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Lipid profile and lipogenic capacity of the seaweed *Ulva lactuca* (Chlorophyta)

. use as potential ingredient for fish aquaculture .

Tese de Mestrado em Biotecnologia Vegetal
orientada por Professor Dr. Leonel C. T. Pereira e Dr. Ivan Viegas
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Julho 2016

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Vegetal, realizada sob a orientação científica do Professor Doutor Leonel C. T. Pereira (Universidade de Coimbra) e do Doutor Ivan Viegas (Universidade de Coimbra)

University of Coimbra

2016

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Acknowledgements/Agradecimentos

Ao Ivan, pelos conhecimentos transmitidos, por toda a inspiração, pelo respeito que me incutiu sobre ciência e por toda a paciência. Por me tranquilizar nos momentos certos, por toda a confiança depositada, ajuda e disponibilidade. Por encorajar um constante espirito critico e principalmente por não me dar todas as respostas.

Ao professor Leonel Pereira, por ter introduzindo ao mundo algas marinhas e ter fomentado o meu interesse em querer saber mais. Pelo incentivo a procurar, pelo estimulo à pro-atividade, pelos conselhos, pelo apoio às minhas escolhas e por me ter ajudado sempre que precisei.

Ao João Rito, que abriu a primeira porta á realização deste projeto, pela orientação inicial e por toda a ajuda durante o trabalho laboratorial. Ao Dr. John Jones, por me ter recebido no laboratório do Biocant e por ter permitido trabalhar ao lado da sua equipa. Ao professor Miguel Pardal, pelo espaço cedido no patronato para a realização desta experiência, pela disponibilidade demostrada e por todo o apoio dado. À Cátia Marques, pelas dicas nos lípidos e NMR. A todo o pessoal do Patronato, pela orientação no local, pela simpatia e disponibilidade demonstrada por todos.

À Ana Carolina, por Estar Lá em todas as típicas fases de uma tese e não tendo sido uma *plausible cause* nem um *little bit,* foi sem dúvida fundamental no *crossfit* que foi este ano (*per si)*! À Maria João, pelas dicas e por ser a pessoa que me compreendeu no dialeto lípido-alga. À Diana, ao André, a Alhinho, ao Xavier e ao Bruno que me orientaram sempre que precisei em territórios de plantas. Ao pessoal das algas marinhas, Pedro Br, Pedro Branco, Tiago, João Cotas e Nídia, por toda a entreajuda e união.

A toda a bitcharada da Fortaleza! Andreia, Capela, Sara, Nando, Nicole e Teresa por todo o apoio, compreensão, pela preciosa sabedoria tésica transmitida ao bicho mais novo (ajudou bastante!) e por serem a minha casa em qualquer sítio de Coimbra.

Às minhas redheads: Marina e Leonor.

Para a minha família,

à minha Mãe, ao meu Pai e ao meu Irmão, a quem dedico a minha tese.

Abbreviations

-N	Nutrient deprived
RGR% day^{-1}	Percentage of relative growth rate per day
+N	Nutrient supplemented
$^1H/^2H$	Hydrogen / Deuterium
^2H_2O	Deuterated water
BW	Body water
DHA	Docosahexaenoic acid
DNL	*De novo* lipogenesis
FA	Fatty acids
FAME	Fatty acid methyl esters
FFA	Free fatty acids
FTG	Triglyceride fraction
MTBE	Methyl-*tert*-butyl ether
MUFA	Monounsaturated fatty acids
n-3	Omega 3 fatty acids
n-6	Omega 6 fatty acids
NMR	Nuclear magnetic resonance
PES	Provasoli Enriched Medium
ppm	Parts per million
PUFA	Polyunsaturated fatty acids
SD	Standard deviation
SFA	Saturated fatty acids
T	Temperature
TG	Triglyceride
TW	Tank water
USD	United States Dollars
DT	Direct transesterification
SPE	Solid Phase Extraction

Summary

Ulva lactuca is an opportunistic green seaweed (Chlorophyta) with high growth rates being found in marine and estuarine environments. This is one of the most studied seaweed and it has revealed to be suited for numerous commercial applications. One of the potential and more recent applications is the incorporation in the feed sector for fish aquaculture. Seaweeds are presented as an alternative source for several ingredients used in feed formulations that are becoming increasingly unsustainable, namely fishmeal and fish oil. Unlike fish, seaweeds are capable of synthetizing polyunsaturated fatty acids (PUFA), including omega 3 fatty acids (n-3) that are essential to fish and, therefore, need to be provided through their diet in order to ensure a healthy development. Despite having a low lipid content, *Ulva lactuca*, reveals an interesting qualitative lipid profile, having a high PUFA concentration, particularly n-3, when compared to saturated fatty acids (SFA) and monosaturated fatty acids (MUFA). This seaweed has a well-studied nutritional profile but its lipid synthesis and metabolism are still undeveloped topics.

Deuterium (^2H) is a stable isotope of hydrogen (^1H) that can be delivered as a tracer, by incorporation in seaweed tank water, as deuterated water (^2H_2O). The tracer incorporates into lipid metabolism allowing the determination of the lipid flux through $^1H/^2H$ nuclear magnetic resonance (NMR). This technique has been successfully demonstrated in mice and fish to determine *de novo* lipogenesis (DNL), elongation and desaturation rates.

In the present study different protocols were tested for pretreatment of the samples (oven drying and lyophilization), lipid extraction (Folch, MTBE, Direct Transesterification) and lipid purification (solid phase extraction with silica columns and solid phase extraction with sodium carbonate). Regarding lipid pretreatment, oven drying and lyophilization were both suited for lipid analysis, not revealing differences between the two treatments. The extraction method that provided the best results for NMR analysis was Direct Transesterification (DT) of fatty acids, revealing a more uniform lipid resonance, with less interference and overlapping signals when compared to Folch and MTBE. Moreover, with DT protocol lipid purification was unnecessary.

Ulva lactuca discs growth could be determined either by area or weight. A correlation between the two parameters was established ($y = 114.52x^{1.0068}$). Nevertheless, weight measurement was preferred for practical reasons. Tree distinct trials were made, in which *Ulva lactuca* discs were exposed to ^2H_2O for 6 days, dried and lipids were extracted. The lipidic content and lipid metabolic fluxes were determined by $^1H/^2H$ NMR.

It was proved for the first time that seaweeds could incorporate ^{2}H in their metabolism and that lipid metabolic fluxes, such as n-3 DNL, could be studied through ^{1}H/^{2}H NMR. The highest values of n-3 FA were observed in seaweeds grown at a normal rate of 7.90 ± 1.26% day^{-1}, which presented a content of 63.68 ± 1.61% of PUFA, of which 30.45 ± 2.58% were n-3 FA, while MUFA and SFA were estimated to be 19.34 ± 4.00% and 16.98 ± 5.61%, respectively. These seaweeds synthetized n-3 FA *de novo* at a 2.82 ± 0.45% day^{-1} rate. Differences in lipid metabolism between seaweeds cultivated with nutrient supplemented medium (+N) and nutrient depleted medium (-N) were tested and PUFA content revealed to be significantly higher, by 6.1%, in +N conditions.

The effect of two different culture temperatures (18 ^{o}C and 23 ^{o}C) in *Ulva lactuca* lipid metabolism was tested and differences were observed between the two treatments. Comparing to higher temperature, discs cultured at lower temperatures revealed an increase in unsaturation, reflected by a decrease of 5.71% in SFA, 6.54% in MUFA and consequently non n-3 FA, which decreased 9.14%, followed by increase of 12.24% PUFA and consequently n-3 FA, that increased by 9.14%.

Overall, these results showed that lipid metabolism of *Ulva lactuca* can be further explored through ^{1}H/^{2}H NMR following the optimizations made to the analysis. As a partial alternative to fishmeal this seaweed seems to be a good source of n-3 FA (30.45 ± 2.58% of total FA) suited for fish feed supplementation, being synthetized *de novo* at a 2.82 ± 0.45% day^{-1} rate.

Keywords: Direct Transesterification, *de novo* lipogenesis, n-3 fatty acids, NMR, *Ulva lactuca*

Resumo

Ulva lactuca é uma macroalga verde (Chlorophyta) oportunista que apresenta elevadas taxas de crescimento podendo ser encontrada em ambientes marinhos e estuarinos. Esta é uma das algas mais estudadas e tem demonstrado aplicação em diversas utilizações comerciais. Uma das potenciais utilizações mais recentemente estudadas é a sua incorporação no sector das rações para aquacultura de peixes. As macroalgas podem ser utilizadas como fonte alternativa a vários ingredientes aplicados na formulação das rações cuja utilização se tem tornado cada vez mais, não sustentável, nomeadamente a farinha e óleo de peixe. Ao contrário dos peixes, as algas têm a capacidade de sintetizar ácidos gordos polinsaturados (PUFA), incluindo ácidos gordos ómega 3 (n-3), que são essenciais para os peixes e por isso necessitam de ser incluídos na sua dieta de modo a garantir um desenvolvimento saudável. Apesar de ter um baixo conteúdo total de lípidos, *Ulva lactuca* apresenta um perfil lipídico qualitativo interessante, apresentando altas concentrações de PUFA, particularmente n-3, comparando com o conteúdo apresentado em ácidos gordos saturados (SFA) e monoinsaturados (MUFA). O perfil nutricional desta alga tem sido extensamente estudado, contudo a informação sobre o seu metabolismo e síntese lipídica é escassa.

O deutério (^{2}H) é um isótopo estável de hidrogénio (^{1}H), que pode ser incorporado como um marcador na água do tanque das macroalgas, sob a forma de água deuterada ($^{2}H_2O$). Este marcador é incorporado no metabolismo lipídico permitindo deste modo o cálculo do fluxo lipídico através de ressonância magnética nuclear (RMN) $^{1}H/^{2}H$. Esta técnica foi utilizada com sucesso em ratos e peixes para determinar as taxas de lipogénese *de novo* (DNL), elongamento e desaturação dos ácidos gordos.

Nesta dissertação foram testados diferentes protocolos de preparação das amostras (desidratação em estufa e liofilização), extração lipídica (Folch, MTBE e Transesterificação Direta) e de purificação lipídica (extração em fase sólida com colunas de sílica e extração em fase sólida com carbonato de sódio). Tendo em conta a preparação da amostra, a desidratação em estufa e a liofilização revelaram ser igualmente adequadas para a análise lipídica, não apresentando diferenças entre os dois tratamentos. O método de extração que apresentou os melhores resultados para análise foi a Transesterificação Direta dos ácidos gordos que revelou um espectro com ressonâncias mais uniforme, com menos interferências e menos sobreposição de sinais, quando comparado com Folch e MTBE. Utilizando o protocolo de Transesterificação Direta a purificação lipídica foi desnecessária.

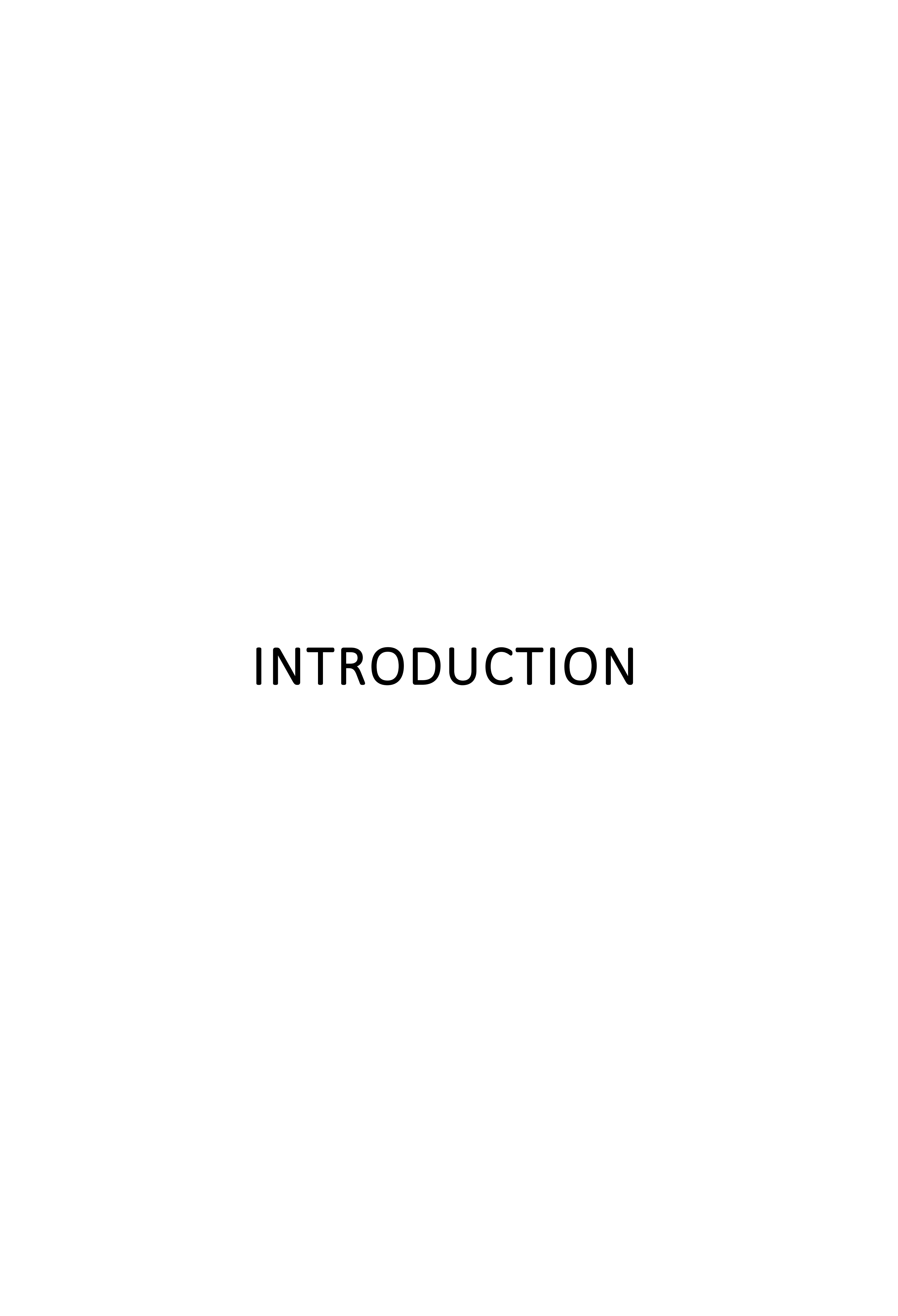
O crescimento dos discos de *Ulva lactuca* pode ser determinado através da medição da área ou do peso. Foi estabelecida uma correlação entre os dois parâmetros, explicada pela seguinte equação, $y = 114.52x^{1.0068}$. Contudo, a pesagem foi preferida por ser mais prática. Foram realizados três testes distintos, em que discos cortados de lâminas de *Ulva lactuca* foram expostos a ^2H_2O durante 6 dias, sendo posteriormente desidratados e a fração lipídica extraída. O conteúdo em espécies lipídicas e os fluxos metabólicos lipídicos foram determinados por $^1H/^2H$ NMR.

Foi provado, pela primeira vez, que as macroalgas conseguem incorporar ^2H no seu metabolismo lipídico e que os fluxos lipídicos, como a DNL de n-3, podem ser determinados por $^1H/^2H$ RMN. Os valores mais elevados de n-3 foram observados em macroalgas que apresentaram um crescimento de 7.90 ± 1.26% por dia, revelando um conteúdo de 63.68 ± 1.61% de PUFA, dos quais 30.45 ± 2.58% eram n-3, enquanto que os MUFA e SFA apresentaram conteúdos de 19.34 ± 4.00% e 16.98 ± 5.61%, respetivamente. Foram também testadas as diferenças no metabolismo lipídico em macroalgas, geradas pelo cultivo em meio enriquecido com nutrientes (+N) e em meio de cultivo sem nutrientes (-N) e o conteúdo de PUFA revelou ser significativamente maior (6.1%) em condições de cultivo +N.

Foi estudado o efeito do cultivo desta alga a diferentes temperaturas (18 ºC e 23 ºC) no metabolismo lipídico, sendo observadas diferenças entre macroalgas sujeitas a diferentes tratamentos. Comparando, com a diminuição da temperatura de cultivo foi observado um aumento de insaturação, refletido por uma redução de 5.71% em SFA, 6.54% em MUFA e consequentemente em ácidos gordos não n-3 de 9.14%, seguido por um aumento de 12.24% de PUFA e em particular n-3, que aumentaram 9.14%.

Em suma, estes resultados revelaram que o metabolismo lipídico de *Ulva lactuca* pode ser estudado por $^1H/^2H$ RMN seguindo as otimizações realizadas para análise. Considerando esta macroalga como alternativa parcial à farinha de peixe, esta aparenta ser uma boa fonte de n-3 (30.45 ± 2.58% de ácidos gordos totais) adequada para suplemento de rações, sendo sintetizado *de novo* a uma taxa de 2.82 ± 0.45% por dia.

Palavras-chave: ácidos gordos n-3, lipogénese de novo, RMN, Transesterificação Direta, *Ulva lactuca*



INTRODUCTION

Overview

Seaweeds are aquatic photosynthetic organisms that belong to the domain Eukaryota. Green and red seaweeds are part of the kingdom Plantae and brown seaweeds are from kingdom Chromista. Green seaweeds are similar to vascular plants and belong to phylum Chlorophyta, red seaweeds are included in phylum Rhodophyta and brown seaweeds in phylum Heterokontophyta/Ochrophyta [1]. The first recorded use of seaweeds for man's advantage dates from 3000 BC in China, where they were harvested for medicinal use, as described in the compilation of "Chinese herbs" [2]. Moreover, seaweeds have been part of Japanese diet since 300 BC. It is not clear when seaweed farming begun but it is believed that it started in Japan around 400 years ago, with *Porphyra* sp. (Rhodophyta) being the first farmed seaweed used for human consumption. Since then, seaweed farming developed and new species gained relevance in different industrial sectors from medicine, to cosmetics, food, feed and even biofuels, seaweeds have gained economical interest and developed into innovative applications [3].

Aquaculture is defined as farming of aquatic organisms including fish, mollusks, crustaceans and aquatic plants [4]. This is one of the fastest growing sectors of food production industry and plays a key role in providing food for the growing human population, expected to increase to 9.7 billion people by 2050 [5]. In the recent analysis of the world aquaculture production [6] the production of finfishes, crustaceans, mollusks, amphibians, freshwater turtles and other aquatic animals for human consumption was defined as "food fish" and was distinguished from aquatic algae, that are mostly represented by seaweeds. According to this report, from 2002 to 2012, aquaculture grew at an average annual rate of 5.6% and reached 90.4 million tones, representing 144.4 billion USD of market value, seaweed represented 24 million tonnes, with a market value estimated of 6.4 billion USD, and "food fish" 137.7 billion USD. Seaweed production has been exponentially growing during the last fifty years and more than doubled from 2000 to 2012 (Fig. 1), with China being the major producer, responsible for more than 50% of the world's seaweed production [6]. Nowadays there are six main cultivated seaweeds in the world, that provide for 98.9% of total production: *Kappaphycus alvarezii* and *Eucheuma denticulatum* exploited for carrageenan extraction, a phycocolloid largely used by the food industry as a gelling, stabilizer and viscosity building agents [7], *Laminaria japonica, Undaria pinnatifida,* and *Porphyra* spp. (Rhodophyta) for direct human consumption and *Gracilaria* spp. mainly for animal feed [6].

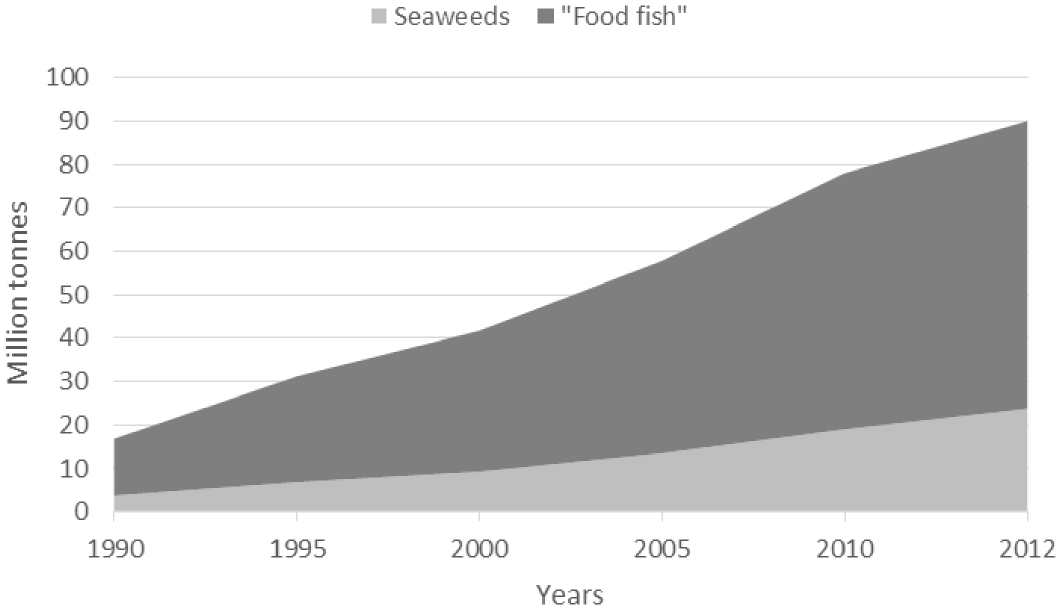


Fig. 1 - Overall aquaculture production from 1990 to 2012 of seaweeds and “food fish” in million tones [6].

Extreme variations in habitat conditions such as salinity, light, temperature and nutrients, have forced seaweeds to quickly adapt to environmental changes to insure survival, consequently producing unique metabolites that cannot be produced by other living organisms [8]. These metabolites include essential vitamins, minerals, pigments, polysaccharides, dietary fibers, polyphenols, amino acids and lipids that provide seaweeds antitumoral, antiviral, antibacterial, antioxidant and other biological proprieties, having economic value when exploited and applied to the different industry sectors [9], [10].

Ulva lactuca

Ulva lactuca L. is a green seaweed that belongs to phylum Chlorophyta (order Ulvales) [11], commonly known as sea lettuce since its soft and translucent green fronds resemble common lettuce leaves. This seaweed has a two cell layers, a small discoid holdfast and absent stipe. It is a fast growing opportunistic seaweed, capable of rapid colonization when conditions are favorable, it can detach from substrate, continuing to grow, and frequently forms blooms in eutrophic environments. *Ulva lactuca* can be found throughout the year, being more abundant in summer and fall. Normally grows in the intertidal and subtidal zone and is commonly found on estuaries [12] . This seaweed has potential for different commercial applications: it is the most studied species used as a biofilter in aquaculture systems due to its great capacity for nutrient intake having a good bioremediation potential [13–16]; it is one of the most ancient seaweeds used as crop fertilizer [17]; it has also shown results in cosmetics [18] and in the pharmaceutical industry, since its polysaccharides (ulvans) were proven to have dietary, antitumor, immune modulation and anticoagulant activities [19]. It also shows potential in biofuels sector, mainly in methane production [20] and promising results for human and animal nutrition [21–23].

This species has a haplodiplontic life cycle, consisting on the alternation of two isomorphic generations. The haploid gametophyte (n) produces gametes by mitosis, a female and a male gamete from distinct fronds fuse and create a zygote (2n) which can grow and form a diploid sporophyte (2n). The sporophyte produces zoospores by meiosis that are released to water and grow into the following generation (gametophyte) [24]. Some fronds may present a yellowish-brown region near the margins representing reproductive areas. This seaweed is rich in dietary fiber, representing approximately 60% of dry weight, of which nearly half is insoluble, and this characteristic is mainly attributed to polysaccharides. The ash content is estimated to be 11% being higher than in land plants. It reveals relatively high protein content (27%) similar to common protein sources such as legumes and grains, containing a high level of essential amino acids such as lysine, phenylalanine, methionine, leucine and valine. Being a source of low nutritional energy, seaweeds have generally low lipid content, and *Ulva lactuca* is not an exception with only 1% of lipids per dry weight [21]. Nevertheless, besides the low total content, it has a good qualitative profile, further explored below, with great potential. It is also important to refer that the nutritional value assessed may vary, considering that, according to environmental factors such as temperature, salinity, light, nutrients and mineral availability, this seaweed chemical composition has been found to change drastically [25]. As an example of the stated,

fronds collected from the same species in Tunisia revealed a total lipid content estimated in 7.87% per dry weight [26]. It must also be noted that chemical composition evaluated in different studies can differ significantly depending on the extraction methods used for this purpose [27–30]. Overall, *Ulva lactuca* is considered to have a great economic potential, particularly if taken into consideration its application to human and animal nutrition [21].

Seaweed as Fish Feed

Fish consumption is estimated to grow 27% by 2030 and aquaculture is projected to supply more than 60% of fish destined for direct human consumption [31]. Consequently, fish feed production is expected to increase. The provenance and sustainability of fish feed is of crucial importance since feed may be responsible for up to 50% of the operating costs in intensive aquaculture [32].

Farmed fish have specific nutritional requirements for normal growth and development. Considering that they do not have access to a diverse variety of organisms as wild fish do, essential ingredients have to be provided through feeds. The type and amounts of nutrient required for optimal performance are essentially known for the major cultured species and the most crucial feed ingredients that compose fish feed are fishmeal and fish oil. These ingredients are produced from captured fish (mainly anchoveta, *Engraulis* spp.) and supply essential nutrients, such as amino acids, fatty acids, minerals and trace elements for optimal growth, health, reproduction and physiological well-being of fish. Over the past years these ingredients' availability have been decreasing due to increasing capture and demand, but also as consequence of natural phenomena like the El Niño [33]. Altogether, the effects have impacted the species captured for its production, thus prices have been rising and estimates suggest an increase in price of 90% by 2030 [34, 31]. With an expected increase in fish demand for consumption, mainly produced trough aquaculture, simultaneous increase in fish feed demand and decline of main ingredients such as fishmeal, there is an obvious supply/demand gap for specific fish ingredients and urgent need in research for low-cost sustainable alternative feed ingredients in order to decrease dependence on fishmeal and fish oil [33].

Seaweeds are one of the alternatives as they reveal high levels of protein and fiber, have a well-balanced amino acid profile and rich content in minerals and vitamins, as other unique bioactive compounds. Seaweeds have also been studied as protein source in other animals such as chickens and pigs to reduce soybean meal, due to the current limited agriculture resources, arable land and

freshwater. They can provide complete or partial protein nutrition for abalone, sea urchins or shrimp [35] and compared to other studied plant alternatives, seaweeds do not require land space, fertilizers, pesticides, or freshwater, having good relative growth rates [36].

One of the first experiments made with seaweed supplementation in fish feed, from the 80's, suggested that growth and feed efficiency of Red seabream (*Chrysophrys major)* were improved when adding 5% of *Undaria pinnatifida* (Ochrophyta) [37]. Supplementation with 10% of *Ulva* meal activated fish lipid metabolism in Black seabream (*Acanthopagrus schlegelii*) triggering responses such as accumulation and mobilization [38]. Since then, several studies have evaluated the incorporation of different seaweeds in fish feed. Distinct seaweed genera found in the Portuguese coast like *Porphyra* (Rhodophyta), *Gracilaria* (Rhodophyta)*, Ulva* (Chlorophyta), *Sargassum* (Ochrophyta)*, Laminaria* (Ochrophyta) and *Fucus* (Ochrophyta) have been tested at different levels of inclusion in European seabass (*Dicentrarchus labrax*), Nile tilapia (*Oreochromis niloticus*), Rainbow trout (*Oncorhynchus mykiss*) and Gilthead seabream (*Sparus aurata*). European sea bass juveniles were fed with *Gracilaria bursa-pastoris* and *Ulva rigida* up to 10% inclusion and 5% of *Gracilaria cornea* with no negative consequences on growth performance, nutrient utilization or body composition [39]. *Gracilaria* and a mix of *Gracilaria, Fucus* and *Ulva* at 7.5% were found to modulate metabolic rate, innate immune, and antioxidant responses in European seabass [40]. Rainbow trout's apparent digestibility coefficient seemed to improve with *Gracilaria vermiculophylla* and tilapia with *Ulva* spp. and *Sargassum muticum* [41]*.* In Nile tilapia *Ulva* spp. meal was incorporated up to 10% without compromising growth, protein utilization, protein retention or flesh organoleptic proprieties, resulting in enhanced fish immune response [42–44]. Efficient iodine fortification of Gilthead seabream, with no negative effect on growth, was possible with inclusion of 10% of *Laminaria digitata* [45]. Other studies had similar results when supplementing fish feed with different seaweeds. Rainbow trout fed with *Porphyra dioica* meal presented dark orange pigmentation of flesh when compared to control group and it could be incorporated up to 10% without significant negative effects on weight gain and growth performance [46]. Nile tilapia fed with *Ulva* meal at 5% improved growth performance, feed efficiency, nutrient utilization, and body composition of fish [47]. *Ulva* meal could also be incorporated up to 4% in Gilthead seabream [48]. In Barramundi (*Lates calcarifer*) cultured on diets containing ≥20% of *Ulva ohnoi*, the organoleptic profile of fillets were improved when compared to fish on a standard commercial diet, developing a richer and complex seafood-like flavor [49].

Currently there is at least one seaweed meal brand in the market (OceanFeed™) made of a blend of different seaweeds sold for incorporation in salmon and trout species feed. This brand claims to improve health and help in the control of infections and chronic disease associated with farmed salmonids, reducing cost of feed and improving nutritional value of fish. A study with Atlantic salmon (*Salmo* s*alar*) fed with this product revealed that feeding with 12% of OceanFeed™ improved final concentration of valuable fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by 62% when compared with control groups with no seaweed meal [50]. This brand incorporates 40-75% of *Ulva lactuca* in its formulation [51]. These and other studies, that have shown positive results for seaweeds incorporation in feeds, lead to believe that this ingredient may possibly play a part as a sustainable source in the animal feed sector [52] that was estimated to reach close to 1 billion tonnes annually with a market value estimation of 400 billion UDS in 2015 [53].

Lipids and fatty acids in seaweeds

Fatty acids (FA) are carboxylic acids with hydrocarbon chains (aliphatic chain) ranging from 4 to 36 carbons long (C_4 to C_{36}). In one end of the molecule there is a methyl group ($-CH_3$, Fig.2), designated omega (ω), and in the other end a carboxyl group (-COOH). The carbon atom next to the –COOH group is the α carbon and the subsequent one is the β carbon. The position of the carbon atoms is usually indicated from the carboxyl group. The aliphatic chain can be saturated or unsaturated. If the FA is saturated (SFA) it only has single bonds between the carbons of the chain. Unsaturated FA (UFA) can have an aliphatic chain with only one double bond between two carbons, these are called monounsaturated FA (MUFA), or two or more double bonds, named polyunsaturated FA (PUFA). FA nomenclature indicates the number of the aliphatic chain carbons and the number and position of double bonds. Specifically, in PUFA nomenclature, the position of a double bond is usually indicated from the methyl end and the letter *n* is often used instead of ω. In PUFA the first double bond may be found between the 3rd and 4th carbon counting from the methyl end, these are called n-3 FA, commonly called omega 3 (*e.g.* α linoleic acids – 18:3 n-3). If the first double bond is located between carbon 6 and 7, they are called n-6 FA.

The aliphatic chain of FA can be separated in functional groups (Fig. 2). The carbons bound to hydrogens from the double bond represent an olefin group (-CH_2=CH_2-). The double bonds are separated from each other by a bisallylic group (CH=CH**-CH_2-**CH=CH). A carbon with a single bond with a carbon that is in turn linked with a double bond to another carbon is called an allylic group (**-CH_2-**CH=CH). When a carbon, bound to two hydrogens, is connected with two other atoms by single bonds is called a methylene group (**-CH_2-**).

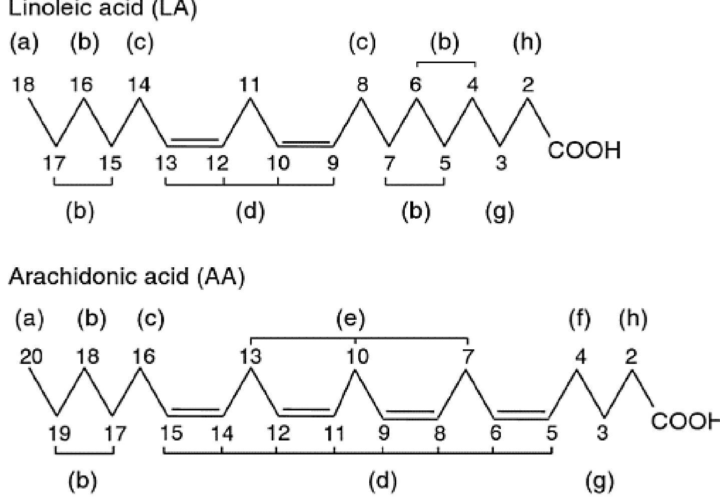


Fig. 2 - Linoleic acid (LA) and arachidonic acid (AA) chemical structure [128] with functional groups represented by letters: (a) methyl group; (b) aliphatic chain methylenes; (c) allylic group; (d) olefinic group; (e): bisallylic group; (h): α methylenes; (g): β methylenes.

FA can be esterified to different polar head groups (*e.g.* glycerol) or non-esterified, also called free fatty acids (FFA). Seaweed lipids consist of non-polar/neutral lipids, such as glycerides mainly in form of triglycerides (TG), and FFA, they are also rich in polar lipids such as phospholipids, glycolipids and betaine lipids [54, 55]. TG are composed of three FA linked to a glycerol backbone through three ester linkages (sn1, sn2, sn3), this is the most common neutral lipid accumulated in algae as a storage product and energy reservoir that provides metabolic energy to the cell when needed, it also acts as a reservoir for FA and donates acyl groups for polar lipid biosynthesis.

Polar lipids are important structural components of the cell membrane acting as a selective but permeable barrier, responsible for maintaining crucial membrane functions, but also can play as intermediates in cell signaling pathways, and respond to changes in environment. Phospholipids detected in *Ulva lactuca* are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA) [56], they are present in extra-chloroplast membranes with the exception of PG which occurs in significant amounts in thylakoid membranes. These lipids are characterized by having a phosphate group at the sn3 position which is linked to a specific head group, that could be enthanolamine (PE), glycerol (PG), serine (PS) or myo-inositol (PI), and two FA ester linked to the sn1 and sn2 positions. Glycolipids as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) are also present in *Ulva lactuca* [56]. MGDG and SQDG are strictly restricted to the thylakoid membranes of the chloroplast while DGDG is also found in extraplastidial membranes. They contain 1,2-diacylglycerol moiety with mono or oligosaccharide groups attached at sn3 position of the glycerol backbone. MGDG contains one galactose molecule while DGDG contains two, and SQDG contains a sulfonated glucose residue joined to diacylglycerol. The main betaine lipid in *Ulva* is 1,2-diacylglyceryl-3-O-4'-(N,N,N-trimethyl)-homoserine (DGTS) [56], this lipid is not present in higher plants and often substitutes phosphatidylcholine (PC) in green algae, it contains a betaine moiety linked to the sn3 position of glycerol backbone by an ether bond and two FA esterified at sn1 and sn2 positions. Normally storage lipids, such as TG are mainly composed by SFA content and glycolipids, phospholipids and betaines are richer in PUFA [57].

Marine macroalgae are considered to be a good source of these essential PUFA, and although the total lipid content is generally low, their PUFA percentage is equivalent or even higher than those of terrestrial vegetables [58]. According to Kumari et al. [58] Phaeophyceae and Rodophyta seaweeds species seem to be rich in arachidonic acid (20:4 n-6) and eicosapentaenoic acid (20:5 n-3), and Ulvales order rich in docosahexaenoic acid (22:6 n-3).

The genus *Ulva* generally has high PUFA concentrations (41.8%) when compared to SFA (35.3%) (Fig. 3). UFA account for more than half of total FA. The major SFA is palmitic acid (16:0). The most abundant MUFA are oleic (18:1) and palmitoleic acid (16:1). The dominant PUFA is α-linolenic acid (18:3 n-3), and eicosapentaenoic acid (20:5 n-3) is one of the main long-chain PUFA [59].

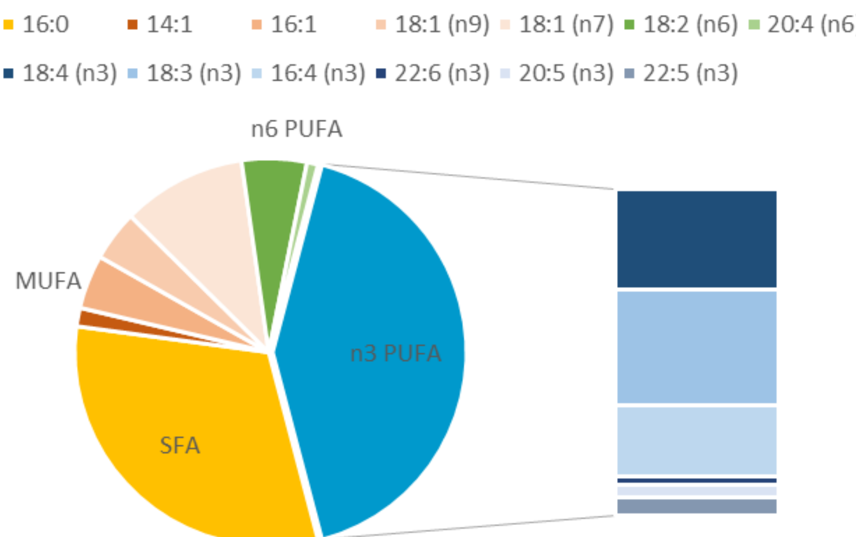


Fig. 3 - Representation of characteristic fatty acids and respective abundance from a review of 40 profiles analyzed (23 references) of the genus *Ulva,* as percentage of total FA [59]

Western diets have excessive amounts of n-6 FA and are deficient in n-3 FA having a ratio of ~16:1 when the normal ratio is supposed to be ~1:1. This excess in n-6 FA promotes the development of many diseases like cardiovascular diseases, cancer, and inflammatory and autoimmune diseases, while increased levels of n-3 PUFA suppress these effects [60]. These specific FA need to be incorporated into human diet since humans are unable to synthetize them, and fish consumption is one of the main sources of n-3 FA [61], which are also mainly acquired through their diet. Therefore, regarding seaweeds as potential ingredient in fish feed, its lipidic fraction contribution is one of the aspects that must be considered.

Lipid metabolism

Lipid metabolism and particularly FA and TG synthesis pathways are less understood in algae than in higher plants, where most of the knowledge comes from studies in plant models such as *Arabidopsis thaliana*. Nevertheless, land plants evolved from a green algae ancestor [62] and genomic and transcriptomic data from microalgae, identifying algal counterparts of plant genes involved in lipid metabolism, suggests that central metabolic pathways are conserved within photosynthetic organisms [63]. The haploid green algae *Chlamydomonas reinhardtii* is considered as a model organism to study algae lipid metabolism and functions in photosynthetic organisms [64].

In *C. reinhardtii* the biosynthesis of FA (Fig. 4) occurs in the chloroplast, as in higher plants. Lipid biosynthesis starts with carboxylation of acetyl-CoA catalyzed by a multi-enzyme complex named acetyl-CoA carboxylase, yielding malonyl-CoA at the expense of one molecule of ATP. Then malonyl-CoA is transferred to an acyl carrier protein (ACP), and from there it enters FA synthesis cycle. The carbon chain of FA is assembled in a repeating sequence of four reactions catalyzed by FA synthase. In the first reaction, an acetyl group from acetyl-CoA and two carbons from malonyl-CoA are condensed, with elimination of CO_2 from the malonyl group (condensation). In the second reaction, the carbonyl group is reduced to an alcohol, while NADPH is oxidized (reduction). In the third reaction, elimination of H_2O creates a double bond (dehydration). In fourth reaction, the double bond is reduced to form the saturated fatty acyl group while NADPH is oxidized (reduction). In the second cycle the butiryl group acts as the acetyl group from the first reaction, being condensed with a new malonyl-CoA, with loss of CO_2.

In each reaction cycle, the growing acyl chain is elongated by two carbon atoms, derived from acetyl-CoA via malonyl-CoA. When the chain length reaches 16 carbons (palmitate; 16:0) the product leaves the cycle still bound to ACP. The resulting FA can be used directly in the chloroplast, leading to the production of diacylglycerol (DAG) and phosphatidic acid (PA) that serve as precursors for structural lipids of the photosynthetic membrane, MGDG, DGDG, SQDG and PG respectively. Alternatively, FA can form PA that generates PG and PI, and is then dephosphorylated to DAG to produce other membrane lipids (DGTS and PE) and storage lipids (TG) (Fig. 4). Although it was previously believed that this process was exclusive of the endoplasmic reticulum (ER) [64] recent studies indicated synthesis of TG occurring in the chloroplast and storage in the chloroplast and cytosol [65, 66]. TG can be degraded by TG lipases releasing FFA and DAG. After cleaving of glycerol backbone by lipases FFA can be further metabolized via beta oxidation in the peroxisome. Membrane lipids could also be degraded to produce TG.

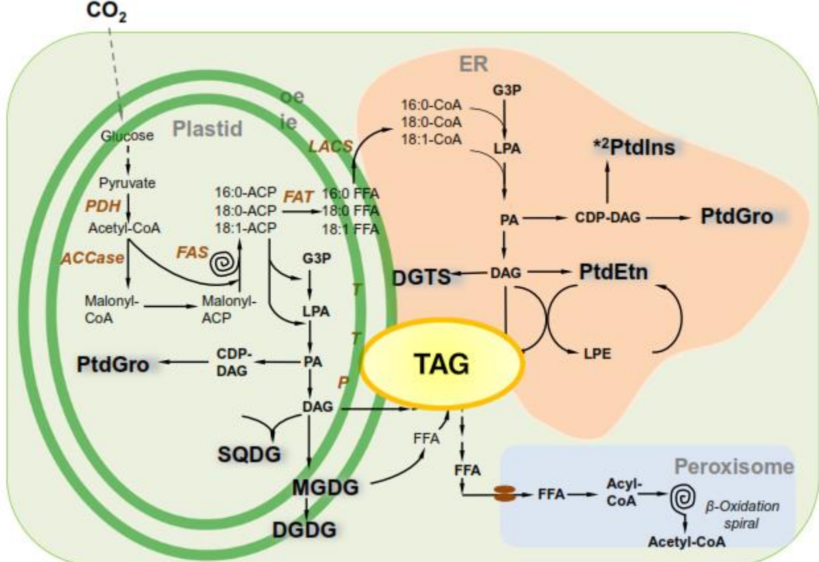


Fig. 4 – Representation of lipid metabolism of *Chlamydomonas reinhardtii* adapted from Li-Beisson et al. [66].

End products are in bold; Enzymes are in brown and italic. ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; CoA, coenzyme A; CDP, cytidine 50-diphosphate; DAG, diacylglycerol; ER, endoplasmic reticulum; FAT, fatty acyl-ACP thioesterase; FA, fatty acid synthase; FFA, free fatty acid; G3P, glycerol 3- phosphate; GPAT, glycerol 3-phosphate acyltransferase; PA, phosphatidic acid; TAG (TG), triacylglycerol (triglycerides).

During lipid synthesis, FA can be desaturated and elongated (Fig. 5). Desaturation is catalyzed by FA desaturases that convert a single bond between two carbon atoms (C–C) to a double bond (C=C) at specific positions of a FA chain. Mammals and also fish can synthetize long-chain FA through elongation and desaturation process. Having α-linolenic acid (18:3 n-3) fish can generate long chain FA such as eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3) through Δ5 and Δ6 desaturases and elongases, but they are unable to synthetize α-linolenic acid (18:3 n-3) (Fig. 5). Marine fish have revealed limited Δ5 desaturase and C18 to C20 elongase activity, and consequent limited conversion of C18 FA into longer UFA. Therefore FA such as EPA, DHA and α-linolenic are considered essential for these fish and need to be incorporated in their diet to meet dietary requirements for normal development [67] . On the other hand, algae can synthetize α-linolenic acid since they have Δ12 and Δ15 desaturases. They also express Δ4 desaturases that allow a more direct pathway for DHA synthesis comparing to vertebrates. The presence of Δ15 and Δ17 desaturases allow FA from the n-6 pathway to be converted in FA from the n-3 pathway.

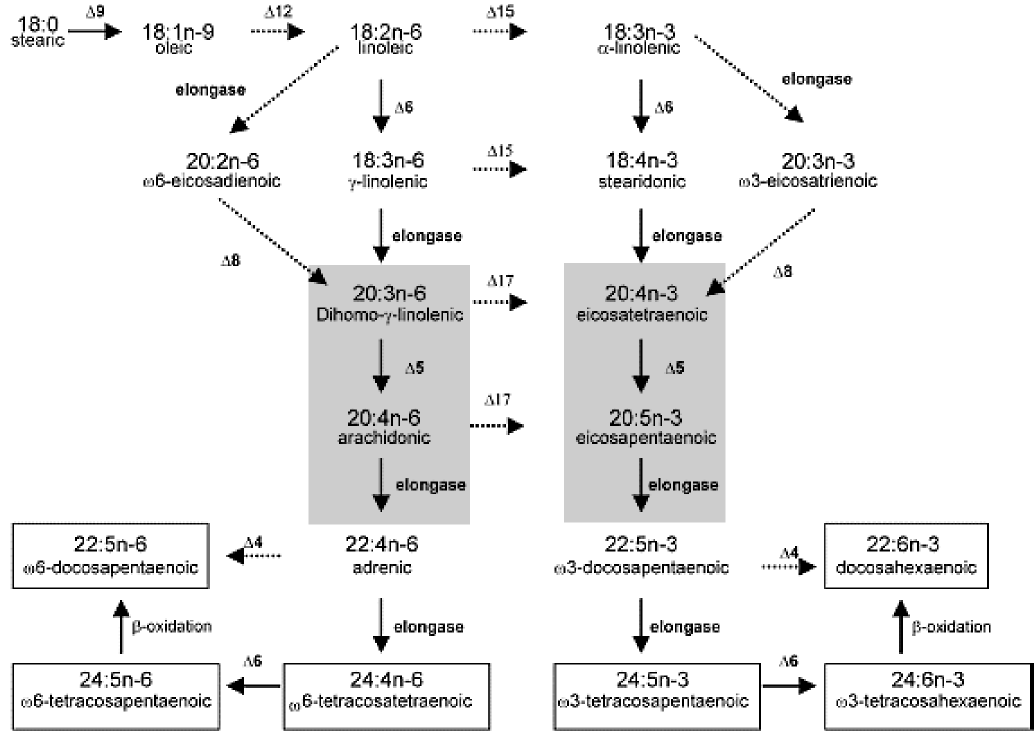


Fig. 5 - Biosynthesis of long chain PUFA schematisation. Arrows in solid line are found in mammals and fish, arrows with dotted line are exclusive of plants and algae. Plain boxes represent exclusive pathways in mammals and fish. Shaded boxes are limited pathways in marine fish. [129, 130]

Environment variations on algae lipid metabolism

The lipid metabolism of photosynthetic organisms is known to be modulated according to environmental changes such as light, temperature, nutrients or salinity. The most relevant and well documented changes in lipid metabolism are triggered by light intensity and temperature [55]. Generally, lipid metabolic changes alter the membrane bilayer physical proprieties in order to continue its normal functions. The most commonly observed change in membrane lipids following adverse environmental conditions in algae is alteration in FA unsaturation [68].

Temperature

When algae are exposed to lower temperatures FA unsaturation seems to increase as higher temperatures promote saturation, this pattern has also been specifically observed in macroalgae. *Egregia menziesii, Chondracanthus canaliculatus* and *Ulva lobata* have shown highest total lipid content in winter, and a specific increase in PUFA comparing to summer, when there was an increase in SFA. TG

was also higher in summer than winter and polar lipids have shown reverse scenario [69]. In *Saccharina japonica* (formerly *Laminaria japonica*), *Sargassum pallidum* and *Ulva lactuca* the same was observed in polar lipids and the increase in unsaturation was related to increase in n-3/n-6 PUFA ratio, suggesting substitution of n-6 by n-3 PUFA [70]. *Fucus serratus* [71] and *Grateloupia turuturu* [72] also revealed higher unsaturation in winter and higher saturation in summer. However, other authors did not find an universal pattern when FA profile of different macroalgae was compared between November and June [73]. *Ulva australis* (formerly *Ulva pertusa*) had higher PUFA when cultivated at 15 ^{o}C when compared to cultivation at 25 ^{o}C [74]. The change in unsaturation is believed to facilitate membrane fluidity, specifically in the thylakoid membrane to enhance electron transport between the two photosystems [54, 55, 70, 75].

Nutrients

In terms of nutrients, nitrogen and phosphate play a crucial role in algae metabolism, regulation and growth. Nitrogen limitation usually causes reduced cell division in algae, and is associated with activation of biosynthesis of TG, generally associated with increase in saturation and decrease in PUFA [68]. Most of the research on this matter has been done in microalgae with potential for the biodiesel industry which benefits from algae cultivation that provides a higher TG content [76, 77]. *Ulva rigida* FA synthesis has shown to continue during nitrogen deprivation but its composition changed, SFA and MUFA increased to a final percentage of 72.3% and PUFA decreased to 27.7%, suggesting a conversion of 24% PUFA into SFA+MUFA. In nitrogen supplemented medium the opposite was observed [78]. *Ulva lactuca* also showed an increase in PUFA in nitrate and phosphate supplemented medium, and SFA and MUFA decreased comparing to medium with no supplement, that showed an increase of SFA, mainly palmitic acid (16:0). Neutral lipids were higher when *Ulva lactuca* was nutrient-deprived and polar lipids increased when the seaweed was cultured with nutrients [56]. Different nitrogen sources (nitrate and ammonia) also reflected different lipid accumulation in *Ulva australis* (formerly *Ulva pertusa*) [74]. Photosynthesis is mainly controlled by light and provides energy for lipid synthesis. When cell division is limited, photosynthesis is not shut down completely and lipids continue to be synthetized, but once there is no need to generate membrane compounds a decrease in structural lipids is expected and newly synthetized FA are stored as TG until they are needed for energy or other FA formation.

Light

It is generally accepted that high light intensity leads to oxidative damage of PUFA [68]. In microalgae the degree of unsaturation of FA seems to decrease with increasing radiance, especially the percentages of total n-3 FA [79]. At low irradiance PUFA increase due to membrane lipids synthesis in order to maximize light capture, instead of storage lipids. The following has been observed in different seaweeds subjected to low and high light treatments with higher storage FA observed at high light intensities in *Ulva australis* (formerly *Ulva pertusa*) and *Sargassum* sp. [74, 80]. In *Ulva fenestrata* cultivated at 80% PAR (photosynthetically active radiation) TG were dominant with palmitic acid being the major FA and when the same seaweed was grown at 24% PAR polar lipids increased 2 - 3.5% when compared to 80% PAR, suggesting that when energy input from sunlight exceeds cell capacity for energy utilization FA are stored in TG [81]. Algae cultured in different photoperiod sets have also resulted in changes in FA profile. Under 24:0 LD (light/dark) the marine diatom *Thalassiosira pseudonana* revealed an overall increase in SFA and MUFA and a decrease in total PUFA when compared to 12:12 LD set at the same light intensity, and the same occur in high light intensity when compared to low light intensity, with the same photoperiod set [82].

Other conditions

Other parameters have revealed to modulate lipid metabolism, such as salinity and carbon availability. Salinity influences algae altering membrane permeability and fluidity. Polar lipids in particular play once again an important role in regulating these functions under different salinities. When algae suffer increase in salinity the normal response is the increase in unsaturation of lipids. The green microalga *Dunatiella salina* proliferates within a wide range of salinity, which makes this species a great model for lipid metabolic studies. It has been proved that this algae, when transferred from low NaCl concentration to seven times higher concentrations, induces the expression of β-ketoacyl-coenzyme A synthase (KCS) which catalyzes the first step in FA elongation, and also overall high unsaturation was observed, suggesting also desaturase activation [83]. This desaturase activation was also suggested for high salinity levels in the marine seaweed *Gracilaria corticata* [84]. It was demonstrated high PUFA production in two microalgae when cultivated at high levels of CO_2 (30 - 50%) [85].

Lipidomic analysis

Metabolomics is defined as the comprehensive and quantitative analysis of all, or a subset of metabolites in a biological system at a specific time point. This type of analysis is useful to understand how living organisms respond to the environmental dynamic conditions, since it can provide information of the physiological status of a biological system at a given moment helping to decipher metabolic responses involved in plant and environment interactions [86]. Lipidomic database information for marine macrophytes is still very undeveloped when compared to terrestrial plants [87]. Although there is considerable research in lipid and FA profile through classic techniques, information on metabolic pathway flux changes, such as *de novo* lipogenesis, elongation or desaturation of lipids is still scarce in algae. In microalgae, these type of studies have been increasing and gaining more relevance due to the potential for the biodiesel industry of selected oleaginous algae. In seaweeds no information was found regarding these specific metabolic pathways.

Conventional methods used to study lipid profiles generally involve lipid separation and identification through chromatography allied to mass spectrometric techniques, providing detailed information of lipid composition based on their mass to charge ratio. Some of the more frequent techniques used are LC-MS that combines liquid chromatography to mass spectrometry and GC-MS, gas chromatography coupled with mass spectrometry. These methods provide highly detailed data about identification and abundance of different lipid classes and compounds, and are normally preferred because of their high sensitivity, also allowing individual FA quantification. However these techniques are also time consuming, and require complex chemical manipulation [86]. In algae these are also the more frequent techniques used for FA profiling [88].

Nuclear Magnetic Resonance Spectroscopy (NMR) is a non-destructive and non-selective analytical technique that provides information about molecular structure of specific metabolites. In ^{1}H NMR samples are subjected to a magnetic field with a specific frequency (MHz) that excites the proton of the hydrogen nucleus, which then relaxes, traducing into a NMR signal, given by the nuclear spin of the proton. The molecular structure is translated in a spectrum of peaks with specific chemical shifts can be quantified against a standard compound. This technique can be applied to assess the molecular structure of FA, once its structure is mainly formed by hydrogen and carbon. Hydrogens have specific resonances according to their position on the FA molecule and chemical shifts of the different FA groups are well documented in literature allowing its identification. One of the main advantages of this technique is the relatively easy sample preparation and the fact that is non-destructive which allows

samples to be used for further analyses. ^{1}H NMR has been used in seaweeds mainly to reveal the structure of economically important polysaccharide such as fucoidan in brown algae [89, 90], carrageenan in red algae [91, 92] and ulvan in green algae of *Ulva* genus [19, 93, 94]. Regarding lipids the majority of research in ^{1}H NMR is made in microalgae for the biofuel industry [95, 96], but there are still very few studies in seaweed lipids with ^{1}H NMR. Specific lipid structure of different brown macroalgae were confirmed with this technique [97]. In other studies an attempt to determine lipid biotechnology potential of two seaweeds was made [98, 99]. FA, TG and sterols were identified through ^{1}H NMR in seaweed extracts studied for antioxidant purposes [100].

^{2}H as a tracer

A metabolic tracer is as substrate used to follow the biological transformation of an endogenous substrate (tracee). A tracer must have a distinct propriety to allow differential detection, and be chemically identical to the tracee. Isotopes can be used as tracers that provide information on the rate of conversion between molecules over time, as they produce a change in the metabolite molecular mass without disturbing metabolism. Labeling indicates the formation and turnover through the movement of the isotope through metabolites providing flux descriptions which are relevant to understand metabolic operation, regulation and control [101].

Deuterium (^{2}H) is a non-radioactive (stable) isotope of hydrogen (^{1}H) that has a nucleus composed of one proton and one neutron, while ^{1}H only has one proton. This isotope can be delivered as a tracer by the administration of deuterated water (^2H_2O), that is ~11% denser than regular water. The isotope incorporates in molecules such as FA, glucose or other hydrogen containing molecules that are being synthetized during exposure to ^2H_2O allowing to follow its synthesis. One of the advantages that labeled water has over labeled substrates is that rapidly equilibrates with total body water (BW) [102]. The presence of the ^{2}H tracer in molecules is possible to differentiate from ^{1}H tracee through NMR because it resonates at a different frequency. Its metabolism is not exactly equivalent to ^{1}H once the strength between C-^{2}H bond is stronger than a C-^{1}H bond. Therefore ^{2}H makes the bonds harder to break, and this can generate apparently slower rates of transformation of ^{2}H enriched metabolites compared to ^{1}H. Deuterium is relatively inexpensive tracer easily delivered into body water of organism, like seaweeds by immersion in ^2H_2O. This approach has been successfully used to study diverse aspects of carbohydrate metabolism in humans [102–104] and other mammals [105–107] and more recently fish [108–110].

^{1}H NMR and ^{2}H NMR together can provide information from metabolic fluxes occurring in organisms during $^{2}H_2O$ administration, once the chemical resolution of lipid functional groups provides the positional enrichment as already demonstrated in mice [107] and fish [110]. Lipid synthesis through *de novo* lipogenesis (DNL), or lipid modification through desaturation or elongation can be assessed using $^{1}H/^{2}H$ NMR [107] DNL can be followed by the enrichment of the methyl end protons that mark the beginning of the synthesis of a new FA chain as described earlier. This FA moiety remains unchanged during synthesis and these hydrogens have a specific resonance that allow their identification and quantification by NMR. In FA elongation hydrogen units are added to the carboxyl terminal region of the FA and during ^{2}H exposure this processes can be followed through the enrichment of the α protons. Desaturation is estimated by the enrichment of monounsaturated allylic hydrogens, since these are the hydrogens that surround the double bond inserted by Δ9 desaturase.

In ^{1}H NMR spectra, n-3 FA are easily identified by their methyl end protons that appear slightly upfield when compared to non n-3 FA protons. As stated before, mammals and fish are not able to synthetize *de novo* these FA, therefore studies regarding these organisms can only determine non n-3 FA synthesis. However, if grown in the presence of ^{2}H, spectra from seaweeds with ability to synthesize n-3 are expected to reveal the correspondent peak in a ^{2}H NMR spectrum.

Objectives

To have an accurate estimate of the lipid metabolism it is crucial to determine the best method of lipid extraction in seaweeds for quantification by NMR analysis. Since this type of analysis in seaweed lipids is still undeveloped one of the goals was to test a) different sample pretreatments b) distinct lipid extraction protocols c) lipid purification protocols for NMR spectra optimization. It was also evaluated the best method to determine seaweed growth.

The main goal of this dissertation was to determine the lipid flux of *Ulva lactuca* using $^{2}H_2O$ and how different environment changes (temperature and nutrients) affect lipid profile and lipid flux with particular focus on n-3 FA *de novo* lipogenesis, in order to provide a better understanding of this seaweed potential as a fish feed ingredient.

To the author's knowledge, it is the first time that rates of *de novo* lipogenesis, elongation and desaturation of FA are quantified in seaweeds, and particularly n-3 *de novo* lipogenesis.

Materials and Methods

Lipid extraction

To determine the best method for extraction of lipid content of *Ulva lactuca* for NMR analysis, three different extraction methods were tested: Methyl-*tert*-butyl ether (MTBE), Folch extraction, extraction and a Direct Transesterification method (DT). This was tested in order to obtain the highest lipid purity and signal-to-noise ratio with the less interference by contamination and overlapping signals possible. In samples with low lipid content, the results of lipid extraction using the more popular Blight and Dyer method do not differ from Folch extraction [111], therefore this method was not tested. The effects of two different drying methods, lyophilization and oven-drying, were also tested.

Folch extraction is a frequently used method in algae for separation of total lipids [112]. More recent MTBE extraction was tested since it was reported to perform a faster and cleaner lipid recovery, similar or better, when compared with more traditional methods, but providing a simpler lipid collection. MTBE has low density and lipid organic phase forms in the upper layer, whereas Folch chloroform is denser and organic lipid phase stays in down layer (Fig. 6) [113]. Two procedures for lipid purification after extraction were also tested: solid phase extraction (SPE) with a silica column [114] and SPE with sodium carbonate [115].

Transesterification is a process that involves the displacement of alcohol from an ester by another alcohol (catalyst) (Fig. 7). In TG and all other ester linked lipids (phospholipids and glycolipids) esterification results in the separation of the polar (water soluble) head groups from the non-polar FA chains that are converted to fatty acid methyl esters (FAME). DT is a method that allows lipid extraction and transesterification in one single step, avoiding lipid losses in small samples, showing better recoveries that two step procedures [116].

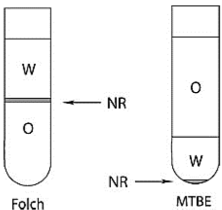


Fig. 6 - Phase distribution in Folch vs MTBE methods.

NR: insoluble (protein) residue; O: organic phase; W: water phase [113]

$$\begin{array}{l} CH_2-OCOR^1 \\ | \\ CH-OCOR^2 \\ | \\ CH_2-OCOR^3 \end{array} + 3CH_3OH \xrightleftharpoons{\text{Catalyst}} \begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array} + \begin{array}{l} R^1COOCH_3 \\ R^2COOCH_3 \\ R^3COOCH_3 \end{array}$$

Triglyceride Methanol Glycerol Methyl esters

Fig. 7 - General equation for transesterification of triglycerides [131]

Seaweed pretreatment

For the initial extraction testes, different seaweeds were collected during low tide: *Codium tomentosum* (Chlorophyta), *Grateloupia turuturu* (Rhodophyta), *Sacchoriza polyschides* (Ochrophyta) and *Porphyra* spp. (Rhodophyta), were collected in Buarcos Bay (40°17'N, 8°88'W) and *Ulva lactuca* (Chlorophyta) was collected in Mondego Estuary (40°08'N, 8°50'W). At the collection site they were briefly cleaned with seawater to remove major epiphytes and incrusting material. Prior to extraction seaweeds were brought to the lab and were carefully cleaned with running tap water and deionized water, blotted and weighed (FW). After, they were oven-dried at 60 ^{o}C for 48 h and equal amount of the same algae was lyophilized until constant weight. Dry weight was registered (DW) and samples were grinded to powder with a coffee grinder and stored in a sealed falcon until lipid extraction.

Lipid extraction

For MTBE extraction approximately 1 g of seaweed powder was incubated for 1 h at room temperature under agitation with MTBE and methanol. Samples were covered with parafilm to avoid evaporation of solvents. After, they were centrifuged for 10 min, at 6500g, and liquid fraction was separated from pellet. To induce phase separation distilled water was added, the mixture rested for 10 min and was then centrifuged at 1000g for 10 min. The upper phase that contained lipids and MTBE (Fig. 6) was collected. The ratio of solvents used was MTBE/methanol/water – 10:3:2.5 v/v/v and solvent/sample – 20 mL/g [113].

In Folch extraction seaweed powder (1 g) was incubated for 1 h at room temperature under agitation with chloroform and methanol. Samples were covered with parafilm, after centrifuged, liquid fraction separated, phase separation induced and centrifuged again as in MTBE. The lower phase contained lipids and was collected (Fig. 6). The ratio of solvents used was chloroform/methanol/water - 8:4:3 v/v/v and solvent/sample – 20 mL/g [112].

Direct Transesterification (Fig. 8) was carried with modifications to Lepage & Roy method [117]. Seaweed powder (0.1 g) was incubated for 1 h at 80 ^{o}C with acetyl chloride (acid catalyst) and methanol (1:19 v/v) and samples were vortexed each 20 min. Samples were covered with aluminum foil to avoid lipid oxidation and in the end vials were cooled in ice. Water and hexane were added to the mixture, samples were vortexed three times and left to rest for 10 min to allow phase separation. Vials were centrifuged at 2057g for 5 min and upper phase containing hexane and FAME was collected.

All samples were collected in amber vials to prevent lipid oxidation, dried in fumehood (hotte) under nitrogen stream and frozen at -20 ^{o}C until further NMR analysis.

Fig. 8 - Direct Transesterification process. A: lyophilization, B: grinding; C: samples for extraction; D: sample with methanol and acetyl chloride; E: heating (80 ^{o}C); F: complete transesterification with phase separation (upper phase contains n-hexane and FAME, lower phase contains other metabolites, water and methanol); G: FAME phase collection to amber vial; H: evaporation of n-hexane under nitrogen stream; I: sample vials storage.

Sample Purification

SPE with silica column: seaweed lipid extract

SPE with silica pre-packed columns (Sigma Discovery® DSC-Si SPE Tubes - 2 g) was carried out for separation of TG and FFA fractions as described by Hamilton et al. [114], with some adaptations.

The column was first washed with 8 mL of hexane/MTBE (96:4) and 24 mL of hexane, prior to sample loading, and the eluted solvent was discarded. Seaweed lipid extract was previously dissolved in 3 mL of hexane/MTBE (200:3) and applied to the column. The vial that contained the extract was washed once with 1 mL of the same previous solution, and applied to the column. For TG elution 24 mL of hexane/MTBE (96:4) was used and the fraction collected saved (FTG). The column was then acidified with 24 mL hexane/acetic acid (500:1) (Facid). FFA were eluted with 24 mL hexane/MTBE/acetic acid (500:10:1) (FFFA). Finally, the columns were washed with 12 mL hexane/MTBE (200:3) (Flav), to be reused. The four separate fractions (FTG, FFFA, Facid and Flav) were dried in fumehood (hotte) under nitrogen stream and frozen at -20 ºC, for further NMR quantification.

SPE with silica column: standard solution

Silica column purification efficiency was also tested with a standard lipid solution consisting of two known lipids: glyceryl trioleate (50 µL), a TG consisting of 3 oleic FA (18:1) esterified to a glycerol molecule and palmitic acid (16:0), a SFA, applying the same protocol described above.

SPE with sodium carbonate

Algae lipid extract was dissolved in 60 µL toluene, then added to sodium carbonate (60 mg) with 0.1M KOH (100 µL), as described by Paik et al. [115]. For TG extraction, 2 mL of dichloromethane/n-hexane (1:4) was added and after vigorous vortex and brief centrifugation (2 min, 6000g) the upper phase was collected to a separate vessel. The column was then acidified with 1M HCl (pH<2), and after FFA were extracted adding 2 mL of binary solvent, followed by vortex and centrifugation, the upper phase was collected. Both TG and FFA extractions were prepared in triplicate and extracts were combined, dried in fumehood (hotte) under nitrogen stream and frozen at -20 ºC, for further NMR quantification.

Lipogenic capacity and lipid synthesis

Three distinct cultivation trials (Fig. 10) were conducted from October of 2015 to June of 2016. The first trial started in October; this was the first experiment in which seaweed were cultivated in ^2H_2O and for the first time it was tested if they could incorporate ^2H in their lipid metabolism. A second trial was initiated in December and two different conditions were tested: one batch of seaweeds was exposed to ^2H_2O enriched with nutrient solution, and another batch was deprived of nutrients. The third and final trial was conducted in April and May and two different culture temperatures with 5 ^oC of difference (18 ^oC and 23 ^oC) were tested.

Prior to harvesting Provasoli enriched medium (PES) was made following [118] protocol. For cultivation trials *Ulva lactuca* fronds were collected in the Mondego Estuary (40°08'N, 8°50'W) during low tide. Seawater for cultivation was also collected, transported to the laboratory, filtered (0.45 µm) and stored at room temperature for further use. Seaweeds were brought to the laboratory and were carefully cleaned with filtered seawater to remove epiphytes and other residues from the thalli surface. Superficial water from seaweed fronds was soaked with paper (blotted) and total fresh weight was measured before acclimation.

Algae acclimation (Fig. 9) was done in a 40 L tanks with a stoking density of 2.5 g/L of seaweed. In the different trials this phase lasted from one to three weeks and during this period seawater enriched with PES to a final concentration of 20 mL/L [118] was renewed weekly. Fluorescent light and constant aeration were provided and tank water temperature, salinity, pH, and dissolved O_2 were measured regularly. These procedures were performed in accordance with Leston et al., Kalita et al. and Kumari et al. [14, 119, 120].

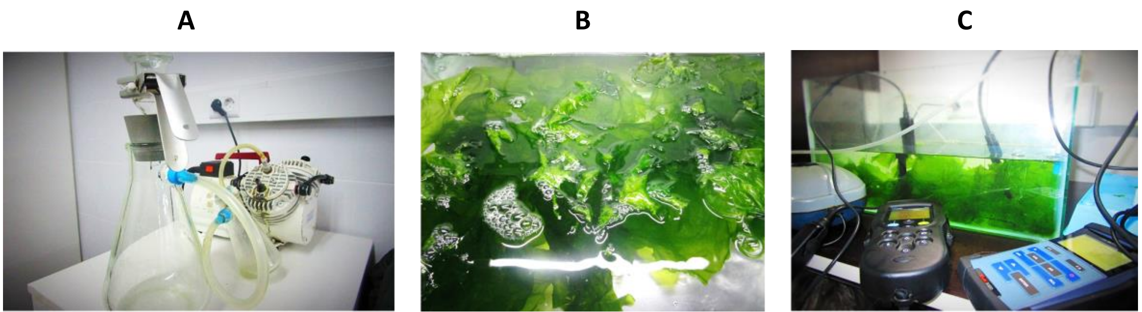


Fig. 9 - *Ulva lactuca* acclimation. A: water filtration, B: fronds in acclimation, C: pH, salt, T ^oC and O^2 measurements.

Collected filtered seawater (~4.5 L) was mixed with the ^2H (~0.5 L) to make five liters of ^2H_2O with 10% of ^2H-enrichment. The same water was used in all trials, and at the end of each trial it was filtered and stored at 4 °C for future use. Temperature, salinity, pH and dissolved oxygen were always measured before and in the end of ^2H exposure to make adjustments if needed. In the third trial additional 500 mL were made to adjust seawater salinity that was always kept at 30. Tank water (TW) was collected in each trial to verify seawater ^2H enrichment and the value obtained was used for lipogenic flux determination, instead of BW.

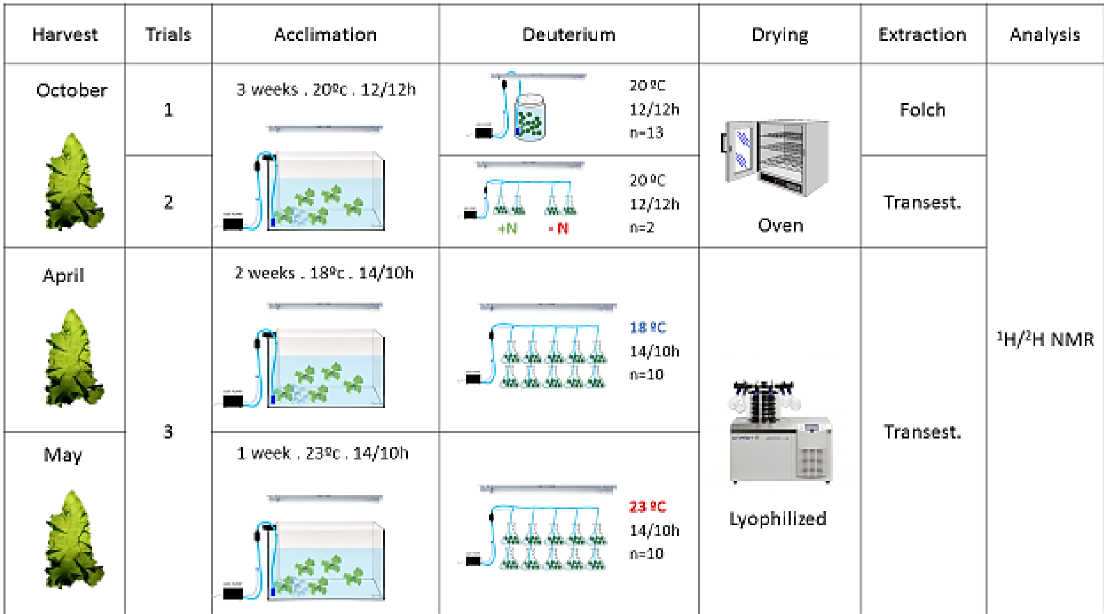


Fig. 10 - General *Ulva lactuca* “Lipogenic capacity and lipid synthesis” experimental designs

Trial 1 – Preliminary deuterated water test

Seaweed culture

In this trial algae were kept at a controlled temperature of 20 °C, salinity ~ 35, pH ranged from 7.88 to 8.71, and dissolved oxygen at ~100%, these parameters were measured regularly during acclimation that lasted 3 weeks. A yellow light was provided with a photoperiod set of 12:12 LD exposure. Algae discs of 3 cm in diameter were cut from fronds, blotted and weighed, previous to ^2H exposure. All discs were cultured ^2H_2O with PES (20 mL/L) for six days in one flask with 1.5 L capacity covered with parafilm to avoid water evaporation, with small perforations to ensure gas exchanges. Stocking density and all

the parameters were kept equal to acclimation, only salinity was lowered to 30 due to ^2H addition to seawater. Discs were also photographed in the beginning and end of ^2H exposure, and the area was determined using “ImageJ” software to determine the best way to evaluate algae growth. *Ulva lactuca* fronds that were not used in this experiment were kept in culture maintenance, under the same conditions as the experiment for future tests.

Lipid extraction

After 6 days, discs were removed from ^2H_2O, photographed, blotted, weighed, after they were paired in groups of three (each group representing one replicate) and oven-dried at 60 °C for 48h. Dried replicates were weighed and grinded to smaller particles. In this first trial lipid lipids were extracted by the Folch method as previously described.

Trial 2 – Nutrient variations

Seaweed culture

Ulva lactuca fronds from the culture maintenance were used for this trial. Prior to ^2H exposure fronds were deprived of nutrient for one week. As in trial 1, algae discs of 3 cm in diameter were cut from fronds, blotted and weighed previous to ^2H exposure. This time discs were paired in groups of six, and were cultured in individual Erlenmeyer flasks with 250 mL capacity in ^2H_2O for six days, sealed with parafilm to avoid water evaporation, with small perforations to ensure gas exchanges. Half of the replicates were given nutrients at normal concentration PES (20 mL/L) and the other half remained deprived of nutrient. All the parameters (temperature, light, salinity, pH, O_2) were maintained as in the first trial.

Lipid extraction

Procedures were made as in Trial 1, only this time lipids were extracted by DT method, as previously described.

Trial 3 – Temperature variations

Seaweed culture

This trial was separated in two phases, in April cultivation at 18 ºC was tested and in May and in June it was tested cultivation at 23 ºC. New *Ulva lactuca* fronds were harvested for each of the tests and prepared for acclimation as explained above.

Fronds collected in April were acclimated for 2 weeks at 18 ºC, salinity was kept at 30, pH ranged from 8.14 to 8.49, dissolved oxygen was kept at ~100%, and parameters were measured regularly during acclimation. Constant aeration and white fluorescent light were provided with a natural photoperiod set to 14L:10 LD. Fronds collected in May were acclimated for 1 week at 23 ºC, pH ranged from 8.17 to 8.49 and the remaining parameters were kept equal as the previous.

After acclimation discs with 4 cm in diameter were cut from fronds, blotted, weighed and grouped in six, cultured in separate Erlenmeyer flasks with 250 mL capacity in in ^2H_2O for six days, each flask representing one replicate, sealed with parafilm to avoid water evaporation, with small perforations to ensure gas exchanges (Fig. 11). Extra seaweed was cultured in the same conditions to extract body water (BW) to assess BW enrichment.

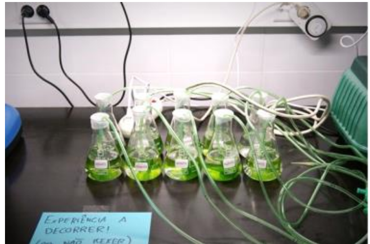


Fig. 11 – *Ulva lactuca* discs in ^2H_2O, with provided photoperiod (14L:10 LD), aeration, and nutrients (n=10).

Lipid extraction

After 6 days discs were removed from ^2H_2O, blotted, weighed and lyophilized to constant weight. Dried replicates were weighed and grinded to smaller particles. Lipids were extracted using DT protocol. All samples were labeled, saved in amber vials to prevent lipid oxidation, dried in fumehood (hotte) under nitrogen stream and frozen at -20 ºC until ^2H analysis.

Body water enrichment

Ulva lactuca fronds are very thin, having only two cell layers, which makes water collection from small portions of the fronds very difficult through maceration of fresh material. Therefore, body water extraction was tested using non-conventional methods. The first attempt was made by collecting the water that was deposited in the lyophilizer serpentine after seaweeds were dried. In the second test a small portion of the macroalgae was dried in a glass flask in the microwave for 1 min at 900 W and the water that condensed in the flask was collected (Fig. 12). The two methods were tested with seaweeds exposed to ^{2}H for 3 h and 6 h to understand how fast they would equilibrate body water. The microwave test was also carried using seaweeds from the last cultivation trial to confirm body water enrichment. The enrichment was analyzed in ^{2}H NMR in duplicates using 10 µL of sample for each that were added to acetone standard [102].

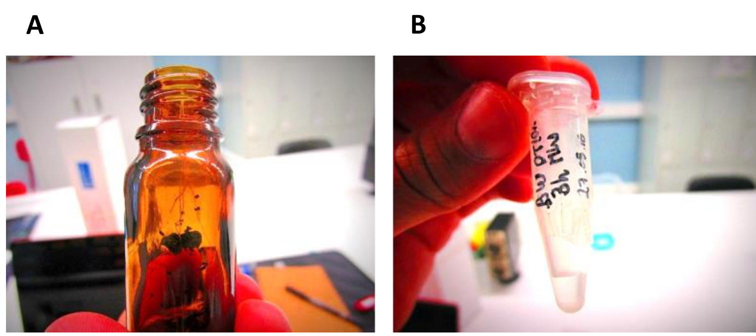


Fig. 12 – Body water (BW) collection from *Ulva lactuca* fronds by microwave drying.

^{1}H/^{2}H analysis

Lipids were reconstituted in chloroform and mixed with pyrazine standard and hexafluorobenzene. The samples were analyzed by ^{1}H and ^{2}H-NMR spectroscopy at 25 ^{o}C using a Bruker Avance III HD at 500 MHz equipped with a 5-mm broadband probe. ^{1}H NMR spectra were acquired with a 90° pulse, 3 s of acquisition time and 8 s of delay, making 11 s of recycling time and 16 scans. ^{2}H NMR spectra at 76.8 MHz were acquired with a 90° pulse, 0.67 s of acquisition time and 8 s of delay, making 8.67 s of recycling time and the number of scans ranged from 1577 to 4096 (T1), 3416 to 4922 (T2) and 2249 to 5144 (T3), corresponding to approximately 4.30 to 10 (T1), 8.30 to 12 (T2) and 5.40 to 12.30 (T3) hours of collection time.

Spectra were analyzed using the curve-fitting routine supplied with the ACD/Labs 1D NMR processor software. Chemical shift assignments were assessed according to literature [107, 121–124], and summarized in Table 1.

Table 1
^{1}H and ^{2}H chemical shifts assignments

Code Letter	Protons	^{1}H δ (ppm)	^{2}H δ (ppm)
A+B	Non n-3 methyls	0.88 (t)	0.89
C	n-3 methyls	0.98 (t)	0.99
D	Aliphatic chain methylenes	1.15 – 1.45	1.27
E	β methylenes	1.63 (m)	1.63
F	MUFA allylic	2.00 – 2.04	2.06/2.02
G	PUFA allylic	2.05 – 2.11	
H	α methylenes	2.30 (t)	2.31
I	DHA α and β methylenes	2.39 (t)	2.38
K	Bisallylic	2.76 – 2.86	2.80-2.86
M	Methoxy (FAME)	3.67 (s)	*
N	Olefinic	5.37 (m)	5.40
O	Chloroform	7.27	7.27
P	Pirazine standard	8.60	8.65

* Methoxy protons (M) derive from methanol used in the transesterification process which converts lipids to FAME, therefore they do not appear in ^{2}H NMR spectra. Chemical shift values can vary from spectrum up to ± 0.02ppm. (m): multiplet; (s): singlet; (t): triplet.

Formulas for lipid quantification and algae growth

Algae relative growth rate was determined by the following formula, which according to Yong et al. [125] introduced the least error in growth measurement in seaweeds. Where “W0” is the initial weight, “Wt” is the final weight and “t” are the days of cultivation.

$$RGR\ (\%\ day) = \left[\left(\frac{W\mathrm{t}}{W0}\right)^{\frac{1}{t}} - 1\right] \times 100\%$$

Lipidomic analysis

The formulas used for lipid quantifications were adapted from Duarte et al. [107].

n-3 FA

The terminal group of n-3 FA is slightly downfield comparing to non n-3 FA. The distinct resonance allows the determination of the % of n-3 FA:

$$\%\ \text{of n} - 3\ \text{FA}\ = 100 \times \frac{C_{1H}}{C_{1H} + AB_{1H}}\ (eq. 1)$$

Where C_{1H} is the ^{1}H area of n-3 FA and AB_{1H} is the ^{1}H area of non n-3 FA.

PUFA and MUFA

The % of mono and polyunsaturated FA is given by:

$$\%\ \text{PUFA} = 100 \times \frac{G_{1H}}{(2 \times H_{1H}) + I_{1H}}\ (\text{eq. 2})$$

Where G_{1H} is the ^{1}H areas of all polyunsaturated FA allylic protons, H_{1H} is the ^{1}H area of all FA α protons and I_{1H} is the ^{1}H area of DHA α and β protons.

The percentage of MUFA is given by:

$$\%\ \text{MUFA} = 100 \times \frac{F_{1H}}{(2 \times H_{1H}) + I_{1H}}\ (\text{eq. 3})$$

Where F_{1H} is the ^{1}H area of all monounsaturated FA allylic protons, H_{1H} is the ^{1}H area of all FA α protons and I_{1H} is the ^{1}H area of DHA α and β protons.

The % of unsaturated FA is therefore:

$$\%\ \text{unsaturated FA} = \%\ \text{PUFA} + \%\ \text{MUFA}\ (\text{eq. 4})$$

And the amount of saturated FA is then:

$$\%\ \text{saturated FA} = 100 - \ \%\ \text{unsaturated FA}\ (\text{eq. 5})$$

Docosahexaenoic acid

Linoleic acid (18:2 n-6) and DHA (22:6 n-3) have resolved ^{1}H resonances. DHA α and β protons overlap and appear slightly upfield of the other α protons. Therefore, the % of DHA is given by:

$$\%\ \mathrm{DHA} = \frac{\mathrm{I_{1H}}}{(2 \times \mathrm{H_{1H}}) + \mathrm{I_{1H}}}\ (\mathrm{eq.\,6})$$

Where I_{1H} is the ^{1}H area of DHA α and β protons and H_{1H} is the ^{1}H area of all FA α protons.

Calculation of average chain length and molecular weight

In order to calculate average FA chain length, all FA were considered polymers of a methylenic (CH_2) and/or a olefinic (HC=CH) subunit, i.e.: $^-OOC\text{-}(CH_2)_x\text{-}(HC{=}CH)_y\text{-}CH_3$. The average number of protons (ANP) per FA:

$$\mathrm{ANP} = \frac{\sum_{\mathrm{A}}^{\mathrm{K}}(\mathrm{X_{1H}})}{\frac{\mathrm{H_{1H}}}{2} + \frac{\mathrm{I_{1H}}}{4}}\ (\mathrm{eq.\,7})$$

Where $\sum_{\mathrm{A}}^{\mathrm{K}}(\mathrm{X_{1H}})$ is the sum of all corrected areas of peaks arising from the fatty acyl moieties, H_{1H} is the ^{1}H area of all FA α protons and I_{1H} is the ^{1}H area of DHA α and β protons.

Since all olefinic protons are represented in peak N, the number of olefinic protons (y) is:

$$\mathrm{y} = \frac{\mathrm{N_{1H}}}{\frac{\mathrm{H_{1H}}}{2} + \frac{\mathrm{I_{1H}}}{4}}\ (\mathrm{eq.\,8})$$

Where N_{1H} is the ^{1}H area of all olefinic protons, H_{1H} is the ^{1}H area of all FA α protons and I_{1H} is the ^{1}H area of DHA α and β protons.

If, the general structure of the FA is considered $^-OOC\text{-}(CH_2)_x\text{-}(HC{=}CH)_y\text{-}CH_3$, the following equation applies

$$\mathrm{ANP} = \mathrm{x} \times 2 + \mathrm{y} \times 2 + 3\ (\mathrm{eq.\,9})$$

which along with eq. 7 and 8, solve for x.

The x and y factors calculated can then be applied to the following equation to yield the average number of carbons (ANC) per FA.

$$\mathrm{ANC} = \mathrm{x} + \mathrm{y} \times 2 + 2 \ (\mathrm{eq.} 10)$$

ANC and ANP allow the determination of the average molecular weight (AMW) of the fatty acyl moieties:

$$\mathrm{AMW} = \mathrm{ANP} \times 1{,}00794 + \mathrm{ANC} \times 12{,}0107 + 2 \times 15{,}9994 \ (\mathrm{eq.} 11)$$

Determination of lipid methyl 2H enrichment:

For a certain peak X enrichment (X_e) is given by:

$$\mathrm{X_e} = 100 \times \frac{\mathrm{X_{2H}} \times \% \ 2\mathrm{HS} \times \mathrm{P_{1H}}}{(\mathrm{X_{2H}} \times \%2\mathrm{HS} \times \mathrm{P_{1H}}) + (\mathrm{X_{1H}} \times \%1\mathrm{HS} \times \mathrm{P_{2H}})} \ (\mathrm{eq.} 12)$$

Where X_{1H} is its ^1H area, X_{2H} is the its ^2H area, %1HS is the percentage of deuterium unlabeled standard (pyrazine), %2HS is the percentage of deuterium labeled standard (pyrazine), P_{1H} is the ^1H area of the pyrazine standard and P_{2H} is the ^2H area of the pyrazine standard. If divided by the percentage of body water enrichment in ^2H the fractional synthetic rate (FSR_X) is obtained during the exposure to ^2H_2O. FSR can be then divided by unit of time (day or hour).

De novo lipogenesis (DNL)

Fractional synthetic rate for non n-3 FA:

$$\%\mathrm{FSR_{AB}} = 100 \times \frac{\mathrm{AB_e}}{\% \ body \ water \ enrichment} \ (eq. 13)$$

Where AB_e is the enrichment for the terminal methyl hydrogens of non n-3 FA

Fractional synthetic rate for n-3 FA:

$$\%\mathrm{FSR_C} = 100 \times \frac{\mathrm{C_e}}{\% \ body \ water \ enrichment} \ (eq. 14)$$

Where C_e is the enrichment for the terminal methyl hydrogens of n-3 FA

meaning that $FSR_{AB} + FSR_C$ = overall *de novo* lipogenesis

FA desaturation:

Desaturase activity was determined in a similar FAhion as DNL:

$$\%\ \text{Desat. activity} = 100 \times \frac{F_e}{\%\ \text{body water enrichment}}\ (\text{eq. } 15)$$

Where F_e is the deuterium enrichment of the monounsaturated FA' allylic protons.

FA elongation:

The α protons are enriched during either DNL or chain elongation. The first pathway involves the complete synthesis of a new FA chain starting from acetyl-CoA while the second adds acetyl-coA to preexisting FA chains. Medium chain FA pre-existing the treatment with ^2H_2O will be unlabeled, but if these FA are elongated during tracer exposure, the subsequently added methylene hydrogens will be labeled. Comparison of the ^2H area at the terminal methyl end of the fatty acyl moieties with that of the α protons reports the fractional contribution of elongation (F_{Elong}) to total lipid synthesis:

$$F_{Elong} = 1 - \left[\frac{(AB_e + C_e) \times 2}{H_e \times 3}\right]\ (\text{eq. } 16)$$

Where AB_e is the enrichment for the terminal methyl hydrogens of non n-3 FA, C_e is the enrichment for the terminal methyl hydrogens of n-3 FA and H_e is the deuterium enrichment of the α protons of FA.

The total percentage of FA that underwent any degree of elongation can be calculated by the equation:

$$\%\text{Elong} = F_{Elong} \times \left(\frac{H_e}{\%\ body\ water\ enrichment}\right) \times 100\ (eq. 17)$$

Statistics

Differences between groups with different treatments were analyzed using unpaired t-test. Differences were considered statistically significant at $p<0.05$. Values are presented as mean ± standard deviation (SD). Statistical analysis was conducted with STATISTICA 7 software (StatSoft, OK, USA).

Results and Discussion

Lipid extraction

Seaweed pretreatment

Lyophilization and oven drying did not seem to induce differences in the basic molecular structure of *Ulva* lipids, as observed by McCauley et al. [59]. ^{1}H NMR spectrum of both oven-dried and lyophilized fronds (Fig. 13), subjected to the same extraction method (MTBE), presented similar peak resonances. Besides *Ulva lactuca*, in all the other seaweeds collected (*Codium tomentosum, Grateloupia turuturu, Sacchoriza polyschides* and *Porphyra* spp) subjected to the different pretreatments it was possible to identify representative peaks of all expected functional groups from non n-3 methyls (A+B) to bisallylic protons (K). Olefinic protons (N) also appear in both spectra in 5.37 ppm position, as well as chloroform (O) and pyrazine standard (P) in the expected positions (Table 1). Although the major groups can be identified by representative peaks, most of the regions do not appear clearly defined, especially the methyl end and PUFA and MUFA methylene regions.

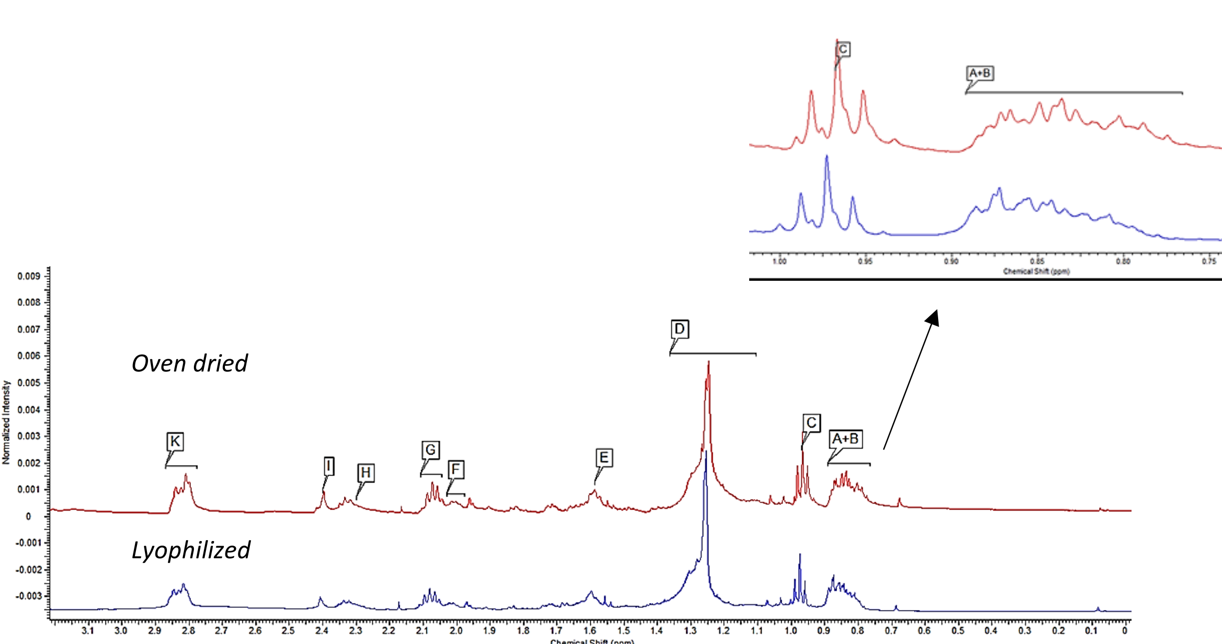


Fig. 13 - Representative ^{1}H NMR spectra of lipids (MTBE extracted) from *Ulva lactuca* fronds previously dried in oven at 60 ºC and lyophilized. The letters above represent peak assignments of protons according to Table 1.

Lipid extraction

Comparing the different methods used, DT delivered the best results. Overall, ^{1}H NMR spectra of DT, Folch and MTBE extraction (Fig. 14) revealed the main functional groups of lipids expected, from non n-3 methyls (A+B) to olefinic protons (N). Nevertheless, in the DT spectrum the functional groups appear more individualized, showing less interference and overlapping signals.

It is crucial that specific regions such as methyl ends and α methylenes, which are key regions for lipogenesis quantification, have the best definition possible in order to avoid misestimating lipogenesis. When spectra are amplified in these specific regions (Fig.15 and 16) it is possible to see clear differences between each extraction method. N-3 methyl end triplet (C) and non n-3 methyl protons (A+B) appear clearly defined in the DT spectrum when compared to Folch and MTBE spectra that reveal lipid overlapping (Fig. 15). The overlapping signals in this region suggest the presence of different classes of lipids. These distinct resonances may have been caused by the esterification of FA to larger molecules of the head groups that normally generate broader undefined signals. In Fig. 16 it is also possible to see the clear superior definition of α methylenes (H) and DHA α and β methylenes (I) when lipids are transesterified. In this process the head group molecules are isolated from the FA chains and the functional groups resonate equally since all lipid classes are converted to FAME.

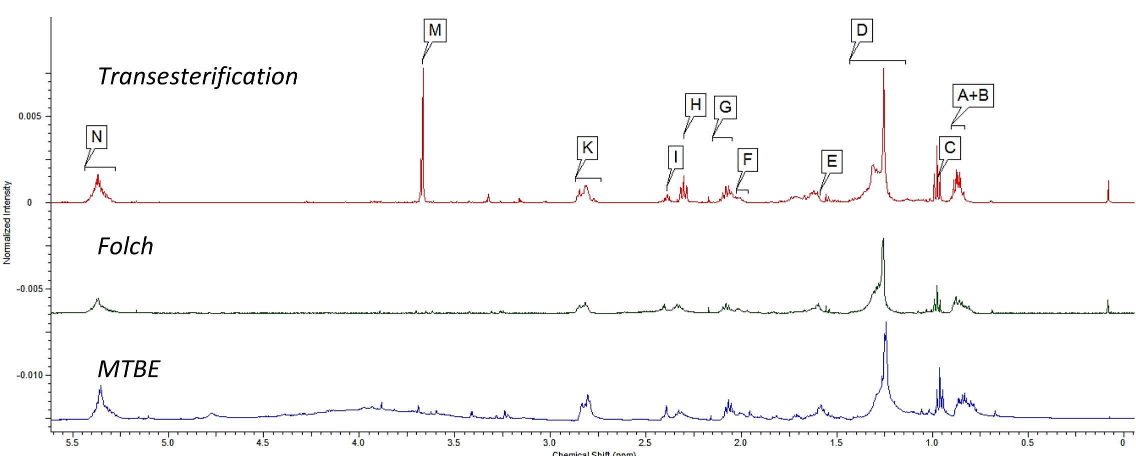


Fig. 14 - Representative ^{1}H NMR spectra of lipids from *Ulva lactuca* fronds submitted to Transesterification, Folch and MTBE extraction. The letters above represent peak assignments of protons according to Table 1.

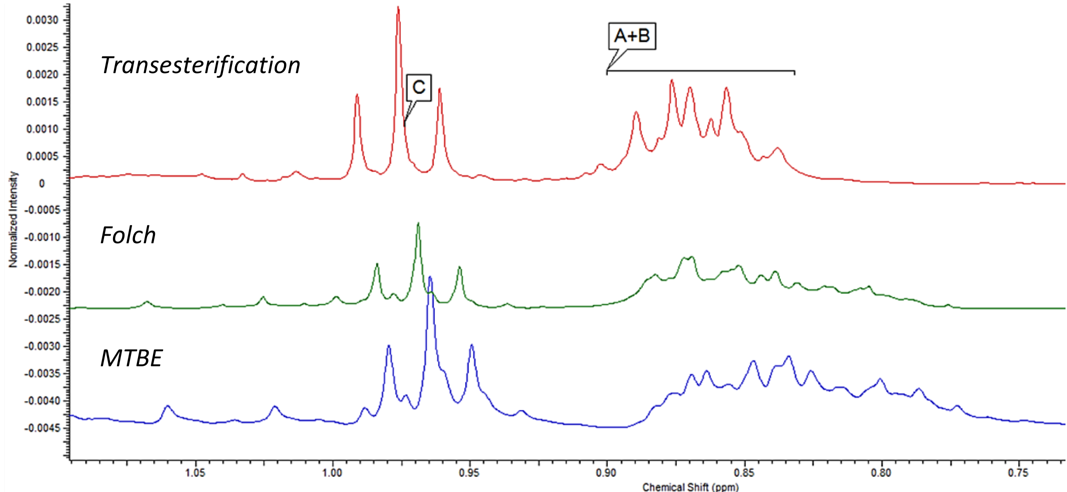


Fig. 15 - Amplified methyl end region (A+B and C) of representative ^{1}H NMR spectra of lipids from *Ulva lactuca* fronds submitted to Transesterification, Folch and MTBE extraction. The letters above represent peak assignments of protons according to Table 1.

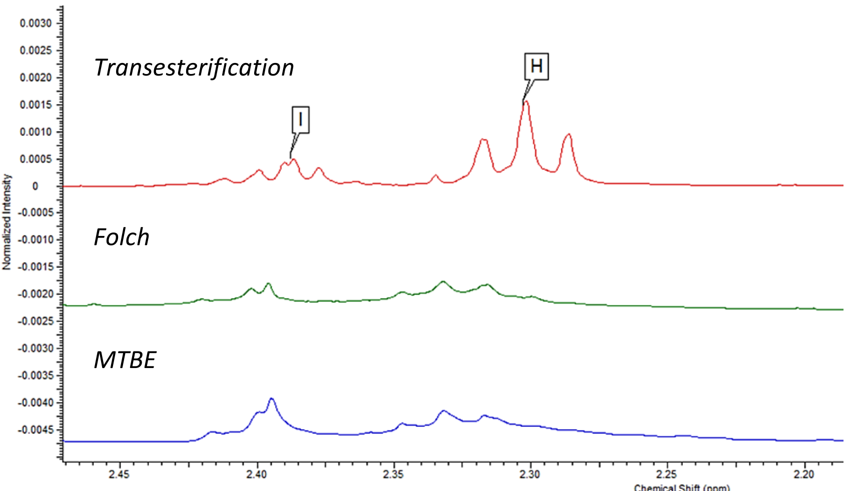


Fig. 16 - Amplified α methylenes (H) and DHA α and β methylenes (I) region of the representative ^{1}H NMR spectra of lipids from *Ulva lactuca* fronds submitted to Transesterification, Folch and MTBE extraction. The letters above represent peak assignments of protons according to Table 1.

The better definition of DT spectrum may also represent a higher yield of lipids being extracted, as reported by Kumari et al. [28] who verified a higher FA recovery by DT method when compared to conventional methods like Folch or Blight and Dyer in *Ulva fasciata*. Suganaya et al. [30] also optimized extraction of *Ulva lactuca* oil with n-hexane as the extraction solvent used in the transesterification process, which provided a better oil extraction yield when compared to other solvents such as MTBE, or chloroform used in Folch method. The rise in temperature increases the dissolution capacity of solvents generating a superior oil yield, which also confirms the better results of transesterification, performed at 80 ^{o}C while Foch and MTBE were carried out at room temperature, also verified in *Enteromorpha intestinalis* [29]. The use of an acid catalyst in transesterification (acetyl chloride) helps improving lipid extraction since they catalyze transesterification of all FA, whether free or linked to other molecules. This conversion is shown in the presented results by the presence of methoxy group peak at 3.67 ppm that is created from the resonance of the hydrogens derived from the alcohol (methanol) used in the process.

Sample Purification

SPE with silica column: seaweed lipid extract

SPE with silica columns was not effective in sample purification. The different SPE lipid fractions (FTG, Facid, FFFA and Flav) revealed basically the same resonances that are amplified in FTG (Fig. 17): 4.28 ppm; 1.74 ppm; 1.68 ppm; 1.47 ppm; 1.34 ppm; 1.29 ppm; 1.36 ppm; 0.96 ppm; 0.91 ppm. These peaks do not represent any FA functional group from *Ulva lactuca* lipids. The chemical shifts presented were not found on the common laboratory solvent impurities list [126] therefore these are believed to be a contamination from de silica columns or other material involved in the process.

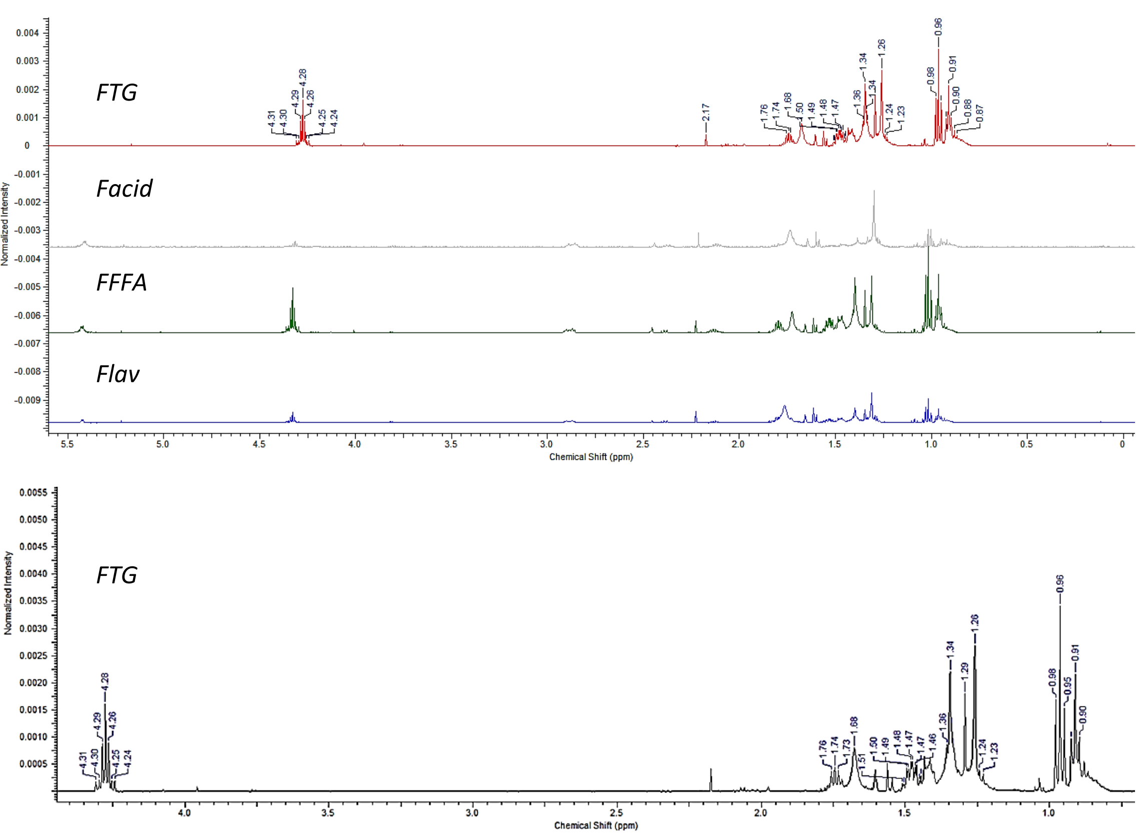


Fig. 17 - Representative ^{1}H NMR spectra of lipid fractions from *Ulva lactuca* fronds, Folch extracted, and submitted to SPE with silica pre-packed columns (on top) and amplified FTG fraction (on bottom). *FTG*: triglyceride fraction; *Facid*: acidification fraction; *FFFA*: Free fatty acid fraction; *Flav*: washing fraction.

SPE with silica column: standard solution

SPE with standard solution confirmed the inadequacy of the silica columns. ^{1}H NMR spectrum of purified lipids from the standard solution of glyceryl trioleate and palmitic acid did not reveal the expected peaks. FTG was supposed to reveal the characteristic glyceryl trioleate proton resonances, such as the glycerol backbone sn1 and sn3 position at 4.1-4.4 ppm witch were not observed in any fractions. Moreover the characteristic peaks of the double bond (1.97-2.05 ppm) of oleic acid (Fig. 18A), the FA that constitutes glyceryl trioleate, also did not appear in the FTG spectrum (Fig. 18B), instead this fraction mainly revealed the characteristic peaks of palmitic acid (Fig. 19A) and some interference signals possibly from the same contamination above described.

Oleic acid (18:1)

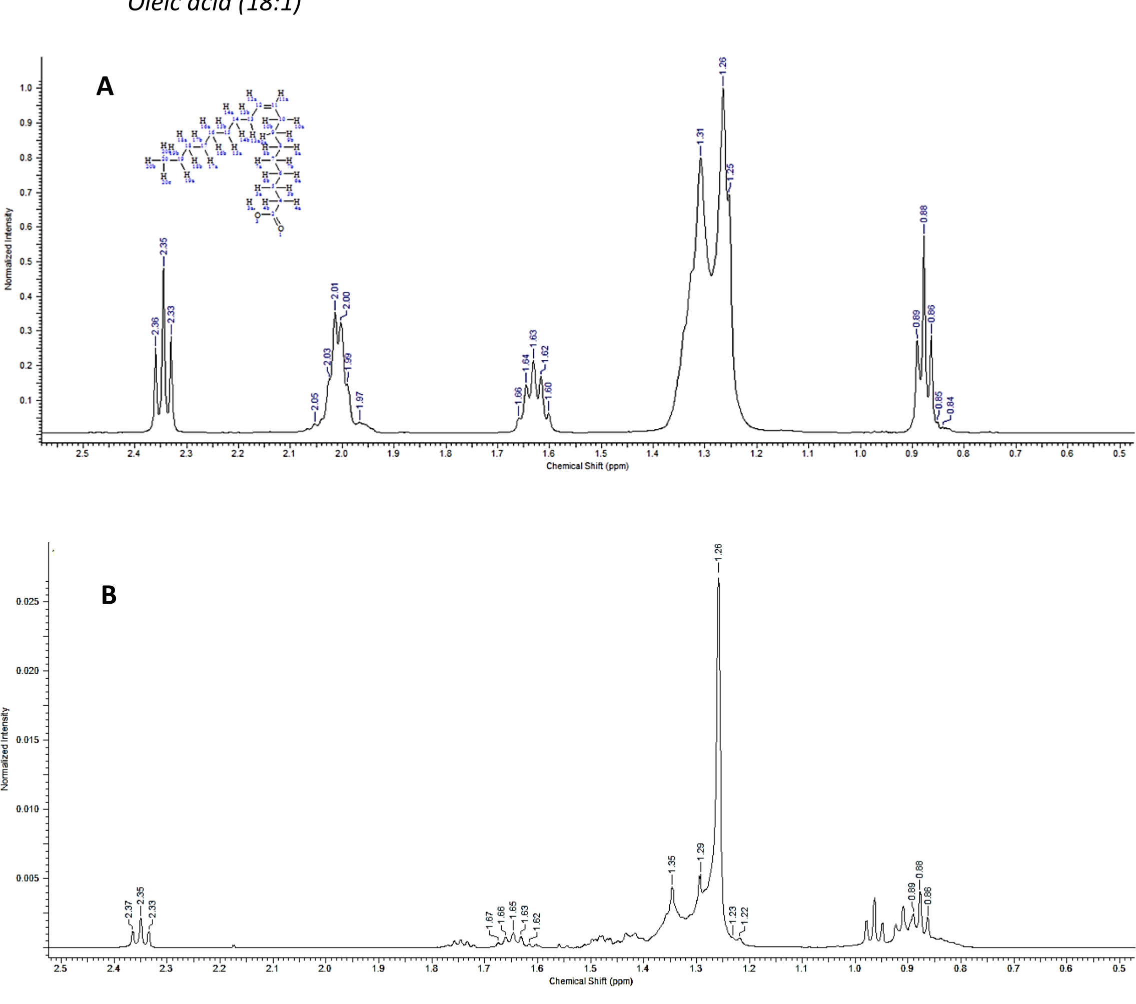


Fig. 18 - A: ^{1}H NMR amplified spectrum of pure oleic acid (18:1) (Wishart et al. 2013). B: ^{1}H NMR amplified spectrum of FTG of standard solution purified with silica pre-packed columns.

FFFA spectrum revealed the expected palmitic acid peaks (Fig. 19) and also the same interference peaks described. In sum these results indicate that the columns using Hamilton et al. [114] protocol were not purifying the lipid fraction as expected, once the standard TG did not elute and the SFA eluted in all fractions including Facid and Flav.

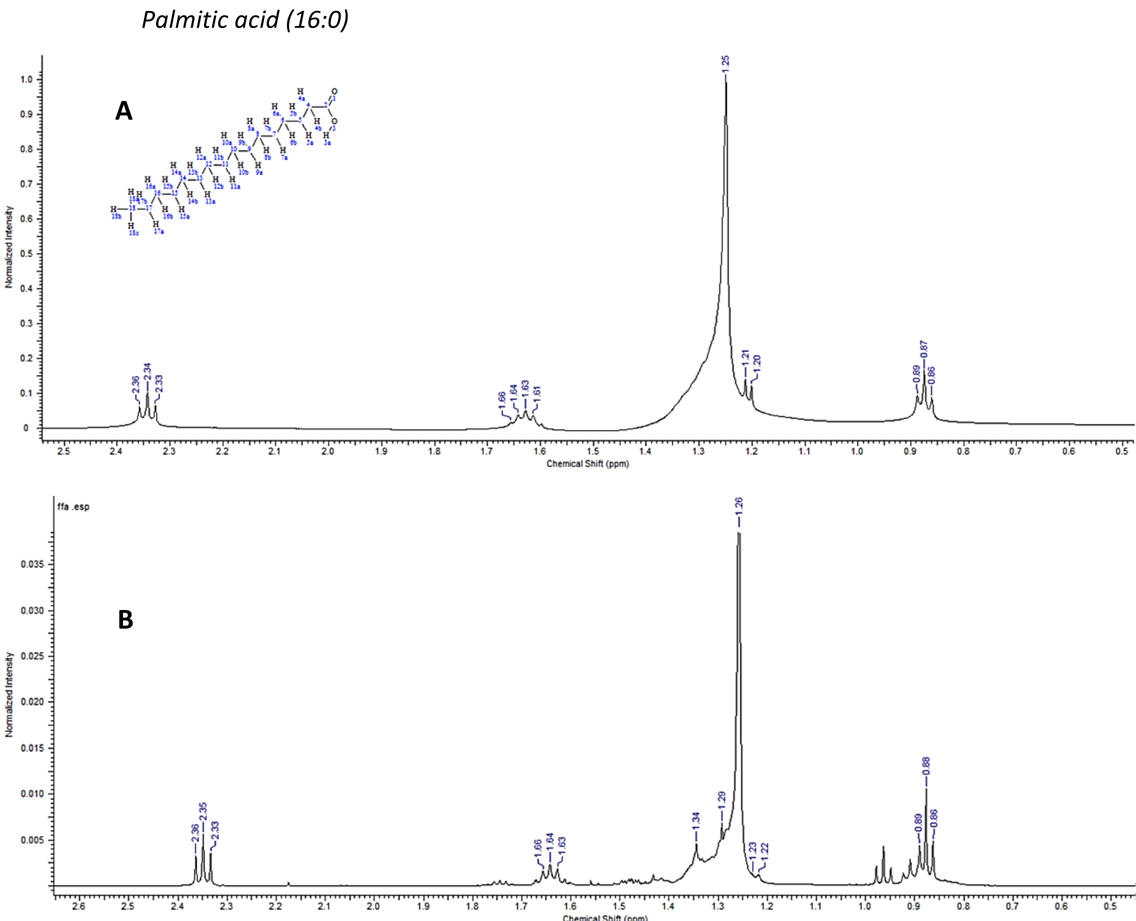


Fig. 19 - A: ^{1}H NMR amplified spectrum of pure palmitic acid (16:0) (Wishart et al. 2013b). B: ^{1}H NMR amplified spectrum of FFFA of standard solution purified with silica pre-packed columns.

SPE with sodium carbonate

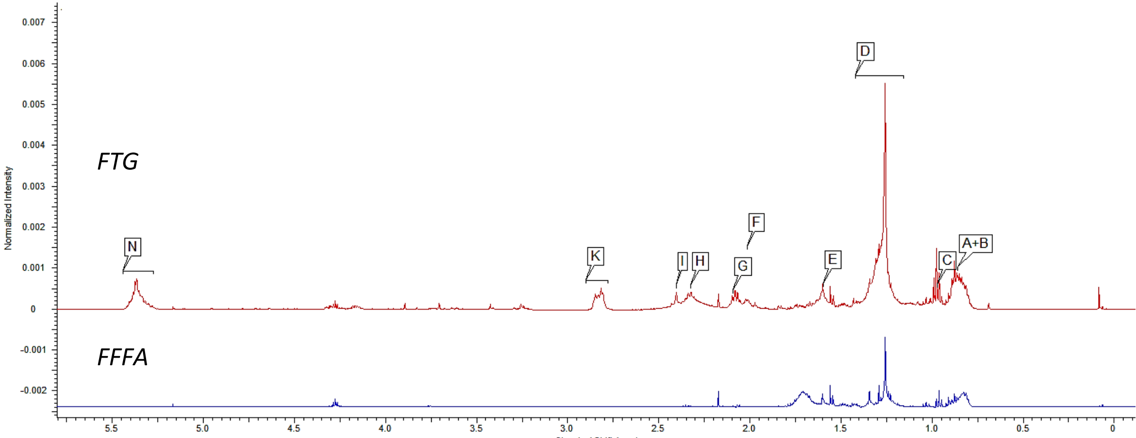


Fig. 20 - Representative ^{1}H NMR spectra of lipid fractions from *Ulva lactuca* fronds, Folch extracted, and submitted to SPE with sodium carbonate. *FTG*: triglyceride fraction; *FFFA*: Free fatty acid fraction. The letters above represent peak assignments of protons according to Table 1.

SPE with sodium carbonate did not separate TG from FFA. ^{1}H NMR spectrum of FTG shows all typical peaks of *Ulva lactuca* crude lipid extract as in the original Folch extraction, and FFFA only reveals the interference peaks that were present in the silica column spectra (Fig. 20). This leads to the conclusion that the origin of the contamination is not in the columns but in the material (glass vials) for collecting the different lipid fractions that were used in all the sample purification tests, including sodium carbonate SPE. Apart from the contamination, this purification method was not successful considering that no signals of purification of the sample were observed and FTG revealed resonances from FA but no peaks from the glycerol moiety, which are expected in this fraction.

Lipogenic capacity and lipid synthesis

Trial 1 – Preliminary deuterated water test

The first trial proved that seaweeds could incorporate ^2H in their lipid metabolism considering that for the first time a ^2H spectrum was generated from the lipidic fraction in the end of ^2H_2O exposure (Fig. 23B).

The mean disc weight (0.07 g) almost doubled during 6 days culture and in average *Ulva lactuca* presented a normal RGR of 11.5% day^{-1} regarding culture conditions [56, 127]. Growth was possible to observe by the visible increment in disc area (Fig. 21) and although it could be accessed by the measurement of the area, using "ImageJ" software (Fig. 22B), which presented a lower coefficient of variation when compared to weight (Fig. 22A) this method was more laborious and time consuming, therefore it was decided that RGR% day^{-1} should be evaluated through weight measurement instead of area. Additionally, a correlation curve between the two measurements was created ($y = 114.52x^{1.0068}$) (Fig. 22C). Discs revealed 75.6 ± 2.05% of water content and the mean weight of sample for analysis was 0.09 ± 0.01 g (Table 2).

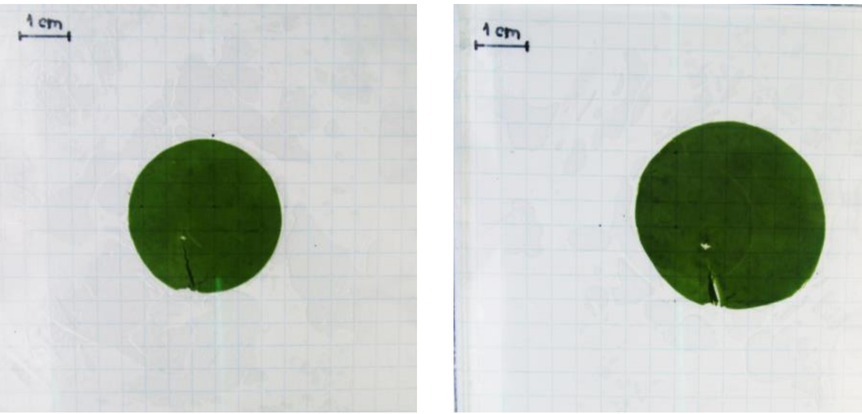
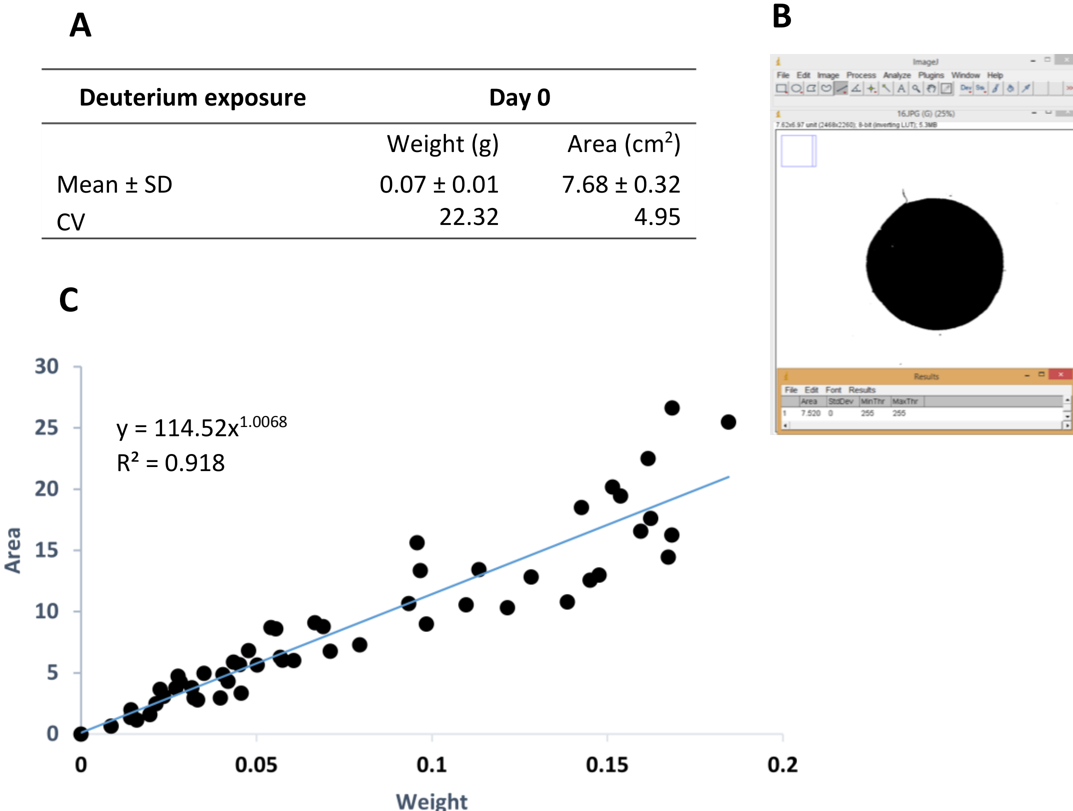


Fig. 21 - Representative *Ulva lactuca* discs before (left) and six days after (right) ^2H_2O exposure.

Table 2
Analyzed parameters in T1 (Mean ± SD), n=13

TW (% ^2H)	10	± 0.05
RGR (% day^{-1})	11.5	
Water content (%)	75.6	± 2.05
Sample dry weight (g)	0.09	± 0.01



A

Deuterium exposure	Day 0	
	Weight (g)	Area (cm^2)
Mean ± SD	0.07 ± 0.01	7.68 ± 0.32
CV	22.32	4.95

Fig. 22 - A: Mean ± SD of weight and area of discs before ^2H_2O exposure (n=45); B: *Ulva lactuca* discs processed with “ImageJ” software; C: Area vs Weight correlation curve (n=52).

The cultivated *Ulva lactuca* revealed a higher concentration of non n-3 FA (83.16 ± 3.46%) than n-3 FA (16.84 ± 3.46%) (Fig. 24A). This quantification is expected to have an error associated, due to the overlapping signals in the non-n3 methyl end region (A+B) that is used for n-3 and non n-3 FA quantification, possibly causing underestimation of n-3 FA in this trial. The extraction method performed in this trial was Folch and this problem has been described earlier when lipid extraction methods were tested. Nevertheless, lipid content can vary depending on environmental and culture conditions, and some studies revealed a content of n-3 FA in collected *Ulva lactuca* under 10% [26, 58].

Despite the fact that every FA functional group could be identified in the spectra (Fig. 23A,B) the overall ^1H spectrum (Fig. 23A) of the samples had many overlapping and interference signals, including in the regions used for PUFA and MUFA quantification which were discarded, and also made desaturation and elongation rates quantification impossible, since F and G region were unreadable in most samples.

Using an adaptation in the methodology applied in mice to determine lipid flux [107] it was possible to quantify n-3 DNL in seaweeds for the first time. *Ulva lactuca* synthetized *de novo* 5.09 ± 1.31% day^{-1}

of n-3 FA and non n-3 FA presented an inferior DNL estimated in 3.95 ± 0.81% day^{-1} (Fig. 24B) resulting in a total DNL of 9.04 ± 1.42% day^{-1}. In the ^2H spectrum (Fig. 23B) overlapping is less pronounced, but considering that the lipogenesis rates are calculated using both spectra information there is still likely a great margin of error also associated to lipogenesis quantification caused by the extraction method.

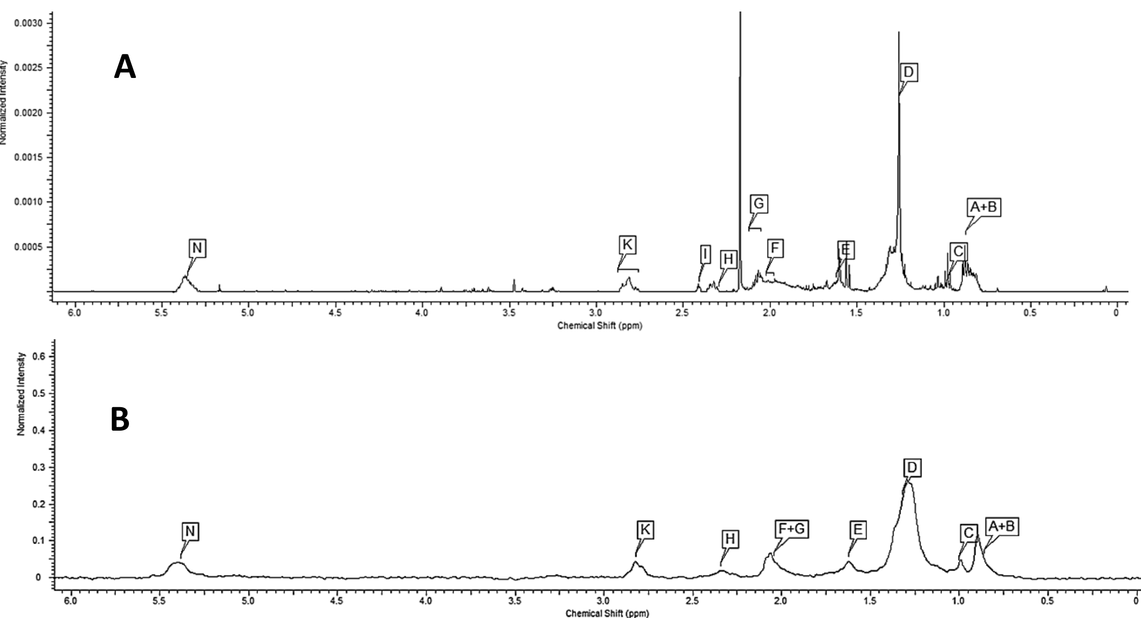


Fig. 23 - ^1H NMR (A) and ^2H NMR (B) representative spectrum of Folch extracted lipids from *Ulva lactuca* discs after 6 days of ^2H_2O exposure. The letters above represent peak assignments of functional groups in ^1H and ^2H according to Table 1.

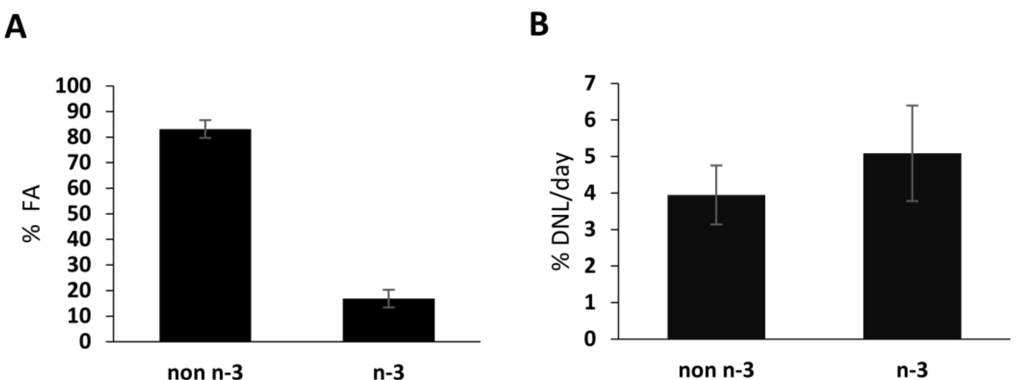


Fig. 24 - Lipidomic analysis of *Ulva lactuca* fronds. A: lipid species quantification determined by ^1H NMR. B: percent contribution of DNL day^{-1} during ^2H_2O exposure. Mean values ± S.D (n=13) are presented.

Trial 2 – Nutrient variation

Although not being statistically significant, RGR% day^{-1} of -N discs was relatively lower (6.13 ± 0.38) when compared to +N discs (7.94 ± 1.26). The fact that growth rates were generally lower when compared to Trial 1 is probably due to the prolonged time of culture maintenance of the fronds (5 months) that could have decreased growth. The water content of the seaweeds was within normal values and an average of 0.1 g of dry matter were analyzed per sample (Table 3).

Table 3
Analyzed parameters in T2 (Mean ± SD), n=2

	-N	**+N**
TW (% ^2H)	9.90	
RGR (% day^{-1})	6.13 ± 0.38	7.94 ± 1.26
Water content (%)	75.60 ± 2.73	78.91 ± 0.25
Sample dry weight (g)	0.1 ± 0.02	0.1 ± 0.04

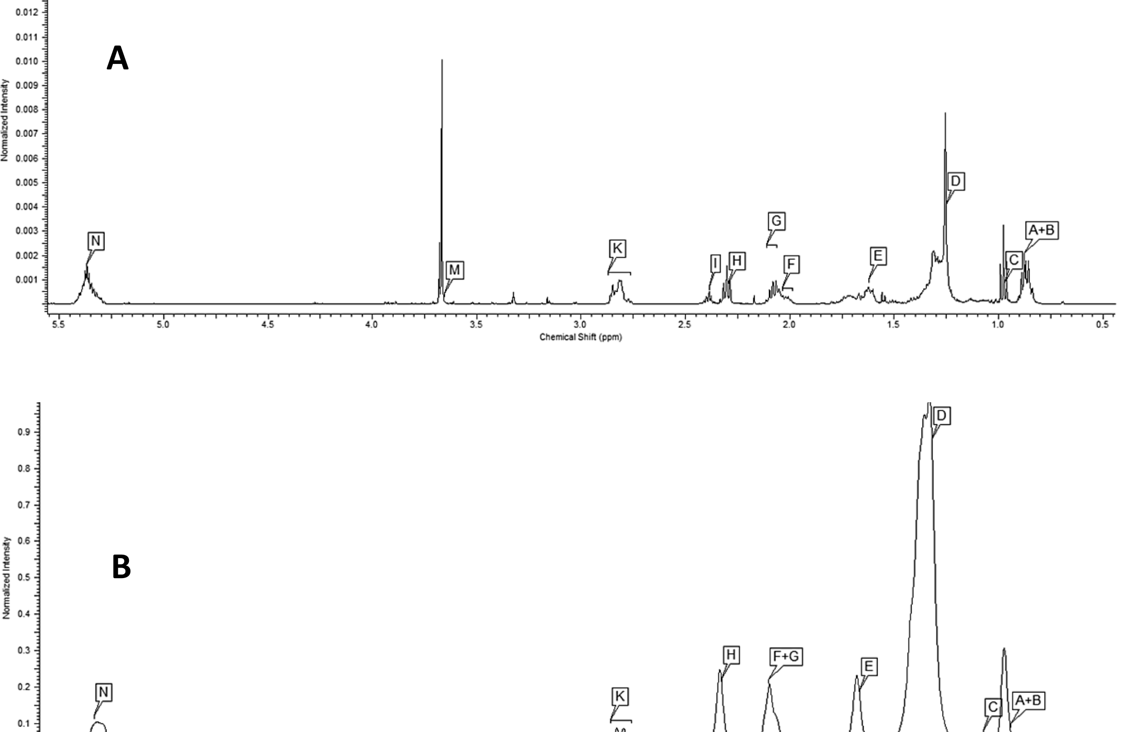


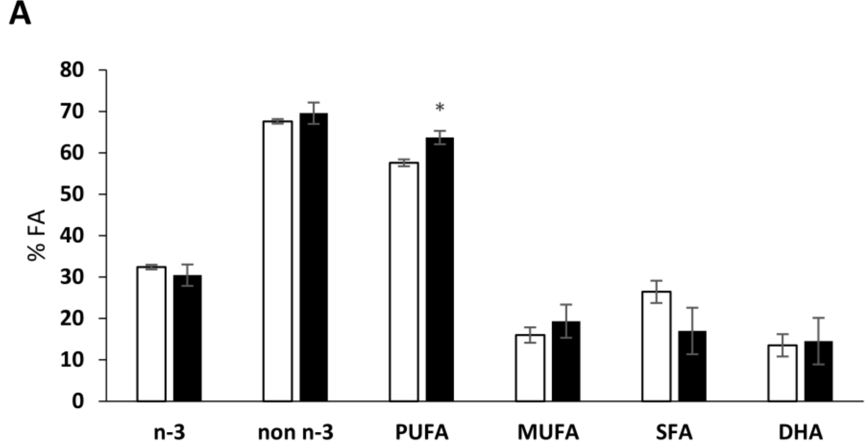
Fig. 25 - ^1H NMR (A) and ^2H NMR (B) representative spectra of transesterified lipids from *Ulva lactuca* discs after 6 days of ^2H_2O exposure. The letters above represent peak assignments of functional groups in ^1H and ^2H according to Table 1.

Both ^1H and ^2H spectra had good resolution, presenting improved lipid purity with less interference and overlapping signals due to transesterification of FA. Therefore all functional groups were easily identified (Fig. 25A,B) and could be more accurately quantified. Comparison between treatments (+N and -N) did not reveal significant statistical differences for most of the lipid species and lipid flux quantification (Fig. 26A,B). Nevertheless, in +N treatment it was possible to observe an increase in PUFA content (63.68 ± 1.61%) compared to -N (57.58 ± 0.84%) (Fig. 26A), being in accordance to other studies made in *Ulva lactuca* and *Ulva rigida* that revealed 17.3% [56] and 28.5% [78] increase in PUFA content in +N culture conditions. This could be explained by an enhancement in membrane lipid synthesis pathways in nutrient supplemented medium, since cell division and formation of new photosynthetic tissue is supposedly higher.

In -N treatment lipid synthesis is expected to continues but cellular division should be lower, therefore it is expected an enhancement of TG synthesis pathway rather than membrane lipid synthesis, which would be visible by an increment in SFA, which mainly constitute storage lipids, MUFA and non n-3 FA as observed in other studies [56, 76, 78], and probably non n-3 DNL, with decrease in n-3 content and also a lower n-3 DNL. Nevertheless, in these study this differences were not observed. The lack of statistical significant differences is believed to be caused by the low number of replicates per treatment (n=2) and not to the actual effects of the treatments, since PUFA content was shown significantly different, which can indicate that the different treatments generate distinct lipid metabolic responses.

In the present trial +N seaweed lipids (Fig. 26A), revealed a PUFA content of 63.68% ± 1.61%, which constituted the major portion of FA, as expected, being higher than SFA (16.98 ± 5.61%) and MUFA (19.34 ± 4.00%). From the PUFA fraction, n-3 FA content presented values closer to what was expected (30.45 ± 2.58%), however DHA (14.52 ± 0.31%) reported values significantly higher than those found in literature for *Ulva,* normally ranging from 0.1% to 2.2% [26, 56, 58, 59, 69]. Regarding that the extraction protocol used in this trial was Direct Transesterification, which promoted better lipid quantification, it was assumed that this trial presented more accurate lipid metabolism quantification in comparison to trial 1. In fact the percentages observed for SFA, MUFA, PUFA and n-3 FA were in accordance to literature, regarding culture conditions [56, 59].

Opposite to the first trial estimates of DNL of non-n3 FA (3.44 ± 0.40% day^{-1}) was higher than DNL of n-3 FA (2.82 ± 0.45% day^{-1}) (Fig. 26B), this could be expected since in the first trial the possible overestimation of non n-3 FA in the ^1H spectra may have caused an underestimation of the non n-3 FA that were being synthetized. Total % of DNL was estimated in 6.26 ± 0.85% day^{-1} being lower than the first trial, due to the lower growth rate observed. Interestingly it was found that the rate between total DNL day^{-1} and RGR% day^{-1} was the same for both trials (~0.78) indicating a possible correlation between both parameters. Desaturation was estimated in 17.62 ± 3.76% day^{-1} and elongation was 0.07 ± 0.1% day^{-1}. The desaturation flux presented the highest value, leading to believe that this was the main metabolic process occurring, but it is important to note that this high rate can have some associated



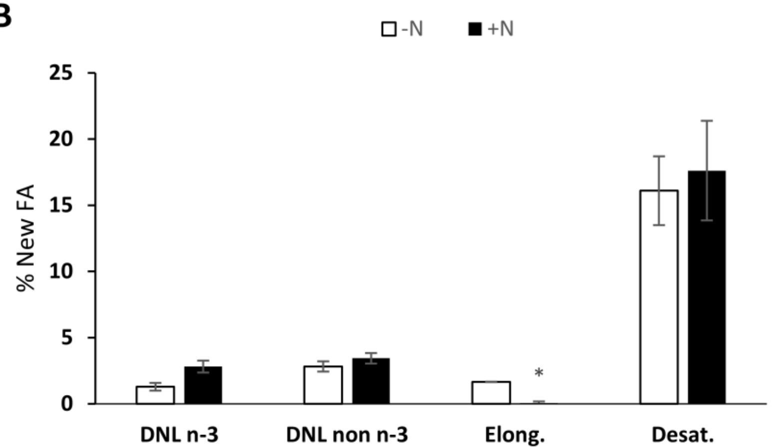


Fig. 26 - Lipidomic analysis of *Ulva lactuca* deprived of nutrients (-N) and supplemented with nutrients (+N). A: lipid species quantification determined by ^1H NMR. B: percent contribution of n-3 DNL, non n-3 DNL, desaturation, and elongation per day during ^2H_2O exposure. Mean values ± S.D (n=2) are presented. Significant differences between culture conditions (+N, -N) are indicated by asterisks (t-test, *p<0.05).

error, that is causing an overestimation in quantification, once MUFA and PUFA. ^{2}H signals overlap in the ^{2}H spectrum and are being quantified together for the desaturation rate, instead of just MUFA (eq.17 – materials and methods). The elongation rate could be considered null, since the standard deviation revealed to be higher than mean.

Trial 3 – Temperature variation

In this final trial *Ulva lactuca* did not grow as expected. At 18 °C it was observed a minimal growth of 0.90 ± 0.48% day^{-1} and at 23 °C no growth was observed. This could be due to the shortening of the acclimation period, since fronds were growing at lower rates when passed from acclimation phase to experimental phase, also when fronds were taken at the end of $^{2}H_2O$ exposure, some discoloration was observed suggesting that the seaweeds were under stress, caused by an unknown factor. Water content fitted the expected range in both temperatures and an average of 0.08 ± 0.01 g for T18 °C and 0.09 ± 0.01 g for T23 °C of dry matter per sample was analyzed (Table 4).

Despite the low growth rates obtained, NMR analysis was able to detect significant lipid metabolism changes in *Ulva lactuca* regarding different temperature conditions (Fig.28 A,B) with all peak signals of interest being clearly identified and quantified (Fig. 27A,B). MUFA (26.12 ± 2.21%) and SFA (35.87 ± 2.51%) revealed a decrease of 6.54% and 5.71%, respectively, when fronds were cultivated at lower temperatures, as expected non-n3 FA (83.05 ± 1.90%) content also decreased, by 9.14%. PUFA content (38.01 ± 3.24%) suffered a significant increase of 12.2% at 18 °C that was also observed specifically in the increase of n-3 content (16.95 ± 1.90%), by approximately 9.14% (Fig. 28A). Values presented in parenthesis refer to values assessed at 23 °C. The effect of temperature was also observed in *Ulva lobata* [69] that reveled decrease of 7.1% of SFA and increase of 8.5% of PUFA in fronds collected in spring when compared to fronds collected in summer in southern California. Higher unsaturation was also observed in *Ulva lactuca* (formerly *U. fenestrata*) and *Ulva australis* (formerly *Ulva pertusa*) collected and cultivated at lower temperatures [70, 74]. Together the increase in PUFA, n-3, and n-3 DNL suggest a higher membrane lipid synthesis (which are mainly composed by PUFA) at lower temperatures that could be a way to increase membrane fluidity as a response to decreased temperatures. But since seaweeds seem to be under some kind of stress and normal growth rates were not observed, the results were not conclusive.

Table 4
Analyzed parameters in T3 (Mean ± SD)

	18 ºC	23 ºC
TW (% ^{2}H)	9.45	8.15 ± 0.11
RGR (% day^{-1})	0.90 ± 0.48	-
water content	79.57 ± 1.69	83.74 ± 1.61
sample dry weight	0.08 ± 0.01	0.09 ± 0.01

Significant differences could also be observed in seaweed lipid fluxes (Fig. 28B). Although DNL of n-3 FA represent 0.34 ± 0.27% at 18 ºC, since the standard deviation represented 80% of the mean this rate was considered to be null, as well as elongation at 23 ºC. Therefore, only non n-3 synthesis per day and desaturation of lipids were considered positive regarding lipid fluxes. DNL of non-n3 FA was not altered. Desaturation was higher in T23 ºC (5.94 ± 1.49% day^{-1}) when compared to T18 ºC (4.00 ± 0.95% day^{-1}), evidenced by the higher MUFA content measured at higher temperatures. Flux rates were significantly lower, with inexistent n-3 and elongation, probably due to the minimal growth obtained, which limits the present analysis.

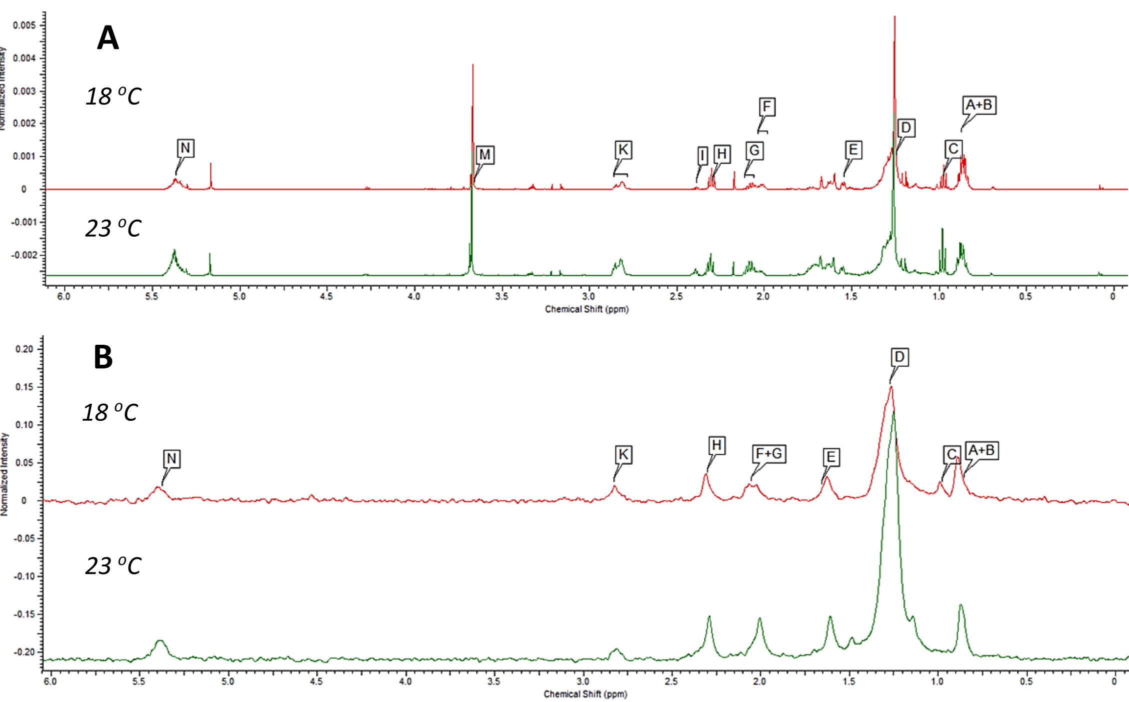
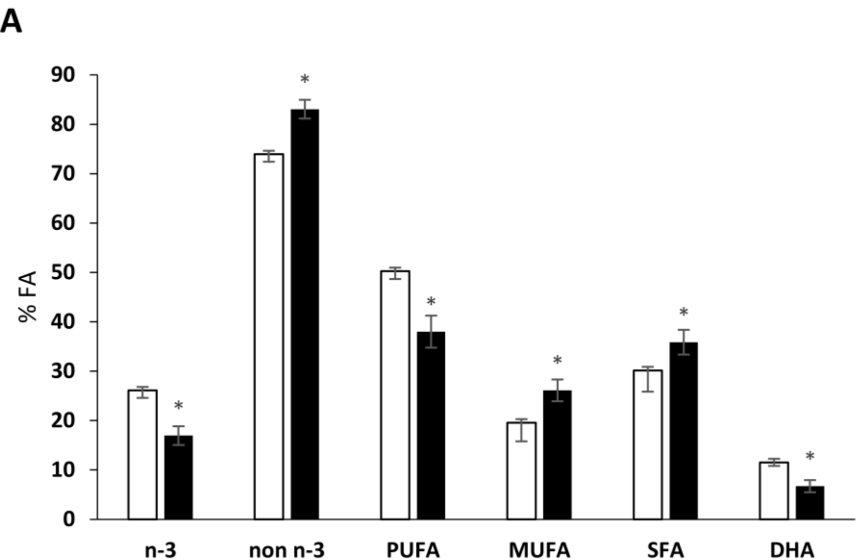


Fig. 27 - ^{1}H NMR (A) and ^{2}H NMR (B) representative spectra of FAME from *Ulva lactuca* after 6 days of ^2H_2O exposure at 18 ºC (red) and 23 ºC (green). The letters above represent peak assignments of functional groups in ^{1}H and ^{2}H according to Table 1.



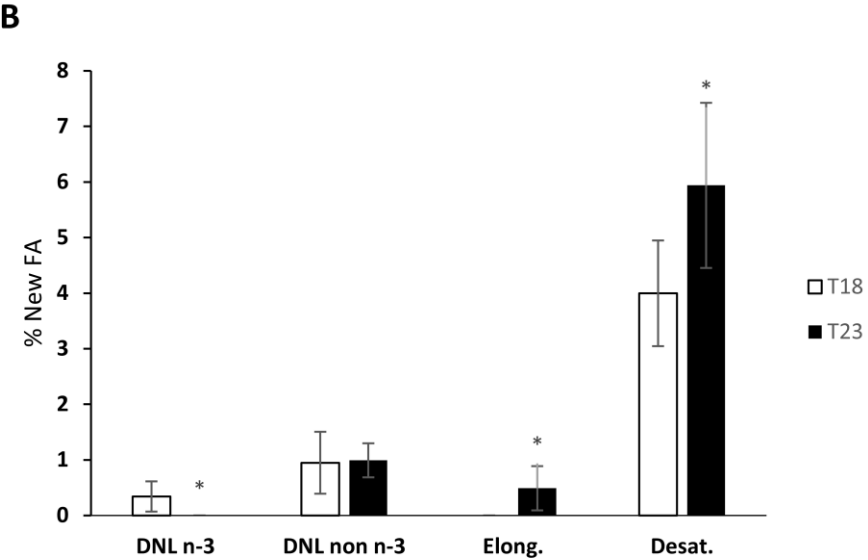


Fig. 28 - Lipidomic analysis of *Ulva lactuca* cultured at different temperatures (18 °C and 23 °C). A: lipid species quantification determined by ^{1}H NMR. B: percent contribution of DNL, desaturation, and elongation to FA per day during $^{2}H_2O$ exposure. Mean values ± S.D (n=10) are presented. Significant differences between culture conditions (18 °C and 23 °C) are indicated by asterisks (t-test, *p<0.05).

Body water enrichment

For BW enrichment the best results were obtained with microwave extraction of the water content from the seaweed. The enrichment measured by the water deposited in the lyophilizer, after seaweeds were dried, was not successful due to an air leak that enabled proper vacuum and probably sucked air moisture, resulting in a very low enrichment measured.

Seaweeds from which BW was extracted trough microwave revealed ^{2}H enrichment values close to the TW enrichment measured. *Ulva lactuca* fronds exposed to $^{2}H_2O$ for 3 h revealed 6.96 ± 0.79% of ^{2}H enrichment and when exposed for 6 h an enrichment of 7.86 ± 0.08% was observed (Table 5). The differences observed by TW and BW ^{2}H enrichment could be caused by evaporation of preferentially H_2O in spite of $^{2}H_2O$, considering that H_2O has a lower boiling point than $^{2}H_2O$ (Table 6). These results suggest that this seaweed is incorporating $^{2}H_2O$ being fully enriched with ^{2}H.

Table 5
BW and TW ^{2}H% enrichment of *Ulva lactuca* exposed to $^{2}H_2O$ for 3 h and 6 h

^{2}H %	3 h	6 h
BW	6.96 ± 0.79	7.86 ± 0.08
TW	8.15 ± 0.11	

Table 6
$^{2}H_2O$ and H_2O chemical proprieties

Property	$^{2}H_2O$	H^2O
Freezing point	3.82 ºC	0.0 ºC
Boiling point	101.4 ºC	100.0 ºC

CONCLUSIONS

Regarding lipid extraction it is possible to conclude that for *Ulva lactuca* lipid analysis fronds can be lyophilized or oven-dried, since both pre-treatments showed similar results. The best method of extraction for posterior NMR analysis is a method that involves transesterification of FA into FAME, in this case the DT method that removes lipid class resonances, generated by the polar head groups which create overlapping signals and lead to error in quantification. Therefore, Folch and MTBE extraction method are not adequate for this studies, neither purification of samples. For seaweed growth assessment, weight measurement was more practical and was therefore preferential.

In trial 1, for the first time, it was proved that seaweeds could incorporate ^{2}H in their metabolism and lipid metabolic fluxes, such as DNL, could be determined through $^{1}H/^{2}H$ NMR. Quantification of n-3 FA, non-n3 FA content and DNL, was however probably misestimated due to the interferences caused by extraction method, which also enabled quantification of SFA, MUFA, PUFA, DHA, elongation and desaturation rates.

In trial 2 quantification of all lipid species was possible, as well as n-3 DNL and non-n3 DNL, elongation and desaturation rates. The lipid species content was in accordance with literature, except for DHA which presented higher values. In this trial the only differences observed between the different treatments was in PUFA content, which was superior in +N medium compared to -N. The low number of replicates affected this analysis.

In trial 3 it was possible to observe differences in the lipid metabolism of seaweeds tested under different temperatures. Overall, seaweeds cultured at lower temperatures revealed higher instauration reflected by a decrease in MUFA and SFA, followed by decrease of non n-3 FA and increase in PUFA content, followed by increase of n-3 FA and DHA. *De novo* lipogenesis of n-3 FA was practically inexistent as well as elongation. Desaturation was significantly higher at higher temperatures and DNL of non-n3 was similar in both treatments. This trial was affected by the minimal growth rates observed.

This seaweed presented the higher n-3 content in trial 2, in +N conditions, revealing to be a good source of these FA for fish nutrition as alternative to fishmeal. With this work we could determine the rate of formation of this essential fatty acid. Nevertheless, further research is needed to better develop to consolidate this knowledge.

Future Perspectives

The need to develop alternatives to fish feeds, regarding fishmeal and fish oil substitution, requires a deep understanding of the alternative ingredient potential. Considering seaweeds as a potential substitute, research efforts should be focused on the improvement of nutritional profile and considering the metabolic process that control it, to obtain the best desirable profile for fish nutrition.

Regarding lipid metabolism, now that lipid content and flux analysis have been proven to be possible to determine by $^{1}H/^{2}H$ NMR, the priority is in establishing solid baseline values for lipid synthesis of *Ulva lactuca* and eventually other economically interesting species (*e.g. Porphyra* sp.), by increasing the number of replicates, assuring a standard growth rate of the tested seaweed. Besides nutrients and temperature, other environmental factors that can influence the normal lipid metabolism (salinity, light, CO_2) would also be interesting to analyze within a wider range of values.

Regarding NMR analysis, it would also be important to optimize some particular aspects. For instance, body water extraction methods described in this study (by microwave) should be further studied to determine, more precisely, the accuracy of this method and also to analyze the evolution of the enrichment of seaweeds exposed to $^{2}H_2O$. In this study, DHA presented abnormal values, being ~10 times higher than expected, therefore these peak resonances, of lipids extracted from *Ulva lactuca,* should be compared with a standard solution of DHA, to confirm if the assessment of the region as DHA was correct. A method for lipid yield measurement, would also have added value to the experiment, and evaluation of specific FA content with other techniques (*e.g.* GC) to compare to NMR values, would enrich lipid profile analysis.

Finally, it would be interesting to perform a study in seaweeds farmed trough an Integrated Multitrophic Aquaculture System (IMTA), which allies' fish farming with seaweed farming. This way, it would be possible to understand how lipid metabolism would fluctuate in a final product (regarding integration in fish feed) of a specific commercial activity.

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