results from **papers III-IV** show that the heavy metal content of *U. fenestrata* was below the EU maximum levels (MLs) in foodstuffs (European Commission, 2006; 2022a; **Table 4**). Although the tAs content in TUB cultivated *U. fenestrata* in **paper III** was high, iAs only made up 0.02-0.72% of tAs throughout all the tested biomass, which resulted in a very low amount of iAs in the biomass. These results correspond well with the general assumption that the proportion of iAs in seaweeds is typically one percent of the tAs (Duinker et al., 2020; Blikra et al., 2021).

As of today, there are no MLs established for As, Hg, Pb, or Cd in seaweeds. Instead, the MLs reflect the levels, size of consumption, and toxicity in foodstuffs occurring at the market. Therefore, the MLs are not permanent but constantly evaluated and adjusted as new foodstuffs are being added. It is currently being assessed if the MLs should be modified to include seaweeds (EFSA, 2023). It is important to consider that the levels of heavy metals can vary greatly both between and within seaweed species. For example, the levels can be affected by age, growing conditions, and processing methods, making it hard to categorize seaweeds into one group (Duinker et al., 2016, 2020; Anbazhagan et al., 2021; Véliz et al., 2023).

Table 4. Summarized heavy metal content in *Ulva fenestrata* cultivated in seawater control (SW), salt brine (SAL), and tub water (TUB) from **papers III-IV**. The range of maximum allowed levels in foodstuffs (MLs) is set by the European Union's Commission Regulation (EC) No. 1881/2006 (version 03/05/2022).

	Content in biomass μg g dw^{-1}			MLs in foodstuffs μg g^{-1}
	SW	SAL	TUB	
Total arsenic (tAs)	2.42	2.11-4.85	3.02-106.95	Not established
Inorganic arsenic (iAs)	0.01	0.02	0.01-0.02	0.1*-0.3
Mercury (Hg)	<0.036	0.04-0.21	<0.036	0.1-1
Lead (Pb)	0.13	0.34-1.45	0.35-0.97	0.02*-3**
Cadmium (Cd)	0.03	0.13-0.21	0.14-0.39	0.01*-3**

*Food destined for babies and young children (for iAs rice intended for baby food, Pb cereal based food, and for Cd young children formulae).

**Food supplements as sold, consisting exclusively or mainly of dried seaweeds, products derived from seaweeds, or of dried bivalve mollusks.

In **papers III-IV**, simple exposure assessments were used to estimate how much of the cultivated biomass that can be consumed per day before exceeding reference doses (RfDs) set by the US EPA (US EPA, 2007). These reference doses are more conservative than the values from the Joint FAO/WHO Expert Committee on Food Additives (JECFA., 2011), but less conservative than Pb and Cd values from EFSA (EFSA, 2009, 2010). The US EPA recommendations were chosen for easier comparisons with other publications within the field. The results show that a daily consumption of 90-450g dw from biomass cultivated in HPPWs can be consumed before exceeding the RfDs. This translates to a maximum of more than two kg fresh weight, given a dw:fw ratio of 20:80. Using the more restrictive reference dose for Pb from EFSA, around 30-120g dw can be consumed daily. These values can be considered conservative, as they are based on an adult body weight of 63.3kg, which is lower than the average weight of over 75kg in America, Australia, and Europe. It also assumes that all heavy metals in the biomass are bioavailable, which is probably not true. Heavy metals often bind to polysaccharides in seaweeds (Duinker et al., 2016; Ortiz-Calderon et al., 2017; Khandaker et al., 2021) which are typically not easily digestible for humans (Fleurence, 2016; Trigo et al., 2021). Hence, the bioavailability of heavy metals needs to be investigated to establish how readily they are absorbed into the bloodstream during digestion. Furthermore, processing of biomass, such as blanching, washing, boiling, and protein extraction may reduce the content of some heavy metals in seaweed biomass (Stévant et al., 2018; Blikra et al., 2021; Véliz et al., 2023). This would further increase the possible amount of biomass that can safely be consumed.

DETERMINATION OF NITROGEN CONTENT BY COLOR IMAGE ANALYSIS

The composition of the seaweed biomass depends on seasonal growing conditions. Hence, the timing of harvesting is important for the quality of the seaweed crop (Steinhagen et al., 2022). Linear regression models based on color image analysis have been used to estimate the physiological status and nitrogen content of many terrestrial plants (Mercado-Luna et al., 2010; Tewari et al., 2013; Riccardi et al., 2014; Zhang et al., 2022). However, although it has been widely documented that the color of seaweed thallus can change due to variations in tissue nitrogen content (Nagler et al., 2003; Yu and Yang, 2008; Robertson-Andersson et al., 2009; Ashkenazi et al., 2022), prior to **paper VI**, no models had been developed to evaluate the nitrogen content of seaweeds based on their color. Therefore, in **paper VI**, non-destructive, labor- and cost-efficient models were developed to estimate the nitrogen content (and hence protein content) in the crop seaweed *U. fenestrata* by color image analysis. It was shown that the three band colors R, G, and B were highly correlated with nitrogen content (**Figure 10**) and that they can be used in both simple and multiple linear regression models to predict the nitrogen content of *U. fenestrata*. These results show that color can be a powerful tool for seaweed farmers to quickly and cost-efficiently assess the quality of their crop, without having to use time-consuming and expensive laboratory procedures. This could potentially help overcome some of the barriers farmers face in producing sustainable and high-quality seaweeds.

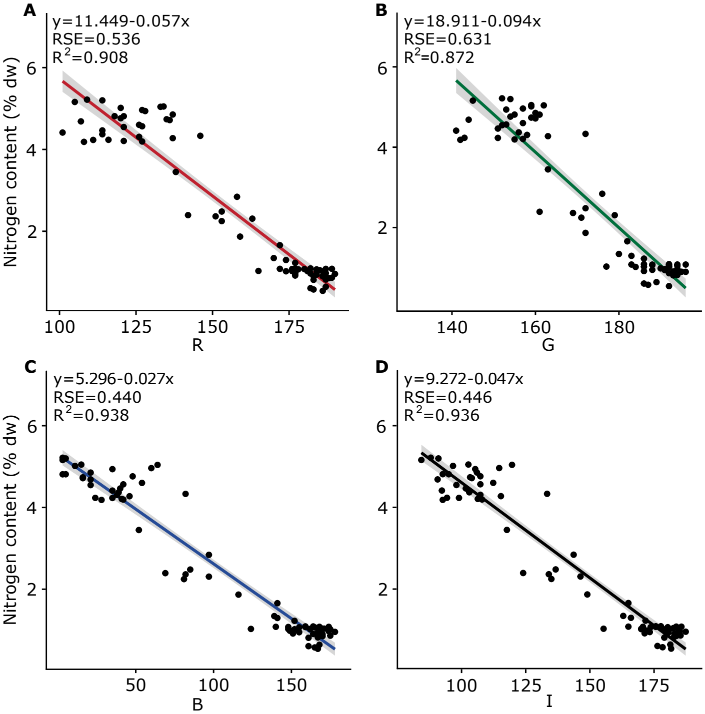

Figure 10. Nitrogen content (% dw) as a function of the color variables **(A)** red, **(B)** green, **(C)** blue, and **(D)** intensity ((R+G+B)/3) for fresh tissue of *Ulva fenestrata*.

CONCLUSION

This thesis shows that seaweeds are promising alternative food protein sources for the ongoing dietary protein shift. It provides insights into the different steps of seaweed cultivation, with a focus on enhancing the biomass yield and composition of the crop, while also ensuring its safety and evaluating its sensory aspects as food. The results show that green seaweed species are especially suitable to cultivate in a wide variety of industrial FPPWs, resulting in increased seaweed growth and protein content (**papers I-V**). After an initial screening of different seaweed species (**paper II**), this thesis focuses on the green seaweed species *U. fenestrata*. By cultivating *U. fenestrata* in HPPWs, this thesis provides what is, to my knowledge, the highest crude protein content (>37% dw) achieved in *Ulva* biomass when compared to previous studies (**paper V**). The HPPWs provided seaweeds with favorable amino acid profiles containing all the EAA, and the increased protein content also translates to a significantly higher content of EAA in the biomass (**papers III-V**). Furthermore, **papers III-IV** show that the biomass cultivated in HPPWs is safe to consume in large quantities without exceeding health-based reference points for the analyzed heavy metals. However, the iodine content of *U. fenestrata* should be investigated, as it has been identified in other studies as a potential limiting factor for safe intake levels. Additionally, cultivation in HPPWs did not negatively affect any sensory attributes, underscoring the potential of this method for safe and palatable biomass production.

Even though the extraction process of proteins from seaweeds will most likely become more efficient in the near future, ensuring a high protein content in the input biomass will remain crucial for seaweeds to become a viable protein source moving forward. To increase seaweeds' contribution to the ongoing dietary protein shift, the industry must expand, and cultivation in FPPWs can play a role, particularly when targeting crops with high protein content. Therefore, further upscaling of the concept, with for example year-round production in FPPWs, should be investigated to assess the annual protein output from these systems.

In conclusion, this thesis clearly shows the possibility for a novel nutrient loop in which the costly disposal of food production process waters can be turned into economic revenue by sustainable production of protein-enriched seaweeds. This is an important step for the continued downstream application of the biomass in the food value chain. The process water offers a way to incorporate seaweeds into land-based cultivation, considering water quality and clean effluents as a bonus in a system where the main products are seaweed biomass yields with high protein content.

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