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PROSPECTION OF BIOACTIVITIES, BIOACCESSIBILITY, AND BIOCHEMICAL CHARACTERIZATION OF GREEN SEAWEEDS GROWN IN INTEGRATED MULTI-TROPHIC AQUACULTURE ENVIRONMENTS

A THESIS

For the Degree of Master of Science in Marine Biology

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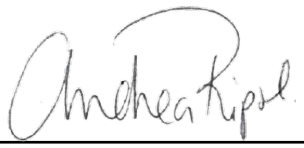
September 2017

Prospection of Bioactivities, Bioaccessibility, and Biochemical Characterization of Green Seaweeds Grown in Integrated Multi-Trophic Aquaculture Environments

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



(Andrea Ripol Malo)

Acknowledgments

Foremost, I would like to express my sincere gratitude to my advisor Dr. Carlos Cardoso for the continuous support, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis, making of it an enjoyable journey; I could not have imagined having a better advisor and mentor for my Master Thesis. You are a very special person!

Besides my advisor, I would of course like to thank my two supervisors: Dr. Narcisa Bandarra and Prof. João Varela for the opportunity and trust they put on me. Their encouragement, insightful comments, and experience have made this work truly meaningful.

In addition, I would like to thank all the colleagues that contributed to the work: Claudia Afonso, Hugo Quental-Ferreira, Pedro Pousão-Ferreira and the colleagues of National Health Institute Doutor Ricardo Jorge.

I would also like to thank my parents for their wise counsel and constant support. You are always there for me.

Finally, there are my friends and boyfriend. We were not only able to support each other by deliberating over our problems and findings, but also by happily talking about things other than just our papers and work.

Thank you very much, everyone!

I. Abstract

The nutritional composition of five species of green seaweeds (*Rhizoclonium riparium, Ulva lactuca, Ulva prolifera, Chaetomorpha linum, Ulva intestinalis*) grown in multi-trophic aquaculture systems were studied. Firstly, fucose and total polyphenols, as relevant bioactive constituents, were analyzed and antioxidant and anti-inflammatory activities were measured. The effects of bioaccessibility on these aspects were also assessed. Though lipid content was very low (less than 3 g/100 g dry weight), there were qualitative differences between lipid fractions, since fatty acid profiles varied considerably between the five seaweed species. The fucose content also depended on the particular species. Total polyphenol content and antioxidant activity presented a significant correlation. *U. prolifera* had the highest total polyphenol content and antioxidant activity, whereas no polyphenol or antioxidant activity was found in the bioaccessible fraction. The anti-inflammatory activity was highest in *U. prolifera* and *C. linum* extracts with high COX-2 inhibition (ranging between 18 and 27 %) at a concentration of 100 µg/mL. Despite the compounds causing this anti-inflammatory activity were not rendered bioaccessible, *U. prolifera* seems to be a potential source of bioactive substances, provided that adequate methods for their extraction are used or tisanes are developed that are able to enhance their bioaccessibility. Secondly, the lipid composition of the five species of green seaweeds was studied. In particular, the overall fatty acid (FA) profile and the FA profile of each main lipid class found in these seaweed species were thoroughly analysed. It was found that every seaweed had a specific FA profile, whose specificities were rendered more obvious with the study of the FA profile per lipid class. However, between *U. lactuca* and *U. intestinalis*, there were only minor differences. Nonetheless, it was possible to identify significant differences between the palmitic acid content in the PL class of each seaweed. A clear distinction between the FA profiles of *R. riparium* and *C. linum* (Cladophorales) and those of *Ulva* (Ulvales) was also determined. Moreover, there were also differences among lipid classes, yielding large contrasts between PL and TAG as well as between MAG and FFA. This study also found evidence supporting the location of particular FA in specific TAG positions. Finally, the mineral composition was studied. The elemental bioaccessibility in these species was also investigated through the application of an innovative *in vitro* digestive model. It was observed that *R. riparium* had the highest levels of Mn, Sr, Cd, Sn, and I and that *U. lactuca* had the highest Ni and Cu concentrations. The daily amounts of dried green seaweed required for achieving specific dietary intakes were calculated: 7 g of dried *U. lactuca* (for meeting Cu Recommended Daily Allowance, RDA); 173 g of dried *U. lactuca* (Zn RDA); 78 g of dried *C. linum* (Se RDA); 41 g of dried *C. linum* (Mo RDA); and 0.5 g of dried *R. riparium* (I Dietary Reference Intake, DRI). Mn and Cu had the highest values of elemental bioaccessibility, always above 50 %, whereas I was always poorly bioaccessible, in the range of 14-31 %. The bioaccessibility range of *R. riparium* (31-100 %) was higher than the ranges for other species, particularly that of *C. linum* (≤ 56 %). The bioaccessibility results entailed higher quantities of dried seaweed for reaching dietary intakes: 10 g of dried *U. lactuca* (Cu RDA); 290 g of dried *R. riparium* (Zn RDA); and 2 g of dried *R. riparium* (I DRI). Accordingly, *R. riparium* is a very rich I source. This study showed the importance of taking into account bioaccessibility results in estimating dietary intakes.

Keywords: Green seaweed; IMTA; antioxidant activity; anti-inflammatory activity; lipid classes; fatty acid composition; mineral composition; bioaccessibility.

II. Resumo

A composição nutricional de cinco espécies de algas verdes *(Rhizoclonium riparium, Ulva lactuca, Ulva prolifera, Chaetomorpha linum, Ulva intestinalis*) em sistemas de aquicultura multitróficos foi estudada. Em primeiro lugar, a fucose e os polifenóis totais, como constituintes bioativos relevantes, foram analisados e as atividades antioxidantes e anti-inflamatórias foram medidas. Os efeitos da bioacessibilidade nesses aspectos também foram avaliados. Embora o teor de lipídios tenha sido muito baixo (menos de 3 g / 100 g de peso seco), houve diferenças qualitativas entre as frações lipídicas, uma vez que os perfis de ácidos graxos variaram consideravelmente entre as cinco espécies de algas marinhas. O teor de fucose também dependeu das espécies específicas. O conteúdo total de polifenóis e a atividade antioxidante apresentaram correlação significativa. *U. prolifera* apresentou o maior teor total de polifenóis e atividade antioxidante, enquanto que nenhuma atividade de polifenol ou antioxidante foi encontrada na fração bioacessível. A atividade antiinflamatória foi maior nos extratos *U. prolifera* e *C. linum* com alta inibição de COX-2 (variando entre 18 e 27%) a uma concentração de 100 μg / mL. Apesar dos compostos que causam essa atividade antiinflamatória não serem tornados bioaccessíveis, a *U. prolifera* parece ser uma fonte potencial de substâncias bioativas, desde que sejam utilizados métodos adequados para sua extração ou desenvolvidos desenvolvendo tisanas que possam aumentar sua bioacessibilidade. Em segundo lugar, estudou-se a composição lipídica das cinco espécies de algas verdes. Em particular, o perfil geral de ácidos graxos (FA) e o perfil FA de cada classe principal de lipídeos encontrados nestas espécies de algas marinhas foram cuidadosamente analisados. Verificou-se que todas as algas tinham um perfil FA específico, cujas especificidades foram mais evidentes com o estudo do perfil FA por classe de lipídios. No entanto, entre *U. lactuca* e *U. intestinalis*, houve apenas pequenas diferenças. No entanto, foi possível identificar diferenças significativas entre o teor de ácido palmítico na classe PL de cada alga. Uma clara distinção entre os perfis de *R. riparium* e *C. linum* (Cladophorales) e os de *Ulva* (Ulvales) também foi determinada. Além disso, houve diferenças entre as classes de lipídios, produzindo grandes contrastes entre PLs e TAG, bem como entre MAGs e FFA. Este estudo também encontrou evidências que suportam a localização de FA específicas em posições TAG específicas. Finalmente, a composição mineral foi estudada. A bioacessibilidade elementar nessas espécies também foi investigada através da aplicação de um modelo digestivo in vitro inovador. Observou-se que *R. riparium* apresentou os níveis mais altos de Mn, Sr, Cd, Sn e I e que a *U. lactuca* apresentou as maiores concentrações de Ni e Cu. As quantidades diárias de algas verdes secas necessárias para a obtenção de ingestão dietética específica foram calculadas: 7 g de *U. lactuca* secas (para a reunião Cu recomendado diariamente, RDA); 173 g de *U. lactuca* seca (Zn RDA); 78 g de *C. linum* seca (Se RDA); 41 g de *C. linum* seca (Mo RDA); e 0,5 g de *R. riparium* seca (I Dietary Reference Intake, DRI). Mn e Cu tiveram os valores mais elevados de bioacessibilidade elementar, sempre acima de 50%, enquanto que sempre fui praticamente bioacessível, na faixa de 14-31%. A faixa de bioacessibilidade de *R. riparium* (31-100%) foi maior do que as faixas para outras espécies, particularmente a de *C. linum* (≤ 56%). Os resultados de bioacessibilidade implicaram maiores quantidades de algas secas para atingir a ingestão dietética: 10 g de *U. lactuca*

seca (Cu RDA); 290 g de *R. riparium* seca (Zn RDA); e 2 g de *R. riparium* seca (I DRI). Consequentemente, *R. riparium* é uma fonte muito rica. Este estudo mostrou a importância de levar em consideração resultados de bioacessibilidade na estimativa de ingestão dietética.

Palavras-chave: Algas verdes; IMTA; atividade antioxidante; atividade anti-inflamatória; classes de lipídios; composição de ácidos graxos; composição mineral; bioacessibilidade.

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1. Introduction

1.1 Integrated multi-trophic aquaculture (IMTA)

The aquaculture production has grown dramatically worldwide. Over the past three decades, aquaculture production has increased from 6.2 million t in 1983 to 70.2 million t in 2013 (FAO, 2015). In 2013, aquaculture surpassed the supplies from the capture fisheries and contributed nearly 51% to the global fish production. This growth in marine aquaculture industry has introduced many concerns about the environmental impacts of aquaculture waste on the ecosystems (Mente et al., 2006; Reid et al., 2008; Tacon & Forster, 2014). Intensive fish farming can release significant quantities of nutrients to the vicinity of the farm site in the form of uneaten feed, feces and other excretory products. These metabolic wastes from farm effluents, mostly ammonia, may contribute to an increment of nutrients and, consequently, eutrophication in the farm. One of the major challenges for the sustainable development of aquaculture industry is to minimize environmental degradation concurrently with its expansion.

In many monoculture farming systems, the fed aquaculture species (e.g. carnivorous fish, shrimps) and the organic/inorganic extractive aquaculture species (e.g. bivalves, herbivorous fishes and aquatic plants) are independently farmed in different geographical locations, resulting in a pronounced shift in the environmental processes (Sasikumar & Viji, 2006). Integrated multi-trophic aquaculture (IMTA) is a practice in which the by-products (wastes) from one species are recycled by becoming inputs (fertilizers, food and energy) for another species (Fig. 1). IMTA involves cultivating in the appropriate proportions, fed aquaculture species (e.g. finfish/shrimps) with organic extractive aquaculture species (e.g. suspension feeders/deposit feeders/herbivorous fish) and inorganic extractive aquaculture species (e.g. seaweeds) for a balanced ecosystem management approach that takes into consideration site specificity, operational limits, and food safety guidelines and regulations. The goals are to achieve environmental sustainability through biomitigation, economic stability through product diversification and risk reduction, and social acceptability through better management practices (Barrington et al., 2009).

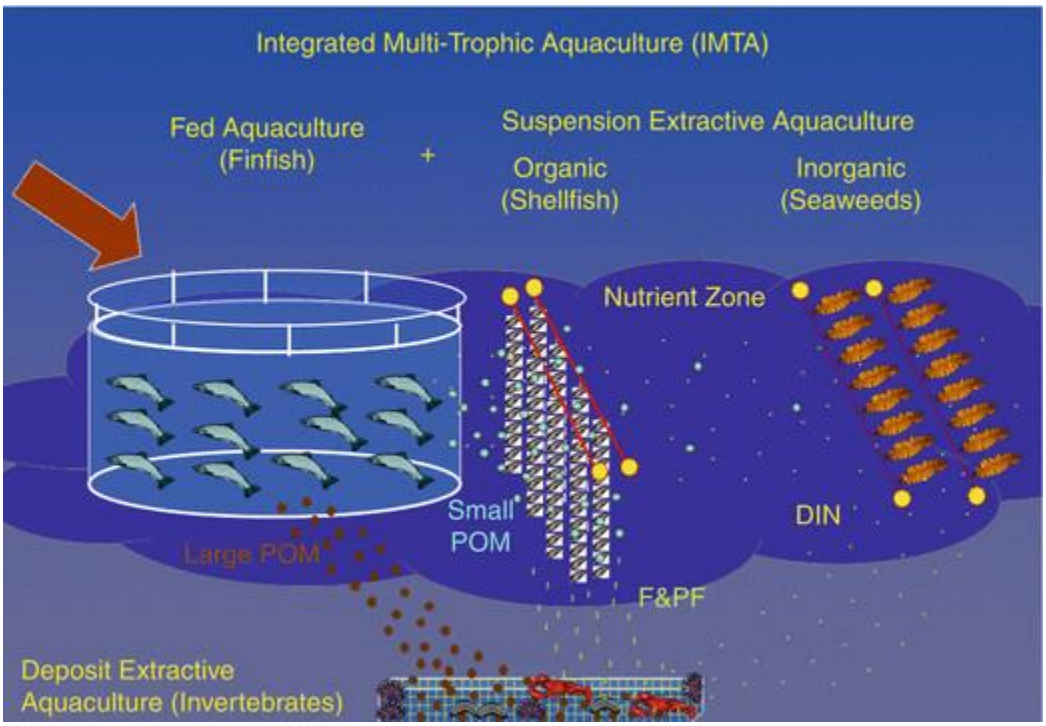

Figure 1: Conceptual diagram of an Integrated Multi-Trophic Aquaculture (IMTA) where the organic extractive aquaculture species (e.g., shellfish) take advantage of the enrichment in small particulate organic matter (POM) coming from the excretion products of fed aquaculture (e.g., finfish); inorganic extractive aquaculture species (e.g., seaweeds) take advantage of the enrichment in dissolved inorganic nutrients (DIN). Deposit organic extractive aquaculture species (e.g., echinoids, holothuroids, and polychaetes), take advantage of the enrichment in large particulate organic matter (POM) and feces and pseudo-feces (F&PF) from suspension-feeding organisms. The bioturbation on the bottom also regenerates some DIN, which becomes available to the seaweeds (Chopin, 2013).

The appropriate selection and proportions of the different species will provide different ecosystem functions and it is crucial for the correct balance of the biological and chemical processes in an IMTA. However, the co-cultured species should be more than just biofilters; they should also be harvestable crops of commercial value (Barrington et al., 2009). Seaweed aquaculture is a rapidly growing component of marine aquaculture, with about 0.17% of all named marine seaweed having been cultured to date and a growth rate of global marine seaweed production at 7.5% per year. In parallel, the range of sectors demanding products of seaweed farming has widened, from an initial focus to direct food supply to humans, to include bio-energy, cosmetics, biomedical applications, and formulation of feeds for aquaculture animals (Mazarrasa et al., 2014). Marine macroalgae are considered to be important reservoirs of several bioactive compounds that display important biological activities, which may be relevant for the improvement of human health, including antioxidant, anti-inflammatory and antitumor activities (Custódio et al., 2016). Several authors have referred to the nutritional high value of several seaweeds and suggest that they may be

included as a food ingredient. For example, *Phaeophyta* seaweeds can be a good source of biological active compounds, such as antioxidants, fatty acids, polysaccharides, vitamins (A, B12, C, D and E) and minerals (iron, Fe, magnesium, Mg, iodine, I, calcium, Ca) among others (Cian et al., 2014a).

In the Far East and Pacific, there has been a long tradition of consuming seaweeds as sea vegetables, while in Western countries, commercial applications of seaweed products have been restricted to the manufacture of gelling agents, stabilizers and food additives, pharmaceuticals and fertilizers (Burtin, 2003). However, with the increased awareness for the need of a healthy diet, the interest in seaweeds for food consumption is on the rise in Western countries. Depending on the concentration of metals in the environment and the bioavailability ratio from the seaweed, macroalgae can accumulate metals at levels several thousand times higher than those found in the surrounding seawater. Although seaweeds are a source of essential minerals for humans, they also might present a risk for human health because they are accumulators of non-essential elements, some of them widely recognized for their high toxicity, such as arsenic (Kim, 2015). Bioaccesibility will be nevertheless the final determining factor affecting bioavailability and human health. In order to exert their beneficial or harmful effects, nutrient content in seaweeds need firstly to be released from the food matrix and secondly to be absorbed at intestinal level (Moreda-Piñeiro et al., 2012).

1.2 Seaweeds properties

1.2.1 Antioxidants

Antioxidant molecules are important to the living organisms since they act against reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), nitric oxide (NO), superoxide anion (O_2^-) and hydroxyl radical ($OH^\bullet$). These ROS are produced during the cellular metabolism with harmful effects, since they are highly reactive and tend to initiate chain reactions that promote irreversible damage to proteins, lipids, and DNA (Balboa et al., 2013; Hamed et al., 2015).

Several seaweeds species can be found belonging to three different groups on the basis of their color: Chlorophyta (green), Rhodophyta (red) and Phaeophyta (brown). The color in green seaweeds is due to the presence of chlorophyll *a* and *b* in the same proportions as in 'higher' plants. Phycoerythrin and phycocyanin are responsible for the color of red seaweeds by masking the pigments such as chlorophyll

a. The brown coloration is due to the presence of xanthophylls such as fucoxanthin that will mask, like in red algae, the chlorophylls present in the seaweed (Gupta and Abu-Ghannam, 2011).

Brown and red seaweeds are considered rich sources of antioxidants, including pigments such as fucoxanthin and polyphenols such as phlorotannins (Chakraborty et al., 2013). However, in a study with *Sargassum siliquastrum*, a brown seaweed, the total phenolic content did not correlate with antioxidant activity. These inconsistent results imply that not only the total phenolic content, but also other constituents, such as chlorophyll and carotenoids, may affect the antioxidant activity of extracts from marine algae. Despite extensive research on the antioxidant potential of extracts from various types of marine algae, few studies have been performed on the antioxidant compounds originated from green seaweeds, which are ubiquitous, easily cultivated and important natural resources (Cho et al., 2011). The antioxidants levels present in algae can be affected by a number of parameters such as location, salinity, sun exposure, season, and seaweed age (Holdt and Kraan, 2011).

1.2.2 Lipids and fatty acids

The total lipid (TL) contents in seaweeds is, in general, in the range of 1-6% dry weight (dw), varying in green seaweeds according to its phylogeny (Fleurence and Levine, 2016). The major lipids found in seaweeds are glycoglycerolipids, representing more than half of the total lipid content in some species, followed by phospholipids, triacylglycerols, sterols and pigments (Holdt and Kraan, 2011; Miyashita et al., 2013).

Glycoglycerolipids are very important to seaweeds and high plants since they collaborate in the photosynthesis and serve as markers for cellular recognition because of their association with cell membranes (Holdt and Kraan, 2011; Miyashita et al., 2013). Glycoglycerolipids are abundant in chloroplasts whose composition is rarely rich in highly unsaturated fatty acids. On the contrary, chloroplasts are rich in polyunsaturated fatty acids (PUFA). Polyunsaturated fatty acids (PUFA) are essential nutrients which cannot, or only to a limited extent, be synthesized by mammals. Therefore, they must be ingested via dietary sources. The two main PUFA groups are omega-3 (*n*-3) and omega-6 (*n*-6). The *n*-3 PUFA are provided by fish and microalgae, whereas the *n*-6 PUFA are ingested mainly via vegetable oil.

It is important to maintain an appropriate balance of n-3 and n-6 in the diet as these Fatty Acids (FA) work together to promote health: some n-3 PUFA, especially DHA, are major components of brain cells and crucial for proper development and functioning of the brain and the nervous system. Besides this, n-3 FA, particularly EPA, have been recognized to exhibit anti-inflammatory and antioxidant activity. Until now the major source of n-3 long-chain PUFAs, EPA and DHA, is fish oil. However, it is noteworthy that the original source of these long-chain PUFA is not the fish itself, but marine algae and phytoplankton which form their major dietary source (van Ginneken et al., 2011). The most common PUFA found in green seaweeds is alpha linolenic acid (ω3 C18:3), followed by EPA and DHA (Burtin, 2003).

1.2.3 Polysaccharides

Seaweeds contain large amounts of cell wall structural polysaccharides and storage polysaccharides (Davis, Volesky and Mucci, 2003; Holdt and Kraan, 2011). Polysaccharides are polymers of simple sugars (monosaccharides) linked together by glycosidic bonds, and have numerous commercial applications as food/feed additives (Holdt and Kraan, 2011). The total polysaccharide concentrations in the seaweed species range from 4% to 76% of dry weight, which makes them of major interest to the industry (Holdt and Kraan, 2011).

Polysaccharides are classified according to their biological function in two groups: energy storage and structural polysaccharides (Stadnik & Freitas, 2014). The principal cell wall polysaccharides in green seaweeds are ulvans, representing 8 to 29% of the algal dry weight (Vera at al., 2011). Because of its texturizing properties (gelling, thickening) and chemical specificities (presence of sulphate groups and rare sugars), ulvan offers numerous opportunities for applications in different industrial sectors: agri-food, cosmetics, pharmaceuticals, agriculture, chemicals industry, etc (Lahaye & Robic, 2007; Yanagisawa, Kawai, & Murata, 2013).

Sulfated fucans are one of the most well-studied sulfated polysaccharide and contain the monomer fucose, being present in all brown algae. Nevertheless, these polysaccharides may occur in minor amounts in green algae (Chlorophyta), red algae (Rhodophyta), and golden algae (Xanthophyta) (Mao, Zang, Li, & Zhang, 2006; Pomin & Mourão, 2008). One of the reasons why fucoidan has been so intensively studied is the numerous biological properties with potential human health applications that have

been shown such as: antitumor, anticoagulant, antioxidant activities, antiviral, antithrombotic, in addition to the impact on the inflammatory and immune systems (Davis et al., 2003; Holdt and Kraan, 2011; Hamed et al., 2015).

1.2.4 Essential elements

The mineral content of seaweeds is usually high enough (8–40% w/dw) to fulfill the recommended daily intakes of essential macroelements and trace elements for human nutrition (Kumar et al., 2011). This elemental content includes both macro- and trace elements such as magnesium, calcium, iron, copper, sodium, potassium, zinc, manganese, cobalt and especially iodine that are usually present in higher concentrations in seaweeds than in higher plants (Mabeau and Fleurence, 1993; Mohamed at al., 2012). Seaweeds are high in minerals due to their marine habitat, and the diversity of the minerals they absorb is wide. Important minerals, such as Ca, accumulate in seaweeds at much higher levels than in terrestrial foodstuffs. This is illustrated in an 8 g portion of *Ulva lactuca*, which provides 260 mg of Ca, equaling approximately 37% of the RDA of Ca for an adult male (Macartain et al., 2007). Nevertheless, mineral composition deeply varies according to the phylum, season, environment, geography, and physiology (El-Said & El-Sikaily, 2013; Kumar et al., 2011; Moreda-Piñeiro et al., 2012).

Having a diet rich in minerals is really important for human health, since they can interfere and/or be a part of key pathways necessary for the well-functioning of the human body. For example, Mg that is present in high quantities in seaweeds, works as co-factor for DNA and protein synthesis, oxidative phosphorylation, neuro-muscular excitability, enzyme activity and regulation of parathyroid hormone secretion (Saris et al., 2000; Romani, 2011). Mg deficiency or hypomagnesaemia is fairly common, with an estimated prevalence in the general population ranging from 2.5 to 15%, with higher rating, up to 65%, in patients in intensive care settings (Ayuk and Gittoes, 2014).

Iodine is also present in high quantities in seaweeds, and it essential for the production of the thyroid hormones thyroxine and triiodothyronine, which regulate many important physiological processes in humans. An iodine deficiency can cause several problems including an effect on growth and development due to insufficient formation of the thyroid hormones leading to spontaneous abortion, stillbirth, cretinism, goiter, and mental defects (Andersson at al., 2012; Hamed et al., 2015).

Trace elements, such as Zn, are present in seaweeds and some of these, such as arsenic, have negative health effects (Macartain et al., 2007). Severe Zn deficiency causes thymic atrophy, severe depression of immunity and acrodermatitis. Growth failure, delayed puberty, pregnancy complications, teratology, poor healing and decreased immunity occur in less severe Zn deficiency (Sahdstead & Smith, 1996). In the case of As, further analysis of speciation indicates that the type of As is important in assessing toxicity; since many types of As are not metabolized, these do not pose a risk to health. For the vast majority of seaweeds, the levels of heavy metals are below food safety limits naturally (Macartain et al., 2007).

Copper is an essential micronutrient that forms part of several proteins involved in a variety of biological processes indispensable to sustain life (Olivares et al., 2011). Minerals such as Cu and Fe are present in seaweeds at higher levels than in many well-known terrestrial sources of minerals, like meats and spinach. Therefore, regular seaweed consumption can help regulate Cu and other minerals dietary requirements (Macartain et al., 2007).

1.2.5 Toxic elements

Because of increasing levels of pollution in the oceans, seaweeds also tend to bioaccumulate contaminants like lead, cadmium, mercury, and arsenic, which makes them good environmental biomonitors (Reis et al., 2014). The toxicity of these elements is related to reactions that end up in electron transfer, formation of oxygen free radicals that can damage DNA, leading to increased mutagenicity, teratogenicity, genotoxicity, and carcinogenicity (Afonso, 2009).

Cadmium pollution may result from mining operations, metallurgical industries, corrosion of galvanized zinc batteries and the use of fertilizers (Afonso, 2009). In the human body, Cd competes with Zn, Cu and Fe which might lead to inhibiting their absorption. Cadmium can also replace Ca leading to bone changes that can provoke bone pain, osteoporosis, and osteomalacia (Del Ramo at al., 1993; Goyer, 1997).

Contamination of the environment by Pb is due to industrialization and the use of petrol (leaded) as fuel, as well as, the use of lead in cooking utensils, in pipes, paints, in pottery glazed with lead or industrial emissions, among others (Goyer and Clarkson, 1996). Nevertheless, the replacement of leaded petrol by unleaded fuel resulted in a decrease of the Pb contamination levels (Afonso, 2009). In individuals subjected to

occupational exposure, the most common effects are observed on the hematopoietic system and central nervous system which lead to encephalopathies. In extreme cases, Pb intoxication can lead to coma and death (Del Ramo at al., 1993; Goyer and Clarkson, 2001).

Arsenic is a ubiquitous element in the environment and can be derived from natural or anthropogenic sources. Anthropogenic causes comprise the release of As from primary Cu, Zn, and lead smelters, glass manufacturers that add As to the raw materials and chemical manufactures (Goyer and Clarkson, 2001). Arsenic is most toxic in its inorganic form as As(III) and As(V) but their methylated metabolites, monomethylarsonic acid and dimethylarsinic acid, are less toxic, while other major organoarsenicals found in seafood, arsenobetaine, trimethylarsine oxide, and arsenocholine have low or negligible toxicity (Moreda-Piñeiro et al., 2011). Chronic exposure to inorganic As compounds may lead to neurotoxicity of both the peripheral and central nervous systems, liver injuries, cardiovascular diseases, increased risk of diabetes mellitus, skin and lung cancer (Goyer and Clarkson, 2001).

Precisely, the wide range of constituents found in seaweeds has stimulated research into their potential as a source of bioactives and their ability to yield practical applications in animal and human food, cosmetics, and other biotechnological fields. This has, in turn, fostered the interest in producing seaweeds in larger quantities and under controlled conditions, thus leading to the study of aquaculture systems with a seaweed production component. In particular, green seaweeds are frequently part of aquaculture systems mainly to their spontaneous growth. Particularly, *Ulva* sp. is usually chosen to integrate aquaculture environments due to it fast growth and high uptake rates (Winberg et al., 2009). This study is going to focus on five different species of green seaweeds that spontaneously grow in the ponds of an Integrated Multitrophic Aquaculture system.

1.3 *Chaetomorpha linum*

Chaetomorpha linum, also known as spaghetti algae, is a green seaweed that grows as a loosely entangled filamentous mass (Fig. 2). Usually free-floating, it may also be attached to rocks and shells. The filaments themselves are unbranched and usually between 5 and 30 cm in length. The unattached filaments are wiry, stiff and curled in appearance. It is bright light to dark green in color. Spaghetti algae, though not

palatable to many herbivorous species, is popular in reef aquariums for its ability to remove nitrates, assist in buffering pH, uptake carbon dioxide producing oxygen, and assist in balancing trace elements. It also provides hiding spaces for small creatures. *Chaetomorpha linum* is an intertidal and supralittoral species that can be found in groups of hundreds or thousands of individuals in sandy areas, on rocks or around tide pools (Barnes, 2008).

Figure 2: *Chaetomorpha linum* in Mar Menor, Murcia, Spain. 11 Jul 2011. Isabel Rubio Perez (Guiry & Guiry 2017).

1.4 *Rhizoclonium riparium*

Rhizoclonium riparium is a cosmopolitan filamentous alga, which occurs in a variety of habitats including semienclosed intertidal zones, marshy areas of estuaries and abandoned aquaculture ponds (Fig. 3). It prefers brackish water, such as the intertidal zone, to full marine conditions and is especially abundant in standing water (Chao et al., 2005). It consists of mats or loose fine entangled threads 50-100 mm long that can be attached to other substrates by rhizoids or occur as floating mats (Fig. 4). It

is a common species and has a broad geographical distribution (Guiry & Guiry, 2017; Z, Huan, L, & H, 2016).-

Figure 3: Sample from a floating mat of *Rhizoclonium riparium* from Aquaculture Research Station, Olhão (EPPO), Portuguese Institute for the Sea and Atmosphere (IPMA, IP).

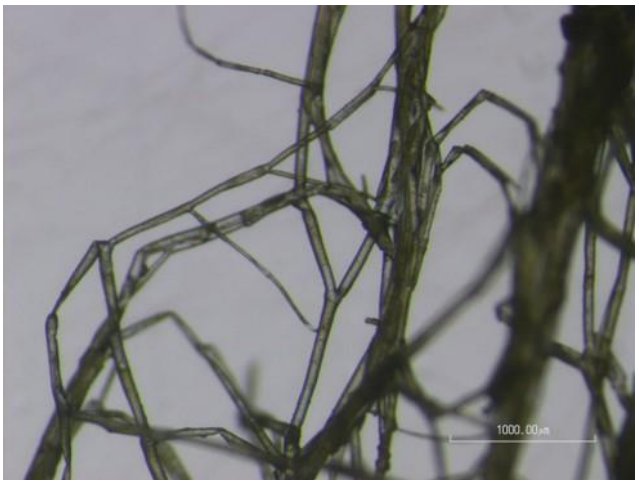

Figure 4: Detail of *Rhizoclonium riparium* from EPPO (Olhão) in 2015.

1.5 *Ulva intestinalis*

Ulva intestinalis is a green macroalgal species, frequently found in the coastal zone of seas and oceans. It is found also in sweetened out habitats connected with estuary waters (Messyasz & Rybak, 2008). *U. intestinalis* is a well-known bright grass-green seaweed, consisting of irregularly constricted tubular fronds that grow from a small discoid base. Fronds are typically unbranched with the tips usually rounded (Fig. 5). Fronds may be 10-30 cm or more in length and 6-18 mm in diameter. Like other members of the genus, *Ulva intestinalis* is a summer annual, decaying and forming masses of bleached white fronds towards the end of the season (Fig. 6). It occurs in a

wide range of habitats on all levels of the shore. Where suitable support is available, it will grow on rocks, mud, sand and in rock pools. The seaweed may become detached from the substratum and rise to the surface, where it continues to grow in floating masses (Budd & Pizzola 2008).

Figure 5: Studied sample of *Ulva intestinalis* from Aquaculture Research Station, Olhão (EPPO), Portuguese Institute for the Sea and Atmosphere (IPMA, IP).

Figure 6: *Ulva intestinalis* floating before sampling at EPPO (Olhão). Under the surface we can discern large quantities of *Chaetomorpha linum.*

1.6 *Ulva lactuca*

Ulva lactuca, also known as sea lettuce, is a small green alga (up to 30 cm across) with a broad, crumpled frond that is tough, translucent and membranous (Fig. 7). The sea lettuce is found at all levels of the intertidal, although in more northerly latitudes and in brackish habitats it is found in the shallow sublittoral. Usually it is attached to rocks but in very sheltered conditions. Plants that have become detached from the substrate can continue to grow, forming extensive floating communities. The plant tolerates brackish conditions and can be found on suitable substrata in estuaries. It presents green to dark green in color.

Figure 7: *Ulva lactuca* in Zeeland delta, Netherlands. 26 Mar 2011. Mat Vestjens & Anne Frijsinger (Guiry & Guiry 2017).

1.7 *Ulva prolifera*

Ulva prolifera is a common green alga which grows near the top of the shore, on rocks (Fig. 8) or other algae. It can be found on open coasts or in estuaries and harbors, where it may grow mixed with *U. intestinalis* or other species of the genus. The fronds are tubular, tough and often more or less flattened (Brodie et al, 2007) (Fig. 9).

Free-floating *Ulva prolifera* is one of the causative species of green tides that occur along the shoreline in many countries which not only seriously affects the inshore environment, but also threatens the offshore environment and the ecological balance of the marine community. The very high growth rate in addition to the rapid proliferation

of produced spores causes the rapid biomass accumulation characteristic of green tides (Huan et al 2016).

Figure 8: *Ulva prolifera* grown on a rock from the Aquaculture Research Station in Olhão.

Figure 9: Studied sample of *Ulva prolifera* from the Aquaculture Research Station, Olhão (EPPO), Portuguese Institute for the Sea and Atmosphere (IPMA, IP).

Although green seaweeds represent a food group that is not normally ingested in Western societies, currently they are attracting increasing attention as a valuable food source. As we have seen, the potential of green seaweeds is large, with high levels of carbohydrates as well as minerals, vitamins, and trace elements such as iodine (Macartain et al., 2007). However, not only the amount of the components of a food is important, but also, they must be available for absorption after the digestive process.

1.8 Digestive process

The digestion is a physiologic process (through mechanic movements, chemicals and enzymes) that allows the release of nutrients and phytochemicals, among others, from the food matrix, allowing them to be later absorbed by the organism (Tagliazucchi et al., 2010; Bouayed et al., 2011).

1.8.1 Human digestion

Human digestion is considered to be extracellular and happens in what is known as "digestive tract". In the latter, mechanical processes like mastication, swallowing and peristalsis movements occur. These are accompanied by the chemical component of digestion entailing pH variation and enzyme action. pH variation will promote the enzymatic hydrolysis of the food proteins, lipids, carbohydrates and nucleic acids. Afterwards, the monomers will be absorbed at the intestinal level to the blood stream (Diagram Group, 2005).

The digestive tract is composed of mouth, pharynx, esophagus, stomach, small intestine, large intestine, and anus. Besides these ones, other organs and glands are also associated to the digestion system such as salivary glands, liver, pancreas, and gall bladder (Lidon and Silvestre, 2010). The digestive process begins in the mouth or oral cavity, where the mechanical and chemical disaggregation of the food takes place. The initial degradation of, for instance, polysaccharides and triacylglycerols occur during mastication and salivation, with the help of teeth and tongue, which tend to facilitate the enzymatic action. Saliva is composed mainly of water and salivary amylase that initiates the digestion of carbohydrates (Diagram Group, 2005; Bouayed et al., 2011). Subsequently, the bolus is swallowed entering the esophagus through involuntary movement (rhythmic wave-like muscle contractions and relaxations) to the stomach.

In the stomach, the gastric juices, produced by glands in the stomach wall, dissolve the intercellular substances from the ingested food, helping the mechanical fragmentation initiated by the chewing process. The acid facilitates the fragmentation of various macromolecules, provides an optimum pH for protein digestion, contributes to the activation of enzymes present in the gastric juice, and exerts germicidal action. The enzymes of the gastric juice are pepsin, gastric lipase and gastric amylase. Pepsin is a proteolytic enzyme having a maximum activity at acidic pH (pH 2.0) and becoming

inactive at pH values above 5.0. With the action of gastric juice on food bolus it gives rise to chyme (Diagram Group, 2005; Lidon and Silvestre, 2010).

Stomach chyme passes into the small intestine stimulating duodenal mucosa to produce the hormones secretin and pancreosin, which in turn stimulate the pancreas to secrete pancreatic juice containing water, enzymes (trypsin, chymotrypsin, pancreatic amylase, pancreatic lipase, deoxyribonuclease and ribonuclease), and large amounts of sodium bicarbonate to neutralize the acidity of the chyme and thus ensure the action of pancreatic enzymes (Lidon and Silvestre, 2010). In the duodenum, the bile is also discharged from the gallbladder. Bile has no digestive enzymes but have bile salts, sodium glycocholate and taurocholate, to emulsify lipids, thereby fostering lipid digestion. In the intestine, chyme is transformed into chyle, a fluid rich in simple sugars, amino acids, fatty acids and glycerol (Lidon and Silvestre, 2010; Bouayed et al., 2011; Gião et al., 2012).

The nutrients in their simplest forms are then absorbed through the intestinal wall as water is reabsorbed. In the large intestine (which has no villi and does not secrete digestive juices) occurs the water and salts absorption, and by the action of numerous bacteria that make up the intestinal flora, proceeds to the dissolution of food remains unassimilable, thus leading to the formation of faeces. The bacterial fermentation that occurs in the large colonic intestine also plays a key role in the release of nutrients, making them available for absorption through the gut barrier (Diagram Group, 2005; Bouayed et al., 2011; Gião et al., 2012).

1.8.2 Bioaccessibility and bioavailability of nutrients and contaminants

Bioaccessibility is defined as the fraction of a compound that is released from the food matrix to the gastrointestinal tract, so it can be absorbed by the intestine. On the other hand, the bioavailability is the fraction of a bioaccessible compound that reaches the systemic circulation and becomes available to be absorbed by the various cells in any tissue of the human organism, stored and/or used in metabolic functions (Moreda-Piñeiro et al., 2011). Bioavailability and bioaccessibility of a compound can be affected by several factors, including:

- Possible interactions with other food components;
- Formation of stable compounds that are slowly metabolized;
- Special physiological conditions of the consumer, like age and health;

- The release from the food matrix;
- The chemical state of the nutrient.

Thus, the total amount ingested of a compound may not provide an adequate guidance for the amount that is bioaccessible and bioavailable.

1.8.3 Evaluation of the bioaccessibility *in vitro* digestion model

The compound mobilization in the food matrix to the gastrointestinal tract is a dynamic process with constant changes in physiological conditions. In the *in vitro* digestion model, the digestive process is simulated in a simplified manner by applying/simulating physiological conditions that replicate the chemical composition of the digestive fluids, pH and typical residence time in each digestive step (Versantvoort and Rompelberg, 2004).

The *in vitro* digestion model developed by Versantvoort & Rompelberg (2004) allows to simulate the digestive process in the gastrointestinal tract (mouth, stomach and small intestine). In each compartment, the matrix is incubated at 37±2 °C. Digestion is started by adding artificial saliva to the matrix under investigation. Subsequently, gastric and duodenal juices and bile are added to simulate the digestive process in the stomach and small intestine, respectively. Subsequently, concentration of the compound of interest is determined in the bioaccessible and undigested fractions (Versantvoort and Rompelberg, 2004; C Afonso et al., 2015).

Although there are *in vivo* methods to estimate the bioavailability of a nutrient/contaminant, *in vitro* methods are preferred, even with their limitation, since are less expensive, easier to reproduce and do not raise ethical problems (García-Sartal et al., 2013).

2. Objective

Over the last few years, marine organisms, particularly seaweeds, have proved to be a unique source of molecules with high biotechnological interest, providing new compounds with the most diverse pharmacological and food properties.

The Aquaculture Research Station (EPPO) from the Portuguese Institute for the Sea and Atmosphere (IPMA) owns several Earth Ponds Aquaculture Systems with a Seaweed Production component where seaweeds grow spontaneously. Hence, the main objective of this work corresponded to the biochemical characterization of five seaweed species from IMTA systems and the determination of relevant bioactivities such as antioxidant and anti-inflammatory properties. This study enabled an assessment of the potential of this significant aquatic resource for future applications in the areas of human and animal nutrition, nutraceuticals, or cosmetics. Moreover, the bioaccessibility of the green seaweeds nutritional composition was evaluated, which is an area still poorly explored.

3. Materials and Methods

3.1 Cultivation conditions

At the Aquaculture Research Station, Olhão (EPPO), earth ponds with 0.2 ha and 2500 m^3 in volume were used for meagre (*Argyrosomus regius*) experimental grow-out from 10 g to 1 kg and, in some tanks, till 2.5 kg in fish weight. All ponds had constant water renovation, with a daily average of 30 %, using pumped water from a reservoir connected directly to the Ria Formosa Lagoon. Dry feed is distributed to fish daily, starting with 2.3 (winter, cold water, low feed consumption by the fish) and increasing progressively to 44 kg/day (summer, warm water, high feed consumption by the fish), thereby reaching a total of 5,125 kg. No algicide (such as copper sulfate) was used during the grow-out and the presence of seaweed-feeders like gilthead seabream, *Sparus aurata*, was low (less than 500 specimens per pond). Seaweed biomass in the ponds was allowed to grow naturally until covering around 20 % of water surface area and was collected weekly.

3.2 Samples

Samples of five species of green seaweeds (*Chaetomorpha linum*, *Rhizoclonium riparium*, *Ulva intestinalis*, *Ulva lactuca*, *Ulva prolifera*) were collected manually and transported immediately in seawater to a nearby lab (< 100 m). This harvest was carried out in the summer (July). Each sample was thoroughly washed with seawater to eliminate any biofouling organisms. After washing, the frond samples were kept moist in a 20-L bucket and transported to the IPMA Lisbon Lab. Seaweeds were then finely minced. The processed biological material was frozen, freeze-dried, and stored at – 20 °C.

3.3 Proximate composition

The moisture and ash contents were determined according to AOAC methods (AOAC 2000). The protein level was quantified according to the Dumas method (Saint-Denis and Goupy 2004) and a conversion factor of nitrogen into protein of 5 was used. Crude fat was determined following the Folch extraction method (Folch et al., 1957). Carbohydrate content was determined by difference between 100 % and the sum of the moisture, protein, crude fat, and ash contents.

3.4 Lipid extraction

Bligh and Dyer (1959) method was used for extraction of total lipid content from the fresh seaweeds. Briefly, 5 mL methanol:chloroform (2:1), 1 ml of saturated NaCl solution and 2 ml of chloroform were sequentially added and homogenized with 1 g of sample. After centrifugation (2,000×*g* at 4 ºC for 10 min) (Fig. 10), organic phase was filtered (Fig. 11) through anhydrous sodium sulfate and evaporated in an RE 121 model rotary evaporator (Büchi, Flawil, Switzerland). Extractions were done in duplicate. Samples were stored at -20 ºC until further analyses.

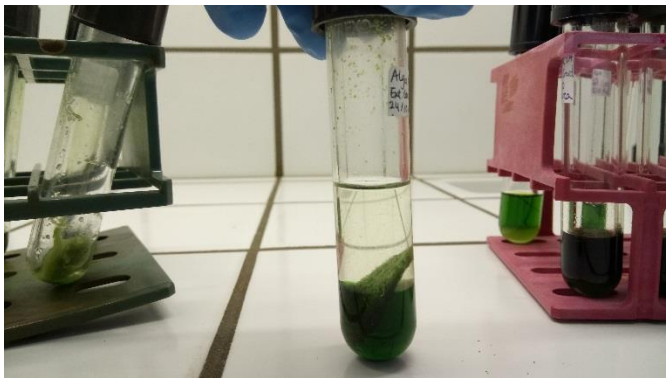

Figure 10: Sample after centrifugation. Three phases can be differentiated: water, seaweed fibers and organic phase containing the lipids.

Figure 11: Organic phase containing lipids after filtration

3.5 Lipid class analysis

The main lipid classes were separated by analytical thin-layer chromatography (TLC) in plates coated with 0.25 mm silica gel G and developed with a mixture of hexane:diethylether:acetic acid (50:50:2 by volume), based on the method described by Bandarra et al. (1997). Extracted lipids were dissolved in chloroform (10 mg/ml concentration). A mixture of standards (Sigma Chemical Co., St. Louis, Mo) was also prepared in chloroform with the same concentration. Specifically, glyceryltrioleate (TAG), glyceryl 1,3-dipalmitate (diacylglycerol, DAG), DL-α-monoolein (MAG), oleic acid (FFA), L-α-phosphatidylcholine (PL), and monogalactosyl diacylglycerol (GL) were used. The samples and standards (10 μL) were applied to the plates and each plate was immersed in 100 mL of the elution mixture inside a developing chamber. The elution front was followed visually. After elution front reached the upper limit, plates were taken out from the chamber. The developed plates were then sprayed with 10 %

phosphomolybdic acid in ethanol (w/v). Identification of lipid classes (polar and non polar) was done by comparison with standards. Quantification was performed using a scanner and version 4.5.2 of Quantity One 1-D Analysis software from Bio-Rad (Hercules, CA, USA) (Fig. 12). There were always two replicates.

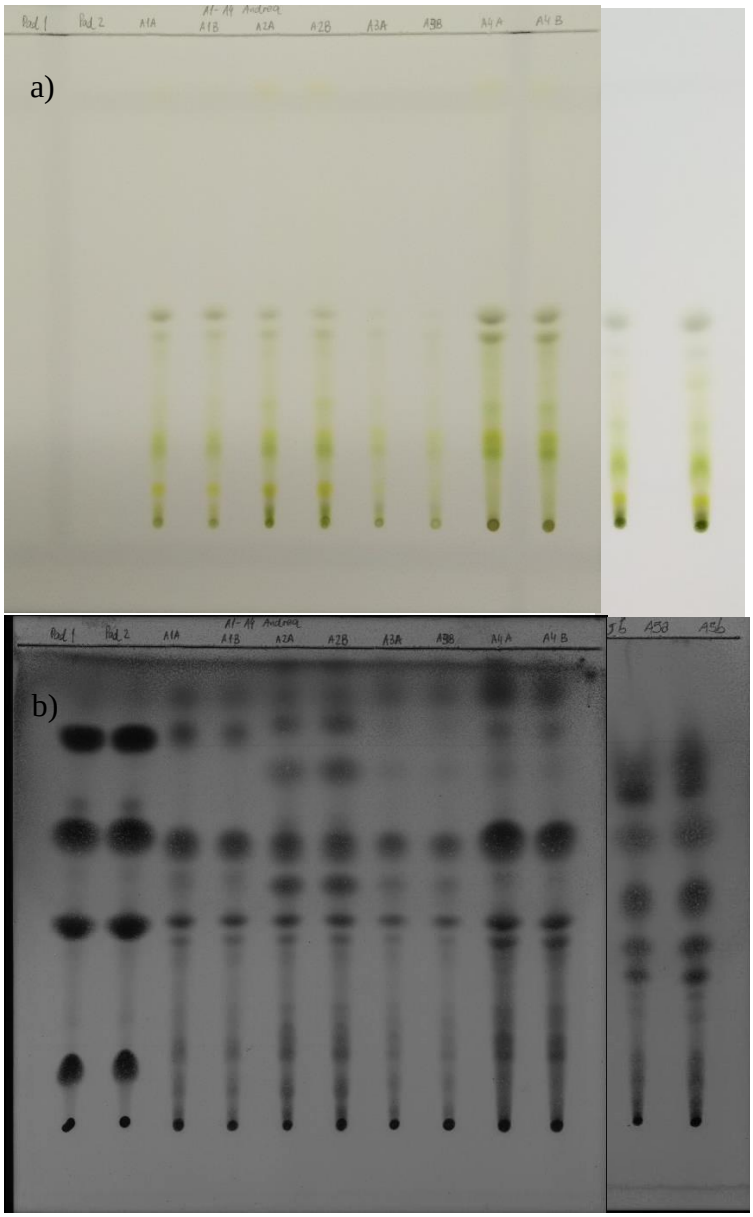

Figure 12: Distribution of the lipid classes through analytical TLC for the five seaweeds: a) before spraying with 10 % phosphomolybdic acid in ethanol and b) when imaged at a digital scanner.

3.6 Lipid classes separation for fatty acid analysis

The different lipid classes were fractionated using a preparative TLC. This involved applying 25 µL of a 50 mg/mL chloroform solution on several points of the

TLC. The plate was placed in an elution vessel containing hexane:diethyl ether:acetic acid (50:50:2) and afterwards elution plates were sprayed with a 0.2 % solution of 2',7'-dichlorofluorescein (Sigma, St. Louis, MO, USA) in ethanol. Visualization was achieved in a cabinet II model UV chamber (CAMAG, Muttenz, Switzerland). Lipid fractions were identified using sigma standards (St. Louis, MO, USA) — glyceryltrioleate (TAG), glyceryl 1,3-dipalmitate (DAG), DL-α-monoolein (MAG), oleic acid (FFA), L-α-phosphatidylcholine (phospholipid, PL), and monogalactosyl diacylglycerol (GL). There were always two replicates.

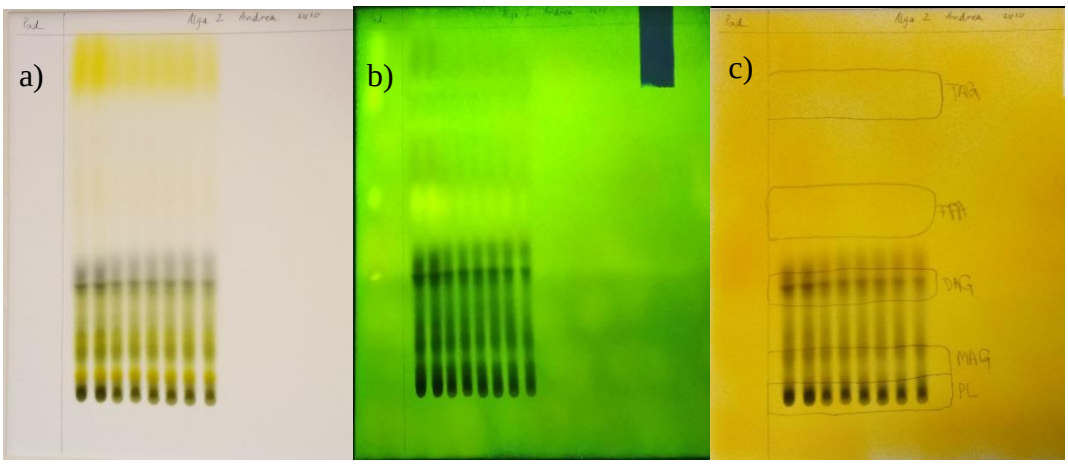

Figure 13: Preparative TLC for *Chaetomorpha linum* (a) after hexane:diethyl ether:acetic acid solution (b) in UV chamber after dichlorofluorescein (c) with lipid fractions identified using sigma standards (TAG - glyceryltrioleate, DAG - glyceryl 1,3-dipalmitate, MAG - DL-α-monoolein, FFA - oleic acid, and PL - L-α-phosphatidylcholine).

3.7 Fatty acid profile

Fatty acid methyl esters (FAMEs) were prepared by acid-catalyzed transesterification using the methodology described by Bandarra et al. (1997). To 150 mg extracted crude fat present in a screw cap glass tube, 5 mL of a 5 % acetyl chloride methanolic solution (prepared immediately before addition) were added. These glass tubes, after vigorous agitation, were placed in a hot bath (80 °C) and left there 1 hour, in accordance with the method described by Lepage and Roy (1986), modified by Cohen et al. (1988). Upon reaction completion, the solution was cooled, diluted with 1 ml water and 2 mL *n*-heptane and vigorously mixed, the last addition produced an organic phase (Fig. 14) that was filtered through anhydrous sodium sulfate. The resultant methyl esters were applied to a DB-WAX (Agilent Technologies, Santa Clara, USA) capillary column (film

thickness, 0.25 μm, 30 m × 0.25 mm i.d.), integrated in a Varian Star 3800 CP gas chromatograph (Walnut Creek, CA, USA), equipped with an auto sampler with a split injector (100:1) and a flame ionization detector, both at 250 °C. The separation of the FAMEs was carried out with helium as the carrier gas and using a temperature program for the column starting at 180 ºC and increasing to 200 °C at 4 °C/min, holding for 10 min at 200 °C, heating to 210 °C at the same rate, and holding at this temperature for 14.5 min. FAMEs were identified by comparing their retention time with those of Sigma–Aldrich standards (PUFA-3, Menhaden oil, and PUFA-1, Marine source from Supelco Analytical). Analyses were always done in triplicate.

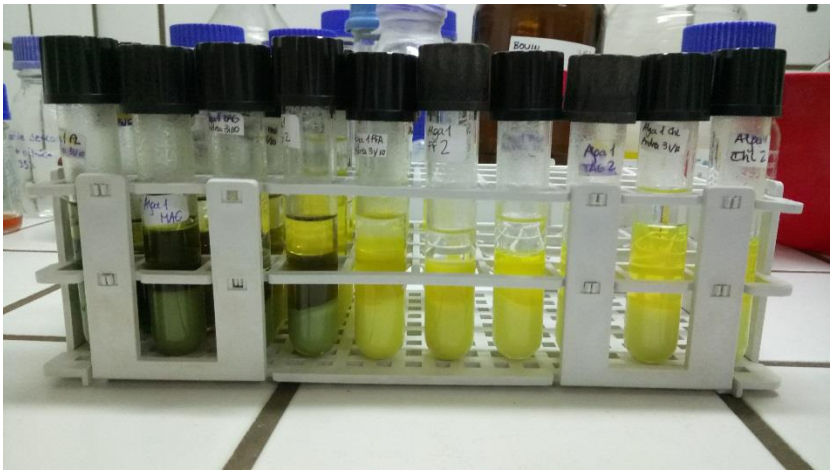

Figure 14: Differentiation of phases during acid-catalyzed transesterification

3.8 Fucose content

Free fucose was determined by the cysteine-sulfursulfuric acid method for methyl pentoses. Triplicates (50 mg) of fresh seaweed were placed into separate test tubes and mixed and homogenized at 30,000 rpm with 1 ml of Milli-Q water using a model Polytron PT 6100 homogenizer (Kinematica, Luzern, Switzerland). Afterwards, samples were subjected to an ultrasound treatment at 25 ºC for 15 min in a Sonorex Super 10 P model (Bandelin Electric, Berlin, Germany). Commercial L-fucose attained from Sigma (St. Louis, MO, USA) was used as the standard. 4.5 ml of sulfursulfuric acid (prepared by adding six volumes of concentrated sulfuric acid with one volume of water) was added into each tube (including tubes containing 1 mL of bioaccessible fraction of each seaweed) and mixed. Tubes were then put into a boiling water bath for 3 minutes. Afterwards, tubes were cooled and 0.1 mL cysteine hydrochloride solution (5

% cysteine hydrochloride in Milli-Q water) was added to each tube and mixed. Absorbance was read at 396 nm and 427 nm, after zeroing the spectrophotometer with a water blank treated in the same way. Fucose-specific absorbance values were calculated according to the following expression: Absorbance = A396nm – A427nm (Dische and Shettles, 1948). Interference by solutions and digestive enzymes used in the bioaccessibility method was accounted for by subtracting absorbance of the bioaccessibility blank from the absorbance measured with the bioaccessible fraction samples.

3.9 Total polyphenol content

Phenolic compounds were extracted by an appropriate solvent mixture (Siriwoharn et al., 2004) (Fig. 15) and determined by the Singleton and Rossi method using the Folin-Ciocalteu reagent (Singleton and Rossi 1965). A volume of 100 μL of each seaweed or bioaccessible extract was added to a vial. To each vial, 600 μL of MiliQ water plus 150 μL of twice-diluted Folin-Ciocalteau reagent were added and allowed to stand for 5 min at room temperature. Then, 750 μL of a 2 % w/v sodium carbonate solution were added. After 15 min reaction in the dark at room temperature, absorbance at 750 nm was measured in a Helios Alpha model (Unicam, Leeds, UK) UV-Vis spectrophotometer. Gallic acid (GA) was used as standard and phenolic content was expressed as gallic acid equivalents (mg GAE/g) through the calibration curve of gallic acid (Sigma, Steinheim, Germany). Interference by solutions and digestive enzymes used in the bioaccessibility method (see section 3.12) was taken into account by subtracting absorbance of the bioaccessibility blank from the absorbance measured with the bioaccessible fraction samples.

Figure 15: Final result of the polyphenol extraction

3.10 Antioxidant activity

The antioxidant activity was measured through the determination of the radical scavenging activity using DPPH (Miliauskas et al., 2004). In order to prepare the extracts, approximately 0.5 g of freeze-dried green seaweed was weighed or 5 mL of bioaccessible fraction (see section 3.12) was measured, homogenized with 25 mL of methanol 50 % v/v using a model Polytron PT 6100 homogenizer (Kinematica, Luzern, Switzerland) at a velocity of 30,000 rpm during 1 min, and agitated for 1 h on an orbital shaker. After centrifugation (5,000×*g* at room temperature during 10 min), the supernatant was filtered and diluted 1:5. A volume of 1 mL of the extract was prepared in triplicate for each sample and 2 ml of DPPH (Sigma, Steinheim, Germany) 0.15 mM methanolic solution was added and thoroughly mixed (Fig. 16). After 30 min of incubation at room temperature in the dark, absorbance was measured at 517 nm in a Helios Alpha model (Unicam, Leeds, UK) UV/visible light spectrophotometer. A solution containing methanol 50 % v/v was the blank.

Radical scavenging activity was calculated by the following formula:

$$\% \text{ Inhibition} = (A_0 - A_{sample})/A_0 \times 100$$

where:

A_0 – Absorbance of the blank.

A_{sample} – Absorbance of the sample.

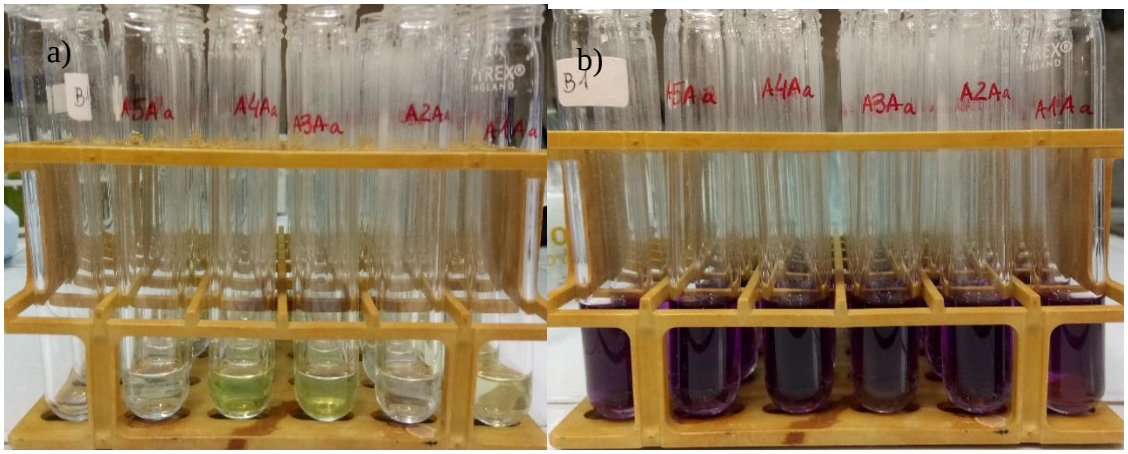

Figure 16: Sample solutions (a) before and (b) after adding DPPH.

Interference by solutions and digestive enzymes used in the bioaccessibility method was taken into account by adjusting absorbance measured with the bioaccessible fraction samples with the absorbance of the bioaccessibility blank.

3.11 Anti-inflammatory activity

3.11.1 Extract preparation for in vitro anti-inflammatory activity

For each green seaweed and each respective bioaccessible fraction (see section 3.12), an aqueous extract was prepared with the purpose of attaining a fraction with anti-inflammatory properties to be tested *in vitro*. Accordingly, approximately 200 mg of freeze-dried green seaweed was weighed or 5 mL bioaccessible fraction was measured and homogenized with 2 mL of Milli-Q water using a model Polytron PT 6100 homogenizer (Kinematica, Luzern, Switzerland) at a velocity of 30,000 rpm during 1 min. Afterwards, the mixture was subjected to a thermal treatment (at 80 °C for 1 h). Both the seaweed and bioaccessible extraction mixtures were centrifuged (3,000×*g* at 4 ºC during 10 min) and the respective supernatant was evaporated using vacuum rotary evaporator with the water bath temperature at 65 °C and inert gas (nitrogen) stream.

3.11.2 Cyclooxygenase (COX-2) inhibition assay

The cyclooxygenase (COX-2) inhibition assay is a practical and quick screening method for assessing the anti-inflammatory activity. The prepared extracts were dissolved in 100 % dimethyl sulfoxide (DMSO) to prepare a stock preparation with a concentration of 10 mg/mL. The extract was tested at 1 mg/ml and 100 µg/mL using a commercial cyclooxygenase (COX) inhibitory screening assay kit Cayman test kit-560131 (Cayman Chemical Company, Ann Arbor, MI, USA). The COX inhibitor screening assay directly measures the amount of Prostaglandin F2α generated from arachidonic acid (AA) in the cyclooxygenase reaction. A volume of 10 µL each of test extract or DMSO was used. The reaction was initiated by addition of 10 µL 10 mM AA and each reaction tube was incubated at 37 °C for 2 minutes. Reaction was terminated by addition of 50 µL 1 N HCl and saturated stannous chloride. Assays were performed using 100 units of human recombinant COX-2. An aliquot was removed and the

prostanoid produced was quantified spectrophotometrically (412 nm) via enzyme immunoassay (ELISA) after 18 h incubation, washing, addition of Ellman's reagent, and further 90 min incubation (Fig. 17). Interference by solutions and digestive enzymes used in the bioaccessibility method was taken into account by subtracting COX-2 inhibition of the bioaccessibility blank from the COX-2 inhibition measured with the bioaccessible fraction samples.

Figure 17: Spectrophotometer with plate for prostanoid reading

3.12 *In vitro* digestion model

An *in vitro* digestion model was chosen for the determination of bioaccessibility in each of the five seaweed species (Fig. 18). Such model comprises three sections, which enable the simulation of digestion in three different parts of the GI tract: mouth, stomach, and small intestine (Afonso et al., 2015).

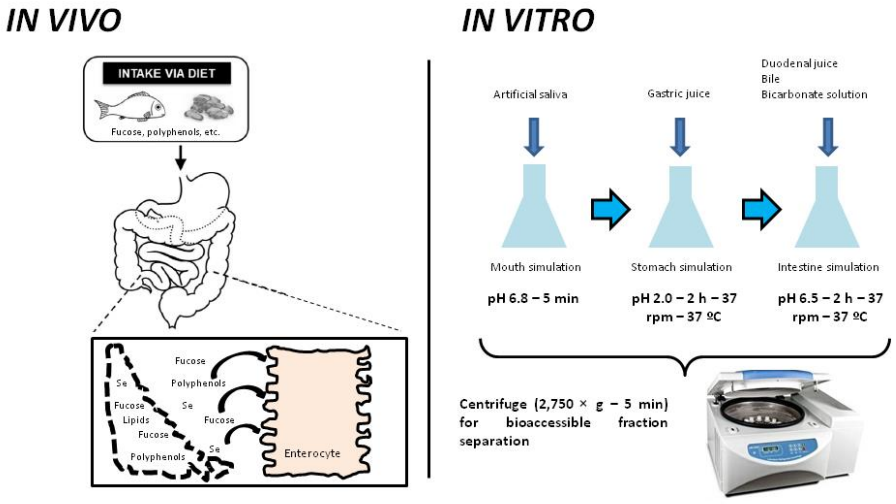

Figure Figure 18: Comparison between *in vivo* and *in vitro* digestion model applied

The composition of digestive juices (saliva, gastric, duodenal and bile) was the same described by Versantvoort et al. (2005). The chemicals KCl, NaH_2PO_4, Na_2SO_4, NaCl, $NaHCO_3$, HCl, $CaCl_2.2H_2O$, KH_2PO_4 and $MgCl_2$ used for preparation of the digestive fluids, were obtained from Merck (Darmstadt, Germany). NH_4Cl was obtained from Fluka (Buchs, Switzerland) and all other chemicals were obtained from Sigma (St. Louis, MO, USA). In the case of duodenal juice, trypsin and α-chymotrypsin from Sigma (St. Louis, MO, USA) were also added. The quantities of these two enzymes (0.08 g trypsin and 0.87 g α-chymotrypsin in 500 ml of duodenal juice) were estimated on the basis of the work by Gatellier and Santé-Lhoutellier (2009).

Briefly, approximately 1.5 g homogenized and hydrated (with up to 1.5 mL water) seaweed was weighed. For the bioaccessibility blank, 1.5 mL of Milli-Q water was used instead. Sample was mixed with 4 ml of artificial saliva at a pH 6.8 ± 0.2 for 5 min, then 8 ml of artificial gastric juice (pH 1.3 ± 0.02 at 37 ± 2 ºC) was added, and pH was lowered to 2.0 ± 0.1. The mixing lasted 2 h in a head-over-heels movement (37 rpm at 37 ± 2 ºC). Finally, 8 ml of artificial duodenal juice (pH 8.1 ± 0.2 at 37 ± 2 ºC), 4 mL of bile (pH 8.2 ± 0.2 at 37 ± 2 ºC), and 1.33 mL of HCO_3^- solution (1 M) was added. The pH of the mixture was set at 6.5 ± 0.5 and agitation for 2 h was identical to gastric conditions. The mixture generated in the *in vitro* model was subjected to centrifugation at 2750×*g* for 5 min, thus yielding a non-digested portion and the bioaccessible fraction. While chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany), enzymes were attained from Sigma (St. Louis, MO, USA).

3.12.1 Calculation of bioaccessibility

The percentage (%) of each seaweed constituent (C) in the bioaccessible and in the non-digested fraction was estimated as follows:

$$\% \text{ C bioaccessible} = [C]_{\text{bioaccessible}} \times 100/[S]$$

and

$$\% \text{ C non-digested} = [C]_{\text{non-digested}} \times 100/[S]$$

Being:

[C] = Concentration of constituent.

[S] = [C] in the bioaccessible fraction + [C] in the non-digested fraction.

3.13 Mineral composition analysis

With the exception of iodine, for elemental determination, freeze-dried samples of seaweed were weighed (0.5 g) in triplicate and digested using a closed-vessel microwave digestion system (Milestone ETHOS 1 Series, Shelton, USA). Microwave digestion method was as specified in Nascimento et al. (2014). Samples were diluted to 25 ml with deionized water. For I determination, 0.2 g of freeze-dried sample was weighed into a 50-mL tube. Extraction was obtained using a graphite block system for 3 hours at 90 °C. All samples were centrifuged and filtered through 0.45-μm filters. Elemental composition of the bioaccessible fraction was obtained by making a minimum 10-fold dilution of this fraction using deionized water. Dilutions were adjusted according to elemental content.

Blank solutions and certified reference materials were prepared with the same procedure of the samples. Standard curves were used for the determination of analysed elements. All elements were analyzed by an inductively coupled plasma mass spectrometer, ICP-MS Thermo X series II (Thermo Fisher Scientific, Waltham, MA, USA). ICP-MS instrumental setting was as specified in Nascimento et al. (2014). Iodine was analysed separately from the remaining elements. The elemental concentration was expressed in mg/kg dry weight.

3.14 Statistical analysis

In order to test normality and variance homogeneity, the Kolmogorov-Smirnov's test and Levene's F-test, respectively, were used. Data fulfilled the assumptions of both parametric tests. The seaweed species as well as the contrast between initial and bioaccessible contents were the two the studied factors in the first paper (section 4). The seaweed species (*C. linum*, *R. riparium*, *U. intestinalis*, *U. lactuca*, and *U. prolifera*) and the contrast between different lipid classes (TAG, PL+GL, MAG, and FFA) were the two factors studied on the second paper (section 5). All statistically analyzed values were in percentage. Whereas, in the case of FA profiles, values were percentage of total FA in the whole fat fraction or in a specific lipid class, in the case of lipid class distribution, values were percentage of the total fat. The seaweed species was the studied factor for the third paper (section 6).

The parametric test, Tukey HSD, was carried out with STATISTICA v. 6, (StatSoft, Inc., Tulsa, OK, USA). For all statistical tests, the significance level (α) used was 0.05. Whenever p was lower than α, statistical differences between species (total FA comparison; class distribution; specific class FA comparison) or between lipid classes for the same species (specific group FA comparison) were identified. In the first situation, lowercase letters were used, while, in the second situation, uppercase letters were used.

4. Composition, Biological Activity, and Bioaccessibility of Green Seaweeds from an Integrated Multi-Trophic Aquaculture System[1]

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[1] Ripol Malo, A., Cardoso, C., Afonso, C., Varela, J., Quental-Ferreira, H., Pousão-Ferreira, P., Bandarra, N. (2017) Composition, Biological Activity, and Bioaccessibility of Green Seaweeds from Fish Pond Aquaculture. *Natural Product Communications*, (Submitted 29 November 2017, Under Review).

4.1 Introduction

Seaweeds are experiencing a growing market interest and their production has increased in the Asia-Pacific region. Though most seaweed is harvested offshore, they can also be produced in separate ponds or as co-products in fish farming (Chopin et al., 2001), for instance, in meagre (*Argyrosomus regius*) farming in earth ponds. Particularly, fish pond aquaculture production systems are a new and promising scientific field that brings together fish farming and production of seaweeds and other marine organisms (Chopin et al., 2001). This is environmentally valuable and may usher in economic advantages. The composition and economic value of seaweeds may vary between species and, for a given species, parameters depend on abiotic/biotic conditions. Hence, it is worthwhile to study the composition and properties of seaweeds from systems of fish pond aquaculture where integrated multi-trophic aquaculture can be implemented.

Regarding green seaweed nutritional composition, moisture content is typically high, protein levels are significant, and lipid content is low, even on dry matter basis (Kendel et al., 2015; Maehre et al., 2014). Though fatty acid profiles may vary, they are usually rich in polyunsaturated fatty acids (PUFA), with a typically high level of some ω3 PUFA, such as 16:4ω3 and 18:4ω3, which are not so abundant in other organisms (Kendel et al., 2015). Moreover, seaweed polysaccharides are a potential source of soluble and insoluble dietary fibers. Many of these compounds exhibit high water holding capacity. Soluble dietary fibers demonstrate an ability to increase viscosity, form gels, and/or act as emulsifiers (Elleuch et al., 2011).

Concerning biological activities, there are reports of anti-inflammatory activity (Khan et al., 2008). Namely, methanol extracts of *Ulva linza* at a concentration of 40 mg/ml showed strong suppression of edema, with a relative inhibition of 84 %, and suppression of erythema, with an inhibition of 70 % (Khan et al., 2008).

Given the aforementioned components and bioactivities of green seaweeds, they may be worth further research aiming at nutritional and pharmacological applications. However, it must be taken into account that the absorbable quantity of a compound in the gastrointestinal (GI) tract is not accurately predicted by its total content in the seaweed. Bioaccessibility corresponds to the share of the initial content that is rendered free from the seaweed structure into the GI tract (Afonso et al., 2015). Thus, determining bioaccessibility may contribute to the assessment of the effective

nutraceutical/pharmacological potential of a specific seaweed. A bioaccessibility study requires the utilization of an adequate *in vitro* digestion model that reliably simulates human digestion. Indeed, several methodologies have been developed (Cardoso et al., 2015), being the static model with digestive compartment distinction and complete digestive juices, including enzymes in all steps, one of the best models (Cardoso et al., 2015; Versantvoort et al., 2005). In recent years, these *in vitro* techniques for assessing human bioaccessibility have been improved (Afonso et al., 2015).

Therefore, experimental work was carried out in order to determine the nutritional composition, biological activities, and critical bioaccessibility effects on these properties for a significant group of green seaweeds grown under fish pond aquaculture conditions.

4.2 Results and Discussion

4.2.1 Seaweed proximate composition

The proximate composition of studied green seaweed species is displayed in Table 1. It was observed that there were differences between species. In particular, *U. prolifera* and *C. linum* had very high moisture content. The dry matter of these species (as well as of the other species) was mainly composed of protein and carbohydrates. The differences between wet weight ash, fat, protein, and carbohydrate contents changed when dry weight was considered due to differences in moisture between algae. The ash content of *U. prolifera* and *C. linum* in both wet and dry basis was lower than that of the other three species. This was not observed for protein, since *C. linum* had the highest protein content once converted to dry matter. On the other hand, *C. linum* had the lowest carbohydrate content. Fat levels were always very low for all species, not surpassing 3 % w/dw.

These results are similar to those reported for green seaweeds by previous studies (Khotimchenko et al., 2002; Satpati and Pal, 2011; Setthamongkol et al., 2015; Wong and Cheung, 2000) in that moisture content is very high (> 80 % wet weight) and lipid content is very low even on a dry weight basis (< 5 % dry weight). However, protein content on a dry weight basis was significantly higher than the values reported previously (up to 2-fold). For instance, in *C. linum*, protein content was clearly higher than values found in the literature (Setthamongkol et al., 2015). Therefore, the main

nutritional value of studied green seaweeds from fish pond aquaculture lies in their protein and carbohydrate contents.

Table 1 - Proximate crude composition (g/100 g wet weight and for ash, protein, fat, and carbohydrate also g/100 g dry weight) in the five studied green seaweed species

	R. riparium	*U. lactuca*	*U. prolifera*	*C. linum*	*U. intestinalis*
Moisture	87.2 ± 0.0^{a}	88.9 ± 0.4^{ab}	95.7 ± 0.1^{d}	93.8 ± 0.2^{c}	90.1 ± 0.1^{b}
Ash (g/100 g ww)	2.2 ± 0.1^{a}	1.9 ± 0.1^{a}	0.5 ± 0.0^{c}	0.9 ± 0.1^{b}	2.0 ± 0.0^{a}
Ash (g/100 g dw)	17.5 ± 0.4^{a}	17.5 ± 0.7^{a}	11.3 ± 0.7^{c}	14.4 ± 1.5^{b}	20.3 ± 0.2^{d}
Protein (g/100 g ww)	5.4 ± 0.7^{a}	3.5 ± 0.6^{b}	1.8 ± 0.4^{c}	3.7 ± 0.3^{b}	3.4 ± 1.0^{b}
Protein (g/100 g dw)	42.4 ± 5.1^{a}	31.6 ± 5.7^{b}	41.8 ± 9.6^{a}	59.9 ± 4.7^{c}	34.4± 10.1^{ab}
Fat (g/100 g ww)	0.3 ± 0.0^{a}	0.2 ± 0.0^{ab}	0.1 ± 0.0^{b}	0.1 ± 0.1^{b}	0.1 ± 0.0^{b}
Fat (g/100 g dw)	1.9 ± 0.0^{a}	1.5 ± 0.1^{ab}	2.0 ± 0.3^{a}	2.2 ± 1.0^{a}	1.0 ± 0.0^{b}
Carbohydrate (g/100 g ww)	4.9 ± 0.6^{a}	5.5 ± 0.3^{a}	2.0 ± 0.5^{b}	1.5 ± 0.4^{b}	4.4 ± 0.9^{a}
Carbohydrate (g/100 g dw)	38.2 ± 4.6^{a}	49.4 ± 3.1^{b}	45.0 ± 12.0^{ab}	23.6 ± 6.1^{c}	44.3 ± 9.4^{ab}

Values are presented as average±standard deviation. Different letters within a row correspond to statistical differences ($p<0.05$).

4.2.2 Seaweed fucose content

The fucose content in the studied green seaweed species as well as the bioaccessible fraction of fucose in these species is shown in Table 2. The highest level was determined in *U. intestinalis* with 23.6 ± 9.1 mg/g dw, which was significantly ($p<0.05$) higher than in *C. linum*, whose fucose content was the lowest of all, 8.7 ± 0.6 mg/g dw. Concerning bioaccessible fucose contents, no detectable level was measured with exception of *R. riparium*. Thus, bioaccessibility percentage was only determined for this seaweed species and it was low, 38 %.

It is worth noting that the lowest fucose content in *C. linum* correlates with the lowest carbohydrate content on a dry matter basis. However, fucose contents may be quite variable, even within samples of the same species (Mao et al., 2006). In *U. lactuca*, it has been reported the presence of fucose in the structural carbohydrates of the cell wall (Mao et al., 2006). Fucose importance in green seaweeds is small if compared with brown seaweeds (Percival, 1979). Nevertheless, the biological activities of fucose polysaccharides, especially the sulphated ones, for instance, as anti-diabetic agents (Sharifuddin et al., 2015), justifies studying them also in other seaweed groups. However, such polysaccharides would need to be extracted from seaweed for

nutraceutical and pharmacological applications since their bioaccessibility was low. This may be related to their presence in the cell wall structures, which are resistant to the digestive process, as well as to their resistance to the enzymes of the human digestive system.

Table 2 – Fucose content (mg/g dry weight) in the five studied green seaweed species before (initial) and after digestion (bioaccessible) and fucose bioaccessibility (%).

		R. riparium	*U. lactuca*	*U. prolifera*	*C. linum*	*U. intestinalis*
Fucose (mg/g dw)	Initial	22.3 ± 6.5^{ab}	15.7 ± 0.8^{ab}	19.6 ± 4.7^{ab}	8.7 ± 0.6^{a}	23.6 ± 9.1^{b}
	Bioaccessible	8.5 ± 7.3^{a}	nd^{b}	nd^{b}	nd^{b}	nd^{b}
	Bioaccessibility (%)	38^{a}	nd^{b}	nd^{b}	nd^{b}	nd^{b}

nd – Not detected. Values are presented as average±standard deviation. Different letters within a row correspond to statistical differences ($p<0.05$).

4.2.3 Seaweed total polyphenol content and antioxidant activity

The total polyphenol content and the antioxidant activity are displayed in Table 3. The green seaweed *U. prolifera* had the highest total polyphenol content and antioxidant activity. There was also some significant antioxidant activity by the *R. riparium*, but much lower than that of *U. prolifera*. The lowest antioxidant activity was determined in extracts from *U. lactuca* and *U.intestinalis*. There was some correlation between polyphenol content and the antioxidant activity (measured as radical scavenging activity) with a r^2 of 0.88 (p = 0.018). Moreover, no polyphenol and antioxidant activity were detected in the bioaccessible fractions attained from the studied seaweeds.

Table 3 – Total polyphenol content (mg GAE/g dry weight) and antioxidant activity (% inhibition) in the five studied green seaweed species before (initial) and after digestion (bioaccessible).

		R. riparium	*U. lactuca*	*U. prolifera*	*C. linum*	*U. intestinalis*
Total polyphenol content (mg GAE/g dw)	Initial	1.04 ± 0.12^{a}	0.78 ± 0.11^{a}	2.69 ± 0.49^{b}	1.49 ± 0.05^{a}	1.02 ± 0.16^{a}
	Bioaccessible	nd	nd	nd	nd	nd
Antioxidant activity (% inhibition)	Initial	13.5 ± 4.3^{b}	1.0 ± 0.2^{a}	47.7 ± 0.1^{c}	6.6 ± 1.3^{ab}	1.3 ± 0.4^{a}
	Bioaccessible	nd	nd	nd	nd	nd

nd – Not detected. Values are presented as average±standard deviation. Different letters within a row correspond to statistical differences ($p<0.05$).

The obtained total polyphenol content values are within the range reported in the relevant literature on green seaweeds, 1-5 mg GAE/g dw (Farasat et al., 2014). On the other hand, comparison of the antioxidant activity measured by DPPH radical scavenging activity of methanolic extracts to equivalent data from previous studies show higher activities in these studies (Farasat et al., 2014). Indeed, these authors reported IC_{50} lower than the 4 mg/ml of *U. prolifera* in the current study, thereby suggesting a higher activity.

Differences are also important for the same species. For instance, *U. intestinalis* from the Persian Gulf had 2.0 ± 0.3 mg GAE/g dw and a IC_{50} of 1.88 ± 0.03 mg/ml (Farasat et al., 2014). This contrast with Portuguese results may be ascribed to the UV radiation level in each location, since higher phenolic contents in seaweeds are associated to higher UV exposure (Bischof et al., 2006). Seaweeds synthesize more phenolic substances to scavenge the reactive oxygen species produced by UV radiation. This function is supported by the correlation between total phenolic content and DPPH radical scavenging activity, as observed in this study and elsewhere (Farasat et al., 2014). In fact, *U. prolifera* was more exposed to sunlight in the ponds —floating at the surface— than the other seaweeds. However, it has been reported that the highest free radical scavenging activity and phenolic content in *U. rigida* extracts were observed in samples collected in late winter (February) and early spring (March) (Trigui et al., 2013). Another factor influencing phenolic content and thus antioxidant activity may be the FA composition of the lipid fraction of the seaweed specimens. Indeed, higher PUFA content in *U. prolifera* is associated to higher phenolic content and antioxidant activity. It is known that PUFA are more prone to oxidation than SFA and monounsaturated FA (MUFA) (Tao, 2015). Hence, green seaweeds may produce more phenolic substances as a result of higher oxidation risk of the organism lipids. Other unaccounted factors may also play a role. In this regard, it is worth noting that *U. prolifera* from the Persian Gulf had lower phenolic content (Farasat et al., 2013) than that analysed in the current study.

The absence of bioaccessible phenolic compounds (and antioxidant activity) suggests that human digestion as simulated in the used *in vitro* model is unable to release these compounds from the non-digested protein and carbohydrate material. Extraction of these compounds may be advisable. Nonetheless, there was interference of the bioaccessible blank, which justifies future work in methodological improvement.

4.2.4 Seaweed anti-inflammatory activity

The anti-inflammatory activity values of the five species of green seaweed measured as a percentage of inhibition of the enzyme COX-2 are presented in Table 4. Two different extract concentrations were tested, 1 mg/ml and 100 μg/mL. Furthermore, the anti-inflammatory activity values of the bioaccessible fractions were also determined.

Anti-inflammatory activities were detected for all five species at the higher concentration, ranging between 31 and 45 % of COX-2 inhibition. At 100 μg/mL, however, only the extracts of *U. prolifera* and *C. linum* displayed anti-inflammatory properties. No statistical difference was detected between these two extracts, 27 ± 9 % in *U. prolifera* and 18 ± 12 % in *C. linum*. For the bioaccessible fractions, after eliminating the bioaccessibility blank background interference, values were not significantly different from zero, even in the case of *U. prolifera* and *C. linum*. The correlation between COX-2 inhibition at 1 mg/mL or 100 μg/mL by the seaweed extracts and COX-2 inhibition measured in the bioaccessible extracts was very low (r^2 < 0.30, p>0.05).

Table 4– Anti-inflammatory activity (% inhibition of COX-2) in the five studied green seaweed species before (initial) and after digestion (bioaccessible).

Anti-inflammatory activity (% inhibition of COX-2)	Extract concentration	*R. riparium*	*U. lactuca*	*U. prolifera*	*C. linum*	*U. intestinalis*
Initial	1 mg/mL	45 ± 10[a]	31 ± 13[a]	34 ± 13[a]	36 ± 9[a]	34 ± 22[a]
	100 μg/mL	nd[a]	nd[a]	27 ± 9[b]	18 ± 12[b]	nd[a]
Bioaccessible	1 mg/mL	nd[a]	3 ± 3[a]	2 ± 3[a]	10 ± 10[a]	1 ± 2[a]

nd – Not detected. Values are presented as average±standard deviation. Different letters within a row correspond to statistical differences (p<0.05).

There are few studies on the anti-inflammatory activity of green seaweeds and methodologies are different, ranging from *in vitro* assays to *in vivo* models (Bitencourt et al., 2015; Jin et al., 2006; Margret et al., 2009; McCauley et al., 2015; Renju et al., 2013). This makes comparison among studies difficult. Nevertheless, several studies point to the existence of anti-inflammatory activity in green seaweeds (McCauley et al., 2015). Different compounds may be involved, such as phenolic compounds, carotenoids, phytosterols, alkaloids or polysaccharides, in particular a sulphated

polysaccharide from *Caulerpa cupressoides*, whose anti-inflammatory action has been reported by Rodrigues et al. (2012). In addition, even though ω3 PUFA have also anti-inflammatory effects (Calder 2010), their action requires *in vivo* systems, which was not the case in the COX-2 inhibition assay used in current study. Some species of the genera *Caulerpa* and *Ulva* (including *U. lactuca*) have been associated to anti-inflammatory properties (McCauley et al., 2015; Lee et al., 2013). However, regarding *U. prolifera* and *C. linum*, no studies are known to the authors. A comparison with other works using the same COX-2 inhibition methodology shows that *U. prolifera* and *C. linum* could provide potent anti-inflammatory activity, since inhibition percentages of 18-27 % for an extract concentration at 100 μg/mL are similar to the inhibition, 25 %, found for a *Polygonum minus* extract at the same concentration (George et al., 2014). In the case of *U. prolifera*, it is possible that high polyphenol concentrations (Table 3) in this seaweed species contributed for the observed anti-inflammatory effect. However, other compounds may be important, as shown by the anti-inflammatory activity of non-polar extracts of *Ulva* seaweeds (McCauley et al., 2015).

The interference of the bioaccessible blank was very important and it may be ascribed to anti-inflammatory compounds in the porcine bile used in the *in vitro* model, such as bilirubin and its derivatives (Joshi et al., 2016). In the literature, to the best of the authors' knowledge, there are few bioaccessibility studies focusing on anti-inflammatory properties after digestion (Dawilai et al., 2013). Given the observed impact of the interference phenomenon, the bioaccessibility methodology needs to be adapted in order to be useful in estimating the effective anti-inflammatory action of bioaccessible fractions from the digestion of seaweeds or other biological materials and foods with potential anti-inflammatory activity. The approach proposed by Dawilai et al. (2013) may be an advisable route, since these authors deleted the bile extract during small intestinal digestion and found a large loss of anti-inflammatory activity (though other aspects besides bile components themselves were possibly involved).

If a low bioaccessibility of the anti-inflammatory compounds in *U. prolifera* and *C. linum* is confirmed, preparation of extracts for nutraceutical and pharmacological applications or seaweed processing through decoction to produce a tisane —especially in the case of *U. prolifera*, which is classified as an edible seaweed— may be advantageous alternatives, thereby rendering these compounds more bioaccessible.

4.3 Conclusions

The performed experimental work represented a first step in the bioprospection of green seaweeds from fish pond aquaculture and integrated aquaculture production and it has identified strengths and weaknesses for each species. Moisture content of the studied seaweed species was very high, exceeding 87 %. The dry matter was mainly composed of protein and carbohydrates. Lipid content was very low (< 3 g/100 g dry weight) with almost no difference between species. However, there were differences between lipid fractions, since fatty acid profiles varied considerably between the five seaweed species. Total polyphenol content and antioxidant activity presented a significant correlation and *U. prolifera* had the highest total polyphenol content and antioxidant activity. No polyphenol or antioxidant activity was found in the bioaccessible fraction. The anti-inflammatory activity was more remarkable in *U. prolifera* and *C. linum*. Indeed, significant COX-2 activity inhibition was found in the extracts at 100 μg/mL. Apparently, the compounds causing this anti-inflammatory activity were not rendered bioaccessible. Future work should focus on the extraction of the bioactive compounds for nutraceutical or even pharmaceutical applications as well as explore the preparation of tisanes and analogous products as strategies to render the bioactives more bioaccessible.

5. Fatty Acid Profiles of the Main Lipid Classes of Green Seaweeds from Fish Pond Aquaculture[2]

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[2] Cardoso, C., Ripol Malo, A., Afonso, C., Freire, M., Varela, J., Quental-Ferreira, H., Pousão-Ferreira, P., Bandarra, N. (2017). Fatty acid profiles of the main lipid classes of green seaweeds from fish pond aquaculture. *Food Sci Nutr.* 2017;00:1–9.

5.1 Introduction

Seaweeds are still a largely undervalued marine resource. Besides, they can be produced in aquaculture systems, enabling a better control of their characteristics and composition. Indeed, they can be produced in separate ponds or as co-products in fish and mollusk farming, for instance, in meagre (*Argyrosomus regius*) farming in earth ponds or in abalone (*Haliotis asinina*) farming in integrated multi-trophic aquaculture (Largo et al., 2016). These aquaculture systems combine marine species that are commercially viable and environmentally sustainable on the basis of the concept that any waste consisting of uneaten feed, feces, and metabolic excretion of one species is an useful input for another species growth, thereby ensuring a natural self-cleansing solution to pollution problems (Chopin et al., 2001).These polluting materials constitute a substantial problem in meagre farming, particularly there are components of fish feed with a low digestibility (Olim et al., 2012). Precisely, seaweeds may be able to operate as natural filters of nitrate and ammonia generated in meagre farming (Largo et al., 2016). This is environmentally valuable and may also provide some economic advantage. The composition and economic value of seaweeds may vary between species and, for a given species, parameters depend on abiotic/biotic conditions. Therefore, it is worthwhile to study the composition and properties of seaweeds from systems of fish pond aquaculture.

Though the lipid fraction has been less studied and typically does not surpass 5 % of the dry seaweed matter in green seaweeds (El Maghraby and Fakhry, 2015; Maehre et al., 2014), it may comprise molecules with valuable bioactivities and may be a tool in differentiating seaweeds themselves and products derived from seaweeds, thereby enhancing traceability and reliability. Indeed, lipid profiling —such as overall and per lipid class fatty acid profiles— may be helpful in the assignment of algal taxonomic position and yield signature profiles for application in organic geochemistry and food studies (Rajasulochana et al., 2010).

In particular, albeit variable, fatty acid profiles in seaweeds are usually rich in polyunsaturated fatty acids (PUFA), but with *n*-3 PUFA predominantly composed of shorter chain FA, such as 16:4 *n*-3 and 18:4 *n*-3 (Kendel et al., 2015). There are also some species with significant amounts (on a dry matter basis) of eicosapentaenoic acid (20:5 *n*-3, EPA) (Dawczynski et al., 2007). Regarding health benefits, the *n*-3 PUFA

class of FA is considered to play an important role in the prevention of cardiovascular and some autoimmune diseases, possessing anti-tumoural and anti-inflammatory properties (Dawczynski et al., 2007; Newton, 1996).

The aforementioned issues show that a study of the lipid fraction of a representative group of green seaweeds grown under fish pond aquaculture conditions is warranted. Precisely, this was the key objective of the performed analyses and data assessment carried out by this study: total FA profiles (for assessing FA quality and chemotaxonomic purposes); polar and non polar lipid distribution (chemotaxonomic purposes); and FA profiles of triacylglycerols (TAG), monoacylglycerols (MAG), free fatty acids (FFA), phospholipids (PL), and glycolipids (GL) (FA quality and chemotaxonomic objectives).

5.2 Results and Discussion

5.2.1 Seaweed fatty acid profile

The FA profile of the five studied seaweed species is presented in Table 5. These profiles encompass all fat present in all studied green seaweeds. A global comparison enables to point to two main aspects: *U. lactuca* and *U. intestinalis* FA profiles are very similar; all other profiles are quite different. Whereas *U. prolifera* is very rich in *n*-6 PUFA, *R. riparium* is much richer in *n*-3 PUFA. On the other hand, concerning *n*-3/ *n*-6 ratio, the highest value is found for *C. linum*. In this species, total PUFA was lower than in *R. riparium* and *U. prolifera*. A high level of saturated FA (SFA) contrasted with the low PUFA content in *C. linum*.

A closer examination of data showed that EPA and docosahexaenoic acid (22:6 *n*-3, DHA) levels were always low in all seaweeds, not exceeding 3-4 % of the total FA. The most abundant ω3 PUFA in *R. riparium* and *C. linum* was α-linolenic acid (18:3 *n*-3). In the other three species, C16 *n*-3 FA were the most abundant *n*-3 PUFA. The seaweed *U. prolifera* displays a high linoleic acid (18:2 *n*-6) content, 22.0 ± 0.8 %, thus differing from other species of the same genus. The seaweed *C. linum* had a high concentration of 18:1, while this FA was less abundant in *U. lactuca* and *U. intestinalis*. Myristic (14:0) and palmitic (16:0) acids were the main SFA. Stearic acid (18:0) exhibited very low levels in the analyzed profiles. Though *C. linum* had high amounts

of SFA, its myristic acid content was the lowest of all. On the other hand, its palmitic acid level was the highest among all studied species.

Table 5 – Overall fatty acid profile (%) in the five studied green seaweed species.

Fatty acid	*R. riparium*	*U. lactuca*	*U. prolifera*	*U. intestinalis*	*C. linum*
14:0	8.9 ± 0.1^{b}	8.6 ± 0.0^{b}	10.9 ± 0.0^{c}	8.5 ± 0.0^{b}	3.5 ± 0.3^{a}
16:0	20.3 ± 0.2^{b}	19.2 ± 0.1^{a}	21.0 ± 0.2^{b}	19.3 ± 0.0^{a}	32.9 ± 0.4^{c}
18:0	0.4 ± 0.0^{ab}	0.3 ± 0.0^{a}	0.2 ± 0.0^{a}	0.3 ± 0.0^{a}	0.6 ± 0.0^{b}
Σ SFA	34.4 ± 0.1^{a}	38.0 ± 0.0^{b}	38.1 ± 0.2^{b}	38.2 ± 0.2^{b}	46.6 ± 1.2^{c}
16:1 *n*-7+ *n*-9	6.4 ± 0.0^{d}	1.2 ± 0.0^{a}	1.6 ± 0.1^{b}	1.2 ± 0.0^{a}	4.3 ± 0.0^{c}
18:1 *n*-7+ *n*-9	15.7 ± 0.2^{c}	7.7 ± 0.0^{a}	11.5 ± 0.2^{b}	7.3 ± 0.0^{a}	17.4 ± 0.2^{d}
20:1 *n*-7+ *n*-9+ *n*-11	0.3 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.5 ± 0.0^{a}
Σ MUFA	23.0 ± 0.2^{c}	16.6 ± 0.1^{b}	14.9 ± 0.1^{a}	16.6 ± 0.1^{b}	23.4 ± 0.2^{c}
18:2 *n*-6	10.8 ± 0.4^{c}	9.5 ± 0.2^{bc}	22.0 ± 0.8^{d}	8.1 ± 0.0^{b}	2.1 ± 0.1^{a}
20:4 *n*-6	0.9 ± 0.0^{b}	1.8 ± 0.0^{a}	1.7 ± 0.0^{a}	1.8 ± 0.0^{a}	0.1 ± 0.0^{c}
16:3 *n*-3+16:4 *n*-3	4.0 ± 0.1^{a}	10.6 ± 0.1^{a}	8.7 ± 0.3^{a}	11.0 ± 0.3^{a}	0.9 ± 0.1^{a}
18:3 *n*-3	10.5 ± 0.0^{c}	0.1 ± 0.0^{a}	0.2 ± 0.1^{a}	0.1 ± 0.0^{a}	4.1 ± 0.1^{b}
18:4 *n*-3	0.4 ± 0.0^{b}	0.2 ± 0.0^{a}	0.5 ± 0.0^{b}	0.2 ± 0.0^{a}	2.3 ± 0.1^{c}
20:4 *n*-3	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.5 ± 0.0^{c}	0.1 ± 0.0^{a}	0.3 ± 0.0^{b}
20:5 *n*-3	2.7 ± 0.0^{d}	1.6 ± 0.0^{b}	2.2 ± 0.1^{c}	1.7 ± 0.1^{b}	0.6 ± 0.0^{a}
22:5 *n*-3	2.1 ± 0.0^{d}	1.4 ± 0.0^{b}	1.9 ± 0.0^{c}	1.5 ± 0.0^{b}	0.7 ± 0.0^{a}
22:6 *n*-3	0.4 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.4 ± 0.0^{a}
Σ PUFA	33.1 ± 0.4^{c}	26.8 ± 0.3^{b}	39.0 ± 0.5^{d}	26.0 ± 0.4^{b}	12.2 ± 0.3^{a}
Σ *n*-3	20.1 ± 0.1^{c}	14.1 ± 0.0^{b}	14.0 ± 0.3^{b}	14.6 ± 0.4^{b}	8.9 ± 0.2^{a}
Σ *n*-6	12.0 ± 0.5^{b}	12.1 ± 0.3^{b}	24.7 ± 0.8^{c}	10.7 ± 0.0^{b}	2.4 ± 0.1^{a}
Σ *n*-3/Σ *n*-6	1.7 ± 0.1^{c}	1.2 ± 0.0^{b}	0.6 ± 0.0^{a}	1.4 ± 0.0^{b}	3.7 ± 0.0^{d}

Values are presented as average±standard deviation. Different lowercase letters within a row correspond to statistical differences ($p<0.05$).

Regarding these results, there are similarities with other green seaweeds (Chopin et al., 2001), but there are also differences, including significant ones among studied species. Concerning similarities, palmitic acid has been claimed to be very abundant in seaweeds (Gressler et al., 2010). Other common traits of the FA profiles of green seaweeds are a high C18/C20 PUFA ratio and an abundance of C16 *n*-3 (Khotimchenko et al., 2002; Sato, 1975). These traits have been observed in the current study. On the

other hand, there are differences between species due to specific aspects. This seems to make the study of the FA profiles a suitable scientific approach to distinguish between different green seaweed species. However, there are also important divergences in the FA composition of specimens of the same species collected from different locations, which jeopardizes the establishment of a straightforward link between a given FA profile and a particular green seaweed species. As an example, *U. lactuca* from North California coast in November presented 11 % α-linolenic acid (18:3 *n*-3), 22 % stearidonic acid (18:4 *n*-3), 1 % oleic acid (18:1 *n*-9), and 24 % 16:0, (palmitic acid) (Khotimchenko et al., 2002), while *U. lactuca* obtained from North Sea in September/October had 20 % 18:3 *n*-3 acid, 8 % 18:4 *n*-3, 20 % 18:1 *n*-9, and 12 % 16:0 (van Ginneken et al., 2011). Accordingly, the application of lipidomics as a tool to differentiate green seaweed species may require a deeper analysis of the FA composition, involving analysis of the FA profile in each main lipid class (TAG, DAG, MAG, FFA, and PL).

5.2.2 Seaweed lipid class distribution

In order to achieve the aforementioned objective, a first essential step is to determine the distribution of the fat substances into lipid classes. There was co-elution of PL and GL. For this reason, it was chosen to group results into two major classes, polar and non polar (Table 6).

Table 6 – Lipid class distribution (%) as determined by TLC of the five studied green seaweed species.

Lipid Class (%)	*R. riparium*	*U. lactuca*	*U. prolifera*	*U. intestinalis*	*C. linum*
Polar lipid	26.1 ± 3.9^{a}	42.1 ± 4.0^{b}	57.3 ± 8.5^{c}	21.5 ± 1.2^{a}	28.0 ± 0.8^{a}
Non polar lipid	73.9 ± 3.9^{c}	57.9 ± 4.0^{b}	42.7 ± 8.5^{a}	78.5 ± 1.2^{c}	72.0 ± 0.8^{c}

Values are presented as average±standard deviation. Different letters within a row correspond to statistical differences (p<0.05).

In *R. riparium*, *U. intestinalis*, and *C. linum*, the percentage of non polar lipids was higher than in the other seaweeds, thereby exceeding the 70 % share of the total lipids. The highest percentage of polar lipids was measured in *U. prolifera*, 57.3 ± 8.5 % of total lipids. With exception of this latter seaweed species, the values for the relative importance of polar and non polar lipids are within the ranges typically reported in the literature (Chopin et al., 2001).

5.2.3 Fatty acid profile of main lipid classes

The FA composition of the main lipid classes in the studied green seaweeds is shown in Tables 7 and 8 (Annex). The PL (also including GL) and TAG profiles are found in the former table and the MAG and FFA are found in the latter. Because of the very low amount of DAGs in the lipid fraction of all seaweeds, it was not possible to determine the FA composition of this class. In the case of some seaweeds, there was poor separation of TAGs, MAGs, and FFA from neighboring bands, thus leading to the exclusion of the FA profile determination for some classes and species.

Within the PL+GL class, *C. linum* presented the highest SFA content (together with *R. riparium*) as well as the lowest PUFA content. Regarding MUFA, the lowest content was observed in *U. lactuca*, being the other seaweeds from the *Ulva* genus also poorer in MUFA than the seaweeds belonging to other genera. On the other hand, the highest percentage of *n*-3 PUFA in PLs and GLs was found in *U. lactuca*, displaying the other *Ulva* species also substantial amounts of *n*-3 PUFA. A similar situation was observed for *n*-6 PUFA except for the highest content being found in another *Ulva* species, *U. prolifera*. The seaweed *C. linum* exhibited the lowest percentages of both *n*-3 PUFA and *n*-6 PUFA. The relative richness in *n*-3 and *n*-6 PUFA in *U. prolifera* led to the lowest *n*-3/ *n*-6 ratio, 0.7 ± 0.1. Contrastingly, *C. linum* displayed the highest ratio, 2.7 ± 0.2. However, most *n*-3 PUFA had low abundance in this species. The main exceptions were the C18 *n*-3 PUFA. Highest DHA level was in *U. intestinalis*, but a low value (< 2 % of the total FA), especially taking into account that it was determined in the PL+GL class. While there was no difference in the EPA content, all other *n*-3 PUFA presented differences among seaweeds, namely, *U. lactuca* was the richest in C16 *n*-3 PUFA. For linoleic acid, highest value was determined in the PLs and GLs of *U. prolifera*, 19.4 ± 0.3 %. On the other hand, the seaweeds *R. riparium* and *C. linum* were rich in C16 and C18 MUFA. Finally, whereas seaweeds from *Ulva* genus were rich in myristic acid (> 10.0 %), palmitic acid was much more abundant in *R. riparium* and, even more, in *C. linum*, reaching 40.6 ± 1.0 %.

The FA profiles of the PLs and GLs had similarities with the global profiles of Table 1. There were also some differences. Namely, some FA, such as palmitic acid, and the total SFA had different abundances in the PL+GL class. The PLs and GLs in *R. riparium* were poorer in *n*-3 PUFA than the total fat fraction in this seaweed. The

opposite was observed in *U. lactuca* and *U. intestinalis*. In the case of *U. lactuca*, this can be mainly ascribed to the accumulation of C16 *n*-3 PUFA in the PL+GL class.

The differences between PLs and GLs and total fat may be ascribed to the other important lipid class in the studied green seaweed species, TAGs, which is the main group of non polar lipids. The comparison between the FA profiles of TAGs of different seaweeds conveys results similar to those found in PLs and GLs. For instance, as in PLs and GLs, *U. prolifera* had to the lowest *n*-3/ *n*-6 ratio, 0.4 ± 0.0, and *C. linum* presented the highest ratio, 2.0 ± 0.4, or the highest content of linoleic acid was found in *U. prolifera*, 35.3 ± 2.5 %. However, FA percentages in TAG differed significantly from those in PL+GL. For all seaweed species, TAGs were poorer in SFA than PLs and GLs. First and foremost, this was due to palmitic acid, but also myristic acid contributed for the SFA contrast between TAGs and PLs + GLs. Regarding MUFA, differences between TAG and PL + GL were smaller. For *n*-3 PUFA and *n*-6 PUFA, differences were also less significant except for *R. riparium* in *n*-3 PUFA —higher in TAG class— and *U. prolifera* in *n*-6 PUFA —higher in TAG. The latter was largely due to a high level of linoleic acid accumulation in the TAG of *U. prolifera*. The former deviation resulted from a high level of 18:3 *n*-3 in *R. riparium* TAG, 13.4 ± 0.1 %.

The FA compositions of the MAG and FFA classes (Table 4) are related to each other since MAG are formed from TAG (and DAG) by hydrolysis, which also generates FFA. Accordingly, a joint analysis of the FA in each of these two classes can provide valuable insight. It was observed that SFA and palmitic acid percentages were higher and MUFA and linoleic acid percentages were lower in the MAG than in FFA, thereby pointing to a preferential hydrolysis of MUFA and linoleic acid. Moreover, a global comparison involving all studied lipid classes shows that SFA were much more abundant in MAG and PL+GL than in TAG and FFA. Concerning other FA, differences were circumscribed to particular species. For instance, PUFA, including both *n*-3 and *n*-6, and, particularly, 18:3 *n*-3 acid were higher in FFA only in the case of *R. riparium*.

According to literature (Kendel et al., 2015), higher palmitic and SFA contents in PLs and GLs than in the total fat fraction were also observed for another green seaweed, *U. armoricana*. A lower level of *n*-3 PUFA in PL (13.8 ± 0.1 %) and even lower in GL (8.5 %) than in total lipids (23.9 ± 0.1 %) was also found in this species by the same authors. This contrasts with other organisms, where *n*-3 PUFA, particularly very long chain *n*-3 PUFA (EPA, DHA), are typically more concentrated in the PL

fraction and other polar lipids fractions (Mendoza et al., 2011), since EPA and DHA are structurally important FA giving fluidity to cell membranes (Valentine and Valentine, 2004). Hence, *R. riparium* represents an uncommon situation characterized by higher *n*-3 PUFA content in TAG than in PL and GL. In *U. lactuca*, *U. prolifera*, and *U. intestinalis*, there was no specific accumulation of α-linolenic acid in PLs, thus differing from other algae (Kumari et al., 2013). This may explain that though α-linolenic acid is considered characteristic of the order Ulvales, reaching 10-20 % of the total FA (Khotimchenko et al., 2002), its content in the studied Ulvales (genus *Ulva*) was low — PLs did not contribute much to the global α-linolenic acid content.

The preferential hydrolysis of MUFA and linoleic acid over SFA and palmitic acid can be related to the selectivity of any lipase that remains active after harvest and during transport and storage of the seaweeds. This selectivity may lead to the formation of some FFA and the relative concentration of certain FA, such as palmitic acid, in the MAGs. Moreover, lipases may operate in a selective way owing to either chemical affinity or sensitivity to the position of the FA chain in the TAG. On the one hand, whereas *n*-3 PUFA such as DHA are very frequently bound at the 2-position (*sn*−2) of TAG molecules, two other mid- or short-chain FA are in the lateral (1- and 3-) positions (*sn*−1/3) (Schuchardt and Hahn, 2013). This makes the rupture of the ester bond of a long-chain fatty acid by a lipase harder to achieve (Schuchardt and Hahn, 2013). On the other hand, the chemical structure of each FA, in particular, the number of double bonds, may be more important than position. Regarding the positional *vs* chemical structure selectivity hypotheses, the enrichment in palmitic acid in the MAG supports the regioselectivity hypothesis. This is a saturated FA and it is not very long, thus any structural selectivity against DHA hydrolysis would not apply to this FA. Moreover, a positional selectivity of the lipase implies that the palmitic acid (and other SFA) is more frequently bound at the 2-position (*sn*−2) of TAGs in seaweeds. Precisely, it has been reported for other eukaryotic organisms a higher proportion of palmitic and other SFA in position *sn*-2 (Brockerhoff et al., 1968). It is also very interesting to note that according to this study that different MUFA are most often found at *sn*-1/3. This agrees with the results of the current study. Therefore, the lipase responsible for the observed hydrolysis seems to display a predominantly regioselective action and the positioning of FA in the green seaweed lipids does not differ much from that of other organisms.

Results seem to enable two main dividing lines: between Ulvales (*U. lactuca*, *U. prolifera*, and *U. intestinalis*) and Cladophorales (*R. riparium* and *C. linum*) and between *U. prolifera* and the group formed by *U. lactuca* and *U. intestinalis*. In particular, for the first dividing line, important discriminating parameters for the Ulvales are: low MUFA content in total fat, PL+GL, and MAG; low C18:1 content in total fat, PL+GL, and MAG; high C16 *n*-3 PUFA content in total fat and PL+GL; and low *n*-3/ *n*-6 ratio in total fat. For the second dividing line, *U. prolifera* differs from the other species of the *Ulva* genus in: high C18:1 content in total fat and PL+GL; low MUFA content in total fat; high linoleic acid content in total fat and PL+GL; high PUFA content in total fat; high *n*-6 PUFA in total fat and PL+GL; and low *n*-3/ *n*-6 ratio in total fat and PL+GL. Regarding this second contrast, it is worth noting that, according to phylogenetic studies on the basis of genetic analysis, *U. lactuca* and *U. intestinalis* (also known as *Enteromorpha intestinalis*) are nearer to each other than to *U. prolifera* (also known as *Enteromorpha prolifera*) (Hayden et al., 2003). Therefore, FA profiles seem to be usable as a chemotaxonomic tool in green seaweeds. Given the simplicity of the FA determination methodology, this can provide a quick and practical route for the verification of seaweed identity in slightly processed foods —for instance, all *Ulva* species are edible (Edwards et al., 2012)—, where seaweed is dried and finely minced. Nonetheless, more research covering multiple influential aspects, such as season, geographical location, cultivation methods, and others, must be carried out in order to consolidate this possibility.

Finally, it should be noted that seaweed quality as a source of essential FA could be monitored through the calculation of critical ratios in the PL+GL fraction as well as in total fat, such as, *n*-3/ *n*-6 ratio, *n*-3(C20+C22)/ *n*-3(C16+C18) ratio, and the atherogenicity (AI) and thrombogenicity (TI) indices (Senso et al., 2007):

$$AI = [(4 \times C14{:}0) + C16{:}0 + C18{:}0]/(\Sigma MUFA + \Sigma\, n\text{-}6PUFA + \Sigma\, n\text{-}3PUFA)$$

$$TI = (C14{:}0 + C16{:}0 + C18{:}0)/(0.5 \times \Sigma MUFA + 0.5 \times \Sigma\, n\text{-}6PUFA + 3 \times \Sigma\, n\text{-}3\ PUFA + n\text{-}3/\ n\text{-}6\ ratio)$$

Regarding these FA quality parameters, different seaweeds presented the best levels: highest *n*-3/ *n*-6 ratio in *C. linum*'s total fat (and PL+GL); highest *n*-

3(C20+C22)/ *n*-3(C16+C18) ratio in *U. prolifera*'s total fat; and lowest AI and TI in *R. riparium*'s total fat.

5.3 Conclusions

The fish pond aquaculture production system showed to enable the rearing of meagre and the growth of different green seaweed species with specific fat fraction characteristics. Indeed, there was a clear distinction between the FA profiles (total FA and per lipid classes) of *R. riparium* and *C. linum*, which belong to the Cladophorales order, and those of *Ulva* genus, Ulvales order. Moreover, every seaweed had a specific FA profile, whose specificities were rendered more obvious with the study of the FA profile per lipid class. However, between *U. lactuca* and *U. intestinalis*, there were only minor differences. On the other hand, *U. prolifera* differed from the other species of the *Ulva* genus. Furthermore, it was possible to identify significant differences between the palmitic acid content in the PL+GL class of each seaweed. Hence, FA profiling may offer a simple and practical tool for distinguishing among seaweed species, for instance, detecting non-edible species in dried and minced seaweed-based foods. Important differences were found among lipid classes, yielding large contrasts between PLs + GLs and TAGs as well as between MAGs and FFA. This study also found evidence supporting the location of particular FA in specific TAG positions. There are still many unknown aspects, such as the effects of season, wild *vs* cultured seaweeds, geographical location and other factors on the FA profiles, thus warranting further study.

6. Mineral Composition and Bioaccessibility of Green Seaweeds from Fish Pond Aquaculture[3]

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[3] Cardoso, C., Afonso, C., Ripol Malo, A., Varela, J., Quental-Ferreira, H., Pousão-Ferreira, P., Ventura, M., Delgado, I., Coelho, I., Castanheira, I., Bandarra, N. (2018) Elemental Composition and Bioaccessibility of Green Seaweeds from Fish Pond Aquaculture. *Food Research International*; Volume 105, March 2018, Pages 271-277.

6.1 Introduction

Green seaweeds are typically green in colour due to the presence of chlorophyll in their chloroplasts. Their colour depends on the interplay between chlorophylls and other pigments, such as xanthophylls and β-carotene. Main genera of green seaweeds include *Ulva, Codium, Enteromorpha, Chaetomorpha*, and *Cladophora*. Green seaweeds are particularly common in areas with abundant light, such as shallow waters in ponds. Though most seaweeds are harvested offshore, they can also be produced in separate ponds or as co-products in fish farming (Chopin et al., 2001). Fish pond aquaculture production systems are a new and fast-developing scientific field that brings together fish farming and production of seaweeds (Chopin et al., 2001). This is environmentally positive and may have economic benefits. The composition and economic value of green seaweeds may vary between species and, for a given species, parameters depend on abiotic/biotic conditions. Accordingly, it is fundamental to study the composition, including elemental profile, of seaweeds from systems of fish pond aquaculture where integrated multi-trophic aquaculture may be a viable future outcome.

Concerning green seaweed elemental composition, in general, levels of Ca, K, Na, and Mg are high, exceeding contents in other seaweed groups except for K, which is much more abundant in some studied brown seaweeds (Makkar et al., 2015). Nonetheless, green seaweeds may be used as organic fertilizers just as brown seaweeds (Abdel-Raouf et al., 2012), owing to their appreciable K content. Other authors have shown that green seaweeds are also a relatively rich source of Fe (Yaich et al., 2011). Seaweeds of *Ulva intestinalis* and *Ulva lactuca* species, among the most relevant green seaweeds, are not rich in I (Nitschke and Stengel, 2015), thus differing of some brown seaweeds, which are excellent sources of I and are usable in the treatment of I deficiency (Basedow's disease, goiter, and hyperthyroidism) or as hypocholesterolemic and hypoglycemic agents (El Gamal, 2012). Nevertheless, studies on green seaweeds are very focused on the genus *Ulva* and, particularly, *Ulva lactuca* (Yaich et al., 2011), being required studies encompassing other green seaweed species for a more judicious assessment of the nutritional value of their elemental components.

Moreover, in considering the elemental composition in terms of nutritional value of green seaweeds, it must be taken into account that the absorbable quantity of an element in the gastrointestinal (GI) tract is not accurately predicted by its total content in the seaweed. Bioaccessibility is expressed by the share of the initial content that is

available for intestinal absorption (Afonso et al., 2015). The assessment of bioaccessibility may help to better define the nutritional value of any given green seaweed. A bioaccessibility study entails the application of an appropriate in vitro digestion model that simulates human GI tract. Regarding this, various methodologies have been developed, with the static model presenting digestive compartment distinction and complete digestive juices, including enzymes in all steps, being one of the most reliable systems (Cardoso et al., 2015; Versantvoort et al., 2005). More recently, these in vitro techniques for assessing human bioaccessibility have been subjected to significant improvements (Afonso et al., 2015).

The current study was intended to provide more information and insight regarding the elemental composition and associated bioaccessibility of a group of relevant green seaweeds grown under fish pond aquaculture conditions.

6.2 Results and Discussion

6.2.1 Elemental composition

The elemental composition of studied green seaweed species on a dry weight basis is presented in Table 9. For several elements, there were differences between species. Specifically, whereas no difference was observed for Cr, for the elements Mn, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Cd, Sn, I, and Pb (arranged in order of increasing atomic weights), differences were detected.

The seaweed *R. riparium* had the highest levels of Mn, Sr, Cd, Sn, and I. In the latter case, a content of 281.5 ± 6.7 mg/kg was determined. Regarding *U. lactuca*, the highest Ni and Cu concentrations were registered, being the copper content in this seaweed more than twofold the second highest content with 146.8 ± 3.0 mg/kg. Concerning *C. linum*, on the one hand, it exhibited the highest levels of Se (0.71 ± 0.04 mg/kg) and Mo (1.10 ± 0.03 mg/kg). On the other hand, this seaweed had the lowest levels of Co (0.62 ± 0.05 mg/kg) and Zn (32.9 ± 4.6 mg/kg). In the case of As, *C. linum* and *U. intestinalis* were the richest sources among the studied green seaweeds, followed by *R. riparium* and, at a lower level, by *U. lactuca* and *U. prolifera*.

The determined levels of Zn are similar to those reported for *U. lactuca* collected in North Africa (Yaich et al., 2011). However, concentrations of other elements such as Mn, Ni, Cu, Cd, and Pb are different from those found in this study. In

particular, whereas Ni (Bikker et al., 2016) as well as Cd and Pb contents (Yaich et al., 2011) are lower in the current study, thus pointing to a lower level of pollution, Mn and Cu levels are higher than those of green seaweeds collected in the African littoral (Yaich et al., 2011) and in the Indian ocean (Al-Shwafi and Rushdi, 2008). Moreover, Cu levels are also higher than the values of green seaweeds from unpolluted sites, 0.1-3 mg/kg dry weight, being within or near the range of polluted sites, 14-134 mg/kg (Wong et al., 1982). The Zn levels are also higher than the Zn range of seaweed taken from uncontaminated sites, 0.5-23 mg/kg dry weight (Wong et al., 1982; Yaich et al., 2011).

Table 9 - Elemental composition (mg/kg dry weight) of the five studied green seaweed species.

Element	*R. riparium*	*U. lactuca*	*U. prolifera*	*C. linum*	*U. intestinalis*
Cr	3.60 ± 0.05a	3.92 ± 0.17a	6.17 ± 2.38a	5.49 ± 0.55a	5.82 ± 0.33a
Mn	135.1 ± 0.4c	93.6 ± 9.0b	102.1 ± 0.1b	32.2 ± 2.2a	29.6 ± 0.7a
Co	1.39 ± 0.01c	1.42 ± 0.07c	1.13 ± 0.02b	0.62 ± 0.05a	1.01 ± 0.03b
Ni	3.03 ± 0.06b	4.69 ± 0.17c	1.96 ± 0.0a	1.83 ± 0.20a	2.96 ± 0.08b
Cu	68.9 ± 0.6c	146.8 ± 3.0d	50.1 ± 0.2b	51.5 ± 0.5b	16.2 ± 0.1a
Zn	59.3 ± 0.9b	63.8 ± 2.0b	63.5 ± 4.5b	32.9 ± 4.6a	53.2 ± 3.3b
As	5.15 ± 0.06b	4.14 ± 0.49a	4.02 ± 0.18a	6.37 ± 0.20c	6.34 ± 0.01c
Se	0.44 ± 0.03c	0.19 ± 0.01ab	0.16 ± 0.02a	0.71 ± 0.04d	0.26 ± 0.02b
Sr	141.4 ± 1.2c	72.3 ± 0.0ab	89.0 ± 1.2b	59.0 ± 11.4a	70.1 ± 0.1ab
Mo	0.36 ± 0.01a	0.26 ± 0.02a	0.52 ± 0.01b	1.10 ± 0.03d	0.63 ± 0.04c
Cd	0.43 ± 0.01d	0.14 ± 0.01c	0.06 ± 0.00b	0.02 ± 0.00a	0.06 ± 0.00b
Sn	0.81 ± 0.03b	0.15 ± 0.00a	0.11 ± 0.00a	0.15 ± 0.02a	0.17 ± 0.01a
I	281.5 ± 6.7c	114.0 ± 0.8b	120.3 ± 14.1b	93.4 ± 5.3b	45.1 ± 0.1a
Pb	1.47 ± 0.05b	1.45 ± 0.17b	0.62 ± 0.01a	1.66 ± 0.12bc	2.03 ± 0.16c

Values are presented as average±standard deviation. Different letters within a row correspond to statistical differences (p<0.05).

Given the fact that green seaweeds of the current study were grown in earth ponds used for meagre experimental grow-out, it is possible that feeds were influential on these results. The low Cd and Pb contents also corroborate this interpretation.

Though feed formulations have low Mn, Cu, and Zn contents (Prabhu et al., 2016), green seaweeds may bioaccumulate these elements (Sánchez-Rodríguez et al., 2001). It is known that seaweeds remove elements from the environment and accumulate in the body cell, reaching concentrations 4,000-20,000 higher than in the surrounding water (Donat and Dryden, 2001; Sánchez-Rodríguez et al., 2001;

Sudharsan et al., 2012). Elemental concentration in algal tissue seems to be controlled by the elemental content in water, being metabolic processes as well as environmental factors specific to a given location and setting modulators of the final concentration of a given element in the seaweed (Sánchez-Rodríguez et al., 2001). There is no consensus regarding the relative uptake of elements by different seaweed groups. Whereas some authors (Sudharsan et al., 2012) claimed that green seaweeds did not seem to be as prone to bioaccumulate elements, such as Mn, Ni, Cu, Zn, Cd, and Pb, as other seaweeds, there are other authors (Al-Shwafi and Rushdi, 2008) who have observed a higher accumulation of such elements by green seaweeds in comparison to brown and red seaweeds.

In addition, the levels of Cr, Co, As, Sr, and Mo in the five studied green seaweed species are near or within the reported values and ranges for green seaweeds from other locations, 3.4-15.3 mg/kg dry weight (Cr), 0.3-1.0 mg/kg dry weight (Co), 5.8 mg/kg dry weight (As), 230 ± 209 mg/kg dry weight (Sr), and 0.2-2.7 mg/kg dry weight (Mo) (Al-Shwafi and Rushdi, 2008; Bikker et al., 2016; Perryman et al., 2017; Saenko et al., 1976). The Sn contents in the studied seaweeds are within the range of Sn in several edible seaweeds, < 0.46 mg/kg dry weight (van Netten et al., 2000), except for *R. riparium*, which clearly is above this value. The source of Cr, Co, and Mo levels may be the feeds, since these elements are important elements for fish (Watanabe et al., 1997) and algae also accumulate them (Al-Shwafi and Rushdi, 2008).

On the basis of the elemental contents in green seaweeds, it is possible to calculate the amounts of dried seaweed for achieving specific intakes. This is particularly relevant for some elements. For Cu, its Recommended Daily Allowance (RDA) is 900 μg/day for men and women (IOM, 2001). This means that 7 g of dried *U. lactuca* (the richest source of Cu among studied seaweeds) everyday ensures the Cu RDA. Regarding Mo, RDA is 45 μg/day for men and women (IOM, 2001). In this case, 41 g of dried *C. linum* on a daily basis is necessary to meet the Mo RDA. Concerning Zn, RDA is set at 11 mg/day for men and 8 mg/day for women (IOM, 2001). As a consequence, in order to guarantee the male Zn RDA, a daily consumption of 173 g of dried *U. lactuca* is required. Accordingly, studied seaweeds are a rich source of Cu, but not so much of Mo and Zn.

The Directive 2002/32 EC (2002) imposes legal limits for heavy metals in seaweeds intended to be used as feed ingredient (EC, 2002). These limits are 40 mg/kg

for As, 1 mg/kg for Cd, and 10 mg/kg for Pb. For all these contaminants, the five studied green seaweeds are below the legal thresholds.

The Se concentration of the seaweeds in present study is higher than the range reported for *U. lactuca* harvested at the Irish coast, < 0.1 mg/kg dry weight (Bikker et al., 2016). The importance of Se to fish justifies its incorporation in feeds (Prabhu et al., 2016) and may have led to its uptake by green seaweeds in the ponds. This element is a natural antagonist for mercury, either for methylmercury (MeHg) or inorganic (Hg), which may counteract or eliminate symptoms of high exposures to this contaminant (Ralston and Raymond, 2010). The Se RDA for individuals aged between 14 and 52 years (excluding the states of pregnancy and lactation) has been set at 55 μg/day (IOM, 2000). For *C. linum*, the richest Se source among studied green seaweeds, in order to meet the Se RDA, 78 g of dried seaweed would be required every day, which makes this seaweed a modest source of Se.

Among the analyzed elements, I was the most abundant one in three seaweed species, *R. riparium*, *U. prolifera*, and *C. linum*. The I results are different of those reported for green seaweeds by other studies, such as *U. intestinalis* and *U. lactuca*, 79 ± 4 mg/kg dry weight and 63 ± 3 mg/kg dry weight, respectively (Nitschke and Stengel, 2015). However, while I content is higher in the *U. lactuca*'s samples of the current study, it is lower in the *U. intestinalis*'s samples of the current study. In fact, values of the current study do not differ much from those of the literature (Nitschke and Stengel, 2015), being in a similar broad range. Brown seaweeds display higher iodine levels, exceeding in some species 1000 mg/kg dry weight (Burtin, 2003; Nitschke and Stengel, 2015). Nonetheless, the studied green seaweeds and, particularly, *R. riparium* are a substantial source of I. This is important, since I may have an anti-tumoural effect (Garcia-Solis et al., 2005) and is required by humans for normal thyroid function (Dunn, 2003). The daily Dietary Reference Intake (DRI) advised by the Institute of Medicine is 150 μg for adults (IOM, 2004), being the Tolerable Upper Intake Leve (TUIL) 1,100 μg/day (NRC, 2000). Hence, a little more than 0.5 g of dried *R. riparium* already covers the I DRI and exceeding a daily consumption of 4 g of this dried seaweed may warrant a note of caution.

All the estimates of advisable dried seaweed consumption amounts and frequencies presuppose that all elements are absorbed by the human organism. However, it is possible that only a minor share of the initial element contents becomes

available for absorption across the intestinal wall after digestion, that is, bioaccessible (Afonso et al., 2015). Hence, in such a study as the current one, it is very important to assess the elemental bioaccessibility.

6.2.2 Elemental bioaccessibility

The bioaccessible elemental contents are presented in Table 10 and the bioaccessibility factors associated to each element are displayed in Figure 19. For some elements (Cr, Co, Ni, Se, Mo, Cd and Sn), the very low bioaccessible contents near the limits of quantification and the interference of the blank (enzyme solutions used in the digestion model) did not allow for a reliable determination of bioaccessibility percentages.

Table 10 – Bioaccessible elemental contents (mg/kg; calculated taking into account the mass of sample input in the *in vitro* digestion and subtracting blank interference) of the five studied green seaweed species.

Element	*R. riparium*	*U. lactuca*	*U. prolifera*	*C. linum*	*U. intestinalis*
Mn	108.0 ± 3.6d	77.8 ± 3.9b	96.8 ± 3.6c	18.1 ± 4.7a	17.6 ± 0.5a
Cu	55.4 ± 3.0d	107.2 ± 1.7e	38.3 ± 2.0c	26.7 ± 3.4b	11.0 ± 3.2a
Zn	38.2 ± 1.1c	8.2 ± 3.3ab	19.0 ± 1.0b	n.d.a	n.d.a
As	5.10 ± 0.02d	2.77 ± 0.03ab	2.92 ± 0.02b	2.55 ± 0.00a	3.55 ± 0.02c
Sr	84.6 ± 3.7d	49.2 ± 1.3b	55.2 ± 0.4c	15.5 ± 1.0a	46.7 ± 5.6b
I	88.6 ± 10.4c	27.7 ± 0.6b	16.7 ± 1.0ab	13.4 ± 1.7a	13.1 ± 2.2a
Pb	0.59 ± 0.07a	1.02 ± 0.12b	0.60 ± 0.02a	0.77 ± 0.04ab	1.61 ± 0.05c

n.d. – not detected. Values are presented as average±standard deviation. Different letters within a row correspond to statistical differences (p<0.05).

The Mn, Zn, As, Sr, and I bioaccessible contents were higher in *R. riparium* compared to the other green seaweeds. The abundance of these elements in *R. riparium* was also registered for the initial (prior to digestion) contents except for Zn and As. Regarding Cu, *U. lactuca* presented the highest bioaccessible content, thereby replicating the results for Cu contents before digestion. For Pb, bioaccessible concentration was higher in *U. intestinalis* than in all other seaweeds.

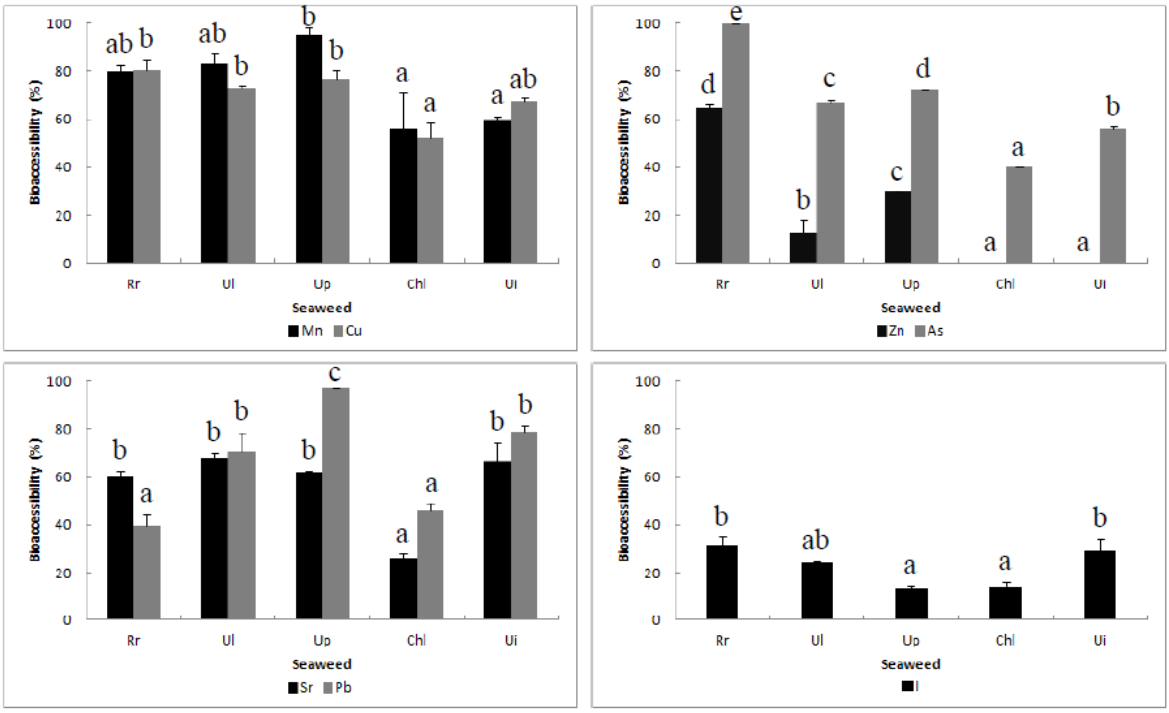

Figure 19 – Bioaccessibility (%) of elements in the five studied green seaweed species (Rr - *Rhizoclonium riparium*, Ul - *Ulva lactuca*, Up - *Ulva prolifera*, Chl - *Chaetomorpha linum*, Ui - *Ulva intestinalis*). Different letters within a series regarding a specific element correspond to statistical differences (p<0.05).

These concentrations enabled the calculation of the bioaccessibility factors for each element in the studied green seaweeds. In particular, the bioaccessibility of Zn and As was higher in *R. riparium* than in all other seaweeds. The highest Pb bioaccessibility was observed for *U. prolifera*. Regarding Mn, its bioaccessibility in *U. prolifera* (95 ± 4 %) was higher than in *C. linum* and *U. intestinalis* (56 ± 15 % and 59 ± 2 %, respectively), being Mn bioaccessibility of the other species intermediate. The bioaccessibility of Cu and Sr in *C. linum* was lower (52 ± 7 % and 26 ± 2 %, respectively) than in all other species except for Cu bioaccessibility in *U. intestinalis*. The elements Mn and Cu had the highest bioaccessibility levels, which were always above 50 %. The bioaccessibility of I was lower (14-31 % in all studied seaweeds) than the bioaccessibility range of Mn, Cu, As, and Pb (40-100 %). Specifically, *R. riparium* and *U. intestinalis* had a I bioaccessibility higher (31 ± 4 % and 29 ± 5 %, respectively) than that of *U. prolifera* and *C. linum* (14 ± 1 % and 14 ± 2 %, respectively), being that of *U. lactuca* in an intermediate position (24 ± 1 %).

A comparison between the five green seaweed species shows that the elemental bioaccessibility range of *R. riparium* (31-100 %) was higher than the ranges for other species, particularly if compared with the range of *C. linum* (≤ 56 %). Therefore, it

seems that the bioaccessibility of any given element may vary considerably within the same class of seaweeds. This may be due to a different capability of the enzymes in the in vitro model for releasing the elements existing in each seaweed or to variations in seaweed composition that affect the elements' affinity for the bioaccessible fraction or their solubility (Laparra et al., 2003). It is also possible that different chemical forms of the elements in each seaweed species play a role as suggested in the case of I, whose distribution between an inorganic form, such as iodide, and organic forms may be influential (Aquaron et al., 2002).

There are only a few studies on elemental bioaccessibility in seaweeds (García Sartal et al., 2011; Laird and Chan, 2013; Laparra et al., 2003; Torres-Escribano et al., 2011). In spite of some methodological differences, the in vitro digestion models are comparable, at least, the static models used in these four experimental works. The studied seaweeds encompassed mainly brown and red seaweeds and only two green seaweed species, *U. rigida* (García-Sartal et al., 2011) and *Enteromorpha sp*. (Laparra et al., 2003). In the former case, an As bioaccessibility of 17 ± 2 % was determined, which is clearly lower than the values calculated in the current study. However, these values varied widely between species and *U. rigida* is another species. In the latter case, As bioaccessibility was found to be 32 ± 2 % (Laparra et al., 2003), which is near to the lowest value that was determined for *C. linum* in this study. In this context, it is important to check whether the relatively high As bioaccessibility values attained in the current study agree with the bioavailability results found in in vivo assays. The studies on this issue point to a concordance between high As bioavailability and high As bioaccessibility values. In a *Hizikia fusiforme* study, when this brown seaweed was administered to mice, between 66 % and 92 % of the As was excreted in urine (Ichikawa et al., 2010), thus indicating a high bioavailability of this element.

The levels of Mn, Cu, and As bioaccessibility in the red seaweed *Porphyra abottae* were 86 ± 8 %, 59 ± 10 %, and 79 ± 7 %, respectively (Laird and Chan, 2013). These values agree with the bioaccessibility factors determined in the green seaweeds, particularly Mn in *R. riparium* and *U. lactuca*, Cu in *C. linum*, and As in *U. prolifera*. Finally, a comparison with *Fucus* sp. submitted to a static digestion model shows more similarity regarding As bioaccessibility values, 72 % (Torres-Escribano et al., 2011). This is very similar to As bioaccessibility in *U. lactuca* and *U. prolifera*. On the other hand, Pb bioaccessibility was only 9 % in *Fucus* sp. (Torres-Escribano et al., 2011), which is much lower than any of the values determined in the present study. Given the

paucity of studies concerning this element as well as others, further research is warranted.

Regarding I, a very relevant study (Romarís-Hortas et al., 2011) on its bioaccessibility encompassing several types of seaweed, including a green seaweed species, *U. rigida*, has shown that bioaccessible I may be only 2 %. These authors used a static model, but their separation of the bioaccessible fraction involved dialysis, thus differing from the method used in the current study (see Materials and Methods). This may account for the differences between studies. Nevertheless, the *U. rigida* study reinforces the conclusion that I bioaccessibility is really low in seaweed, even more so given the low I bioaccessibility in other seaweeds (red and brown), never exceeding 20 % (Romarís-Hortas et al., 2011). Moreover, a comparison to in vivo bioavailability is also possible (Aquaron et al., 2002). These authors reported very high I bioaccessibilities associated to the consumption of a red or a brown seaweed, exceeding 60 %. This contrasts with the bioaccessibility values, which represent a theoretical upper limit for bioavailability. Hence, this issue requires also further study.

The bioaccessibility results lead to the calculation of larger amounts of dried seaweed for reaching the previously discussed dietary recommendations and thresholds. Namely, for Cu and Zn, in order to meet the RDAs (IOM, 2001), 10 g of dried *U. lactuca* and 290 g of dried *R. riparium* would be required on a daily basis, respectively. Moreover, taking into account bioaccessibility, for I, its DRI (IOM, 2004) may require 2 g of dried *R. riparium* and a risk of surpassing its TUIL (NRC, 2000) would arise with 13 g of dried *R. riparium*. These amounts are much larger than those calculated above on the basis of total I contents in seaweed. Therefore, I bioaccessibility results must be taken into account in estimating dietary exposure to I and the risk of exceeding its TUIL. For instance, a study has estimated that the Japanese I intake —largely from seaweeds— averages 1,000 to 3,000 μg /day (Zava and Zava, 2011), which entails a high probability of surpassing the I TUIL. However, this TUIL was defined on the basis of the I effect on the thyroid-stimulating hormone using I supplements (NRC, 2000). Furthermore, high I bioavailability values were presupposed on the basis of previous experimental work (Nath et al., 1992), leading to the claim that, under normal conditions, the absorption of dietary I is greater than 90 %. This is different of current results, given the low I bioaccessibility in the studied seaweeds. This may be due to the enmeshment of I in the matrix of seaweed and its particular chemical form, since the aforementioned study was done with salt (Nath et al., 1992). Of course, a study on I

bioaccessibility with a representative set of edible seaweeds consumed in Japan would be more decisive. Nonetheless, it could be argued that the 1,000-3,000 μg /day I intake do not pose any major risk to the Japanese population.

6.3 Conclusions

This experimental work shed some light onto the elemental composition and bioaccessibility of green seaweeds from fish pond aquaculture and integrated aquaculture production and it has identified advantages and drawbacks for each species. Indeed, there were important differences between seaweed species. Namely, it was observed that *R. riparium* had the highest levels of Mn, Sr, Cd, Sn, and I and that *U. lactuca* had the highest Ni and Cu concentrations. The results of the elemental composition suggest the possibility that this fish pond system may lead to the bioaccumulation of some elements. Moreover, it was possible to calculate the daily amounts of dried green seaweed required for achieving specific dietary intakes: 7 g of dried *U. lactuca* (for meeting Cu RDA); 173 g of dried *U. lactuca* (Zn RDA); 78 g of dried *C. linum* (Se RDA); 41 g of dried *C. linum* (Mo RDA); and 0.5 g of dried *R. riparium* (I DRI). Concerning elemental bioaccessibility, whereas Mn and Cu had the highest values, always above 50 %, I values were always in the low range of 14-31 %. In addition, bioaccessibility range of all studied elements in *R. riparium* (31-100 %) was higher than the ranges for other species, particularly *C. linum* (≤ 56 %). The bioaccessibility results entailed higher quantities of dried seaweed for reaching dietary intakes: 10 g of dried *U. lactuca* (Cu RDA); 290 g of dried *R. riparium* (Zn RDA); and 2 g of dried *R. riparium* (I DRI). Accordingly, this is a very rich source of I. This study has shown the importance of taking into account bioaccessibility results in estimating dietary intakes.

7. General conclusion

Several studies have already shown the biotechnological potential of seaweeds in general. However, this study goes a step forward by looking into the bioprospection of IMTA green seaweeds. This study has enabled to identify strong and weak aspects for each of the five species, as well as to evaluate possible biochemical differences that seaweeds might acquire due to IMTA growing conditions.

The results showed a clear distinction between the FA profiles (total FA and per lipid class) of *R. riparium* and *C. linum*, which belong to the Cladophorales order, and those of *Ulva* genus, Ulvales order. Moreover, every seaweed had a specific FA profile, whose specificities were rendered more obvious with the study of the FA profile per lipid class. In addition to this, the results presented here reinforce the potentialities of green seaweeds from a nutraceutical point of view and prove different bioactivities depending on the particular species. *R. riparium* showed the highest fucose content, whilst *U. prolifera* presented the highest total polyphenol content and antioxidant activity. In extracts from *U. prolifera* and *C. linum* the anti-inflammatory activity was more remarkable. Nevertheless, bioaccesibility seemed to play an essential role. When tested an *in vitro* digestion model, the compounds causing these beneficial bioactivities seemed not to be rendered bioaccessible. From a nutritional point of view, this experimental work shed some light onto the mineral composition and bioaccessibility of IMTA green seaweeds and it has identified advantages and drawbacks for each species. Indeed, there were important differences between seaweed species and the results of the mineral composition suggest the possibility that this fish pond system may lead to the bioaccumulation of some elements incorporated into the feeds. Bioaccessibility results of the seaweed minerals showed a second time the importance of taking into account the effect of the digestion when estimating dietary intakes.

Finally, there is a potential worth exploring in the studied green seaweeds that may lead to applications in different areas, such as nutraceutical (iodine, antioxidant activity, anti-inflammatory activity) and cosmetics (antioxidant activity and anti-inflammatory activity) development, provided that bioaccessibility is enhanced by treatment of the seaweed biomass (enzymatic treatment or extraction of bioactives in tisanes, etc.) prior to incorporation in nutraceuticals or that local bioavailability on the skin is ensured through appropriate cosmetic formulation.

8. Future perspectives

Although this work gave an important insight on composition, bioactivity, and bioaccessibility of different novel compounds from IMTA seaweeds, there are still some questions that require further study. On the one hand, another path of study would encompass the analysis of the effects of season, wild vs cultured seaweeds, geographical location and other factors not only on the FA profiles but also on the production of antioxidants and other bioactive substances, since they have been claimed to fluctuate depending on seasonal aspects and climactic conditions. On the other hand, bioaccessibility has proven to be a limiting factor that affects the possibilities of the seaweed nutritional applications. In order to overcome that, future work should focus on the extraction of the bioactive compounds as well as explore the preparation of tisanes and analogous products as strategies to render the bioactives more bioaccessible. In addition to this, taking into account that, typically, a single human meal is not simply composed of seaweeds, it would be of interest to integrate this seaweed in a food matrix like bread or combine in a typical meal and perform the bioaccessibility assay under such conditions in order to evaluate the outcome. Finally, it would also be worth evaluating the bioavailability of the compounds in study using a cellular model such as Caco-2.

9. References

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10. Annexes

Table 7 – Phospholipid+glycolipid and triacylglycerol fatty acid profile (% of total fatty acids) in the five studied green seaweed species.

Fatty acid	**Phospholipid + Glycolipid Classes**					**Triacylglycerol Class**				
	R. riparium	*U. lactuca*	*U. prolifera*	*U. intestinalis*	*C. linum*	*R. riparium*	*U. lactuca*	*U. prolifera*	*U. intestinalis*	*C. linum*
14:0	9.7 ± 0.1^{bC}	14.1 ± 0.2^{cB}	10.4 ± 0.6^{bB}	10.5 ± 0.0^{b}	2.4 ± 0.1^{aC}	7.0 ± 0.1^{cA}	n.d.	4.5 ± 0.4^{bA}	n.d.	0.0 ± 0.0^{aA}
16:0	30.8 ± 0.3^{dC}	14.2 ± 0.3^{aA}	23.6 ± 0.6^{cB}	21.2 ± 0.0^{b}	40.6 ± 1.0^{eB}	17.2 ± 0.3^{bB}	n.d.	7.3 ± 1.3^{aA}	n.d.	25.6 ± 3.5^{bA}
18:0	0.8 ± 0.0^{bA}	0.4 ± 0.0^{aA}	2.7 ± 0.0^{eA}	1.6 ± 0.0^{d}	1.3 ± 0.0^{cA}	0.6 ± 0.0^{aA}	n.d.	2.2 ± 0.7^{aA}	n.d.	8.0 ± 1.6^{bB}
Σ SFA	$46.6 \pm$	44.1 ± 2.2^{aAB}	42.2 ± 2.9^{aB}	39.8 ± 0.0^{a}	53.0 ± 1.2^{bB}	27.2 ± 0.0^{abB}	n.d.	19.8 ± 0.4^{aA}	n.d.	37.4 ± 5.4^{bA}
16:1 *n*-7+ *n*-9	4.8 ± 0.1^{dA}	1.1 ± 0.0^{bB}	0.8 ± 0.0^{aA}	1.4 ± 0.0^{c}	5.1 ± 0.2^{eA}	6.4 ± 0.0^{bC}	n.d.	2.9 ± 0.3^{aB}	n.d.	4.1 ± 0.6^{aA}
18:1 *n*-7+ *n*-9	12.3 ± 0.1^{dC}	2.5 ± 0.1^{aB}	11.7 ± 0.5^{cAB}	7.6 ± 0.0^{b}	12.8 ± 0.2^{eA}	2.1 ± 0.0^{aA}	n.d.	15.2 ± 0.4^{bB}	n.d.	20.1 ± 0.1^{cB}
20:1 *n*-7+ *n*-9+ *n*-11	0.6 ± 0.0^{aB}	0.3 ± 0.0^{aB}	0.7 ± 0.1^{aA}	0.9 ± 0.0^{b}	0.3 ± 0.1^{aA}	0.0 ± 0.0^{aA}	n.d.	0.6 ± 0.4^{aA}	n.d.	1.7 ± 2.4^{aA}
Σ MUFA	18.6 ± 0.1^{dC}	4.3 ± 0.2^{aB}	14.4 ± 2.0^{cB}	10.4 ± 0.0^{b}	19.5 ± 0.1^{dA}	9.1 ± 0.0^{aA}	n.d.	19.8 ± 0.4^{bB}	n.d.	27.4 ± 3.6^{bA}
18:2 *n*-6	7.2 ± 0.1^{bB}	13.7 ± 0.2^{dB}	19.4 ± 0.3^{eB}	8.1 ± 0.0^{c}	2.7 ± 0.1^{aA}	8.3 ± 0.1^{aC}	n.d.	35.3 ± 2.5^{bC}	n.d.	3.2 ± 0.1^{aB}
20:4 *n*-6	0.9 ± 0.0^{bA}	0.1 ± 0.2^{aA}	1.4 ± 0.0^{cA}	1.7 ± 0.0^{c}	0.4 ± 0.0^{aC}	1.0 ± 0.0^{bA}	n.d.	1.6 ± 0.3^{bA}	n.d.	0.0 ± 0.0^{aA}
16:3 *n*-3+16:4 *n*-3	3.4 ± 0.0^{bA}	16.3 ± 0.3^{eA}	8.8 ± 0.2^{cB}	11.8 ± 0.0^{d}	1.7 ± 0.0^{aC}	3.3 ± 0.0^{bA}	n.d.	8.3 ± 0.3^{cB}	n.d.	0.0 ± 0.0^{aA}
18:3 *n*-‒3	6.0 ± 0.0^{dB}	0.3 ± 0.0^{bB}	0.1 ± 0.0^{aAB}	0.3 ± 0.0^{b}	4.2 ± 0.1^{cA}	13.4 ± 0.1^{cC}	n.d.	0.3 ± 0.1^{aB}	n.d.	6.4 ± 0.3^{bC}
18:4 *n*-3	0.5 ± 0.0^{bA}	0.3 ± 0.0^{aB}	0.4 ± 0.0^{aB}	1.6 ± 0.0^{c}	1.7 ± 0.0^{cA}	0.7 ± 0.0^{aA}	n.d.	0.5 ± 0.1^{aB}	n.d.	4.0 ± 0.5^{bB}
20:4 *n*-3	0.1 ± 0.0^{aB}	0.1 ± 0.1^{aA}	0.4 ± 0.0^{bB}	0.1 ± 0.0^{a}	0.2 ± 0.0^{abB}	0.2 ± 0.0^{aC}	n.d.	0.6 ± 0.1^{bB}	n.d.	0.0 ± 0.0^{aA}
20:5 *n*-3	2.3 ± 0.1^{aA}	1.6 ± 2.0^{aA}	2.4 ± 0.2^{aA}	2.6 ± 0.0^{a}	1.5 ± 0.0^{aB}	3.8 ± 0.1^{bC}	n.d.	2.6 ± 0.5^{bA}	n.d.	0.0 ± 0.0^{aA}
22:5 *n*-3	2.4 ± 0.0^{cB}	3.8 ± 0.1^{dA}	2.1 ± 0.1^{bC}	2.3 ± 0.0^{bc}	0.7 ± 0.0^{aC}	3.4 ± 0.1^{cC}	n.d.	1.2 ± 0.2^{bB}	n.d.	0.0 ± 0.0^{aA}
22:6 *n*-3	0.7 ± 0.2^{bA}	0.3 ± 0.1^{aB}	0.2 ± 0.0^{aB}	1.8 ± 0.0^{d}	1.1 ± 0.1^{cB}	0.3 ± 0.0^{bA}	n.d.	0.2 ± 0.1^{bB}	n.d.	0.0 ± 0.0^{aA}
Σ PUFA	24.8 ± 0.4^{bB}	39.9 ± 0.7^{dA}	37.9 ± 0.9^{dB}	32.0 ± 0.0^{c}	16.0 ± 0.4^{aA}	36.2 ± 0.3^{bC}	n.d.	52.5 ± 4.0^{cB}	n.d.	15.6 ± 2.8^{aA}
Σ *n*-3	15.5 ± 0.4^{bB}	23.9 ± 0.0^{dA}	15.3 ± 1.2^{bB}	20.6 ± 0.0^{c}	11.1 ± 0.0^{aB}	25.7 ± 0.1^{bC}	n.d.	13.6 ± 1.5^{aB}	n.d.	10.4 ± 0.1^{aAB}
Σ *n*-6	8.6 ± 0.1^{bB}	15.6 ± 0.6^{dA}	22.6 ± 0.3^{eB}	11.0 ± 0.0^{c}	4.1 ± 0.3^{aA}	9.9 ± 0.4^{aB}	n.d.	37.7 ± 3.2^{bC}	n.d.	5.2 ± 2.9^{aA}
Σ *n*-3/Σ *n*-6	1.8 ± 0.0^{bA}	1.5 ± 0.1^{bA}	0.7 ± 0.1^{aA}	1.9 ± 0.0^{b}	2.7 ± 0.2^{cA}	2.6 ± 0.1^{aB}	n.d.	0.4 ± 0.0^{aA}	n.d.	2.0 ± 0.4^{aA}

Values are presented as average±standard deviation. Different lowercase letters within a row for each lipid class correspond to statistical differences ($p<0.05$). Different uppercase letters between different lipid classes for each seaweed species (in both Tables 3 and 4) correspond to statistical differences ($p<0.05$). n.d. – not determined.

Table 8– Monoacylglycerol and free fatty acid profile (% of total fatty acids) in the five studied green seaweed species.

Fatty acid	**Monoacylglycerol Class**					**Free Fatty Acid Class**				
	R. riparium	*U. lactuca*	*U. prolifera*	*U. intestinalis*	*C. linum*	*R. riparium*	*U. lactuca*	*U. prolifera*	*U. intestinalis*	*C. linum*
14:0	9.6 ± 0.4^{bC}	6.9 ± 1.6^{abA}	5.3 ± 0.2^{aA}	n.d.	n.d.	8.5 ± 0.0^{cB}	6.8 ± 0.2^{bA}	n.d.	n.d.	1.0 ± 0.0^{aB}
16:0	31.4 ± 1.2^{aC}	31.1 ± 6.6^{aB}	41.4 ± 1.8^{aC}	n.d.	n.d.	9.7 ± 0.0^{bA}	6.6 ± 0.1^{aA}	n.d.	n.d.	36.7 ± 0.0^{cB}
18:0	2.1 ± 0.1^{aB}	3.4 ± 0.8^{aB}	7.4 ± 0.1^{bB}	n.d.	n.d.	0.7 ± 0.0^{aA}	0.6 ± 0.0^{aA}	n.d.	n.d.	1.2 ± 0.0^{bA}
Σ SFA	54.4 ± 1.5^{aD}	50.8 ± 10.5^{aB}	57.7 ± 2.2^{aC}	n.d.	n.d.	23.0 ± 0.0^{aA}	24.4 ± 0.4^{aA}	n.d.	n.d.	44.0 ± 0.7^{bAB}
16:1 *n*-7+ *n*-9	4.9 ± 0.1^{bB}	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}	n.d.	n.d.	6.9 ± 0.0^{cD}	2.9 ± 0.0^{aC}	n.d.	n.d.	6.5 ± 0.0^{bB}
18:1 *n*-7+ *n*-9	5.1 ± 0.1^{bB}	1.0 ± 0.2^{aA}	2.9 ± 4.1^{aA}	n.d.	n.d.	21.1 ± 0.1^{bD}	17.0 ± 0.3^{aC}	n.d.	n.d.	30.6 ± 0.1^{cC}
20:1 *n*-7+ *n*-9+ *n*-11	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}	n.d.	n.d.	0.0 ± 0.0^{aA}	0.4 ± 0.0^{aC}	n.d.	n.d.	0.3 ± 0.2^{aA}
Σ MUFA	10.0 ± 0.3^{bB}	1.0 ± 0.2^{aA}	6.0 ± 2.1^{abA}	n.d.	n.d.	28.2 ± 0.0^{bD}	20.6 ± 0.0^{aC}	n.d.	n.d.	37.5 ± 0.0^{cB}
18:2 *n*-6	2.2 ± 0.1^{bA}	1.5 ± 0.2^{aA}	2.0 ± 0.1^{abA}	n.d.	n.d.	16.2 ± 0.1^{cD}	14.9 ± 0.3^{bC}	n.d.	n.d.	3.7 ± 0.0^{aC}
20:4 *n*-6	1.1 ± 0.0^{aB}	3.8 ± 0.8^{bB}	2.8 ± 0.2^{abB}	n.d.	n.d.	1.0 ± 0.0^{bA}	2.8 ± 0.1^{cB}	n.d.	n.d.	0.2 ± 0.0^{aB}
16:3 *n*-3+16:4 *n*-3	3.8 ± 0.0^{aB}	11.9 ± 2.8^{bA}	2.8 ± 0.1^{aA}	n.d.	n.d.	4.9 ± 0.0^{bC}	10.7 ± 0.1^{cA}	n.d.	n.d.	0.6 ± 0.0^{aB}
18:3 *n*-3	1.8 ± 0.1^{bA}	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}	n.d.	n.d.	13.8 ± 0.2^{cC}	0.3 ± 0.0^{aB}	n.d.	n.d.	5.0 ± 0.0^{bB}
18:4 *n*-3	0.8 ± 0.2^{bA}	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}	n.d.	n.d.	0.5 ± 0.0^{aA}	0.4 ± 0.0^{aC}	n.d.	n.d.	3.4 ± 0.3^{bB}
20:4 *n*-3	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}	n.d.	n.d.	0.1 ± 0.0^{aB}	0.2 ± 0.0^{bA}	n.d.	n.d.	0.2 ± 0.0^{bB}
20:5 *n*-3	3.2 ± 0.2^{aB}	7.4 ± 5.8^{aA}	2.8 ± 0.2^{aA}	n.d.	n.d.	3.0 ± 0.0^{bB}	3.3 ± 0.0^{cA}	n.d.	n.d.	0.0 ± 0.0^{aA}
22:5 *n*-3	0.8 ± 0.0^{aA}	2.4 ± 2.3^{aA}	0.0 ± 0.0^{aA}	n.d.	n.d.	0.7 ± 0.0^{aA}	1.8 ± 0.0^{bA}	n.d.	n.d.	0.6 ± 0.0^{aB}
22:6 *n*-3	0.5 ± 0.0^{bA}	0.0 ± 0.0^{aA}	0.0 ± 0.0^{aA}	n.d.	n.d.	0.3 ± 0.0^{aA}	0.4 ± 0.0^{aB}	n.d.	n.d.	0.0 ± 0.0^{aA}
Σ PUFA	15.6 ± 2.3^{aA}	31.0 ± 9.9^{aA}	13.9 ± 4.5^{aA}	n.d.	n.d.	41.9 ± 0.5^{cD}	36.8 ± 0.1^{bA}	n.d.	n.d.	14.2 ± 0.2^{aA}
Σ *n*-3	$11.3 \pm$	21.7 ± 5.2^{bA}	5.6 ± 0.3^{aA}	n.d.	n.d.	23.6 ± 0.3^{cC}	17.0 ± 0.1^{bA}	n.d.	n.d.	9.9 ± 0.4^{aA}
Σ *n*-6	3.7 ± 0.6^{aA}	9.3 ± 4.7^{aA}	8.4 ± 4.8^{aA}	n.d.	n.d.	17.5 ± 0.2^{bC}	19.0 ± 0.2^{cA}	n.d.	n.d.	4.1 ± 0.2^{aA}
Σ *n*-3/Σ *n*-6	3.1 ± 0.2^{bB}	2.5 ± 0.7^{abA}	0.8 ± 0.5^{aA}	n.d.	n.d.	1.4 ± 0.0^{aA}	0.9 ± 0.0^{aA}	n.d.	n.d.	2.4 ± 0.2^{bA}

Values are presented as average±standard deviation. Different lowercase letters within a row for each lipid class correspond to statistical differences ($p<0.05$). Different uppercase letters between different lipid classes for each seaweed species (in both Tables 3 and 4) correspond to statistical differences ($p<0.05$). n.d. – not determined.