The potential of *Ulva* diversity in southern Portugal for a sustainable food and feed industry

A master thesis

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Presented to the Faculty of Sciences and Technology at the University of Algarve.
For the Degree of Master of Science in Aquaculture and Fisheries.

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Faro, October 2019

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam na listagem de referências incluída.

Universidade do Algarve, 30.09.2019

Acknowledgements

Foremost, I want to thank my supervisor Dr. Aschwin Engelen for his support throughout the entire process from finding my thesis topic to finishing it. I especially appreciate his enthusiasm towards my ambition to include the topic of sustainability in my thesis. I am also grateful for his flexibility to change plans due to my knee surgery. *"Plans are there to be changed"* is a sentence I now truly got to understand the meaning of. Thank you for being supportive and to supervise me even at distance.

I also want to express my greatest appreciation and gratitude to the Marine Biotechnology group and the Marine Ecology and Evolution Group, CCMAR, especially to Tania Aires and Tamára Santos without whom I could not have done the experimental part of my thesis. I learned a lot from you and enjoyed my time in the lab in a great atmosphere.
I thank my fellow Alena Sidow, who did not only make the whole process of planning and conducting my thesis possible but also made it a lot more fun by discussing and being enthusiastic about seaweed together.

Ultimately, I want to say thank you to my friends and family who have supported me the entire time and helped me keep my patience and endurance at times. You are always there for me even when seaweed and aquaculture are far from your everyday topics.

Abstract

The food and feed industry surpass their sustainable boundaries and global food security is one of society's biggest challenges today. Macroalgae of the genus *Ulva* have been identified as a suitable candidate for cultivation, with various applications within the food and feed industry.

This work discusses the sustainability performance of *Ulva* cultivation and identifies the potential of *Ulva* species in southern Portugal for their use in the food and feed industry. It was tested, which species are available in different coastal and lagoon habitats and whether species identity or environmental conditions have a stronger effect on the seaweed's protein content and fatty acid profile, to find out, whether the selection of one *Ulva* species is favorable over another for cultivation.

Ulva species were collected at coastal and lagoon locations in southern Portugal and genetically identified, using the tufA gene sequences. *Ulva rigida, Ulva flexuosa, Ulva fasciata, Ulva australis* and *Ulva compressa* were identified as potential cultivation candidates. *U. australis* has not previously been reported in southern Portugal. Protein content in *U. rigida* sampled in coastal locations was higher ($p < 0.01$) compared to lagoon locations, but not different among species within the Ria Formosa ($p = 0.363$). Fatty acid profiles were not different across locations ($p = 0.739$). However, *U. compressa* had a higher PUFA content than *U. rigida* and *U. fasciata* within the Ria Formosa ($p = 0.0245$). Results suggest that *U. compressa* might be more a more suitable *Ulva* candidate for PUFA production and that protein content in seaweeds may be more susceptible to environmental conditions.
In southern Portugal, *Ulva* is still an underexploited resource but has the potential to be part of the solution to overcome food security challenges in the future.

Keywords: *Ulva;* fatty acids; protein; sustainability; food and feed industry.

Résumo

A indústria de alimentos e rações supera os seus limites sustentáveis e, a segurança alimentar global, é um dos maiores desafios da sociedade hoje. Os sistemas de produção alimentar necessitam de novas soluções e de uma abordagem mais orientada para a sustentabilidade, garantindo produção de alimentos suficiente para as gerações futuras. Neste trabalho, o cultivo de algas é discutido como uma solução para os desafios enfrentados pelo atual sistema de produção alimentar e deverá ser inserido no contexto da sustentabilidade e no debate sobre as mudanças climáticas.

O primeiro capítulo introdutório fornecerá uma visão geral da composição bioquímica de algas marinhas e do uso de biomassa das mesmas na indústria de alimentos para animais, com foco no mercado europeu e no género *Ulva*. *Ulva* contém vários compostos benéficos, como aminoácidos essenciais (EAA), fibras alimentares, ácidos gordos polinsaturados (PUFAs), minerais e vitaminas. As altas taxas de crescimento das algas, a distribuição omnipresente, a sua alta capacidade de absorção de nutrientes, a composição da dieta e a tolerância a diferentes condições ambientais tornam estas algas em potenciais candidatas ao cultivo, atraente para a indústria de alimentos e rações. Atualmente, a produção de algas marinhas ocorre principalmente em países asiáticos e na Europa. Portugal não está listado entre os principais países produtores de algas, com pouca tradição de consumo de algas comestíveis (EUMOFA 2017; Soares *et al.* 2017). Em Portugal continental, a indústria da aquicultura costeira baseia-se principalmente no centro e na costa sul do país e é dominada pela produção de moluscos (50%), dourada, robalo e pregado (Ramalho & Dinis 2011). Diferentes parâmetros de cultivo afetam a taxa de crescimento e a composição química bruta de *Ulva*, portanto a seleção do ambiente de cultivo afeta o rendimento dos compostos-alvo. A literatura sugere que a composição bioquímica das algas marinhas não depende apenas do ambiente de cultivo, mas que as espécies podem mostrar alguma estabilidade no seu perfil nutricional, mesmo entre ambientes (Angell *et al.* 2015; Gosh *et al.* 2012).

O segundo capítulo revelou que o cultivo de algas marinhas se destaca das técnicas atuais de produção de alimentos em termos de desempenho e sustentabilidade. O facto de não haver necessidade de terra arável ou utilização de água doce para produção torna-se cada vez mais relevante, considerando a expectável escassez de água terrestre devido à sobrepopulação e ao aquecimento global. Os benefícios ecológicos do cultivo de algas marinhas são maiores em

sistemas de cultivo aberto ou quando combinados com a produção de outras espécies em uma cultura multi-trófica integrada (IMTA), onde as preocupações ambientais associadas à aquacultura podem ser reduzidas. Considerando que, a pesca global está no seu limite sustentável e a produção de alimentos para animais é discutida de forma controversa, *Ulva* pode então ser considerada uma valiosa fonte de proteínas e PUFAs tonando-se um substituto sustentável da produção agrícola atual.

As condições de cultivo para *Ulva* podem ser otimizadas para obter rendimentos máximos de compostos-alvo para várias aplicações na indústria alimentar e de rações. O objetivo deste trabalho foca-se na identificação do potencial das espécies de *Ulva* no sul de Portugal, quer no seu uso na indústria alimentar, quer como um recurso mais sustentável em comparação com a produção alimentar existente atualmente. Para isso, testou-se se diferentes espécies ou condições ambientais têm um efeito mais pronunciado no conteúdo de proteínas e perfil de ácidos gordos nas algas marinhas, para descobrir se a seleção de uma espécie de *Ulva* é favorável em relação à outra para fins alimentares ou para produção de rações.
Diferentes espécies de *Ulva* foram recolhidas em locais costeiros e lagunares no sul de Portugal e identificadas geneticamente, usando o gene tufA. *Ulva rigida, Ulva flexuosa, Ulva fasciata, Ulva australis* e *Ulva compressa* foram as cinco espécies de *Ulva* identificadas. *U. australis* não foi relatada anteriormente na costa sul portuguesa.

As amostras foram analisadas quanto ao seu teor total de proteínas por combustão térmica. A quantidade de proteína total entre as espécies variou de 2.2% a 9.46% do peso seco (DW), com uma média de 4.35% para todas as espécies nos locais de amostragem costeiros e lagunares. Os números estão dentro, mas na extremidade inferior, do que foi relatado para as espécies de *Ulva.* Observou-se uma quantidade significativamente maior de proteína total em *U. rigida* amostrada em locais costeiros ($p < 0,01$) comparativamente aos locais de amostragem em lagoas, embora não existam diferenças significativas de proteína total entre as diferentes espécies da Ria Formosa ($p = 0.336$), suportando a hipótese de que o ambiente de cultivo tenha um efeito mais forte no perfil nutricional das algas do que na própria espécie.

Os perfis de ácidos gordos foram analisados por cromatografia gasosa - espectrometria de massa. O ácido palmítico (C16:0) foi o ácido gordo mais abundante em todas as espécies de *Ulva*, variando de 36.8% a 85.29% do total de ácidos gordos. Os ácidos gordos polinsaturados

(PUFAs) mais abundantes foram o ácido linoleico (C18: 2n-6) e o ácido α-linoleico (C18:3n-3). Os ácidos gordos saturados (AGS) compuseram a maior proporção de ácidos gordos, seguidos por PUFA e MUFA. No entanto, ao comparar a quantidade de PUFA dentro de *U. rigida* nos locais de amostragem costeira e de lagoa, a diferença não foi significativa ($p = 0.739$), enquanto *U. compressa* apresentou um conteúdo de PUFA significativamente maior que *U. rigida* e *U. fasciata* na Ria Formosa ($p = 0.0245$). Não foram encontradas diferenças significativas entre os perfis de ácidos gordos entre os locais da *U. rigida* ($p = 0.6713$) nem entre as espécies da Ria Formosa ($p = 0.1064$). Os resultados sugerem que *U. compressa* pode ser a espécie mais adequada para produção de PUFA e que o teor de proteínas nas algas pode ser maioritariamente suscetível às condições ambientais, comparativamente à composição de ácidos gordos.

Neste trabalho, as espécies *Ulva U. rigida, U. compressa, U.fasciata e U. flexuosa* foram todas identificadas como um recurso promissor para o cultivo, diversificando e suplementando a atual produção alimentar de forma sustentável no sul de Portugal. Principalmente tendo em conta o cultivo em sistemas de aquacultura multi-trófica integrada (IMTA), uma abordagem de cultivo através da qual a produção de algas marinhas se torna mais económica, podendo acrescentar benefícios ambientais à indústria aquícola nesta região de Portugal. Os serviços de ecossistemas fornecidos através do cultivo em sistema aberto podem ajudar a manter ecossistemas saudáveis no sul de Portugal.

Abbreviations

IMTA	Integrated Multi-Trophic Aquaculture
EAA	Essential amino acids
AA	Amino acids
PUFA	Polyunsaturated fatty acids
MUFA	Monounsaturated fatty acids
FAME	Fatty acid methyl ester
ALA	Alpha linolenic acid
LA	Linolenic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
FAO	Food and Agricultural Organization
DGE	Deutsche Gesellschaft für Ernährung
EUMOFA	European Market Observatory for Fisheries and Aquaculture Products
GHG	Greenhouse gas
FCR	Feed conversion ratio
DW	Dry weight
GC-MS	Gas chromatography - mass spectrometry
OECD	Organization for Economic Cooperation and Development.

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Chapter one - Overview on the properties and application of seaweeds in the food and feed industry

1. Introduction

In light of a growing world population and climate change, combined with an increasing awareness and demand for nutritiously high value food or functional foods, the food industry is facing various challenges for which new solutions have to be found. Due to their high nutritional value and numerous advantages over terrestrial food production, seaweeds have been suggested to be one such solution. The present work aims to identify the potential of cultivating the green macroalgae *Ulva* in southern Portugal as a sustainable resource for the food and feed industry in an environmentally friendly way.

The first introductory chapter will provide a general overview on seaweeds biochemical composition and the use of seaweed biomass in the food and feed industry, with a focus on the European market and the genus *Ulva. Ulva* contains various beneficial compounds such as essential amino acids (EAA), dietary fibers, polyunsaturated fatty acids (PUFAs), minerals and vitamins (Rasyid 2017; Jannat-Alipour *et al.* 2019). The seaweed´s high growth rates, ubiquitous distribution, high nutrient uptake capacity, dietary composition and tolerance to different environmental conditions generally make them attractive cultivation candidates for the food and feed Industry (Wu *et al.* 2018; Wichard *et al.* 2015). Today, seaweed production mainly occurs in Asian countries. In Europe, seaweed cultivation is emerging but Portugal is not listed amongst the major seaweed producing countries, with little tradition in the consumption of edible seaweeds (EUMOFA 2017; Soares *et al.* 2017). In mainland Portugal, the coastal aquaculture industry is mainly based in the center and south coast of the country and dominated by the production of molluscs (50%), seabream, seabass and turbot (Ramalho & Dinis 2011). Different cultivation parameters affect the growth rate and the crude chemical composition of *Ulva*, wherefore selection of the cultivation environment impacts the yield of target compounds. Literature suggests that the seaweed´s biochemical composition does not only depend on the cultivation environment, but that species can show some stability in their nutritional profile, even across environments (Angell *et al.* 2015; Gosh *et al.* 2012).

In chapter two, seaweeds will be embedded into the sustainability discourse. Therefore, the benefits and adverse effects of different cultivation systems will be examined and seaweeds

will be discussed in the context of global food security and climate change. Seaweeds can be cultivated in closed, semi-closed or open systems and in combination with already existing aquaculture structures in IMTA systems. With mainland Portugal's approximately 900km long coastline, the countries existing aquaculture activity, and its coastal lagoons, there are many potential sites for seaweed cultivation in southern Portugal (Ponte Lira *et al.* 2016). An analysis of benefits and adverse effects of cultivating seaweeds for the food and feed industry as compared to current agricultural practices and comparison of different cultivation practices will reveal the potential of *Ulva* as a sustainable food and feed resource and identify the most environmentally friendly cultivation approach.

In Chapter three, for the first time, *Ulva* species from southern Portugal will be genetically identified and screened for their food and feed potential. The hypothesis that the biochemical composition in *Ulva* is species dependent and influenced by environmental parameters, will be tested in this chapter. The aim is to understand whether species selection or environmental conditions have a stronger effect on the seaweed´s biochemical composition and thus it´s value as food or feed. Therefore, barcoding based species identification will be correlated with the amount of total protein and fatty acid (FA) profiles across environments and among species within the same environment. The Ria Formosa is a coastal lagoon system that is connected to the Atlantic Ocean through six major inlets and is located in the south of Portugal. The coastal lagoon is an important habitat for many species but also anthropogenically influenced by the cities, Faro, Olhão and Tavira, which are located at the lagoon *(*Mourade *et al.* 2019). The Ria Formosa as a potential cultivation site for *Ulva* for food and feed purposes will be compared to coastal locations in southern Portugal. Genetic identification of *Ulva* species through barcoding will increase knowledge on the species diversity in southern Portugal.
Overall, results will find implication in cultivating *Ulva* for the food and feed industry by identifying suitable candidates and potential sites for production.

1.1. Seaweeds nutritional components

Seaweeds can be distinguished into three different phyla: Ochrophyta (brown algae), Rhodophyta (red algae) and Chlorophyta, the green algae (Bunker *et al.* 2017). The phyla are not only distinct from each other based on their pigmentations but also possess different biochemical features, leading to variability in their suitability as food or feed. In society, health and wellbeing are closely linked to diet. Health promoting benefits are amongst major food

marketing tools and following dietary regimes to achieve certain body goals is common. A healthy diet includes the intake of all essential nutrients to avoid deficiencies but also to reduce the caloric intake to an extent not exceeding that of personal requirements to avoid obesity (Lichtenstein *et al.* 2006). Bioactive compounds are compounds taken in through the diet, influencing cellular activity (Biesalski *et al.* 2009). Global research effort on the nutritional components in seaweed has proven that they can be abundant sources of these bioactive compounds, containing protein (comprising all EAA), dietary fiber, PUFAs (including n-3 PUFAs), as well as minerals, vitamins and trace elements (Peng *et al.* in Tiwary & Troy 2015, pp. 79-84). These bioactive compounds are said to have antiviral, antibiotic, antioxidant, anti-inflammatory, antiherpetic, anticoagulant, antiangiogenic and antitumoral activity, to name a few (Carlucci *et al.* 1997; Dias *et al.* 2005).

This makes seaweed compounds interesting for vide application in the food and feed industry as well as for medical applications (Cardozo e*t al.* 2007). Associated health benefits derived from regular seaweed consumption are of diverse nature and range from anti-cancer, anti-obesity and anti-inflammatory activity to weight loss and cardiovascular health (Fleurence *et al.* 2012; Hafting et al. 2012; Brownlee *et al.* 2012; SAPEA 2017). Each seaweed, however, is unique in their nutritional profile, determined not only phylogenetically but also by environmental parameters. Changing environmental or cultivation conditions and the seaweeds life-stages cause fluctuation in seaweed growth performance and their nutritional properties (Wells *et al.* 2016). The protein content in a seaweed species for example, can fluctuate between 10-40% DW throughout the year (Pangestuti & Kim in Tiwary & Troy 2015, p. 127). To appraise and enhance the dietary value of the seaweed chosen for cultivation and to accurately position them in the food market, knowledge on the effect of *light, salinity, nutrient availability, aeration, water flow velocity* and *temperature* on the species is needed for successful cultivation.

A combination of identifying a species that obtains high amounts of the targeted compounds and knowledge on the optimal culture conditions for maximization of these is recommendable for selecting a suitable candidate for cultivation.

1.1.1. Proteins in seaweeds

Globally, there is an increasing awareness of the importance of protein in our nutrition. Proteins are essential structural and functional elements of our body to build and repair tissue, maintain a balanced weight etc. (DGE 2017). The amount as well as the quality of protein taken in through the diet is important (Maehre *et al.* 2014).

Proteins consist of amino acids (AA) of which there are twenty in total. Nine of these are so called essential amino acids (EAA), which cannot be synthesized by the human body and have thus to be taken in through food. Namely, these essential amino acids are: Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Taurine, Threonine, Tryptophan and Valine. Arginine, Histidine and Glycine are two AA that are considered additionally essential for some life-stages (Asif *et al.* 2011). The EAA profile of land-based crops is usually not complete and lacks one or more EAA (Hoffman & Falvo 2004). This makes seaweeds a potentially advantageous protein source, with an AA profile that can comprise all EAA (Paiva *et al.* 2017). Whereas protein content of brown seaweeds is generally low (5-15% DW), green seaweeds have moderate levels (9-26% DW) and red seaweeds can reach up to 47% DW. An exception in the green seaweeds is *Ulva* with protein levels of up to 33% DW, equivalent to common vegetable sources (Fleurence *et al.* 2017). Apart from phylogenetic differences, the amount of protein is directly correlated to the availability of nutrients, especially nitrogen. However, fluctuation in protein quantity and quality can also be attributed to seasonal variability and varying environmental factors (Moustafa & Eladel 2015).

Amongst the most common plant-based protein sources today is the protein rich soybean. Soy is not only cultivated as a protein source for human consumption but also as a feed ingredient for livestock or as a replacement of fishmeal in animal feed (Dersjant-Li 2002; Gibney *et al.* 2002, pp. 46-47; Fleurence 1999; Dawcynski *et al.* 2007). Their use as animal feed instead of for direct consumption is often criticized (Gibney *et al.* 2002, pp. 46-47; Fleurence 1999; Dawcynski *et al.* 2007). Studies on the AA profiles of seaweeds have shown that their AA composition can be superior to that of soybeans, justifying their consideration as a soybean meal replacement (Moustafa & Eladel 2015; Dersjant-Li 2002). *Ulva lactuca* e.g., grown with AD manure, had glycine concentrations higher than were found in soybeans and isoleucine, cysteine and serine comparable to that of soybeans (Nielsen *et al.* 2012).

The amount of protein in seaweeds is measured in two ways: either through the direct extraction of protein or through a nitrogen-to-protein conversion factor of 6.25 traditionally (Nielsen *et al.* 2012). However, a new conversion factor has been suggested, because whereas direct extraction methods underestimated protein content by 33%, the nitrogen-to-protein factor of 6.25 over-estimated the protein content by 43%. A new factor of 5 has therefore been suggested to be more accurate (Angell *et al.* 2015).

One major issue of high protein quality derived from seaweeds is their potentially low digestibility. Research on *in vivo* digestibility of protein does not show concordant results

(Holdt & Kraan 2011). The low digestibility of seaweed protein is often associated with high polysaccharide content and phlorotannins, as well as the lack of the proper digestive enzymes (Bleakley & Hayes 2017; Galland-Irmouli *et al.* 1999). Food processing, protein extraction methods and the enzymatic degradation of seaweed fibers have been suggested to improve protein digestibility (Pangestuti & Kim in Tiwary & Troy 2015, p. 129). Ultrasound - assisted extraction (UAW) or the use of pulsed electric field, or enzymes could be possible solutions (Bleakley 2017; Wijesinghe and Jeon 2012). A study comparing sonication, pH shift and accelerated solvent protein extraction methods for different seaweeds concluded that the pH shift method achieved the highest protein yields overall (Harrysson et al. 2018). Even though these methods have shown to improve digestibility and extracts have been successfully incorporated into e.g. pasta or bread, it is also a costly procedure, which could be reduced through product diversification (SAPEA 2017; Bleakley & Hayes 2017). A study on the digestibility of protein of Ulva armoricana showed that not only the composition of amino acids changed by season but also the digestibility, which may be explained by a structural change of protein (Fleurence *et al.* 1999).

1.1.2. Lipids in seaweeds

Lipids are a group of molecules of diverse structures, including fatty acids (Burdge & Calder 2015). Even though the amount of total lipids in seaweeds is low (not exceeding 5% of total dry biomass) seaweed lipid profiles comprise a substantial amount of fatty acids with varying chain lengths (Mišurcová, Ambrožová & Samek 2011). Fatty acids can be distinguished between saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) each differing in their molecular structure and their dietary effect (Mensik 2016). Whereas an excess consumption of SFA has been attributed to negative health effects such as an increased risk of heart disease, the consumption of PUFAs has been attributed to health benefits such as anti-inflammatory, antioxidant and antibacterial activity (White 2009; Pereira *et al.* 2012; Kendel *et al.* 2015). PUFAs can further be distinguished into n-3 and n-6 PUFAs, differentiated by their position of the double bond in the carbon chain. The n-3 PUFA, alpha linolenic acid (ALA) and the n-6 PUFA linolenic acid (LA) are considered important parts of the diet, since they cannot be produced by the body (Simopoulos 2000). ALA is converted to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two omega-3 fatty acids that are mainly found in fish oil. Since the conversion rate from ALA to DHA and EPA is considered low, EPA and DHA should also be taken in through the diet (Wells *et al.* 2017).

Since the same enzyme synthesizes ALA and LA, an optimal ratio of n-3 and n-6 PUFA is often discussed. Views on the importance of an optimal n-6 to n-3 PUFA ratio in the diet differ amongst scientists. On the one hand, an FAO expert consultation came to conclude that no specific ALA to linolenic acid (LA) ratio is necessary if total dietary intake of these PUFAs is met, with fish, seafood and seeds being the most abundant sources of n-3 PUFAs (FAO 2010). On the other hand, studies have shown that the ratio of ALA to LA is important for positive effects on the metabolism. The ratio is often not met due to an insufficient intake of n-3 fatty acids in today's diet (Pereira *et al.* 2012; Simopoulos 2000). Bearing in mind declining fish stocks and a trend towards vegetarian diets, an insufficient n-3 PUFA intake is likely and has increased awareness of the health benefit of n-3 supplementation (Martinson 2016; Swanson *et al.* 2012). Seaweeds have been studied as an alternative source to fish oil for supplementation of n-3 PUFAs and regarded as a good alternative (van Ginneken *et al.* 2011; Schmid *et al.* 2018).

Since the FA profile varies not only amongst phyla but even within the same species under different environmental parameters, the cultivation of seaweed for PUFAs can be optimized through strain selection and optimal cultivation parameters (Schmid *et al.* 2017).

1.1.3. Dietary fibers in seaweeds

Dietary fiber is the term for plant cell components indigestible in the human small intestine. This includes structural and storage type polysaccharides (Gibney 2002, p. 76). In the European food industry, dietary fiber from seaweeds is mainly commercially used as stabilizers, emulsifiers and thickeners (Cherry *et al.* 2019). Carrageenan, agars, agarose and alginates (mainly found in red and brown algae) are the most common storage polysaccharides and differ to those found in terrestrial plants (Stiger-Pouvreau *et al.* in Tiwary & Troy 2015, p.223). These types of carbohydrates are distinguished between water-soluble and insoluble (Pal *et al.* 2014). In seaweeds, polysaccharides, mainly soluble ones, usually make up the highest content of the crude chemical composition (Jiménez-Escrig & Sánchez-Muniz 2000). Polysaccharides have various beneficial effects on human health, especially in the gastrointestinal system. Since they are non-digestible, the dietary fibers pass through the intestinal tract, where their positive health benefits are manifold. Fermentable fibers can e.g. benefit colonic bacteria, ameliorate the gut microbiome or dietary fibers can interact with immune cells and improve immunity (Slavin 2013).

The health benefits associated with dietary fibers are manifold and range from antitumor, antiherpetic and antiviral activity to helping to prevent obesity through controlling appetite

(Mišurcová *et al.* 2012; Lovegrove *et al.* 2017). The Deutsche Gesellschaft für Ernährung (DGE) e.g., suggests a daily intake of 30g of dietary fibers (DGE 2012).

However, polysaccharides act in the gastrointestinal tract where they also have an effect on the digestibility of other nutrients, which can be both positive, e.g. by lowering serum cholesterol or regulating blood sugar. However, polysaccharides can also inhibit the digestibility of other relevant nutrients or influence the digestibility of e.g., minerals or dietary protein (Pal *et al.* 2014; Brown *et al.* 1999; Gudiel-Urbano & Goñi 2002; Jiménez-Escrig & Sánchez-Muniz 2000).

1.1.4. Minerals and vitamins in seaweeds

The mineral content of seaweeds can be highly variable and exceed the concentration of these elements in the surrounding water body due to high accumulation rates (Rupérez 2002). Minerals in marine algae are found to be comparable or even higher to those of land plants and comprise sodium, calcium, magnesium, potassium, chlorine, sulphur and phosphorus, amongst others (Mohamed *et al.* 2012; Paiva *et al.* 2014). The variability in mineral accumulation by different seaweeds offers opportunities to explore seaweed diversity for different mineral supplementation purposes for humans and animal feed (Circuncisao *et al.* 2018).

Iodine and selenium are two minerals in seaweeds that are especially interesting because they are mainly found in fish and not in other plants. Iodine deficiencies often occur in mountainous regions or in areas with iodine poor soils. Supplementation is thus often important, e.g. in the form of iodized salt and can be provided through seaweeds (Gibney *et al.* 2002, p. 211; Vazhiyil 2008). However, if iodine is consumed in high amounts, it may negatively affect thyroid functioning. It is thus important to state the iodine content of edible seaweed products to prevent the risk of overconsumption (Yeh *et al.* 2014).

Generally, seaweeds are a good source for many vitamins, especially water-soluble B Vitamins and the vitamins A, C, D, E and K but also water insoluble Vitamins. This makes seaweeds a valuable candidate as a vitamin source in human nutrition and animal feed (Wells *et al.* 2017; Skrovánková 2011).

1.2. Seaweed nutritional value - challenges and concerns

The nutritional value and potential health benefits associated with the consumption of seaweed is not determined by their nutritional profiles alone. The fate of the nutritional constituents derived from seaweeds, is determined by multiple factors:

First of all, the potential health benefits of seaweed-derived compounds are determined by their bioavailability, the extent to which a nutrient is accessible to the human metabolic system. Bioavailability of seaweed compounds and protein digestibility has been poorly understood and studied little to date, especially in humans (Hambidge 2010; Wells *et al.* 2016; Fleurence 1999). To overcome low in *in vivo* digestibility of seaweed protein, associated with their high dietary fiber content, food processing and protein extraction methods have been suggested (Bleakley & Hayes 2017; Fleurence 1999). Even though these have shown to improve digestibility and extracts have been successfully incorporated into e.g. pasta or bread, it is also a costly procedure, which could be reduced through product diversification (SAPEA 2017; Bleakley & Hayes 2017). Increased research effort on digestibility is required to overcome these uncertainties and improve seaweed quality as food and feed. This can be driven by the increasing interest in seaweeds in western societies (Admassu *et al.* 2015; Hafting *et al.* 2012). Secondly, digestive enzymes in the human gut microbiome can differ among nationalities and can be a determining factor for the health benefits derived from foods, making generalized statements misleading (Chen *et al.* 2016; Wells *et al.* 2017).

Furthermore, the form of processing, for example the drying of the biomass, influences the nutritional composition and thus changes the amount of potential health promoting compounds available from the seaweed (Jiménez-Escrig *et al.* 2001; Cox *et al.* 2011). For budgeting food intake, it thus makes a difference, whether the seaweed is consumed in its whole, fresh or dried form or whether extracts are incorporated into other foods. Processing the seaweed after harvest can thus be an additional means to improve the amount of target compound for a given industry. Lastly, the amount of seaweed consumed should be considered, as the daily recommendation for seaweed consumption does not exceed 8g DW, possibly significantly reducing the uptake of sufficient health promoting compounds (MacArtain *et al.* 2007). An Australian study considered the dietary intake of per-portion amounts of seaweeds and whole foods and came to conclude that dietary fiber, calcium (for *Ulva* spp.), potassium, iron, some vitamins and protein are the nutritionally relevant compounds in seaweeds (Winberg, Gosh & Tapsell 2009). The micronutrient iodine is often discussed in terms of safety of seaweed consumption and amounts can vary widely amongst seaweeds. An iodine deficiency is common in some parts of the world and can negatively affect health, when not supplemented, e.g. through iodine enriched salts. Seaweeds could provide a good source of iodine. An elevated intake of iodine, however, may be a health hazard due to negative effects on the thyroid system (Gunnarsdottir & Dahl 2012; Leung & Braverman 2014). For a safe consumption, the iodine content should be checked for

seaweeds and stated on the packaging and regulations should be established (Circuncisão *et al.* 2018).

Seaweeds are known to have high absorption capacities and have for example proven to be successful candidate in IMTA systems for bioremediation and can be used for biomonitoring (Dadolahi-Sohrab *et al.* 2011; Chung *et al.* 2002).

Toxins, heavy metals and metalloids that have been absorbed by seaweed raise health concerns associated with their consumption. Therefore, careful monitoring is required for safe seaweed consumption, especially when harvested or cultivated in the natural environment. However, health risk assessment conducted for various seaweeds in different geographic locations have resulted in low health risk for the consumer, especially since the amount consumed is usually small (Chen *et al.* 2018; Desideri *et al.* 2015). All the above-mentioned factors should be taken into consideration, when promoting seaweeds as a health food to avoid consumer mislead.

1.3. Utilization of cultivated seaweed in the food and feed industry

Seaweed biomass can either be cultivated or obtained through harvest from the wild. Globally, aquaculture is the major source of seaweeds and over 96.5% of seaweeds used for commercial application are produced in mariculture, the farming at sea (Chopin 2018).

Worldwide, 30.1 million tons of seaweed are produced annually, as compared to a production of 80.0 million tons of food fish. Ninety-six percent of this output has been used for direct consumption or further processing (FAO 2018). In terms of volume, seaweeds dominate the farming activity in inland, marine and coastal aquaculture and is practiced in 50 countries, dominated by Asian countries, with leading farming countries being Indonesia, China, Korea, Japan and the Philippines. About 2220 different species are cultured in these countries (FAO 2016; FAO 2018). Of the about 250 commercially used macroalgae, 150 species have been used for human consumption (Kumari 2010). Of the approximately 150 seaweeds used for human consumption, *Laminaria japonica* (kombu), *Undaria pinnatifida* (wakame), *Palmaria palmata* (dulse), *Porphyra tenera* (nori) *Ulva lactuca* (sea lettuce), and *Ulva pertusa* are some of the most cultivated species with application in the food industry (Chopin 2018). Edible seaweeds can be consumed in their whole form, raw, dried and as a mix of species. Seaweeds nutritional compounds are also extracted and incorporated into other foods, creating so-called "functional foods" that promote health benefits exceeding those of basic nutrition (Gibney *et al*. 2002, pp. 9-10). Extracts of certain compounds can further be sold as food supplements, e.g. for people following a vegetarian or vegan diet.

For commercial application, robust strain selection and new strain development with reduced or absent reproduction, high growth rates, high resilience and abundance of target compounds is desirable and could be achieved through the development of new strains and improved breeding technologies (Kim *et al.* 2017). A study on a sterile *Ulva* strain e.g., showed up to 40% higher N/P uptake rates, higher SGR, higher EAA percentage and an increased lipid (especially PUFAs) and ash content as compared to the wild strains, as well as reduces swelling and oil holding capacity (Gao 2016). This suggests that more research effort in finding robust strains or creating sterile strains can increase the potential of cultivating *Ulva* for the food and feed industry. A study on the effect of processing seaweeds concluded that neither drying nor canning negatively affected the seaweeds nutritional value (Sanchéz-Machado *et al.* 2004). Apart from direct consumption, seaweeds are furthermore utilized in the food and feed industry as animal feed, fodder, fertilizer, fungicides, herbicides, condiments or as a resource of phycocolloids such as agar, alginate, and carrageenan (Elizondo-González *et al.* 2018; López-Mosquera 2011; Yaich *et al.* 2011).

Another way of utilizing the biochemicals of seaweeds is to enhance the nutritional quality of other foodstuff. For meat, dairy products and fish an increase in mineral content has been achieved by including seaweeds into the feed formula (Circuncisao *et al*. 2018).
The future of seaweeds in the food and feed industry will include the development of high value markets for functional foods, calling for improved cultivars, quality control and traceability (Hafting *et al.* 2012; Hafting *et al.* 2015). High growth rates (quantity) and control of the desired compounds (quality) are essential to assure good quality biomass production for the food and feed industry (FAO 2016; Hafting *et al*. 2015).

1.4. Seaweeds in the European food market

In Asian countries such as China and Japan seaweeds are parts of the daily diet (Lahaye 1991). In Europe, seaweed consumption has a tradition in some countries such as France and Britain but seaweed products for consumption mainly serve niche markets and are currently underexplored. They are mainly used for the extraction of thickening agents, which is astonishing, considering their proven health benefit (Fleurence 2012; Paiva *et al.* 2017). Harvest from the wild is currently the primary source of seaweed biomass in Europe. Only 0.3% (93.000 tones) of the biomass produced in the world comes from European production with France (63%), Ireland 32%), Spain (2.3%) and Italy (1.3%) being the main producers. Portugal is not being listed amongst the major producers (EUMOFA 2017).

Recently, the potential of cultivating seaweed in Europe has gained an increasing interest. Root causes of this are: environmental constraints of current food and feed production techniques, declining natural resources, the acknowledgement of the health benefits derived from seaweeds, their economic potential and the influence of Asian cuisine in Europe, such as the use of *Porphyra*, commonly known as "Nori", to wrap sushi (EUMOFA 2017; FAO 2018; Brownlee *et al.* 2012; Chung *et al.* 2017).

A major bottleneck is the lack of knowledge on how to incorporate seaweeds in the daily diet and include them into cooking practice as well as their unaccustomed taste. Seeking inspiration from Asian cooking and experimenting with different tastes and recipes can help to overcome this. Restaurants, especially star cuisines are increasingly incorporating seaweeds into dishes and can further contribute to accustomed seaweed meals (Mouritsen 2012).
Whereas malnutrition is mostly not a concern in Europe, a high awareness of the consumption of micronutrients, functional- and health foods make seaweeds a highly interesting candidate in the European food market (Long, Ortiz-Monasterio & Banziger 2014). France was the first European country to officially authorize the commercialization of 21 seaweeds as vegetables and condiments and the market for seaweeds is expanding (Le Bras *et al.* 2015). Viewing this development and the high potential of an increasing demand for seaweeds in Europe, concerns are being raised regarding harvesting practices that overexploit natural resources and emphasize the importance of implementing seaweed harvesting management (Buschmann *et al*. 2017; Rebours *et al.* 2014). Resource access grant and regulatory bodies differ greatly amongst European coastal countries. In Ireland e.g., Irish authorities must grant seaweed harvesting. Recently, the granting of licenses has stopped due to a lack of compliance with the practice to the Natura 2000 conservation objectives (Fleurence & Levine, p.32, 2016). As has been the case with for fed-fish cultivation as a response to declining natural stocks and conservation measurements, increasing farming activity of seaweed is to be expected in Europe.

Seaweed cultivation has its origins in Asia where immense research effort and experience has contributed to an optimized cultivation. In countries like Korea or Indonesia, low wages and a large coastline suitable for seaweed cultivation facilitates the profitable cultivation of seaweeds. A lack of "know-how", high wages and economic viability are major bottlenecks for seaweed cultivation in Europe. Limited and highly regulated coastal areas in Europe suggest a different cultivation approach as compared to Asian countries (pers. communication).

A more European based production of seaweeds would have various advantages compared to imported products from other continents: A local seaweed industry would reduce emission intensive import. European products would have a higher traceability and can be more regulated under the EU food regulatory body. The local markets can be supported. Research on new strains, technologies and techniques adapted to local conditions through research projects can support this development and overall create new jobs and market opportunity.

1.5. Seaweeds as animal feed

There has been an increased research effort to diversify animal feed for terrestrial as well as for aquatic animals and to find alternative feed sources, wherefore knowledge on nutritional needs of farmed animals has increased. In times of intensive animal production, animal performance and health are of uttermost importance. Currently, a lot of alternative feeds contain protein rich plant sources such as corn, soy, or peas and additional micronutrients, vitamins and minerals (Ayadi *et al.* 2012). The cultivation of these, however, is subject to environmental issues and requires freshwater, arable land and the cultivation of feed crops stands in direct competition for food (Oppenheimer *et al.* 2014; Spierts & Ewert 2009). Furthermore, some plants are not digested easily, due to anti-nutritional components or lack certain amino acids, such as lysine or methionine, wherefore feed often needs to be diversified or processed to meet the animal's requirements (Kaushik & Hemre 2008; Pantalone 2012; Dersjant-Li 2002). Seaweeds have proven to be viable sources of high-value protein, PUFAs and provide important minerals and vitamins in terrestrial as well as aquatic animal feed. Studies on algae supplementation in terrestrial animal feed showed positive effects on physiological and metabolic pathways, gut function and improved overall performance and animal health (Makkar *et al.* 2016; Heim *et al.* 2014; Bendary *et al.* 2013).

Various studies have also investigated the potential of replacing aquatic animal diets by seaweeds. This is especially helpful in times of overfishing and declining fish stocks. Replacing conventional feed partially with *Ulva spp.* has proven successful e.g., in the case of shrimp, gilthead seabream, abalone and sea urchins (Cruz-Suárez *et al.* 2010; Cyrus *et al.* 2015; Bolton *et al.* 2009; Emre *et al.* 2013). However, the successful replacement of fishmeal is depending on the species. In some cases, a partial replacement of fishmeal by *Ulva* did not negatively affect growth performance, whereas higher amounts showed a negatively affect, making a universal feed formula impossible (Diler *et al.* 2007; Abdhel-Wahab 2016).

There are also constraints using seaweeds as livestock feed. The total amount of EAA obtained by seaweeds in their whole form is insufficient to meet the requirement of most livestock, so that concentration and extraction procedures are necessary to obtain sufficient amount (Raymond Angell 2016; Bikker *et al.* 2016). Current high production costs (up to ten times higher for DHA derived from seaweeds as compared DHA derived from fish oil) make the inclusion of seaweeds in aquaculture feed for PUFAs (being the highest expense of farmers) economically not feasible and halts development (Rajauria *et al.* in Tiwari & Troy 2015, p.327). Nevertheless, the increasing interest in seaweeds as a source of biofuel leads to enhanced research and technology optimization in the field. It can contribute to overcome this bottleneck (Mazarrasa *et al.* 2014). An expected increase in crop prices, as well as an increase in cost for fish oil and fish meal may be tipping points to make the incorporation of seaweeds economically feasible in the future (Trostle 2010; Tacon & Metian 2008).

1.6. The genus *Ulva*

Seaweeds of the genus *Ulva* are eukaryotic, photosynthesizing organisms with a wide distribution along the coasts of the world's oceans and can also be found in brackish- and freshwater (Bunker *et al.* 2017, pp. 230-231). The genus *Ulva* belongs to the family *Ulvaceae* and the division *Chlorophyta.* They are characterized by their green color, given by their pigments chlorophyll a and b (Guiry 2019). The green color of *Ulva* is intensified with an increase in tissue nitrogen and fading colors can be an indicator of nutrient poor conditions for farmers (Robertson-Andersson 2009). *Ulva* often thrives in polluted areas where competition with other macrophytes is low. It can form green tides, which can become toxic when hydrogen sulphide is produced. *Ulva* can be free floating or a basal disk can fix the thalli. Ulva morphology is of diverse nature and varies not only among species but also maturity stage of the seaweed. The mature thallus of *Ulva lactuca* e.g., is a flattened, distromatic, lettuce-like blade (see Figure 1) comprised of two cell layers giving the species its common name “sea lettuce” (Bunker *et al.* 2017, p. 218). *Ulva* can also be filamentous and be attached to substrate such as e.g. *Ulva compressa* (see Figure 1).

In algaebase, currently 403 species names are listed in the genus *Ulva* with 132 being accepted taxonomically (Guiry 2019). *Ulva* can be a suitable candidate for cultivation due to high growth rates, ubiquitous distribution, opportunistic nature and tolerance to fluctuation in culture conditions due to a persistent stress response. Their high nutrient uptake rates make them especially interesting candidates for the cultivation in IMTA and can also serve to identify water

quality (Wichard *et al.* 2015, Neori *et al.* 2004; Lawton *et al.* 2013; Vermaad & Sand-Jensen 1987).

1.6.1. Taxonomy and life cycle of Ulva species

For proper selection of *Ulva* for cultivation, correct species taxonomy is essential and often

Figure 1: Lettuce like thalli of *Ulva lactuca* attached at basal disk (left). Image source: http://www.irishseaweeds.com/sea-lettuce-ulva-lactuca/. Fillamentous thalli of *Ulva compressa* (right). Image source: V. Mirgolth - Eigenes Werk, CC BY-SA 3.0.

confused or lacking for *Ulva* species (Bolton *et al.* 2016). The root cause of this being that *Ulva* morphology is highly variable and *Ulva* taxonomy has been solely based on morphology in the past. Morphological changes occur under changing environmental factors and further depend on thallus age, the reproductive stage and some *Ulva* species are morphologically indistinguishable (Bunker *et al.* 2017, p. 218; Blomster *et al.* 2002; Rybak 2018). Recently, new molecular techniques have proven to be efficient for genetic identification and improve species taxonomy (Bunker *et al.* 2017, p. 218). This development also helped to facilitate the distinction of *Ulva* and *Enteromorpha*, two genera whose taxonomy has been often confounded in the past (Hayden & Waaland 2002).

The life cycle of *Ulva* (see Figure 2) is strongly influenced by biotic and abiotic environmental factors resulting in a switch from vegetative to reproductive modes e.g., through temperature drop or change in photoperiod, stimulating germination (Han *et al.* 2002; Hurd 2015). The life cycle of *Ulva* consists of two distinct phases: a haploid (n) gametophyte and a diploid (2n) sporophyte phase which are isomorphic. Haploid plants produce flagellated gametes, which do or do not fuse. The sporophyte phase produces quadri-flagellated zoospores, which develop into the haploid gametophytes that are morphologically identical (isomorph) to the sporophyte and produce male and female gametes. The fusion of male and female gametes forms zygotes

(2n), which grow into the mature sporophyte (Bunker *et al.* 2017, p. 219). Thus, in *Ulva*, reproduction either occurs asexually by quadri-flagellated zoospores or sexually by biflagellate anisogamous (Guiry 2019).

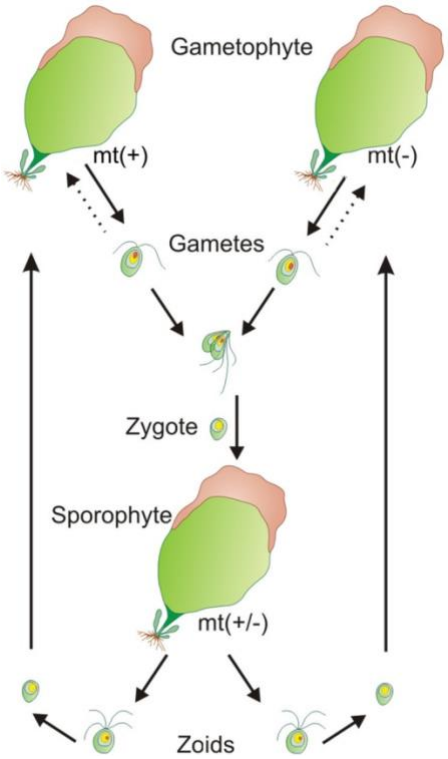

Figure 2: *Ulva* bi-phasic life cycle, distinguished in isomorphic haploid (n) and diploid (2n) stages. Male and female gametes fuse into zygotes, growing into mature sporophytes (mt+/-); mt = mating types. Image available via license: CC BY 4.0.

1.6.2. Dietary composition of Ulva species

Ulva often stands out in the *Chlorophyta* phylum for its favorable biochemical composition, being higher in proteins, dietary fiber, minerals, vitamins and (PUFAs) than most other green algae (Carl *et al.* 2014; Vázquez-Rodríguez & Amaya-Guerra 2016; Yaich *et al.* 2011). Often, the biggest proportion of *Ulva* crude chemical composition is dietary fiber. These can make up to around 60% of the seaweeds DW and represent and abundant source of polysaccharides, containing mostly rhamnose, uronic acids, xylose, arabinose and glucose (Ortiz *et al.* 2006; Yaich *et al.* 2011; Abdel-Fattah & Edrees 1972; Pena-Rodriguez *et al.* 2011). The proportion of soluble and insoluble fraction varies amongst *Ulva* species. For *Ulva clathrate* for example, 8.7-10.7% of insoluble fiber and 15.6 - 16.6% soluble fiber have been reported, whereas *Ulva lactuca* showed more insoluble than soluble fiber at 34.27% and 20.53% respectively (Vázquez-Rodríguez 2016). In *Ulva*, the major water - soluble, sulphated polysaccharide is ulvan and can represent up to 18-29% of the DW (Laheye & Robic 2007; Pérez *et al.* 2016). Ulvan has shown to have, e.g. antioxidants, immunomodulation and tissue-engineering activity but is less

exploited and studied than polysaccharides from red or brown seaweeds (Laheye & Robic 2007; Vázquez-Rodríguez *et al.* 2016; Berri *et al.* 2017). Ulvan also has multiple applications outside the food/feed industry and can be of interest for various applications, such as in the pharmaceutical, agricultural or chemical industry, for example as functional biopolymers or representing an antibiotic alternative in animal feed. This justifies more research attention on ulvan (Lahaye & Robic 2007, Chiellini & Morelli 2010; Cunha & Grenha 2016).

Ulva can show high protein contents of up to 26% DW, protein being the second most abundant component reported for *U. lactuca* (Pirian *et al.* 2018; Amaya-Guerra 2016; Fleurence 1999). Research on *Ulva* derived protein digestibility showed high results with 87% in vitro digestibility for *Ulva pinnatifida*, 95% for *Ulva petusais* and 86% digestibility of protein concentrates from *Ulva lactuca* (Fleurence 1999; Wong & Cheung 2011). *Ulva* protein can also comprise all EAA. This makes *Ulva* seaweed superior to some of the currently used plant protein sources, which often lack one or more EAA such as lysine and methionine, which often have to be supplemented in plant-based animal diets (Nielsen *et al.* 2012; Boland *et al.* 2013). A study by Nielsen (2012) on *Ulva lactuca* grown with AD manure e.g., found Glycine concentrations higher than in soybeans and isoleucine, cysteine and serine comparable to that of soybeans. The total amount of EAA found in *Ulva* has also been reported to be greater than that found e.g. in soybeans (Aquilera-Morales *et al.* 2005).

The total lipid content reported for *Ulva* is generally making up around 1.7% of the DW (Paiva *et al.* 2017; Shanmugam & Palpandi 2008). However, *Ulva* species can show an exceptionally high concentration of n-3 PUFAs within the *Chlorophyte* phyla, particularly of α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Pereira *et al.* 2012). A study that combined the findings of 74 different reported FA profile of *Ulva* showed, that *Ulva* FA profile is comprised of 35.3 ± 13.7% SFA, 15.9 ± 6.6% MUFA and 41.8 ± 15.6% PUFA (McCauley *et al.* 2016). High amounts of PUFA have been confirmed by other studies, e.g. with 29% of total lipids being n-3 PUFAs in *Ulva armoricana* and 33% of lipids being PUFAs in *Ulva clathrate* (Kendel *et al.* 2015; Shanab *et al.* 2018). Furthermore, a health-promoting ratio of n-6 to n-3 PUFA has been reported for *Ulva* (Pirian *et al.* 2018). Amongst seaweeds, *Ulva* can therefore be considered a valuable source of dietary important n-3 FA (Pereira *et al.* 2012).

Ulva seaweeds can also provide a good source of minerals and vitamins, such as vitamin A, B1 and B2, which have been reported e.g. for *Ulva lactuca* (Rasyid 2017). Minerals found in *Ulva* will dependent on the minerals found in the culture medium and are not generalizable. However, an 8g portion of *Ulva* - which is the daily portion of seaweed usually consumed in Asian

countries - can significantly contribute to the recommended daily mineral uptake, since *Ulva* species can be especially rich in Ca, Mg, Fe and Mn (Neto *et al*. 2018; Rasyid 2017; Circuncisão *et al.* 2018).

1.7. Effect of cultivation parameters on the market relevant characteristics of *Ulva*

The quantity (growth performance) and quality (crude chemical composition) of seaweed biomass obtained from culture is not only species dependent but also highly affected by the cultivation conditions. Growth performance and the amount of target compounds fluctuate due to changes in *temperature, light, salinity, nutrient availability* as well *as aeration, water flow velocity, seedling- and culture density*. However, that growth performance in *Ulva* is also species dependent has been suggested by a study, showing that growth of *Ulva prolifera* was higher in growth than naturally co-occuring *Ulva linza* for the same temperatures tested (Luo, Liu & Xu 2012).

In seaweed, growth is mainly affected by sufficient nutrient supply of phosphor, nitrogen and carbon in an optimal ratio, sufficient light, and a tolerable temperature, all of which peak at a certain optimum. For most seaweed, the highest photosynthetic rates, measured by CO_2 consumption or oxygen production per unit time, occur at longer daylengths and higher temperatures and follow a seasonal pattern (Litter, Murry & Arnold 1979; Viaroli *et al.* 2005). For *Ulva* species, reported growth rates range from 9.47 - 22.18% day^{-1} for *Ulva intestinalis* and similar growth rates of 20% day^{-1} were reported for *Ulva rigida* (Ruangchuay *et al.* 2012; Ashkenazi, Israel & Abelson 2019). Temperature alone explained 44% of growth variation in *Ulva curvata* and light, temperature and nitrogen supply combined explained 53% (Duke & Ramus 1989). *Ulva* growth correlates to the natural conditions of the species latitudinal distribution and temperature is an important factor controlling seasonal and annual growth fluctuation (Steffensen 1976).

Temperature also affects the seaweeds life cycle. In *Ulva fasciata,* sporophyte reproduction was observed under higher temperatures than those naturally occurring in the seaweed´s habitat, whereas lower temperatures lead to gametogenesis (Mohsen, Nasr & Metwalli 1973). This was confirmed by a study on *Ulva fenestra*, where optimal vegetative growth was observed at 10°C, whereas an increase or decrease of 5°C lead to the offset of Sporo- and gametogenesis, respectively (Kalit & Tytlianov 2003). So, in order to keep *Ulva* in a vegetative state, favorable

for cultivation, a certain temperature needs to be kept avoiding loss of biomass through offset of reproduction. As is true for temperature, saturation irradiance for *Ulva* depends on the species latitudinal distribution and varies among *Ulva* species. However, through dark respiration, *Ulva* can overcome periods of low light availability, helping the seaweed to thrive in naturally fluctuating conditions (Xiao *et al*. 2016; Sand-Jensen & Borum 1988). Growth of seaweed is further limited by the macro-nutrients nitrogen, phosphorus and carbon (Harrison & Hurd 2001).

Ulva generally has a high nutrient uptake capacity. A study showed that ammonium removal efficiency of *Ulva* was at 97%, and even though ammonium was removed first, almost all nitrate in the water was also removed (Aníbal *et al.* 2014). Salinity tolerance of *Ulva* species is generally considered high and has been confirmed by Ruangchuay (2012) showing that germling clusters of *Ulva intestinalis* grew well in various salinity ranges. Furthermore, aeration and water movement are crucial factors influencing growth, since they ensure sufficient light exposure of the biomass and passing of nutrients over the thallus for increased absorption whilst removing sediments and epiphytes from thalli, reducing boundary layer. Water movement becomes especially relevant in nutrient limited conditions at a Nitrogen supply below 4 g N m^{-2} day^{-1} but showed no further benefit for *Ulva lactuca* under nutrient sufficiency (Msuya 2008; Hurd 2000). It is also the highest cost of production and intermediate aeration has been suggested, with 8h of no aeration at night, showing no negative effect on algae growth (Msuya 2008; Caines *et al.* 2014). Ultimately, density of seedlings and stocking density have an effect on growth of *Ulva* due to matt formation and competition for nutrients, light and dissolved gases of the thalli (Ruangchuay 2012; Sherrington 2003).

Different cultivation parameters may not only affect growth rate but also the crude chemical composition of *Ulva*, thus influencing the nutritional quality of the seaweed. Literature suggests that biochemical parameters vary amongst taxonomic groups and that genotypes can show some stability in their nutritional profile, even across environments (Angell *et al.* 2015; Gosh *et al.* 2012). Understanding the metabolic pathway of the seaweed species in cultivation can be a valuable means to manipulate culture conditions in order to obtain maximum yields of the target compound. The synthesis of protein, carbohydrates, lipids, vitamins and the amount of minerals is strongly influenced by the abiotic factors acting on the seaweed. Certain conditions e.g., result in a stress response in the seaweed, changing the crude chemical composition.

Lipids serve as a storage product in seaweed that can be catabolized as metabolic energy (Guschina & Harwood 2009; Nelson, Phleger & Nichols 2002; Floretto *et al.* 1993). Generally,

at lower temperatures, the amount of total lipid in seaweed increases, whereas a decrease in fatty acid content at an increasing total lipid content has also been reported (Mohsen, Nasr & Metwalli 1973; Gosh *et al.* 2012). A meta study on the effect of different environmental conditions on the fatty acid profiles of *Ulva* seaweeds found that sufficient nutrients in the cultivation medium are beneficial for the production of seaweed oil products with an increase in PUFAs under high nutrient conditions. Nutrient availability has been found to have the strongest influence on the fatty acid composition in *Ulva* (McCauley *et al.* 2016). Light also strongly influences the lipid composition with a decrease of C16:4n-3 and C18:4n-3 fatty acids under low light and an increase of the fatty acid C16:0 under high light conditions reported for *Ulva pertusa* (Harwood 1998; Floreto *et al.* 1993).

As for protein, in *Ulva fasciata* higher amino acids and sugar contents have been observed under higher temperatures (Mohsen, Nasr & Metwalli 1973). Generally, higher temperatures, light and nutrient availability drive photosynthesis and increase the uptake rates in seaweeds, wherefore more nutrients can be absorbed (Harrison, Hurd 2001; Wheeler & Srivistava, 1984). Protein content in seaweed is directly related to nitrogen stored in the tissue. If nitrogen supply exceeds that of demand for growth, it can accumulate as amino acids, especially so in seaweeds with a high surface area to volume ratio (SA:V), thus increasing the protein content (Rosenberg & Ramus 1984). The concentration and quality of protein also varied for different salinities in *Ulva* species. An augmentation in salinity influences protein quality by increasing the total amount of amino acids. However, the amino acid profile changed with an increase of some amino acids whilst decreasing others (Patil & Imchen 2018; Floreto &Teshima 1998).

Carbohydrates in seaweeds are a proxy for photosynthetic rate and are the energy and storage carbon products, serving as photosynthetic reserves (Raven & Beardall 2003). This suggests that when photosynthesis is reduced, the organic solutes will be reduced. The amount of nitrogen available further influences the carbohydrate synthesis and thus the dietary fiber content. Under nitrogen enriched conditions, the C:N ratio is reduced and thereby reduces fiber content in the seaweed, whereas fiber content increased in nitrogen limited culture conditions in *Ulva rigida* (Pinchetti *et al.* 1998).

1.8. Chapter summary

This chapter highlighted some important aspects when debating seaweeds' potential as an alternative food and feed resource in Europe. It has become evident that the crude chemical composition of seaweeds generally has great potential as human food or animal feed, with various potential health benefits for animals and humans. *Ulva* is no exception and especially interesting for cultivation due to its high tolerance to different cultivation parameters. It has been shown that growth performance and the nutritional constituents are strongly influenced by the cultivation parameters. Seaweed quantity and quality can thus be manipulated through culture conditions to optimize production for the target industry. When cultivating *Ulva* for the target compounds protein and PUFAs, sufficient nitrogen supply and optimal temperature have been identified as the two most important cultivation parameters to consider. Ultimately, even though current seaweed cultivation is mostly located in Asian countries, there is a growing interest in seaweeds as food or feed production in Europe. This suggests an opportunity to expand the European cultivation of seaweed in closer proximity to the consumer under the EU regulatory body. Concerns and uncertainties associated with seaweed consumption were highlighted and suggest more in-depth research, consumer information and technology improvement.

Chapter two - Seaweed cultivation under the aspect of sustainable development

The cultivation of seaweeds, as photosynthesizing filter organisms, has various advantages over terrestrial food and feed production in terms of sustainability. This is especially interesting in times when global food security is one of the major challenges that society is facing today. Apart from an overall increase in food demand due to increasing population, there are some general trends that can be observed in the food industry of industrialized, developed countries today. A major trend is the consumption of highly processed, energy dense foods that are easy and ready to prepare for high convenience (Monteiro *et al.* 2013). The current western diets are mostly comprised of saturated fats and simple carbohydrates as their major food components. This diet is said to increase the risk of many health issues such as cardiovascular disease, obesity, type-2 diabetes (Simopoulus 2016; Kanoski & Davidson 2011).

On the other hand, an overall increase in wealth, goes hand in hand with an increasing awareness and demand for a more diversified and nutritionally high value diet. This led to the creation and marketing of so-called "functional foods", being foods that promote health benefits exceeding those of basic nutrition (Siro *et al.* 2008). This development also strengthened the awareness of health benefits derived from a high protein diet, increasing the overall global demand for protein for human consumption but also to feed animals (Henchion *et al.* 2017). Furthermore, higher incomes are positively correlated with a demand for livestock and animal derived products, driving the meat and dairy industry to increase its production (WHO). At the same time, in industrialized nations, a shift to favoring vegetarian and vegan diets, motivated by environmental, health and animal welfare concerns can be observed (Janssen *et al.* 2016). Whereas a diversified plant-based diet can provide all EAA needed for a healthy diet, obtaining sufficient amounts of PUFAs can be challenging. These are often supplemented in vegetarian or vegan diets, especially for omega-3 FA such as DHA and EPA, because they are only found in aquatic organisms (Gladyshev 2013). As has been discussed in the previous chapter, seaweeds can be both, a source of EAA and PUFAs, justifying an examination of their potential for food and feed purposes based on these compounds.

Despite cultivating seaweeds for the food and feed industry, a lot of attention has been drawn to their potential as a renewable energy source and their carbon sink potential, relevant topics in the climate change debate (Alvarado-Morales 2013; Krause-Jensen *et al.* 2018). Seaweed cultivation systems are of diverse nature and will be examined for their sustainability performance in this chapter. Furthermore, benefits and adverse effects of each cultivation

technique in terms of sustainability will be identified. Seaweed cultivation will be embedded into the broader context of sustainable development and climate change. Ultimately, this chapter will allow drawing conclusions on the extent to which seaweed biomass from cultivation can contribute to overcome global food challenges.

2. Sustainable development

In light of finite resources and climate change, the importance of sustainable development, being *"development that meets the needs of the present, without compromising the ability of future generation to meet their own needs"* is long recognized by the United Nations (Brundtland 1987). The three pillars of sustainability (social, environmental and economic sustainability) is a well-known concept that integrates all three pillars to approach and solve sustainability problems (Michelson & Adomßent 2014, p. 28). Environmental sustainability is related to impacts on and cautious handling of ecosystems, resources, and the greater environment we depend upon. To achieve sustainable development, various steps have been taken on national and international level, recognizing and including food and feed production. With the 2030 agenda for sustainable development, the United Nations set the target to *"achieve food security, improved nutrition and promote sustainable agriculture"*, aiming at resilient agricultural practices that increase productivity and production and are adapted to climate change impacts. The aim is further to "*conserve and sustainably use the oceans, seas and marine resources"* and to *"protect, restore and promote the sustainable use of terrestrial ecosystems"*, as well as to ensure *"sustainable consumption and production patterns"* and the sustainable management of water resources (United Nations 2015).

The European Union established the goal of sustainable growth in their 2020 Strategy, being the promotion of a more resource efficient, greener and more competitive economy (European Commission 2010). The blue growth strategy has emerged as a strategy to achieve these goals in the marine and maritime sector, with aquaculture being amongst the sectors of potential for such growth (European Union 2018). The European Commission strategies aim at putting European aquaculture at the forefront of sustainable development and aim at being leaders in the blue revolution (European Commission 2009). Member states are encouraged to find sustainable means of aquatic production, including IMTA systems, which are supported financially by the European Maritime and Fisheries Fund and can incorporate seaweeds (European Commission 2012).

Various European projects working on sustainable seaweed technologies have been funded recently, such as ENalgae or GENIALG. The aim of these projects was and is to discover

seaweeds biotechnological potential e.g. as a biofuel resource and to bring seaweeds into the context of the EU blue economy, by improving cultivation systems to maximize yields. Blue carbon is a term used to describe the potential of the world's oceans and coastal ecosystems to capture carbon. Seaweeds have been discussed in this context. To date, there is no consensus, whether or not to include seaweeds into blue carbon strategies, due to their highly debated role as carbon sinks (Thomas 2014; Krause-Jensen *et al.* 2018).

2.1. Global challenges in the food and feed industry

By 2050, the world population is predicted to reach 9.7 billion people (United Nations 2017). One of today´s major global challenges is food security. An ever-increasing demand for nutrients comes hand in hand with finite resources and a food sector that is greatly impacted by climate change events. At the same time, food production is a major cause of global greenhouse gas emissions (Hart 2017). The IPCC reported that agriculture; forestry and land use account for 24% of global GHG emissions, as the second largest economic sector after electricity and heat production and with agriculture being the main contributor (IPCC 2014). The food production for human consumption comprises meat production and animal derived products such as eggs or milk, the raising of aquatic animals through aquaculture and crop production for food and feed purposes. Given finite resources of fertile land and freshwater, all of these food production practices can become unsustainable, already due to the mere amount needed to meet demand. Each form of food production requires resources and the intensity of production, the location, and the type of product determines their environmental impact. From a production side, responses to the increasing demand for food are either an increase in area (causing the degradation of intact ecosystems) or an increase in yield, often resulting in higher chemical demand or genetic modification techniques, on existing agricultural land (Edgerton 2009).

In light of climate change, the agriculture sector is also facing various challenges. Water shortages, heat and flood events are expected to negatively influence agriculture in the near future and result in yield and quality decrease (OECD 2017). Every year, five to six million hectares of arable land have been estimated to be subject to degradation, affecting agricultural activity (Hamdy 2014).

Agricultural crops, including grains, fruits, vegetables, plants etc., are produced for direct human consumption or for animal feed purpose. Producing crops to feed animals is often discussed controversially, since they could be used as food directly, especially so, since their

cultivation reduces the amount of fertile soils (Sabaté & Soret 2014; Steinfeld *et al.* 2006). In Europe, 68% of the farmland is used for the cultivation of animal feed crops (Leip *et al.* 2015). Producing crops can have negative environmental influences. Through the use of pesticides and nitrogen or phosphorus fertilizers, especially in intensive agriculture, chemicals can end up in the water cycle through runoff. This affects water quality negatively and can lead to eutrophication. Soils are excessively used, so that they become infertile and require even more so the use of chemical treatment (Bennett *et al.* 2001; Smith *et al.* 1999) Withdrawals of water for agriculture accounts for 70% of all withdrawals (Edgerton 2009). This is especially noteworthy in times of freshwater shortages (Jacobsen *et al.* 2013; Cox 2002). Strategies to overcome these challenges, such as monocultures or genetic modification have emerged in the agriculture sector, to make production more efficient and target higher yields. However, these strategies are controversially discussed due to associated risks such as biodiversity loss (Maghari & Ardekani 2011).

Various health organizations around the world, such as the world health organization (WHO) or the Deutsche Gesellschaft für Ernährung (DGE), promote the regular consumption of **animal derived foods,** meat and dairy products, as well as fish for a balanced diet. However, the livestock industry has enormous impacts on the environment. Directly, or indirectly, through the feed crop production such as the protein rich soybeans, occupies 30% of the world´s agriculturally usable surface area (FAO 2012). Land-use and water requirements for animal farming further far exceed that of agricultural crop production (FAO 2012; Steinfield *et al.* 2006). The feed conversion ratio (FCR) to produce one kg of beef is approximately six times fold that of producing one kg of poultry (compare Figure 3).

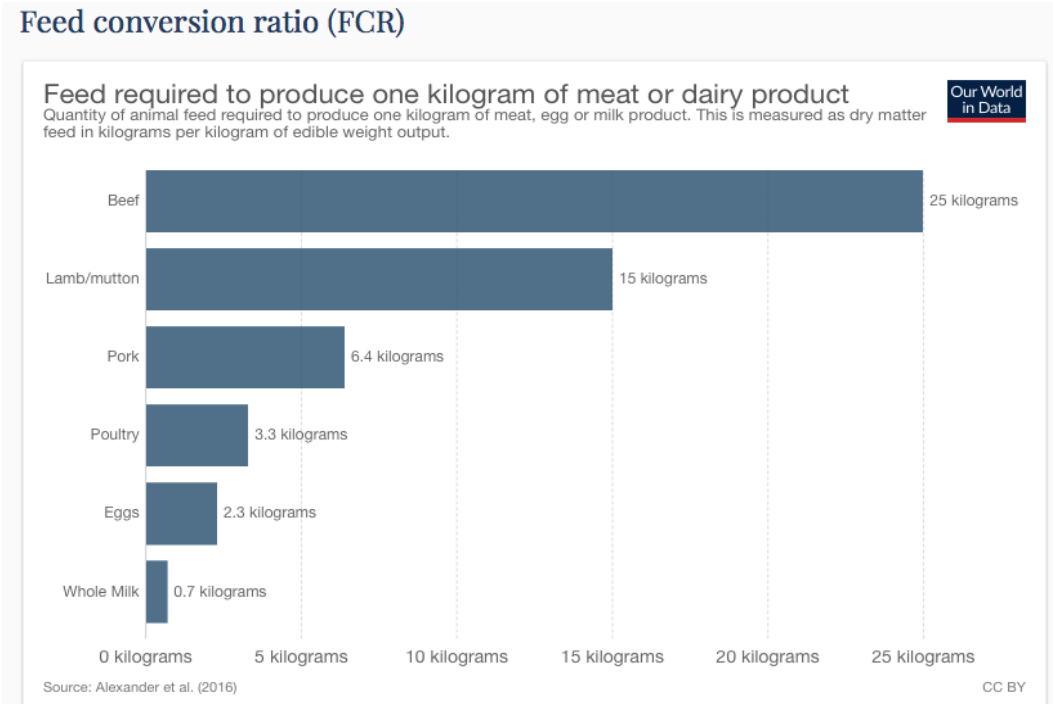

Figure 3: Feed conversion ratio for different animals and animal derived products; image source: https://ourworldindata.org/meat-and-seafood-production-consumption.

The raising of livestock is the number one driver of GHG emissions such as methane or carbon. Beef production is by far the biggest driver of GHG emissions, especially compared to land-crops (compare Figure 4).

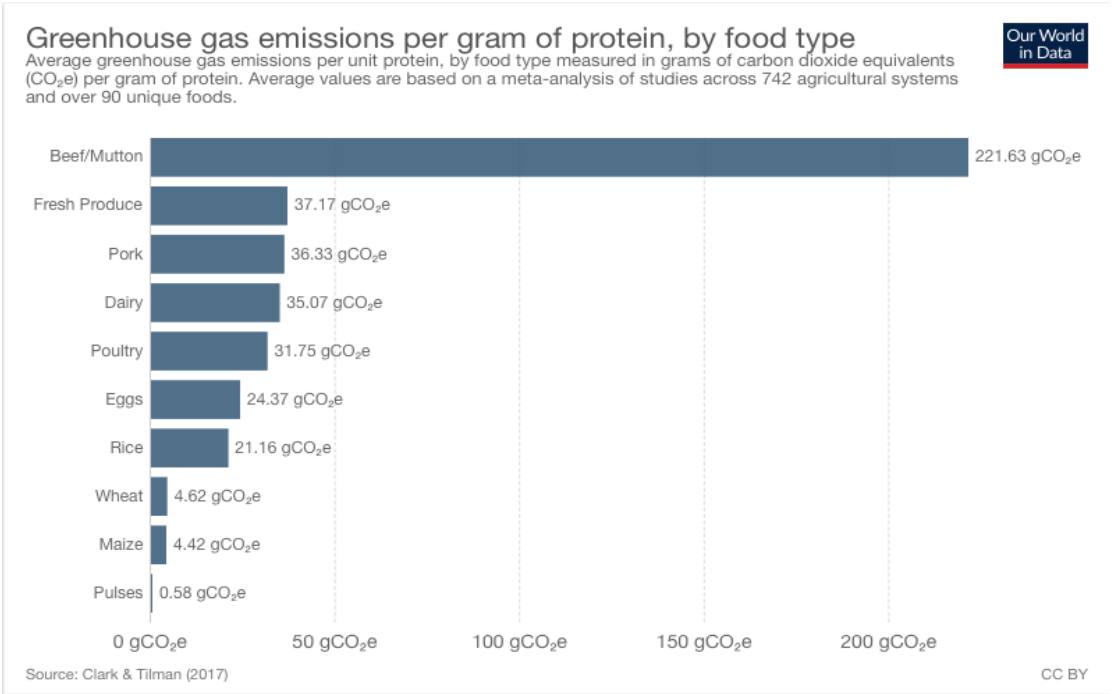

Figure 4: Comparison of greenhouse gas emissions for different protein sources; image source: https://ourworldindata.org/meat-and-seafood-production-consumption.

For both, the production of crops as well as raising livestock, a shortage of space leads to continuous expansion and exploitation of untouched ecosystems, causing deforestation (Erb *et al.* 2016; O´Mara 2011). However, the discourse on the negative effects of raising livestock is also often polarized by environmental organizations. Livestock, for example, can also be farmed extensively and be grass-fed. This way, feed crop usage is reduced and the manure produced by the animals can be used directly as natural fertilizer for the soil.

Aquatic animals are another important source for food. In many coastal areas around the world, fish is the major source of animal protein and provides a unique source of PUFAs (Rice & Garcia 2011). However, capture fisheries experience major challenges due to overfishing and changing species abundance patterns around the globe due to climate change (Roessig *et al.* 2004; FAO 2016, p.6). A 50% increase in current production would be needed to meet the amount of fish required today (Rice & Garcia 2011). However, insufficient fisheries management leads to an increasing decline of fish stocks and global catch levels are at their limit in most regions (Quaas *et al.* 2016). One response to this particular challenge is the development of the aquaculture industry, which today provides about half of the fish that is consumed globally (FAO 2016, p.16). However, the industry itself can have massive negative impact on the ecosystem by releasing organic waste into the environment, destroying habitats or increasing pressure on wild species through fish escape or disease transfer, to name a few (Bunting 2013, p.13). For the aquaculture industry, fishmeal is often used in feed as a source of high-quality protein and lipids. This is controversially discussed since "fishing for feed" additionally stresses wild fish stocks and reduces the amount of fish directly available for human consumption (Chapman & Miles 2018; Goldburg & Naylor 2005).

To sum up, a cascading effect can be observed through the continued exploitation of natural resources and current practices within the food industry: current food and feed production practices can negatively impact the environment and cause biodiversity loss, soil degradation and freshwater scarcity. At the same time, agricultural activity intensifies climate change through GHG emissions and reduces nature's natural climate-change mitigation capacity (Rice & Garcia 2011). What has become evident is, that in order to assure food security in the future, we need to improve the sustainability of current practices, as well as improve cultivation techniques, change consumption behaviors, reduce waste, equalize distribution around the globe and explore alternative food sources (IPCC 2014; Campbell *et al.* 2016). This chapter

intended to outline major trends and challenges the food and feed sector is experiencing, well aware that discussing the full complexity of this topic would exceed the scope of this paper.

2.2. Seaweed cultivation in the context of current food production

In light of the abovementioned, it becomes evident, that apart from changing our consumption habits and increase the efficiency of current farming techniques, we must also look at alternative food sources, to meet the demand for high-value nutrients in a sustainable way for current and future generations (Bleakley & Hayes 2017). Acknowledging that 70% of the earth's surface is covered by water, turning our attention to farming the seas and looking for alternatives there seems to stand to reason. Seaweeds may be one such alternative.

Seaweed culture is considered an extractive form of aquaculture and has various ecological benefits compared to terrestrial production: The cultivation of seaweeds needs no supply of fresh water or arable land, nor are fertilizers or chemicals needed for their production (Radulovich *et al.* in Tiwary & Troy 2015, p. 34). In underprivileged coastal communities, seaweed farming offers alternative livelihoods and can even reduce destructive fishing and increase awareness of the importance of conservation (Crawdord 2002; Link *et al.* 2017). Farming seaweeds can be regarded as a form of nutrient recovery e.g., through the uptake of dissolved nitrogen and phosphorus, which reduces the risk of eutrophication caused by agricultural runoff or effluents of fish farms. When applied in areas of access nutrients, this can be beneficial to the ecosystem. Compared to fed-fish cultivation, seaweed cultivation usually does not require additional feed, when farmed in a nutrient rich environment (Buck *et al*. 2017). As photosynthesizing organisms, seaweeds can furthermore improve primary production, act as carbon sinks and produce oxygen acting against hypoxia (Chung *et al*. 2017; Krause-Jens *et al.* 2018). As a potentially complete source of EAA and PUFAs, as discussed in chapter 1, seaweeds can be an alternative source of these essential nutrients for a plant-based diet and diversify current consumption habits in Europe. Figure 5 illustrates and summarizes the major advantages mentioned that seaweed cultivation has over meat, dairy, aquatic animals and crop production in terms of environmental sustainability.

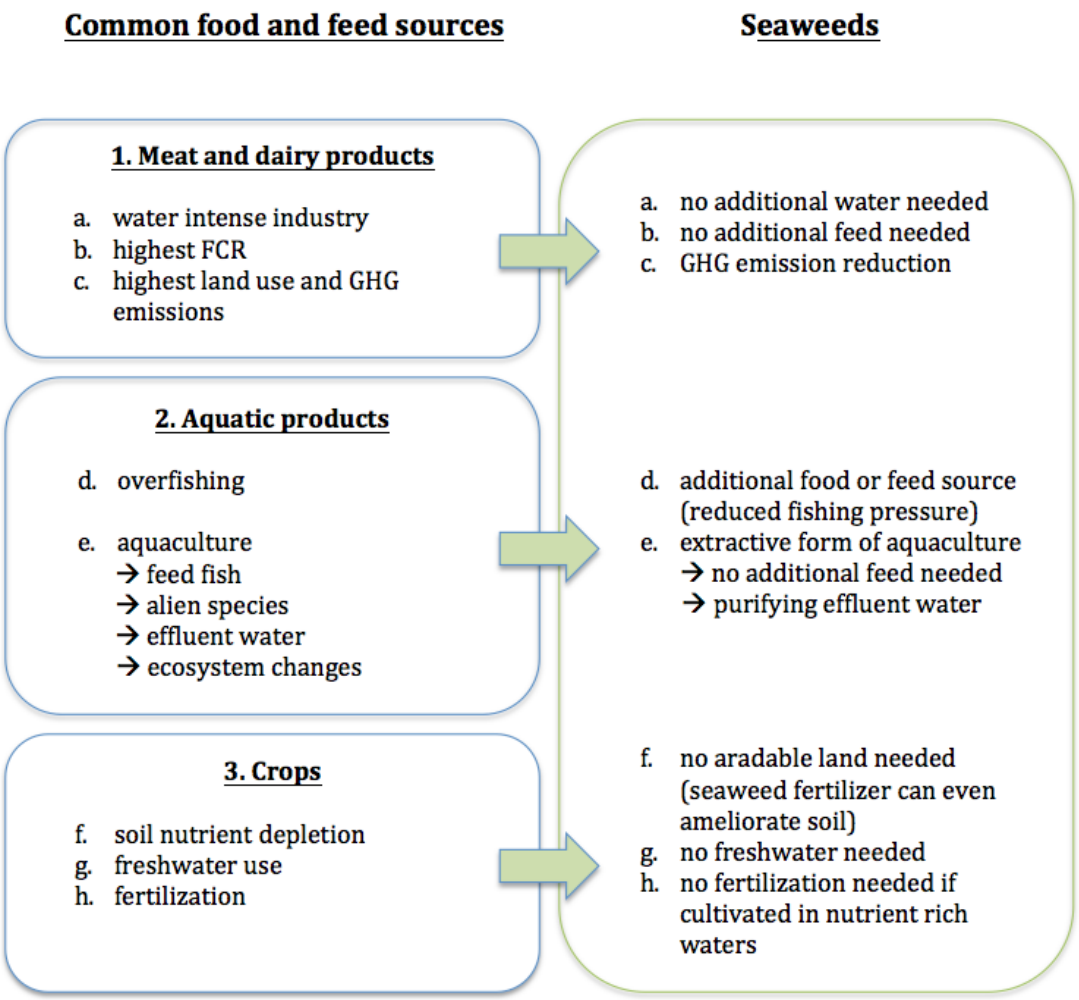

Figure 5: Overview of the ecological benefits of seaweed cultivation compared to traditional production of meat, dairy, aquatic products and crops. The blue boxes show the major ecological concerns of common food and feed sources. The green box shows how seaweed cultivation is superior to the corresponding adverse effects (indicated by letters).

2.3. Seaweed cultivation systems and sustainability

The technique used for seaweed cultivation is very case specific and depends on the purpose, the scale of operation, the species selected for cultivation and the cultivation environment. Generally, it is distinguished between open systems, set up directly in the natural environment, or in land based, closed systems e.g., ponds or tanks. Integrated-multi trophic aquaculture (IMTA) is an approach suitable for both types of cultivation systems (Radulovich *et al.* in Tiwory & Troy 2015, p. 37). For all cultivation types, the right conditions: temperature, salinity, nutrient availability, light duration and intensity, oxygen and CO_2 for optimal growth and yield of target compounds needs to be met for optimal production.

Regarding the seaweed production for food application, a production of high-quality biomass with a stable nutritional profile, low toxins accumulation and high yields of the targeted

compounds is desirable (Magnusson *et al.* 2015). This may generally favor the farming of seaweeds over harvested biomass from the wild, and closed systems over open systems, since culture conditions are more controllable. In all three cultivation cases, species selection must be matched to the cultivation technique and site. Dominating the reproduction cycle and knowing the effect of different culture conditions on the biochemical composition and growth of the selected species improve cultivation success and should be considered a-priori to cultivation. Regardless of the cultivation system, the process associated with seaweed cultivation often starts with seedling production in nurseries. The seedlings are produced in nurseries and directly attach to cultivation structures. Through this type of cultivation higher yields can be obtained, but also increase cost compared to the use of vegetative seedlings. Using vegetative seedlings on the other hand is labor intensive due to selection, cutting and attachment to ropes. Dominating the reproduction pathway of seaweeds requires expertise and a crucial factor for successful cultivation. The cultivation of the seedling then mainly occurs on ropes or in nets but also floating rafts, tank or pond culture (Radulovich in Tiwory & Troy, pp. 37-39). Advantages and adverse effects of different cultivation techniques will be explored from a sustainability perspective and discussed in the following. In all cases, biomass for the food and feed industry can be obtained from a new, additional source and thereby strongly contributes to overcome global food challenges.

2.3.1. Cultivation in open systems

Cultivating seaweeds at sea means that there is a direct impact on the surrounding ecosystem, which can be both positive and negative. The scope of impact of seaweed culture depends on the scale of operation and the farming technique and sustainability assessments of these have been conducted little to date (Hasselström 2018; Loureiro; Gachon & Rebours 2015).

Benefits of cultivating seaweeds in open systems can be provisioning and have shown to provide shelter, habitat and nursery grounds for marine life and to enhance productivity and biodiversity in degraded ecosystems and fisheries (Buschmann *et al.* 2014; Radulovich *et al.* 2015; Walls 2017). As photosynthesizing organisms, seaweed add oxygen to the water, acting against hypoxia (Duarte *et al.* 2017). They also clean aquatic environments from excess nutrients such as phosphate and nitrogen. This becomes especially relevant in areas of high anthropogenic influences, where aquatic systems are often eutrophicated (Buschmann *et al.* 2014). Depending on the structure of the seaweed farm, the farming site can also act as coastal protection by reducing the power of the wave energy (Duarte *et al.* 2017). Lastly, open systems

benefit from natural water availability and water movement through natural currents, so that no water needs to be transported, hold, pumped or aerated, saving resources, energy and money.

Adverse effects and concerns of cultivation of seaweeds in the natural environment have been raised on various different topics. Concerns are being raised, that seaweed cultivation can alter habitats by involuntary spread of cultivars, especially those that are non-endemic, to adjacent areas affecting bacteria, meiofauna, benthic macrofauna, fish and corals (Bindu & Levin 2010; Bergman, Svensson & Öhman 2001). The introduction of invasive species or gene swamping of introduced seaweeds with native stocks should be avoided (Loureiro; Gachon & Rebours 2015). That the spread of species from the cultivation site can also have positive effects for some species has been shown in the case of *Hypnea musciformis,* cultivated in the Hawaiian Islands, now an important food source for an endangered turtle species (Russell & Balasz 1994). A shadowing effect due to the cultivation of seaweed has been reported by Eklöf *et al.* (2006), who observed a negative effect on the local meiofauna. Whereas nutrient uptake can be beneficial for anthropogenically influenced aquatic environments, nutrient depletion of naturally occurring nutrients is also a concern because it may disrupt the natural food cycle (Campbell *et al.* 2019).

Further threats associated with growing seaweed aquaculture activity in Europe are disease outbreak and the introduction of non-indigenous pathogens and pests. It is therefore important to study the epidemiology of potential seaweed pathogens and farm management needs to implement early disease outbreak systems (SAPEA 2017). Cultivation at sea also has physical adverse effects. Farming sites may compete with other use of marine space and can be visually disturbing, especially in sight of the coast (Stévant, Rebours & Chapman 2017).

A recent study on the impacts of seaweed cultivation on ecosystem services in Sweden came to the conclusion that *"no significant impacts are expected, except for the provisioning of space and waterways which is negatively affected"* (Hasselström *et al*. 2018, p.59). A study conducted in Panama on the introduction of seaweed to non-native habitats did observe a spread of algae away from the cultivation site but *"did not observe significant changes in K. alvarezii cover over a six-month period*" (Sellers *et al.* 2015, p.7). Yet another study from Sweden found that *"algal shading, emergence stress and mechanical abrasion were identified as likely contributors"* to lower seagrass epiphyte cover and abundance changes in the taxa (Hasselström *et al.* 2008, p.73). These examples underline that the impact of seaweed farming is very site specific and depends on the cultivation and lastly on the characteristics of the species that is being cultivated and ideally should be analyzed specifically for each farming site. This has been

confirmed by a recent study on the knowledge gaps on environmental risks associated with a growing European seaweed industry, suggesting monitoring to reduce uncertainty (Campbell *et al.* 2019).

Cultivating seaweeds in open systems reduces the possibilities to control the growth conditions and changes in water quality. Varying conditions can directly influence quality and quantity of seaweed biomass and toxins or other hazardous substances can accumulate in the seaweed. However, for the seaweed´s use in the food and feed industry high, steady and safe quality are of uttermost importance. This underlines the need to carefully select a suitable site a-priori to cultivation. Monitoring water quality and testing the seaweed for toxins prior to selling could be additional means to solve this.

In this type of cultivation, economic threats for the farmer are the degradation or loss of biomass through heavy weather events like storms, or through fouling or grazing organisms besetting the seaweeds. Even though money can be saved by avoiding artificial aeration, harvesting of seaweeds cultivated at sea can be labor and energy intensive and may pose additional financial stress (Van den Burg *et al.* 2016).

2.3.2. Cultivation in land-based systems

The ecological benefits associated with seaweed culture at open sea are not all applying for cultivation in land-based systems, since there is no direct interference with the natural environment. This, however, also limits some of the risks and adverse effects associated with this type of culture. An example of a semi-closed system would be cultivating seaweeds in outdoor tanks or ponds (e.g. earth ponds) where *Ulva* can be cultivated unattached (Carl, Nys & Paul 2014). These systems are in direct contact with the atmosphere and can use atmospheric carbon and natural sunlight to grow.

Fully closed systems have the great advantage that all factors influencing the biomass productivity and quality can be optimized. This type of system also allows to control seaweed quality and to reduce the risks that are associated with production in offshore conditions. This is especially important to obtain high value products and assure a steady supply. However, this type of cultivation may be additionally cost intensive, since all parameters have to be artificially created. Tank and pond seaweed culture is often preferred for small-scale intensive cultivation, due to high harvest and risk control (Campbell *et al.* 2019; Pereira, Yarish, Critchley 2015).

However, investment costs are too high at current development stage to be economically feasible. Cost for building infrastructure and for energy often are too high for an economical production (Hafting *et al.* 2012). When reviewing literature on seaweed culture in ponds or

tanks, this almost entirely includes the integration of seaweeds with already existing aquaculture facilities, reducing costs (Friedlander & Levy 1995; Shipgel *et al.* 1993; Neori *et al.* 1996).

2.3.3. Cultivation in Integrated-Multitrophic Aquaculture

Integrated Multi-Trophic Aquaculture (IMTA) is the concept of combining the cultivation of fed species with that of filter organisms such as mollusks, bivalves or seaweeds in one site or in close proximity and can be operated in land-based or offshore systems (see Figure 6). The underlying concept of IMTA is to achieve nutrient cycling of the effluents of fed species and to increase water quality overall (Chopin 2001). Acknowledged benefits of this practice is the reduction of adverse effects of aquaculture and economic benefits by enhancing and diversifying production (Neori 2004). IMTA systems are also energy and water efficient and reduce the ecological footprint of aquatic animal farms (Winberg, Ghosh & Tapsell 2009).

That seaweeds are suitable candidates for IMTA has been proven in various trials. Introducing seaweeds in IMTA systems improves water quality by balancing pH and increases the available oxygen in the water. Nutrient rich effluent waters containing, high levels of e.g. phosphate ammonium, were efficiently removed from the water and improved algae growth. Valuable, marketable biomass for various different industries can be created and reduced the cost of water treatment (Neori *et al.* 1996; Shipgel *et al.* 2017). Biomass can also directly be re-used in the farm as protein-rich fishmeal replacement (Shpigel *et al.* 2017).

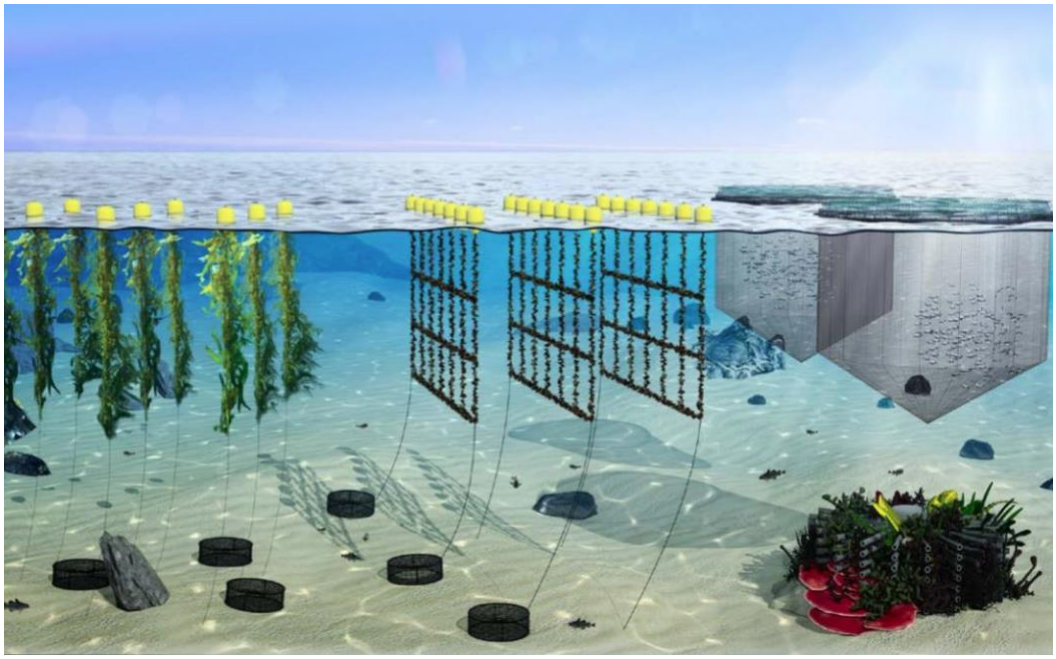

Figure 6: Integrated-multitrophic aquaculture approach in an open cultivation system farming non-fed-species in close proximity to fed-species; image source: https://steemit.com.

The seaweed *Ulva* has been proven to be especially suitable candidates in IMTA systems due to their high growth rates, their capability to outcompete epiphytes, their high nutrient uptake capacity and tolerance to changing culture conditions (Winberg, Ghosh & Tapsell 2009). A test on *Ulva lactuca´*s nutrient removement performance in a land-based IMTA culture showed efficient removal of ammonia. This did not only increase the water residence time but also contributed to the environmental performance of the mariculture system tested (Neori *et al.* 1996). Various studies on replacing conventional feed partially with *Ulva* have proven successful e.g., in the case of gilthead seabream, sea urchin, abalone or shrimp feed and can be directly reused in the farm (Shipgel *et al.* 2017; Laramore *et al.* 2018; Bolton *et al.* 2009).

Table 1 displays and summarizes the major findings on the benefits and adverse effects of the different cultivation systems discussed. It compares open- to closed cultivation systems in terms of ecological impacts and market value. In the third row the additional benefits and adverse effects associated with cultivation of seaweeds in an IMTA are summarized. Grey colour was used to indicate the overlaps between systems.

Table 1: Summary of the findings on the sustainability performance of different seaweed cultivation systems. Grey colour indicates overlap between systems.

	Open - Systems		Closed - Systems	
	Benefits	Adverse effect	Benefits	Adverse effects
Ecological	- Habitat creation - Primary production - Eutrophication regulation - Act against hypoxia - Enhanced water quality - Water movement replaces aeration - Carbon sink - Additional food and feed source - No freshwater - No arable land - No fertilization	- Invasiveness - Epiphytes; grazers & fouling organisms - Shadowing effect - Habitat destruction for farming site creation - Ecosystem changes (nutrient cycling, reduction of phytoplankton..) - Changes in community structure	- Carbon sink - Additional food and feed source - No freshwater - No arable land - No fertilization	-
Market value	- Water movement replaces aeration - Cheaper?	- Subject to extreme weather (mix up) - Less control of culture conditions - Competition for coastal space	- Control of in and outflow water - Control of most cultivation parameters	- Aeration = highest production cost. - Knowledge, manpower, space. - Reduced space in the facilities.
Additional impact of IMTA	- Reduce negative impact of aquaculture - Additional source of income - Innovative activity	- Additional knowledge - Additional manpower - Additional coastal space	- Reduce negative impact of aquaculture - Additional source of income - Innovative activity - Cost reduction for water treatment	- Additional knowledge - Additional manpower - Additional space in facilities

2.4. Seaweed cultivation in the context of global climate change

Discussing seaweed cultivation in the context of global climate change, an interesting interplay of seaweeds both playing a role in climate change mitigation but also being affected by it can be observed (Chung; Sondak & Beardall 2017).

On the one hand, seaweed cultivation can be a means to combat climate change. Seaweed culture can contribute to blue carbon by removing CO_2 from the water and thereby increase the absorption potential of atmospheric CO_2 Seaweed biomass can also reduce emissions from fossil fuels when used as an energy source (Chung *et al.* 2011; Duarte *et al.* 2017). Seaweed cultivation may also reduce negative impacts that are foreseen through increasing climate in the future, by reducing coastal eutrophication, ocean acidification and deoxygenation (Msuya 2008; Duarte *et al.* 2017).

Research on reducing methane emissions from livestock has found that by replacing 2% of livestock feed with seaweed can cut their entire methane emissions (Machado 2015).

On the other hand, the cultivation at sea can be influenced by a changing marine environment in plenty of ways: changing water parameters, such as increasing temperatures, can make a site unsuitable for a certain species and thus hinder production. A study on the effect of climate change on *Ulva rigida* growth and composition showed that elevated temperatures, acidification and nitrate availability accelerated reproduction and thereby shortened the life history. Whereas lipid and protein content increased under these conditions, carbohydrate content decreased (Gao *et al.* 2017). Therefore, to ensure a steady supply of the target compounds, the effect of climate change on the nutritional composition in seaweeds needs to be considered. Under these changing conditions, a steady biomass supply in terms of biochemical composition in open-systems or through harvest becomes even more challenging and climate change effects should be considered by producer and harvester.

Although CO_2 is absorbed by the seaweed, the whole cultivation process, from nursery operation to harvesting and processing or extraction also demands energy and creates emissions. This needs to be taken into consideration when assessing the net carbon sink potential of seaweeds.

2.5. Consumer information - prerequisite for successful marketing of seaweed

In western society, the demand for nutritious, high value food products and increasing environmental awareness has led to renewed research effort and the development of new foodstuff (Admassu *et al.* 2015). *"Novel foods"* is a term describing all aliments newly entering the European food market. To ensure consumer safety, the REGULATION (EU) 2015/2283 on novel foods has been established in 2015, ensuring traceability and safety of new products entering the European food market. Seaweeds and seaweed derived products fall under this umbrella.

The conscious consumer will be confronted with two aspects of seaweed products: that of health aspects associated with their consumption and that of sustainability aspects associated with their production. In both cases, the first step is improving market information by creating awareness of the benefits and adverse effects of seaweed production, which is currently halting commercialization (SAPEA 2017; Hendison 2017). Only well-informed consumers, aware of the health benefits and the possible adverse effects of seaweed consumption have the option to make profound purchasing choices. Therefore, marketing of seaweed products should include consumer safety information and the implication of sustainability standards.

Another bottleneck the consumer is experiencing, is the low knowledge on how to cook with

seaweeds or incorporating them in the diet. Information on their nutritional value as well as cooking recommendations could increase interest and facilitate market entry (Forster & Radulovich in Tivory & Troy 2015, p. 308).

2.5.1. Sustainability standards

According to the International Social and Environmental Accreditation and Labeling Association (ISEAL) *"sustainability standards are a powerful market-based approach for creating positive social, environmental, economic change, and driving transformation in how goods and services are produced"* (ISEAL 2015). The more adverse effects of a practice are covered through a certification process and the more attention is given to them, the more it can be expected that these will be addressed and improved by the suppliers. Seaweed products entering the market can be provided with a certification or seal that allows the consumer to identify products that are compliant with sustainability norms and standards. The organic seal of the EU assures ecological production of food and feed and can be applied to seaweed products. The organic council regulation ((EC) No 834/2007 (1)), describes organic production as *"an overall system of farm management and food production that combines best environmental practices, a high level of biodiversity, the preservation of natural resources, the application of high animal welfare standards and a production method in line with the preference of certain consumers for products produced using natural substances and processes*" (European Union 2007). The aquaculture and marine stewardship councils (ASC/MSC) have recently developed a seaweed standard, certifying harvest operation and farming sites. Standards can be obtained through third party auditing. Certification can also be a means to raise awareness of environmental issues associated with the production of seaweeds. The ASC/MSC standard comprises five guiding principles, each comprising a set of performance indicators. For the certification process, the entire supply chain needs to be transparent. The five guiding principles of the ASC- MSC Seaweed standard are listed in Table 2.

Table 2: ASC/MSC seaweed standard. Source: https://www.asc-aqua.org/wp-content/uploads/2017/06/BC2146_ASC-MSC_A4_6pp_ARTWORK_LRES.pdf.

Principle	Description
Sustainable wild populations	Seaweed harvesting and farming must be conducted in a manner that does not lead to depletion of the exploited wild populations. For depleted populations, harvesting operations must be conducted in a manner that demonstrably leads to their recovery. Where appropriate, stock status, harvest strategy and the genetic impact of the assessment site on the wild stock are also assessed.
Environmental impacts	Seaweed harvesting and farming activities must allow for the maintenance of the structure, productivity, function and diversity of the ecosystem (including habitat and associated dependent and ecologically related species) on which the activity depends. Seaweed operations must also adhere to criteria related to habitat, ecosystem structure and function, species status, species management, waste management and pollution control, energy efficiency, disease and pest management practices, and introduced species management.
Effective management	Seaweed harvesting and farming operations must have an effective management system in place that respects local, national and international laws and standards. Beyond the legal framework, operators are required to review farm/ fishery specific objectives, decision-making processes and compliance and enforcement arrangements.
Social responsibility	Seaweed harvesting and farming activities are required to operate in a socially responsible manner. Operators must ensure that workers are protected from harmful practices including child labor, any degree of forced labor or discrimination, while supported in their rights to collective bargaining, fair disciplinary practices, health and safety, fair and decent wages, and appropriate working hours. Environmental training must also be provided.
Community relations and interaction	Seaweed harvesting and farming activities must operate in a manner that minimizes impacts on other farms, activities and communities. Operations must adhere to strict requirements regulating the appropriate positioning and orientation of farms or water-based structures, identification and recovery of substantial gear, good management of noise, light and odour, and the proper decommissioning of abandoned farms or other water-based structures.

From a European sight there arise a lot of advantages if seaweeds are cultivated in Europe which means that consumer and producer are close together.

Seaweed farmed in the EU can be marketed from a sustainability perspective, by stating that it supports local economy, reduces emissions through proximity of production and consumption and, depending on the cultivation system, benefits local ecosystems, reduces adverse effects of aquaculture and helps mitigate climate change. Farm management, procedures and practices can be adapted to ensure maximal sustainability of the marketed seaweeds. This might be

associated with higher production cost for the farmer, which can be compensated by obtaining a standard that will encourage the consumer to pay a little bit more for a conscious choice. Through good communication and representation of sustainability aspects, European products could become more competitive than imports, e.g. from Asia.
Effective marketing and labeling of sustainable products could result in the following benefits for sustainability:

a: support behavior changes in consumption patterns in favor for sustainably farmed seaweeds.
b: facilitate the right purchasing choices.
c: pressure providers to apply ´*best practices*´ in order to get certified and sell their products.

2.5.2. Consumer health and safety

Seaweed standards, such as the ASC-MSC seaweed standard or the EU organic seal, cover sustainability aspect associated with the production of seaweed. However, they do not provide information about the nutritional value, nor the health hazards associated with seaweed consumption. This can mislead the consumer. The variability in seaweeds nutritional value has been pointed out in the first chapter and shows that generalized statements on the health benefits derived from seaweed consumption can barely be made. The influence of abiotic and biotic factors on seaweed quantity and quality is so big, that for every species and for each cultivation site, the biochemical composition must be individually defined to ensure steady quality. To claim health benefits of seaweed consumption and adequately position them in the food market requires more research and *in vitro* studies. That it is too early to make generalized health claims, can be shown by the example of vitamin B12. Algae have been marketed as a good source of vitamin B12. Whereas this seems to be true for some algae, it is only a pseudo vitamin B12, an inactive coronoid that is found e.g. in Spirulina, a microalga marketed for their vitamin B12 (Watanabe *et al.* 2002). The current state of knowledge does not allow for such generalized statement and can lead to confusion and mistrust on the consumer side.
Regarding toxins that can accumulate in the seaweed tissue, it is important that these are regularly checked, especially for seaweeds harvested from the wild, where water quality is unknown (Chen *et al.* 2018). For consumers, quality certification and information resources are key means to improve marketability of seaweeds. One major health concern is the high iodine content which should be stated on the packaging, together with a daily intake recommendation.

For seaweeds cultivated in the EU an advantage could be higher product traceability and the fact that the production is underlying local food safety regulations.

2.6. Chapter summary

The second chapter has clearly highlighted that the environmental impact of seaweed cultivation varies greatly, depending on the cultivation system and whether or not the cultivation of seaweed co-occurs with other species. Cultivation in open systems is associated with many more ecological benefits than is the cultivation of seaweeds in closed systems (Table 1). However, adverse effects on the ecosystem are also only a concern for open systems. Open systems are mostly reduced in their controllability to proper site selection and subject to natural forces and fluctuations, whereas closed systems are beneficial for controlled cultivation of seaweeds. Whereas open systems reduce availability of coastal space (if not integrated into existing structures), closed systems require higher costs e.g. for aeration and additional space in the facility. Cultivating seaweeds in IMTA systems comes with ecologic and economic benefits but also requires additional work and know-how.

This chapter also discussed seaweed cultivation in Europe as an addition to current food production. It could be shown that that seaweeds have numerous advantages in terms of sustainability but also highlighted difficulties and concerns. The economically viable cultivation of seaweeds for food and feed purpose depends greatly on a combined effort to improve technology, make the entire farming process energy efficient, select for resistant strains with high abundance of target compounds, select suitable cultivation sites and improve cultivation techniques.

Outreach campaigns by NGOs or certification and labeling of seaweed products, according to environmental standards, can contribute to build consumer trust and help build a sustainable European market for seaweeds. The ASC-MSC seaweed standard sets a good incentive and covers all concerns raised in this chapter that can occur when cultivating seaweeds, especially when it comes to environmental impacts of sea-based systems. *Ulva* has been identified as a suitable candidate for production for the food and feed industry, especially in IMTA systems. The findings of this chapter justify the inclusion of seaweed biomass as a resource for a sustainable food and feed industry.

Chapter three – Study on genetic identification, protein content and fatty acid composition in *Ulva* spp.

When choosing an *Ulva* species for cultivation for food and feed purposes, several aspects need to be considered. In the previous chapters, the importance of knowing the nutritional composition of a seaweed for accurate positioning in the food and feed market has been highlighted. Depending on the target industry, a species can be more suitable for production when it contains higher amounts of a target compound. Polyunsaturated fatty acids and proteins are two such target compounds and were chosen to determine the value of *Ulva* species for food and feed purposes in this work. Genetic species identification allowed to see whether there are species specific differences that matter for their production for the food and feed industry based on the target compounds. Knowing what species are naturally occurring in southern Portugal is additionally important to avoid invasiveness when culturing *Ulva* in the area.
The aim was further to understand, whether for cultivation, the selection of a specific *Ulva* species has an additional benefit to the optimization of the cultivation conditions for obtaining higher yields of target compounds. Therefore, the amount of protein and FAs, especially PUFAs, within the same species between coastal and lagoon sampling locations and among species within the same sampling location (Ria Formosa) was compared, to see, whether the species identity or the environment has a stronger effect on the seaweeds market value.

The work presented in this chapter therefore aimed to answer the following main questions:

- What *Ulva* species can be found at southern Portugal's coast (genetic identification)?
- What are their fatty acid profiles?
- How much protein do they have?
- Are there differences in fatty acid profiles and the amount of protein across species and environments?

3. Materials and methods

3.1. Sampling

The collection of 44 samples was conducted during the months of March and April in 2019. The sampling took place at six different coastal and lagoon locations in southern Portugal. Two sampling locations were in the Ria Formosa coastal lagoon (A&B), two at the Algarve coast in Albufeira and Vilamoura (C & D) and two were further north the Portuguese West coast in Praia do Ilha dos Pessegueiro (E) and Arrabida (F). The sampling location are indicated in Figure 7 and further specified in Table 3.

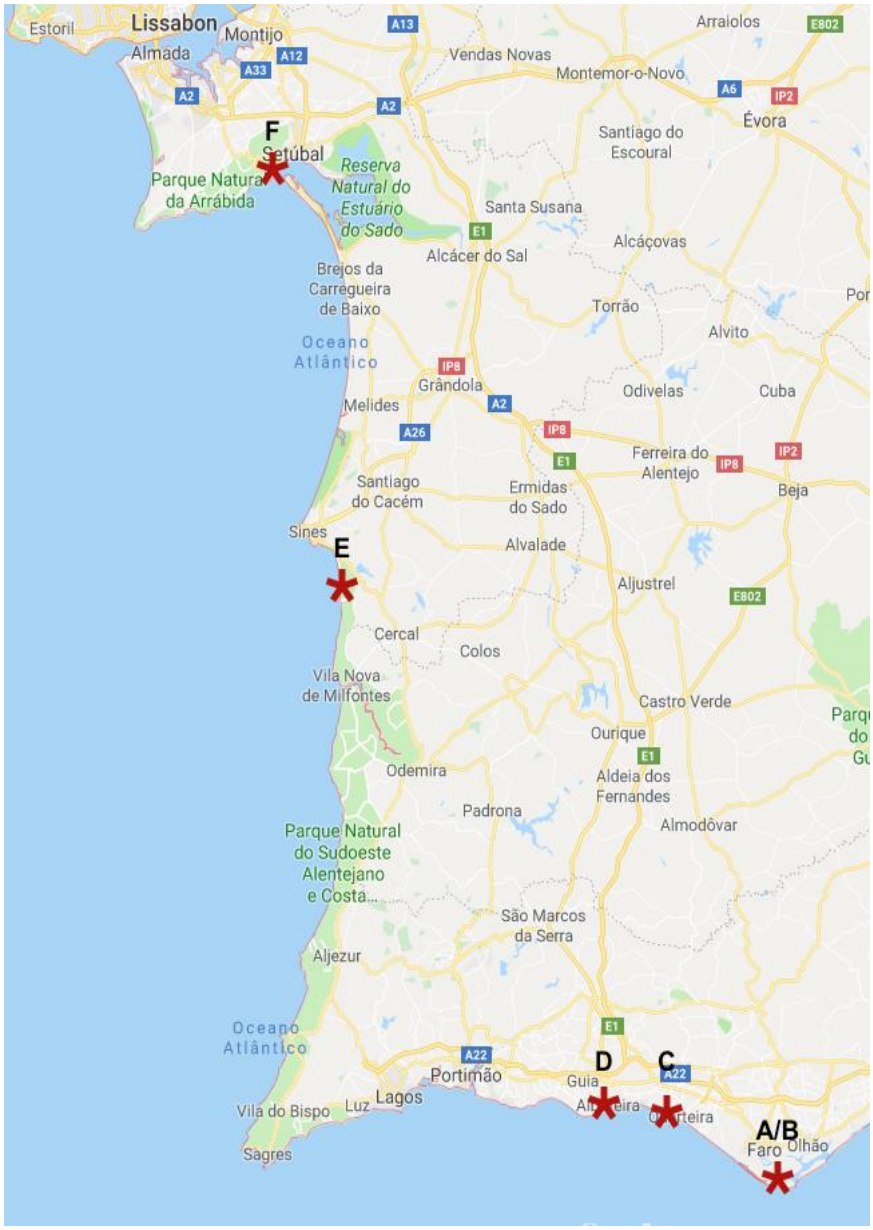

Figure 7: *Ulva* sampling locations in southern Portugal indicated by red stars and further specified by capital letters referred to under # in Table 3.

Table 3: Sampling sites with date of sampling and location. # refers to the different locations marked in Figure 7.

#	Location	Coordinates	Date
A/B	Ramalhette	37°00'19.4"N 7°58'02.8"W	18.03.19
A/B	Ria Formosa, Faro	36°59'53.4"N 7°58'40.5"W	19.03.19
D	Praia de Aveiros, Albufeira	37°5'0.68"N 8°13'53.45"W	08.04.19
C	Vilamoura coast	37°7'19.99"N 8°12'52.24"W	10.04.19
E	Praia do Ilha dos Pessegueiro, westcoast	37°49'42.0"N 8°47'30.6"W	14.04.19
F	Praia da Figueirinha, Arrabida	38°29'06.2"N 8°56'32.5"W	14.04.19

At each sampling location the parameters depth, salinity, temperature, attachment style (rock, sand, free floating, plastic, shell or epiphytic) and substrate type (soft bottom, sandy, rocky pool, ponton) have been determined (see Table 6&7 in Annex A). Collection always took place at low tide at locations accessible by foot. For each species identified on general morphology, two samples were taken.

The first sample of about 1.5cm2 of biomass was taken using nitrile gloves to avoid contamination and was introduced into autoclaved Eppendorfs for DNA barcoding. The second sample for content analysis was put into small ziplock bags. The samples were directly put on ice in a cool box for transportation to the laboratory facilities for biogeography, ecology and evolution of the University of Algarve, Portugal. At each sampling location a seawater sample was collected in a 1l bottle and salinity has been measured after sampling at the Ramalhette field station, by measuring the conductive that is equal to the number of parts per million (ppm), using a salinometer.

3.2. Genetic species identification

3.2.1. Sample preparation

For this work, a total of 44 samples were collected. The tissue for DNA barcoding was stored in the Eppendorfs at -80°C and tissue for content analysis was stored in ziplock bags at -20°C for later processing. Samples for DNA extraction were lyophilized (Modulyo D Freeze Drier) for 48 hours, wherefore the Eppendorfs containing the biomass were prepared by stamping holes into the lid with a heated needle, disinfected after each tube. After freeze-drying, samples

were grinded for 3min at maximum frequency (30 shakes per second) using the TissueLyser II (QIAGEN®).

3.2.2. DNA extraction and samples barcoding

For DNA extraction, the Quick-gDNA Miniprep kit (Zymo Research®) was used, following the manufacturer protocol for solid tissue. A buffer was prepared containing 11.5000µl of genomic lysis buffer and 57.5µl of beta mercapto ethanol. 500µl of buffer was added to each sample and samples were homogenized and then centrifuged for five minutes at 10.000g After centrifuging, 500µl of liquid was transferred from each sample to the corresponding column tube. Subsequently, samples were centrifuged for one minute at 10.000g. The column tubes were placed into new collection tubes and the liquid discarded. In a next step, 200µl of pre-wash buffer was added to the column tubes containing the DNA and again centrifuged for one minute at 10.000g. After centrifuging, 500µl of wash buffer was added and centrifuged again, discarding the buffer afterwards. In a final step, 50µl of elusion buffer was added to release the DNA. After a final round of centrifuging at 10.000g for thirty seconds, the tubes containing the isolated DNA were stored at -20°C.

The DNA extraction was performed in two rounds, including 22 samples each round, to process all 44 samples.

Barcoding was done by amplifying the chloroplast gene tufA, which encodes for elongation factor TU, using algal specific primers developed by Famà *et al.* (2002): forward primer at position 210 (tufAF 5'-TGAAACAGAAMAWCGTCATTATGC-3') and reverse primer at position 1062 (tufAR 5'-CCTTCNCGAATMGCRAAWCGC-3').

PCR reaction was prepared for a total volume of 20µl consisting of 1x of the 2 PCR Buffer mix (already containing $MgCl_2$), 0.25mM of each dNTP, 0.2µM of tufAF and tufAR, 0.2µl of *Taq* DNA Polymerase (Advantage® 2 Clontech™) and 1µl of the DNA template (without dilution). Remaining volume was filled out with water (Sigma-Aldrich®). Amplifications were performed with the following adaptations to the original protocol, using the 2720 Thermal Cycler (Applied Biosystems®) with the following setting: 95°C for 5min for initial denaturation; 37 cycles of: denaturation at 95°C for 45s, annealing at 50°C for 1min, and extension at 72°C for 1min and a final extension of 72°C for 5min.

Amplification success was checked in a 1% gel-electrophoresis. The gel was prepared by using Tris-EDTA-Acidic acid Buffer (TAE) and regular grade agarose. Successfully amplified PCR products were then outsourced for Sanger sequencing and further visualized and edited using

Geneious (http://www.geneious.com, Kearse *et al*. (2012)). The nucleotide sequences obtained were evaluated using the Basic Local Alignment Search Tool (BLAST).

A species name was assigned to the sample based on the highest percentage identity and under the consideration of query coverage, the percentage of alignment to a GenBank sequence.
When results in GenBank had equal percentage identity and query coverage but suggested two different species names that are not used as synonyms, European sources were considered most reliable and sequences were aligned by their identity to check whether differences in nucleotide were correct. The nucleotide BLAST is provided by the National Center for Biotechnology Information (NCBI).

3.3. Content analysis

For content analysis, samples were prepared for total protein analysis by thermal combustion and FAME profile analysis by GC-MS.

3.3.1. Sample preparation

The samples for content analysis were lyophilized for 48 hours in opened ziplock bags. The dry biomass was transferred into small round bottom Eppendorf tubes containing two Tungstein beats for grinding. Each Eppendorf was labeled and weighed empty. After grinding, the beats were removed from the tube with a magnet and the Eppendorfs weighed a second time, to determine the amount of grinded dry biomass obtained per sample. The tubes were stored in a bag with silica.

3.3.2. Total protein analysis

For total protein, each sample was analyzed in duplicates for their nitrogen, carbon and hydrogen content. Per sample, about 1mg of the freeze-dried biomass was weighed into tin containers and loaded into an automatic sampler (Vario EL III). Through external oxygen flash, combustion occurred at a temperature of 1800°C. The gaseous combustion products were carried by helium gas and released separately through a programmed temperature rise in the column.
By use of a thermal conductivity detector (TCD), the target compounds, producing an electric signal proportional to their concentration, were detected. By multiplying the nitrogen content by the conversion factor 5, as proposed by Angell *et al.* (2015), total protein was calculated and expressed as percentage.

3.3.3. Determination of the FAME profile by GC-MS

Fatty acid methyl ester (FAME) profiles of the *Ulva* were obtained through a modified protocol from Lepage and Roy (1984), as described in Pereira *et al.* (2012). Around 10mg of freeze-dried biomass was weighed into vials, in duplicates for each sample. 1.5ml methanol and acetyl chloride (20:1 v/v) was added to the vials and homogenized on ice for 90s by use of an Ultra-Turrax disperser. After adding 1ml of hexane to the homogenized samples, the vials were heated at 70°C for one hour for derivatization. After cooling down the samples, 1ml of distilled water and 4ml of n-hexane was added and vortexed for 60s. The organic phase separated through centrifugation (2000 *g* for 5min), was then pipetted into a new vessel. After the addition of anhydrous sodium sulfate, to remove any residual water, the organic phase was dried and concentrated under a stream of nitrogen and subsequently resuspended in 500µL of gas chromatography-grade hexane.

Samples were analyzed using an Agilent GC-MS (Agilent Technologies 6890 Network GC System coupled with a 5973 inert Mass Selective Detector) using an Agilent Tech DB-5MS column (length: 25m; internal diameter: 0.250mm; film: 0.25µm). Helium was used as the carrier gas. The injection temperature was set for 300ºC through a modified protocol from Lepage and Roy (1984), as described in Pereira *et al.* (2012) to volatilize the molecules.

Compounds were identified by comparison of the retention times of standard samples (Supelco 37 FAME Mix, Sigma-Aldrich) and the mass spectra compared to the NIST library. Results are given in percentage of total FAME.

3.4. Statistical analysis

For all statistical analysis, RStudio software was used. The significance level (α) was determined at 0.05 for all tests. The values for total protein and fatty acids were calculated as mean of the technical duplicates.

Statistical tests were run to test for significant differences in the amount of total protein, PUFA content and fatty acid profiles between regions (Ria Formosa and coastal locations) and among the species *U. compressa* (n=4), *U. rigida* (n=6) and *U. fasciata* (n=3). For comparison of the total protein and PUFA content of *U. rigida* between coastal and lagoon location, only samples from Albufeira (n=8) and Vilamoura (n=1) were joined as coastal locations.

Prior to testing for differences, normality of data was checked with the Shapiro-Wilk test and homogeneity of data with the Fligner-Killeen test of homogeneity of variances.

Data to test for overall differences of protein and PUFA between coastal (n=11) and Ria Formosa (n=14) sampling locations across all *Ulva* species was normally distributed and had homogeneity of variances wherefore a one-way ANOVA was conducted to test for difference between locations.
Data to test for differences in the amount of PUFA between coastal (n=9) and lagoon (n=6) locations for *Ulva rigida* and among the three species *U. compressa* (n=4), *U. rigida* (n=6) and *U. fasciata* (n=3) within the Ria Formosa was also normally distributed and had homogeneity of variances. Therefore, two one-way ANOVAs were conducted, the first, to test whether the amount of PUFA of *U. rigida* between coastal (n=9) and lagoon (n=6) location differs and the second to test, whether the three species *U. compressa* (n=4), *U.rigida* (n=6) and *U. fasciata* (n=3) have a different amount of PUFA within the same sampling site (Ria Formosa).

The data to test for differences in total protein content of *U. rigida* between coastal (n=9) and lagoon (n=6) did not meet the assumption of normality. Therefore, the non-parametric Wilcoxon rank sum test was used for analysis. A one-way ANOVA was used to test for interspecific difference (*U. compressa* (n=4), *U. rigida* (n=6) and *U. fasciata* (n=3)) within the Ria Formosa, since assumption of normality and homogeneity of variance was met.
For comparison of fatty acid profiles among the three Ulva species and between coastal and lagoon locations, a PERMANOVA multivariate analysis was used.

3.5. Results

3.5.1. Genetic species identification

In total, five genetically distinguishable *Ulva* species were identified as well as two samples of the red seaweed *Porphyra umbilicalis* collected at the westcoast. The sampling comprised the *Ulva* species: *Ulva rigida, Ulva flexuosa, Ulva compressa, Ulva fasciata* and *Ulva australis*. The morphotypes of *U. rigida* and *U. australis* were blades whereas *U. flexuosa, U. fasciata* and *U. compressa* were filamentous as visualized in the Herbarium created by Robert Priester (see Annex B).

3.5.2. Total protein & FAME profiles

Total protein across all identified *Ulva* species varied between 2.12% - 9.46% DW at a mean overall protein value of 4.35% DW across all species and sampling sites. Total protein content of *Ulva* measured at the sampling sites Albufeira, Ria Formosa, Vilamoura and the West coast revealed that *Ulva* from the coastal locations had twice more protein than *Ulva* from the Ria

Formosa ($p < 0.01$). The average amount of protein for the locations Albufeira, Vilamoura and West coast (n=10) was 6.38 ± 1.85% and 3.14 ± 1.22% for samples in the Ria Formosa (n=14).

One exception of the low amount of protein measured in the Ria Formosa was *U. flexuosa* with 7.28% total protein, which is notably higher compared to the next highest amount measured for *U. rigida* with 3.87% at an average protein content of 3.19% in the Ria Formosa. Whereas *U. compressa* collected in the Ria Formosa had a protein content around 2.8% for all samples, *U. compressa* collected at the westcoast reached 9.46% protein DW. Protein measured for the sample of *Porphyra umbilicalis* at the westcoast was 6.65% which is just above the average of 6.40% of all samples from coastal locations (see Table 5, Annex C).

Protein content did not differ among the three species *U. compressa*, *U. fasciata* and *U. rigida* within the Ria Formosa ($p = 0.363$). The highest mean amount of protein was measured for *U. rigida* (3.87%), and the lowest for *U. fasciata* (2.12%). Total protein of *U. rigida* differed between coastal and lagoon locations ($p < 0.01$). The amount of total protein of *U. rigida* sampled in coastal locations was more variable and had a higher median of total protein compared to samples from the Ria Formosa (Figure 8). The highest amount of protein measured for *U. rigida* in the Ria Formosa was 3.78%.

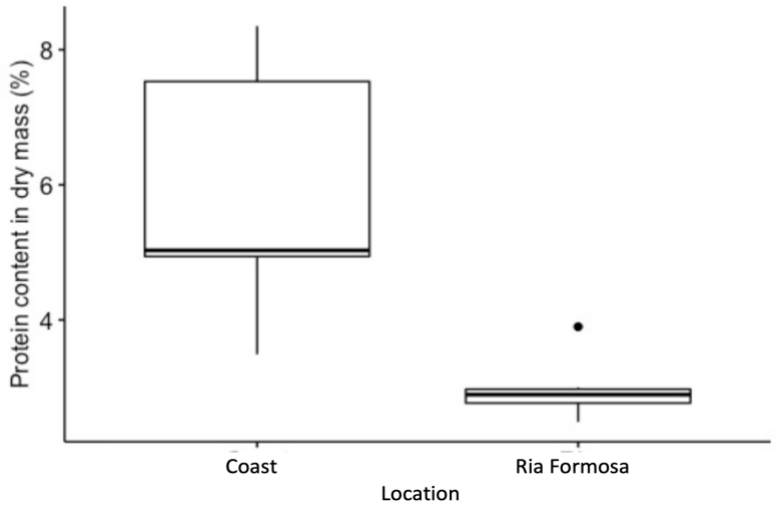

Figure 8: The amount of total protein content in dry mass (%) between coastal (n=9) and lagoon (n=6) location within *U. rigida.* The thick central line in the boxes represent the median amount of protein, the boxes represent 50% of the data and the whiskers show the variation in the dataset. The dot represents an outlier in the dataset. Total protein in coastal locations was significantly higher than in lagoon locations for *U. rigida* ($p < 0.01$).

For all the identified *Ulva*, SFA make up the highest proportion of FA, followed by PUFA and MUFA subsequently (Figure 9). The main fatty acid identified in all *Ulva* species was palmitic

acid (C16:0) ranging between 36.80% - 85.29% of total FA. Another abundant SFA detected in all *Ulva* species was stearic acid (C18:0) ranging between 0.84% - 12.50%. As for MUFAs, oleic acid (C18:1) was the most abundant FA in all species ranging between 4.36% - 18.6 %, followed by palmitoleic acid (C16:1). All PUFAs in *Ulva* ranged between 9.82% - 38.00% of total FA with linoleic (C18:2n-6) and α-linoleic acid (C18:3n-3) as the most abundant PUFAs. C15:0, C20:0, C22:0, C20:1, C18:3n-6, C22:6n-3, C20:5n-3 and C20:4n-6 were also detected in smaller amounts, not exceeding values of 2.73%. For the *Porphyra umbilicalis* sample collected at the West coast, fatty acid composition was quite distinct to that described for *Ulva*. The red seaweed FA profile comprises 51.28% PUFA with C20:5n-3 as the most abundant PUFA (34.51%) followed by MUFA (26.8%) with C18:1 as the most abundant FA and SFA (21.86%) and C16:0 as the most abundant FA (Figure 9 and Table 8, Annex C).

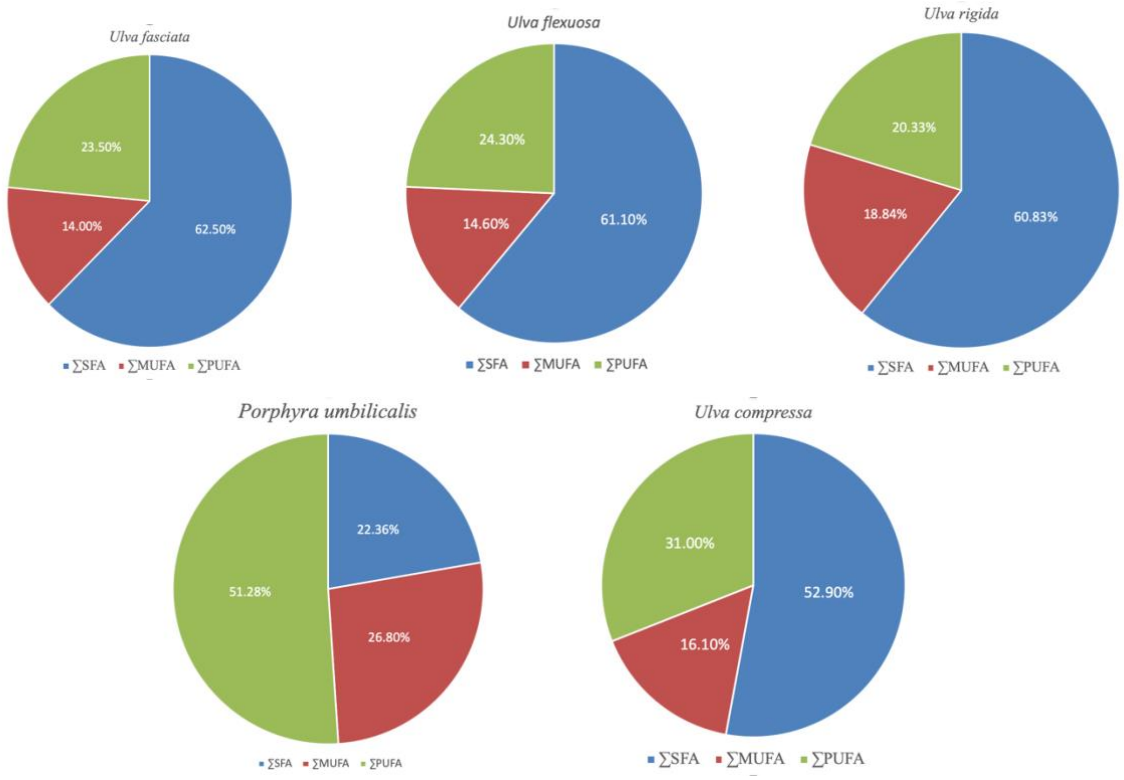

Figure 9: Mean percentage of SFA, MUFA and PUFA of total FAME content of *U. rigida* (n=15), *U. fasciata* (n=4) and *U. compressa (*n=5) as well as for *U. flexuosa* (n=1) and *Porphyra umbilicalis* (n=1).

Within the Ria Formosa, the mean weight percentage of PUFA was 30.18% for *U. compressa*, 20.61% for *U. fasciata* and 20.63% for *U. rigida*. Total FA profiles did not differ among the three species *U. rigida*, *U. compressa* and *U. fasciata* within the Ria Formosa ($p = 0.1064$, Table 4).

Table 4: Mean fatty acid profiles of *U. compressa* (n=4), *U. fasciata* (n=3) and *U. rigida* (n=6) within the Ria Formosa.

Fatty acids (%)	*Ulva compressa*	*Ulva fasciata*	*Ulva rigida*
C14:0	1.02	1.15	1.31
C15:0	0.21	0.99	n.d.
C16:0	44.89	43.53	49.05
C18:0	4.56	7.69	3.78
C20:0	n.d.	3.38	n.d.
C22:0	3.33	6.10	3.13
∑SFA	**54.01**	**62.84**	**57.27**
C18:1	12.17	12.47	15.94
C16:1	3.24	3.28	6.16
C20:1	0.40	0.80	n.d.
∑MUFA	**15.81**	**16.55**	**22.10**
C18:2n-6	11.99	12.71	10.76
C18:3n-3	12.22	4.05	7.30
C18:3n-6	0.82	n.d.	n.d.
C20:5n-3	2.04	1.61	1.06
C22:6n-3	1.40	2.20	1.51
C20:4n-6	1.71	n.d.	n.d.
∑PUFA	**30.18**	**20.61**	**20.63**
∑n-3	15.66	7,91	9.91
∑n-6	14.52	12.71	10.76
∑n-6/∑n-3	**0.92**	**1.60**	**1.08**

PUFA content did not show interspecific differences among *U. compressa*, *U. fasciata* and *U. rigida* within the Ria Formosa ($p = 0.0904$), though there was a trend for higher amount of PUFA in *U. compressa* (Figure 10). When combining the values of *U. fasciata* and *U. rigida* to one group, PUFA content of *U. compressa* was higher compared to *U. fasciata* and *U. rigida* ($p = 0.0245$).

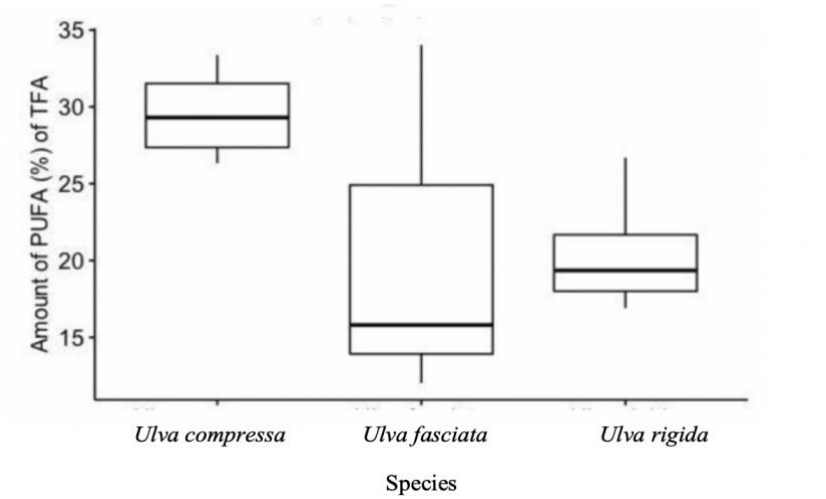

Figure 10: Mean polyunsaturated fatty acids (%) of total fatty acids among the species *U. compressa* (n=4), *U. fasciata* (n=3) and *U. rigida* (n=6) in the Ria Formosa. The thick central line in the boxes represent the median amount of PUFA, the boxes represent 50% of the data and the whiskers show the variation in the dataset.

Comparison of the amount of PUFA (Table 5) and FA profiles for *U. rigida* between lagoon and coastal locations revealed no difference ($p = 0.739$). There was also no difference between locations for the entire FA profile of *U. rigida* ($p = 0.6713$). The total dataset showed regional differences (all samples pooled across species) in fatty acid profiles only between West coast locations and Ria Formosa ($p < 0.01$).

Table 5: Fatty acid profile of *U. rigida* in lagoon and coastal location; sample size: Coast (n=9), Ria Formosa (n=9).

Fatty acids (%)	Coast	Ria Formosa
C14:0	0.90	1.31
C16:0	53.16	49.05
C18:0	5.43	3.78
C22:0	3.55	3.13
∑SFA	**63.04**	**57.27**
C18:1	10.94	15.94
C16:1	5.79	6.16
∑MUFA	**16.73**	**22.10**
C18:2n-6	12.90	10.76
C18:3n-3	5.05	7.34
C20:5n-3	0.99	1.06
C22:6n-3	1.29	1.51
∑PUFA	**20.23**	**20.63**
∑n-3	7.34	9.91
∑n-6	12.90	10.76
∑n-3/∑n-6	**0.87**	**1.08**

3.6. Discussion

3.6.1. Genetic species identification

All species identified, except for *U. australis*, have previously been reported at the Portuguese coast (Araújo *et al*. 2009). In the Mediterranean, *U. australis* was identified as an introduced species from the north-western Pacific region (Verlaque *et al.* 2015). To my knowledge, it is the first time that *U. australis* has been reported at the Portuguese coastal region. Unfortunately, *U. australis* could not be included in further analysis, because the biomass for content analysis was mixed with other species. *U. flexuosa, U. rigida, U. fasciata* and *U. compressa* are identified species that meet the criterion of non-invasiveness and are thus suitable for cultivation in southern Portugal (Krupnik *et al.* 2018).

Ulva identity is no longer based on morphology alone but has been replaced by genetic species identification. The tufA gene is a well-established DNA marker to identify *Ulva* seaweeds on species-level (Kirkendale, Saunders & Winberg 2013). To identify *Ulva* species, the obtained nucleotide sequences through DNA barcoding can be matched to those obtained by other studies in GenBank, a database for genetic sequences.

Matching the obtained nucleotide sequences of the samples with those in GenBank revealed that often there is the same percentage identity and query cover for different species names.

The difficulties in obtaining clear results from GenBank mainly concerned sequences of the species: *U. lactuca*, *U. laetevirens*, *U. rigida* and *U. fasciata*.

In *Ulva* nomenclature, *U. fasciata* is an accepted synonym for *U. lactuca* and *U. rigida* is taxonomically accepted as *U. lactuca var. rigida* (Guiry 2019). Also noteworthy is the difference in species names assigned to matching sequences by country. Generally, species names differed, e.g. for sources from oversea countries as compared to European sources, e.g. *U. rigida* identified by a German research group, has the same sequence an Australian research group used to identify *U. laetevirens*. To bring order into the *Ulva* nomenclature, it seems reasonable to suggest increased effort to genetically identify species and have a sequence assigned to each species accepted in *Ulva* nomenclature. This would also improve comparison of research findings across countries and avoid confusion.

3.6.2. Protein & FAME profiles

The numbers for total protein detected are within, but at the lower end, of what has been reported for *Ulva*, with numbers usually ranging between 8.5% - 27.6% of DW when using the Khiedal-method for protein determination (Yaich *et al.* 2011; Moustafa & Eladel 2015). The reported numbers for total protein used for comparison of data all used the Khiedal-method with the traditional Nitrogen to protein conversion factor of 6.25 which has been adjusted to 5 for this work because it has been proven to give a more accurate estimation total protein in *Ulva* (Angell *et al.* 2015; Shuuluka, Bolton & Anderson 2012). Since numbers are compared to studies using the traditional factor of 6.25, it is an aspect that can to some degree explain the overall low amount of protein found in this study compared to other numbers reported.

As was true for total protein, for *U. fasciata* and *U. rigida*, the mean amount of PUFA found in this study (20.61% and 20.63% respectively) was within but at the lower end of what has been reported for *Ulva* species with numbers ranging from 23.65 ± 0.26 - 41.8 ± 15.6% PUFA (McCauley *et al.* 2016; Pereira *et al.* 2012; Kendel *et al.* 2015; Shanab *et al.* 2018).

The comparably low amount of PUFA detected can be related to the low amount of protein found in these samples. In both cases, temperature (timing of sampling) and nutrient availability (location and season) might explain the overall low values and the regional difference with significantly higher amounts of protein in coastal sampling locations. The amount of protein in *Ulva* is namely directly correlated to the amount of nitrogen stored in the tissue and the FA profile of *Ulva* comprises the highest amount of PUFA under nitrogen sufficiency (Rosenberg & Ramus 1984; McCauley *et al.* 2016).

A study on the nutrient inputs in the Ria Formosa showed that in the Faro area, the input of nutrients through anthropogenic influence is generally low and that nutrients mainly enter the Ria Formosa through rainfall and wastewater treatment plants. During the tourism peaks in summer and rainfalls toward the end of the year, these nutrient sources, especially of nitrogen, are at their peak (Malta *et al.* 2017). Since samples were taken in spring, nutrients might have been low in the Ria Formosa. Explaining the difference in protein content between coastal and lagoon location based on difference in nutrients between the locations remains an assumption, since data supporting higher nutrients in coastal locations is lacking.

Lower water temperatures have also been reported to negatively affect the amount of total protein as well as the amount of fatty acids of total lipids in *Ulva* (Gao *et al.* 2018; McCauley *et al.* 2016).

The water temperature in the Ria Formosa ranger from lowest temperature of 14°C in March to 25°C in September (Malta *et al.* 2017). Again, the timing of sampling earlier in the year and thus at lower water temperatures, might explain the low amounts of protein and PUFA compared to other studies.

Whereas low water temperature and nutrient availability can be regarded as two likely causes of low numbers of protein and PUFA obtained in this study, there seems to be a difference in the effect of environmental conditions on the amount of protein and PUFA in *Ulva*. This statement is based on the findings that the amount of protein was significantly higher in coastal locations than in lagoon locations ($p < 0.01$), whereas PUFA was not different between locations ($p = 0.739$).

Even though the ANOVA comparing PUFA among U. *rigida, U. fasciata* and *U. compressa* revealed no significant difference ($p = 0.0904$), looking at the data (see Figure 13) suggested higher PUFA of *U. compressa* compared to *U. rigida* and *U. fasciata*. To increase group sample size, the two species *U. rigida* and *U. fasciata,* two species closely related, were grouped together and compared, which indeed revealed a significantly higher amount of PUFA ($p = 0.0245$) for *U. compressa* (Krupnik *et al.* 2018). This finding suggests that *U. compressa* is a more suitable candidate for PUFA production and that the environmental conditions have a stronger effect on the amount of protein in the *Ulva* seaweeds than on the PUFA content.

One exception to the significantly lower amount of protein measured in the Ria Formosa compared to coastal areas is the sample identified as *U. flexuosa*, collected in the Ria Formosa. This sample stands out due to its higher protein content in the dataset, with 7.28% total protein. This finding contradicts the above-mentioned, since it shows that equally high amounts of protein can be reached in the Ria Formosa and coastal areas (where highest amount of protein measured was 7.47% for *U. rigida*) and that in fact not the environment but also the *Ulva* species itself might also be important to consider when targeting protein. This might also suggest that *U. flexuosa* is a potentially good *Ulva* candidate for targeting protein. However, sample size is so small and comparative literature is lacking so that this finding only allows for an estimation.

The FAME profiles obtained in this study match those of other studies (Cardoso *et al.* 2017; Pereira *et al.* 2012; Kendel *et al.* 2015). For the food and feed industry, it is especially the PUFAs that are targeted for their health benefits (see Chapter one). All *Ulva* species had a fatty acid profile comprising LA, ALA, DHA and EPA which makes them an exception within the

Chlorophyta (Pereira *et al*. 2012). DHA and EPA are two especially important FAs that are only found in fish oil, justifying the cultivation of *Ulva* for PUFA (Lopez - Huerta 2010).

The *Ulva* fatty acid profile is quite distinct from that of other phyla (Pereira *et al.* 2012). *Porphyra umbilicalis* fatty acid profile comprised about 50% PUFA, including high amounts of C20:5n-3 (34.5%) and C20:3n-3 (6.45%). These results show that there are distinct fatty acid profiles amongst phyla, even if from the same region. The fatty acid profile obtained for the *Porphyra umbilicalis* sample matches those of other studies for the red seaweed (Blouin *et al.* 2006). When targeting PUFAs, especially EPA, *Porphyra* seaweed seems to be the better choice for cultivation than *Ulva.*

Overall, the results might suggest that coastal locations can be regarded as a more suitable site for *Ulva* cultivation for protein than the Ria Formosa and that suitable site selection is more important than species selection when cultivating *Ulva* for protein. The same cannot be said when cultivating Ulva for PUFA, because indeed, *U. compressa* seems to be the most suitable candidate for PUFA production amongst the *Ulva* species identified, whereas generally *Porphyra species* are higher in PUFA and thus favorable.
The results give a first hint that the Faro area of the Ria Formosa in springtime is not ideal for *Ulva* production for the food and feed industry, especially for protein. Industrial activity and rainfall have been identified as the two main sources of nutrients in the Ria Formosa, both of which are low in springtime (Malta *et al.* 2017). This seems reflected in the low amount of protein detected in samples from the Ria Formosa, justifying low nutrient availability as an explanation of low total protein. However, the suitability of the Ria Formosa as a cultivation site for *Ulva* may change due to increased anthropogenic influences and water temperature rise through climate change in the future as well as when cultivation is integrated into existing aquaculture structures through IMTA cultivation.

Throughout this work, it became evident, that repeated sampling throughout the year´s seasons as well as measuring the nutrient composition of the waterbody at time of sampling would have allowed to draw better conclusions on the effect of changing environmental parameters on the protein and fatty acid composition. Without knowing the nutrient profile of the water bodies in which sampling took place, it is not possible to draw final conclusions on the cause of variance in total protein between locations and explain the different finding for PUFA and protein between lagoon and coastal locations. Experiments e.g. of the effect of temperature, light or

nutrient availability on the amount of protein and the fatty acid composition for the identified species would increase knowledge on the cause mechanisms of difference in biochemical profile amongst locations.

As for improving the data, collecting more biomass per sample would have allowed to work with triplicates instead of duplicates for higher statistical accuracy and increased sample size overall would have led larger group size per identified species and thus increased the accuracy of statistical results. Ultimately, if more biomass had been collected, further analysis such as amino acid composition and the amount of total lipids would have been interesting for *Ulva* potential in the food and feed industry.

4. Conclusions

The overarching purpose of this thesis was to identify the potential that the cultivation of *Ulva* in southern Portugal can have to contribute to a more sustainable food and feed production system in the future.

Next to highlighting the importance of understanding the effect of environmental parameters on the biochemical composition of seaweeds to accurately position them in the food and feed market, the first chapter showed that generally, *Ulva* biomass can be used for multiple purposes within the food and feed industry and that their consumption can have various health benefits. Their potentially high amount of protein and their PUFA content, comprising ALA, LA, DHA and EPA, justify their use, e.g. in high value markets, such as the production of algae-based oil products or their use in animal feed formula. The European market is still in its infancy wherefore increased knowledge on species abundances and their biochemical composition can enhance market development in Europe.

The second chapter revealed that seaweed cultivation indeed stands out to current food production techniques in terms of sustainability performance. The fact that there is no need for arable land or freshwater for production becomes increasingly relevant in times of land water scarcity that is expected to increase in times of overpopulation and global warming. Ecological benefits of cultivating seaweeds are greater in open-cultivation systems, or when combined with the production of other species in an IMTA culture, where environmental concerns associated with aquaculture can be reduced. Since global fisheries are at their sustainable limit and feed crop production is controversially discussed, *Ulva* can indeed be regarded as a valuable source of protein and PUFA and as a sustainable substitute to current agricultural production.

Cultivating seaweeds under controlled conditions allows to assure consumer safety and accurate statement of the biochemical composition of the seaweed. Cultivation also does not tap on natural resources so overharvesting is avoided. Since the seaweed industry is still at its early stages in Europe, it seems especially important now to avoid consumer mislead and establish a clear regulatory body for cultivation, labeling systems and consumer information to facilitate the market entry for European seaweed products, assure consumer safety and information to avoid false health claims. Increased effort to make the entire seaweed production process more efficient, improve extraction methods and increase research efforts on understanding the metabolic pathways of the seaweeds bioactive compounds are crucial for further market development in Europe, including Portugal (Chapter 2).

The sampling performed in coastal and lagoon locations in southern Portugal showed the presence of five *Ulva* species across 44 samples: *U. rigida*, *U. flexuosa*, *U. compressa, U. australis* and *U. fasciata. U. australis* has been reported for the first time in southern Portugal and, as an introduced species, was not identified as a potential *Ulva* species for cultivation.
The species-specific differences (More PUFA in *U. compressa* than in *U. fasciata* and *U. rigida* within the same environment) and the variation of the nutritional profile due to different cultivation environments (higher protein in coastal areas than in lagoon locations) that have become evident in this work, support that general statements on seaweeds health benefits should be treated with caution. It also showed that whether one *Ulva* species is favorable for production based on higher protein or PUFA content could only be answered for PUFA, since for *U. compressa* results clearly suggested that when targeting PUFA the species stands out compared to *U. fasciata* and *U. rigida.*
For protein content however, no significant difference among species was observed, whereas protein content was significantly higher in coastal locations compared to Ria Formosa. This finding suggests that site selection and/or cultivation parameter optimization seems to be more important for protein production than for PUFA production, where no difference between locations was observed. The different results for PUFA and protein suggest that protein content in *Ulva* is more susceptible to environmental conditions than PUFA and PUFA being potentially more species dependent.
In the discussion of the results, temperature and nutrient availability were the main factors chosen to explain differences in findings. Here it became evident that whereas the effect of nutrient availability on metabolic pathways in seaweed is well studied and many comparative studies were found, the same cannot be said for the effect of temperature on protein and PUFA

content in *Ulva*. I therefore suggest more research on the effect of temperature on protein and PUFA metabolism in *Ulva*, to enhance understanding on how to optimize *Ulva* cultivation for food and feed purposes.

Due to the low overall amount of protein and PUFA found and the lower numbers in lagoon locations (except for PUFA in *U. compressa*), I suggest that when cultivating *Ulva* for protein and fatty acids in southern Portugal, cultivation in an IMTA system (where nutrient flow is high) or in a closed system (where all cultivation parameters can be controlled) is beneficial compared to cultivating *Ulva* in open-systems alone. Within the marine- and brackish water aquaculture activity in Portugal, 34% of production is intensive and 11.1% semi-intensive (INE 2016). Integrating *Ulva* in existing aquaculture structures in Portugal in the form of IMTA can reduce the adverse effects of aquaculture, whilst creating new valuable biomass and is therefore concluded as the currently best option for production of *Ulva* biomass in Portugal. This does not imply that when integrating *Ulva* in existing aquaculture structures, the entire aquaculture activity becomes biological, since it still depends on other factors such as animal welfare or the fish-feed.

The additional benefit of open-system seaweed cultivation of reducing excess nutrients in highly anthropogenically influenced areas does not seem to apply to the Ria Formosa area around Faro, at least not in springtime. At the same time, seaweed abundance and their biochemical composition are a good bioindicator of the pollution level in the lagoon. However, with increasing anthropogenic influence and temperature rise associated with climate change, the situation may change and make cultivation of *Ulva* in the Ria Formosa more beneficial for the environment, whilst obtaining increased amounts of target compounds through elevated temperature and higher nutrient input into the coastal lagoon.

To conclude, drawing our attention to a more ocean-based food system may not only improve overall human health but also that of our planet. I conclude that *Ulva* is a currently underexploited crop in southern Portugal and that its cultivation is one of the many puzzle pieces of rethinking our food system.

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6. Annexes

Annex A

Table 6: Information on the sampling locations of all 44 samples including date, coordinates, water temperature, salinity, substrate type, water body, depth and attachment.

n	#	Date	Coordinates	Water Temp. (C°)	Salinity	Substrat type	Water body	Depth (cm)	Attachement
1	R1	18.03.19	37°00'19.4"N 7°58'02.8"W	19,4	36,5	Soft bottom(silt)	Salina, Ramalhete	50	Tyre (Plastic)
2	R2	18.03.19	37°00'19.4"N 7°58'02.8"W	18,6	32,7	Sandy	Rising tide, big channel Ramalhete	50	Free floating
3	R3	18.03.19	37°00'19.4"N 7°58'02.8"W	21	40,1	Soft bottom(silt)	Tidal pond limited by vegetation, Ramalhe	10	Free floating
4	R4	18.03.19	37°00'19.4"N 7°58'02.8"W	18,5	33,7	Rock	Rising tide, big channel Quinta do Lago	70	Big Rock
5	R5	19.03.19	36°59'53.4"N 7°58'40.5"W	15,7	35	Sandy	Puddle, low tide, Ilha de Faro	10	Small Rock
6	R6	19.03.19	36°59'53.4"N 7°58'40.5"W	15,7	35	Sandy	Puddle, low tide, Ilha de Faro	10	Shell
7	R7	19.03.19	36°59'53.4"N 7°58'40.5"W	16,4	34,6	Sandy	Puddle, low tide, Ilha de Faro	10	Shell
8	R8	19.03.19	36°59'53.4"N 7°58'40.5"W	16,3	35,4	Sandy	Puddle, low tide, Ilha de Faro	<10	Free floating
9	R9	19.03.19	36°59'53.4"N 7°58'40.5"W	17,6	35,1	Sandy	Puddle, low tide, Ilha de Faro	<10	Shell/burried
10	R10	19.03.19	36°59'53.4"N 7°58'40.5"W	17	34,8	Sandy	Small channel, low tide, Ilha	40	Shell
11	R11	19.03.19	36°59'53.4"N 7°58'40.5"W	17,6	35,5	Ponton	Main channel, lowtide, Ilha	20	Ponton (plastic)
12	R12	19.03.19	36°59'53.4"N 7°58'40.5"W	17,6	35,5	Ponton	Main channel, lowtide, Ilha	20	Ponton (plastic)
13	A13	08.04.19	37°5'0,68"N8°13'53,45"W	17,4	34,5	Rocky pool	Intertidal, low tide, Albufeira coast	1	Rock
14	A14	08.04.19	37°5'0,68"N8°13'53,45"W	17,5	34,5	Rocky pool	Intertidal, low tide, Albufeira coast	1	Rock
15	A15	08.04.19	37°5'0,68"N8°13'53,45"W	16	34,5	Rocky pool	Intertidal, low tide, Albufeira coast	15	Sand on rock
16	A16	08.04.19	37°5'0,68"N8°13'53,45"W	18	34,5	Rocky pool	Intertidal, low tide, Albufeira coast	<1	Epiphytic on *Coralina*
17	A17	08.04.19	37°5'0,68"N8°13'53,45"W	18,6	34,5	Rocky pool	Intertidal, low tide, Albufeira coast	15	Rock
18	A18	08.04.19	37°5'0,68"N8°13'53,45"W	18,5	34,5	Rocky pool	Intertidal, low tide, Albufeira coast	<1	Rock
19	A19	08.04.19	37°5'0,68"N8°13'53,45"W	18,5	34,5	Rocky pool	Intertidal, low tide, Albufeira coast	5	Rock
20	A20	08.04.19	37°5'0,68"N8°13'53,45"W	18,5	34,5	Rocky pool	Intertidal, low tide, Albufeira coast	5	Rock
21	V21	10.04.19	37,071999-8,12522'4	16,5	n.d.	Rocky Pier	Low tide, coast Vilamoura	1	Mussel
22	R22	11.04.19	37°00'19.4"N 7°58'02.8"W	20	n.d.	Soft bottom(silt)	Low tide, Ilha de Faro	10	Sand
23	R23	12.04.19	37°00'19.4"N 7°58'02.8"W	20	n.d.	Soft bottom(silt)	Low tide, Ilha de Faro	10	Sand
24	R24	13.04.19	37°00'19.4"N 7°58'02.8"W	20	n.d.	Soft bottom(silt)	Low tide, Ilha de Faro	10	Sand
25	R25	14.04.19	37°00'19.4"N 7°58'02.8"W	20	n.d.	Soft bottom(silt)	Low tide, Ilha de Faro	10	Sand
26	R26	15.04.19	37°00'19.4"N 7°58'02.8"W	20	n.d.	Soft bottom(silt)	Low tide, Ilha de Faro	10	Sand

Table 7: Table 6 continued.

n	#	Date	Coordinates	Water Temp. (C°)	Salinity	Substrat type	Water body	Depth (cm)	Attachement
26	R26	15.04.19	37°00'19.4"N 7°58'02.8"W	20	n.d.	Soft bottom(silt)	Low tide, Ilha de Faro	10	Sand
27	R27	16.04.19	37°00'19.4"N 7°58'02.8"W	20	n.d.	Soft bottom(silt)	Low tide, Ilha de Faro	10	Sand
28	W28	14.04.19	37°49'42.0"N 8°47'30.6"W	18,4	n.d.	Rocky pool	Decreasing tide, west coast	4	Sand
29	w29	14.04.19	37°49'42.0"N 8°47'30.6"W	18,4	n.d.	Rocky pool	Decreasing tide, west coast	4	Sand
30	w30	14.04.19	37°49'42.0"N 8°47'30.6"W	18,4	n.d.	Rocky pool	Decreasing tide, west coast	4	Sand
31	W31	14.04.19	37°49'42.0"N 8°47'30.6"W	16,6	n.d.	Rocky pool	Decreasing tide, west coast	0	Rock
32	W32	14.04.19	37°49'42.0"N 8°47'30.6"W	16,6	n.d.	Rocky pool	Decreasing tide, west coast	0	Rock
33	W33	14.04.19	37°49'42.0"N 8°47'30.6"W	16,6	n.d.	Rocky pool	Decreasing tide, west coast	0	Rock
34	W34	14.04.19	37°49'42.0"N 8°47'30.6"W	16,6	n.d.	Rocky pool	Decreasing tide, west coast	0	Rock
35	W35	14.04.19	37°49'42.0"N 8°47'30.6"W	16,6	n.d.	Rocky pool	Decreasing tide, west coast	0	Rock
36	W36	14.04.19	37°49'42.0"N 8°47'30.6"W	16,6	n.d.	Rocky pool	Decreasing tide, west coast	0	Rock
37	Ar37	15.04.19	38°29'06.2"N 8°56'32.5"W	16,8	n.d.	Rocky pool	Low tide, Arrábida	0	Rock
38	Ar38	16.04.19	38°29'06.2"N 8°56'32.5"W	16,8	n.d.	Rocky pool	Low tide, Arrábida	0	Rock
39	Ar39	17.04.19	38°29'06.2"N 8°56'32.5"W	16,8	n.d.	Rocky pool	Low tide, Arrábida	0	Rock
40	Ar40	18.04.19	38°29'06.2"N 8°56'32.5"W	16,8	n.d.	Rocky pool	Low tide, Arrábida	0	Rock
41	Ar41	19.04.19	38°29'04.5"N 8°57'20.4"W	16,8	n.d.	Rocky pool	Low tide, Arrábida	0	Rock
42	Ar42	20.04.19	38°29'04.5"N 8°57'20.4"W	16,8	n.d.	Rocky pool	Low tide, Arrábida	0	Rock
43	Ar43	21.04.19	38°29'04.5"N 8°57'20.4"W	16,8	n.d.	Rocky pool	Low tide, Arrábida	0	Rock
44	Ar44	22.04.19	38°29'04.5"N 8°57'20.4"W	16,8	n.d.	Rocky pool	Low tide, Arrábida	0	Sand

Annex B

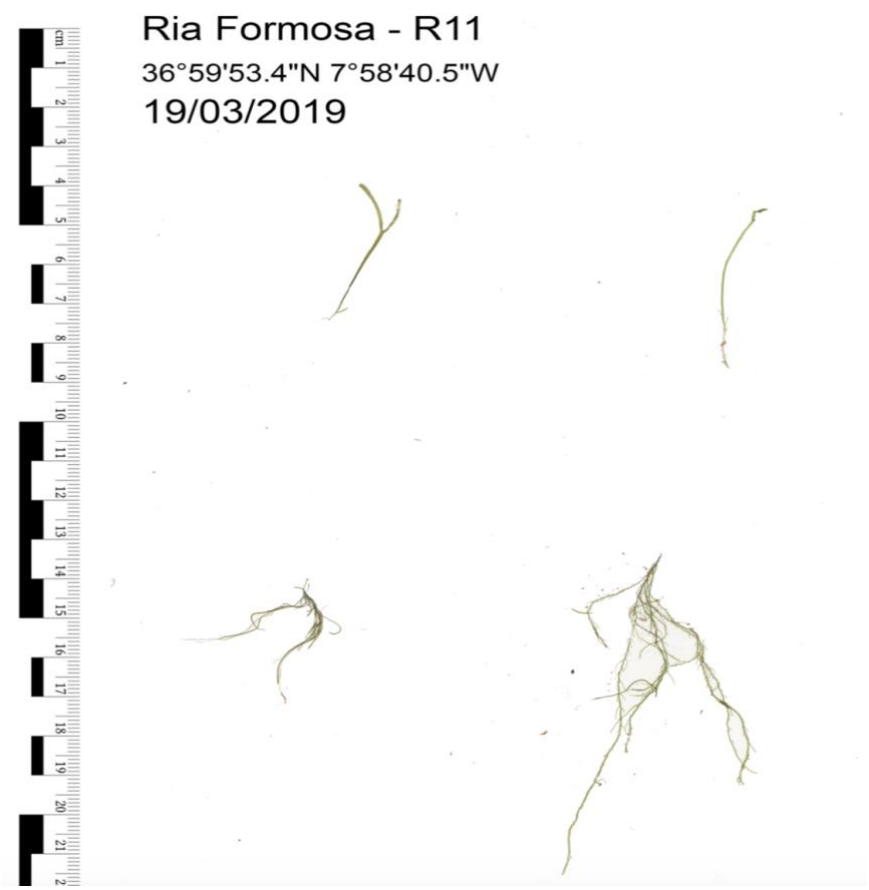
Herbarium of Ulva morphotypes in southern Portugal

by Robert Priester and Alena Sidow

Sample R3 - Identified as *Ulva rigida.*

Sample R1 - Identified as *Ulva flexuosa.*

Sample R7 - Identified as *Ulva compressa.*

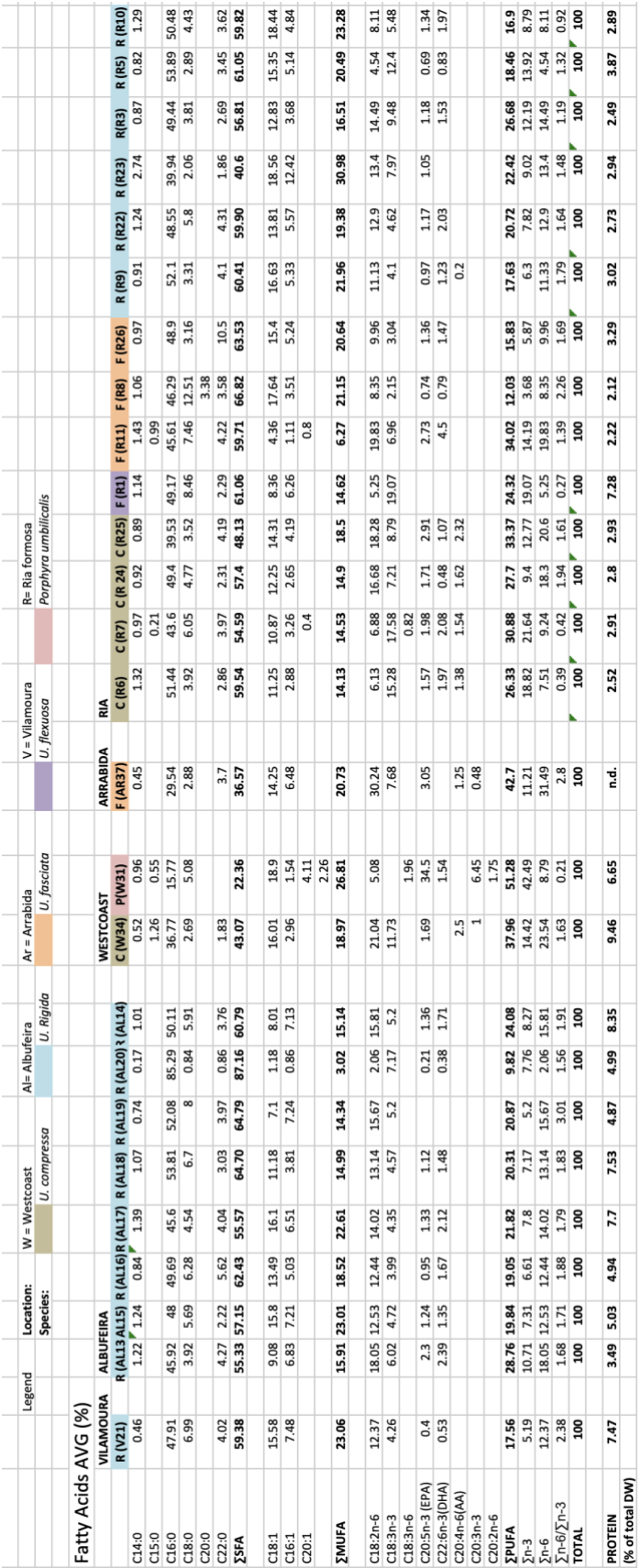


Sample R11 - Identified as *Ulva fasciata.*

Sample Ar41 - 44: Identified as *Ulva australis.*

Annex C

Table 8: Average (%) of the fatty acid composition of each sample expressed in (%) of total fatty acids and total protein (%).



Legend																														
	Location:			W = Westcoast			Al= Albufeira			Ar = Arrabida			V = Vilamoura			R= Ria formosa														
	Species:			*U. compressa*			*U. Rigida*			*U. fasciata*			*U. flexuosa*			*Porphyra umbilicalis*														
Fatty Acids AVG (%)																														
	VILAMOURA	ALBUFEIRA								WESTCOAST		ARRABIDA	RIA																	
	R (V21)	R (AL13	AL15)	R (AL16)	R (AL17)	R (AL18)	R (AL19)	R (AL20)	ϒ (AL14)	C (W34)	P(W31)	F (AR37)	C (R6)	C (R7)	C (R 24)	C (R25)	F (R1)	F (R11)	F (R8)	F (R26)	R (R9)	R (R22)	R (R23)	R(R3)	R (R5)	R (R10)				
C14:0	0.46	1.22	1.24	0.84	1.39	1.07	0.74	0.17	1.01	0.52	0.96	0.45	1.32	0.97	0.92	0.89	1.14	1.43	1.06	0.97	0.91	1.24	2.74	0.87	0.82	1.29				
C15:0										1.26	0.55			0.21				0.99												
C16:0	47.91	45.92	48	49.69	45.6	53.81	52.08	85.29	50.11	36.77	15.77	29.54	51.44	43.6	49.4	39.53	49.17	45.61	46.29	48.9	52.1	48.55	39.94	49.44	53.89	50.48				
C18:0	6.99	3.92	5.69	6.28	4.54	6.7	8	0.84	5.91	2.69	5.08	2.88	3.92	6.05	4.77	3.52	8.46	7.46	12.51	3.16	3.31	5.8	2.06	3.81	2.89	4.43				
C20:0																			3.38											
C22:0	4.02	4.27	2.22	5.62	4.04	3.03	3.97	0.86	3.76	1.83		3.7	2.86	3.97	2.31	4.19	2.29	4.22	3.58	10.5	4.1	4.31	1.86	2.69	3.45	3.62				
ΣSFA	**59.38**	**55.33**	**57.15**	**62.43**	**55.57**	**64.70**	**64.79**	**87.16**	**60.79**	**43.07**	**22.36**	**36.57**	**59.54**	**54.59**	**57.4**	**48.13**	**61.06**	**59.71**	**66.82**	**63.53**	**60.41**	**59.90**	**40.6**	**56.81**	**61.05**	**59.82**				
C18:1	15.58	9.08	15.8	13.49	16.1	11.18	7.1	1.18	8.01	16.01	18.9	14.25	11.25	10.87	12.25	14.31	8.36	4.36	17.64	15.4	16.63	13.81	18.56	12.83	15.35	18.44				
C16:1	7.48	6.83	7.21	5.03	6.51	3.81	7.24	0.86	7.13	2.96	1.54	6.48	2.88	3.26	2.65	4.19	6.26	1.11	3.51	5.24	5.33	5.57	12.42	3.68	5.14	4.84				
C20:1											4.11			0.4				0.8												
											2.26																			
ΣMUFA	**23.06**	**15.91**	**23.01**	**18.52**	**22.61**	**14.99**	**14.34**	**3.02**	**15.14**	**18.97**	**26.81**	**20.73**	**14.13**	**14.53**	**14.9**	**18.5**	**14.62**	**6.27**	**21.15**	**20.64**	**21.96**	**19.38**	**30.98**	**16.51**	**20.49**	**23.28**				
C18:2n-6	12.37	18.05	12.53	12.44	14.02	13.14	15.67	2.06	15.81	21.04	5.08	30.24	6.13	6.88	16.68	18.28	5.25	19.83	8.35	9.96	11.13	12.9	13.4	14.49	4.54	8.11				
C18:3n-3	4.26	6.02	4.72	3.99	4.35	4.57	5.2	7.17	5.2	11.73		7.68	15.28	17.58	7.21	8.79	19.07	6.96	2.15	3.04	4.1	4.62	7.97	9.48	12.4	5.48				
C18:3n-6											1.96			0.82																
C20:5n-3 (EPA)	0.4	2.3	1.24	0.95	1.33	1.12		0.21	1.36	1.69	34.5	3.05	1.57	1.98	1.71	2.91		2.73	0.74	1.36	0.97	1.17	1.05	1.18	0.69	1.34				
C22:6n-3(DHA)	0.53	2.39	1.35	1.67	2.12	1.48		0.38	1.71		1.54		1.97	2.08	0.48	1.07		4.5	0.79	1.47	1.23	2.03		1.53	0.83	1.97				
C20:4n-6(AA)										2.5		1.25	1.38	1.54	1.62	2.32					0.2									
C20:3n-3										1	6.45	0.48																		
C20:2n-6											1.75																			
ϒPUFA	**17.56**	**28.76**	**19.84**	**19.05**	**21.82**	**20.31**	**20.87**	**9.82**	**24.08**	**37.96**	**51.28**	**42.7**	**26.33**	**30.88**	**27.7**	**33.37**	**24.32**	**34.02**	**12.03**	**15.83**	**17.63**	**20.72**	**22.42**	**26.68**	**18.46**	**16.9**				
ϒn-3	5.19	10.71	7.31	6.61	7.8	7.17	5.2	7.76	8.27	14.42	42.49	11.21	18.82	21.64	9.4	12.77	19.07	14.19	3.68	5.87	6.3	7.82	9.02	12.19	13.92	8.79				
ϒn-6	12.37	18.05	12.53	12.44	14.02	13.14	15.67	2.06	15.81	23.54	8.79	31.49	7.51	9.24	18.3	20.6	5.25	19.83	8.35	9.96	11.33	12.9	13.4	14.49	4.54	8.11				
ϒn-6/ϒn-3	2.38	1.68	1.71	1.88	1.79	1.83	3.01	1.56	1.91	1.63	0.21	2.8	0.39	0.42	1.94	1.61	0.27	1.39	2.26	1.69	1.79	1.64	1.48	1.19	1.32	0.92				
TOTAL	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**				
PROTEIN (% of total DW)	**7.47**	**3.49**	**5.03**	**4.94**	**7.7**	**7.53**	**4.87**	**4.99**	**8.35**	**9.46**	**6.65**	**n.d.**	**2.52**	**2.91**	**2.8**	**2.93**	**7.28**	**2.22**	**2.12**	**3.29**	**3.02**	**2.73**	**2.94**	**2.49**	**3.87**	**2.89**				