João Miguel Rocha Salazar

# Optimization of the production of fucoxanthin-rich fractions from the microalga *Isochrysis galbana*

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# Optimization of the production of fucoxanthin-rich fractions from the microalga *Isochrysis galbana*

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## Resumo

As microalgas são organismos microscópicos uni- ou multicelulares com a habilidade de converterem energia solar em energia química num processo conhecido por fotossíntese. Estes seres vivos autotróficos sintetizam ácidos gordos polinsaturados (PUFA) como os acidos eicosapentoenoico (EPA) ou docosahexaenoico (DHA) conhecidos pelas suas aplicações terapêuticas em ensaios clínicos como agentes antioxidantes, anticancerígenos ou no combate à obesidade. Apesar dos benefícios conhecidos do EPA e DHA os seres humanos e outros vertebrados como os peixes não têm capacidade para os sintetizarem e por isso existe uma grande necessidade de encontrar novas fontes sustentáveis destes compostos de maneira a aliviar a pressão sobre os stocks de pesca continuando a dar resposta a um mercado em crescimento. As microalgas utilizam a sua pigmentação não só como forma de capturarem a luz solar, mas também, como uma ferramenta no combate ao stress oxidativo. O pigmento em destaque nesta tese é a fucoxantina, uma xantofila com capacidade para desempenhar as duas funções acima descritas dependendo da espécie de microalga. As bioactividades associadas à fucoxantina fazem com que seja alvo de inúmeros ensaios clínicos demonstrando ser não só um poderoso agente antioxidante bem como um promissor agente no combate a inflamações, diabetes e obesidade, tendo por isso tem um valor de mercado acrescido uma vez que pode ser utilizado em diversas áreas com implicações muito revelantes na saúde humana. A variabilidade da biomassa de microalgas atraiu nos últimos anos inúmeras indústrias que vêm nestes seres vivos a solução para diversos problemas como: a redução de $CO_2$ atmosférico; uma fonte sustentável de biodiesel devido ao alto nível lipídico de algumas espécies; uma nova fonte promissora de proteína ou o potencial para isolar novas moléculas com relevância nas áreas das ciências biomédicas, nas indústrias cosméticas ou farmacêuticas. Na última década a União Europeia tem reunido esforços e financiado projectos nas mais diversas áreas de caracterização de microalgas sendo promotora da aprovação das espécies *Chlorella* e *Tetraselmis* para consumo humano. O desafio prende-se agora com o estabelecimento de protocolos universais que permitam aos diversos países da UE comparar resultados e criar novos consensos levando à regulamentação de cada sector e de um mercado global em crescimento. Assim sendo, a microalga haptofita *Isochrysis galbana* foi escolhida com base no seu perfil bioquímico como uma fonte promissora para fucoxantina, um carotenoide com elevada actividade antioxidante e DHA. Nas instalações da Necton, os ALGEM® environmental labscale photobioreactors foram usados para averiguar quais as melhores condições de crescimento celular da

cultura bem como de acumulação de fucoxantina e DHA. Estes reactores permitem submeter as culturas às condições abióticas desejáveis tendo a capacidade de simular: fotoperíodo, intensidade luminosa (LED), temperatura e agitação. Os ensaios nos reactores ALGEM® revelaram que a estação do ano com maior acumulação de fucoxantina foi o Outono (6.4 ± 0.1 mg/g peso seco) e que o pH mais propicio para a bioacumulação deste carotenoide é pH 7.5 (7.88 ± 0.05 mg/g peso seco). O perfil de ácidos gordos realizado no GC-MS identificou a estação do ano Outono e o pH 8.5 como aqueles que produziram maiores níveis de DHA com 11.9 % e 16.7 % do total de ácidos gordos, respectivamente. A biomassa produzida nos reactores tubulares da Necton continha 17.69 ± 1.06 mg/g peso seco de fucoxantina e foi usada para identificar o melhor método de extracção testando as condições: rácio de biomassa/solvente e tempo de extracção. Os resultados identificaram a proporção 1/2 (10g biomassa humida/ 20 mL etanol) com 1 hora de agitação como a melhor para extrair fucoxantina. Com base neste resultado, vários extractos foram preparados para testar a extracção líquido-líquido (LTPS) na recolha de fucoxantina e DHA. Esta nova técnica permite o fracionamento de um extracto de microalgas separando compostos de elevado valor como o DHA, a fucoxantina e outros ácidos gordos, por isso a eficácia da técnica foi averiguada testando diferentes proporções de hexano e água (H/W: 1/2, 1/1, 2/1) e quantificando a quantidade de fucoxantina e DHA em cada sistema. O LTPS demonstrou ser uma técnica apropriada para a extração destes compostos, com o ratio 2/1 (160/80, H/W) a extrair mais fucoxantina, enquanto a maior quantidade de DHA foi recolhida no sistema 1/1 (120/120, H/W). Por estas razões, conclui-se que o sistema de solventes mais promissor para o LTPS foi o ratio 1/1 (H/W) assumindo a combinação de critérios: quantidade de fucoxantina; quantidade de DHA; peso da fração coloidal. Idealmente, o melhor sistema de solventes será aquele que conseguirá extrair a maior quantidade de fucoxantina sem comprometer significativamente a quantidade de DHA e de fase coloidal. Novos ensaios serão preparados para testar esta e outras condições capazes de influenciar a dinâmica das emulsões.

**Summary**
Microalgae are microscopic unicellular and multicellular organisms capable of harvesting sunlight and convert it to chemical energy in a process known as photosynthesis. These photoautotrophs synthesize PUFA, including eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids known for their therapeutic applications such as antioxidant, anti-cancer or anti-obesity effects. Despite the benefits of these molecules, humans and other vertebrates like fish lack the metabolic pathways to be able to biosynthesize them, therefore, there is an urgent need to find new sustainable sources for antioxidant agents, DHA and other essential PUFAs. The haptophyte *Isochrysis galbana* was chosen based on its biochemical profile, as a promising new feedstock for fucoxanthin and DHA. In Necton facilities, the ALGEM® environmental labscale photobioreactors were used to assess the ideal abiotic conditions for the cultivation of *I.galbana* and its intracellular accumulation of fuoxanthin and DHA. The ALGEM® annual variation trial revealed a significant higher bioaccumulation of fucoxanthin during Autumn season while the pH trial identified the highest value of fucoxanthin at pH 7.5 (7.88 mg/g DW). The fatty acid profile analysis of each Algem® trial identified Autumn season and pH 8.5 as the ones accumulating more DHA, 11.9 % TFA and 16.7 % TFA, respectively. The biomass from the tubular photobioreactors contained 17.69 ± 1.06 mg/g DW of fucoxanthin and it was used to identify the best extraction conditions focusing on: biomass/solvent ratio and time of extraction. The results identified the proportion 1/2 (10g WB/ 20 mL ethanol) at 1 hour mixing as the best for the extraction of fucoxanthin. Several extracts were prepared in this way to test the efficiency of the LTPS in extracting fucoxanthin as well as DHA. This novel liquid-liquid extraction system allows to fractionate a microalgae extract effectively separating high-value compounds such as fucoxanthin, DHA and other PUFA, therefore, the efficiency of LTPS was assessed by testing different solvent systems using hexane and water (H/W: 1/2, 1/1, 2/1) and quantifying the amount of DHA and fucoxanthin in each fraction of all solvent systems. The LTPS revealed to be a suitable technique with the solvent system 2/1 (160,80 H/W) being the one that extracted more fucoxanthin, while the majority of DHA was collected in the ratio 1/1 (120/120, H/W). For these reasons, the best LTPS solvent system was selected based on a combination of the following criteria: quantity of fucoxanthin; amount of DHA and yield of colloidal fraction. The most promising solvent system was considered to be the ratio 1/1 (H/W). Despite that, new trials are being study aiming to

determine a solvent proportion that extracts the highest possible amount of fucoxanthin without significantly compromise the DHA content and the amount of colloidal fraction. Although more trials should be performed, this work revealed to be an important step towards the confirmation of the LTPS technique as a promising protocol for a biorefinery concept.

**Key words: Microalgae, Extract, *Isochrysis galbana*, Fucoxanthin, DHA, PUFA.**

**Theme Justification**

Optimizing an extraction protocol of fucoxanthin and associated high-value molecules such as fatty acids from *Isochrysis galbana* empowers the endorsement of this haptophyte in industrial scale production with a multipurpose application of its biomass. The microalgal carotenoid market is on the rise revealing to be a multi-million dollars industry, which raises the need to understand the biochemistry of these compounds from their production by microalgae to their industrial extraction/purification. Currently, microalgae biomass is applied in food industries mainly for aquaculture purposes but the bioactivity of some of their molecules scratches the surface of more challenging applications such as anti-cancer, anti-obesity or anti-oxidant therapies. The challenge starts by identifying, which abiotic factors provide more intra-cellular accumulation of carotenoids, mainly fucoxanthin, and $\omega$-3 PUFA followed by the need to develop a feasible extraction protocol that can be applied at industrial scale. The application of such method would provide to the applicant the tools to pursue a leading market position by supplying an extremely desirable compound enriched with high-value low molecular weight molecules. The haptophyte *Isochrysis galbana* was chosen based on its biochemical richness with regards to fucoxanthin and DHA contents.

**Glossary**

**ABS** – Absorbance

**ATP** – Adenosine Triphosphate

**BHT -** Butylated Hydroxytoluene

**DHA** – Docosahexaenoic acid

**DW** – Dry Weight

**EPA** – Ecosapentaenoic acid

**FA -** Fatty Acid

**FAME** – Fatty Acid Methyl Ester

**GC-MS** – Gas Chromatography – Mass Spectrometer

**HPLC** – High Performance Liquid Chromatography

**H/W** – Hexane/Water

**LTPS -** Liquid Three Phase System

**MUFA** – Mono Unsaturated Fatty Acid

**PBR -** Photobioreactor

**PUFA -** Polyunsaturated Fatty Acid

**$R^2$** – Coefficient of Correlation

**SFA** – Saturated Fatty Acid

**TAG** – Triacylglycerol

**TFA** – Total Fatty Acid

**WB** – Wet Biomass

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## 1. Introduction

### Microalgae

The term "microalgae" refers to all microscopic unicellular and multicellular organisms capable of harvesting sunlight and convert it to chemical energy in a process known as photosynthesis (Hemaiswarya *et al.*, 2013; Metting, 1996). Consensus still needs to be achieved regarding the inclusion or exclusion of blue-green algae (Cyanophyta) within this term, mainly because they belong to the prokaryotic bacterial domain (Singh *et al.*, 2005; Williams *et al.*, 2010). Even so, it is unanimous that they played a crucial role in the evolutionary path of eukaryotic microalgae (Keeling, 2004). Photoautotrophic microorganisms are the oceans primary producers; they are present throughout the water column and are provided with exceptionally cellular diversity which allows them to survive in both marine and freshwater environments living either isolated or in colonies ( Ahlgren *et al.*, 1992; Kirst, 1990; Renaud *et al.*, 1999; Takabayashi *et al.*, 2006). Different cellular shapes and sizes provide dissimilar advantages or disadvantages mostly regarding nutrient uptake, predator avoidance, light harvesting and position in the water column (Takabayashi *et al.*, 2006). Such differences in cellular size and shape of diatoms, coccolithophores and dinoflagellates translates in different sinking rates in open ocean with implications on the organic matter flow between pelagic and benthic layers (Smayda, 1971).

The cellular variability of these phototrophs is the key to their taxonomy classification, often based on pigment composition (Hemaiswarya *et al.*, 2013), but which can also be assembled according to fatty acid content (Mourente *et al.*, 1990) or thylakoid's membrane organization (Gualtieri, 2001; Metting, 1996). According to evolutionary criteria, microalgae can be divided into cyanobacteria, a prokaryotic phylum of photosynthetic phycobilin-containing bacteria (Lewin, 2002; Newcomb *et al.,* 1975), and several eukaryotic lineages, the most important being *Glaucophyta* (phycobilin-containing freshwater microalgae), *Rhodophyta* (red algae), *Cryptophyta* (cryptophytes or cryptomonads), *Chlorophyta* (green algae), *Euglenophyta* (euglenoids), *Dinoflagellata* (dinoflagelates), *Chrysophyceae* (golden-brown algae), Bacillariophyta (e.g. diatoms) and *Haptophyta* (algae with haptonema) (Gualtieri, 2001; Hemaiswarya *et al.*, 2013; South *et al.*, 1987). Several of the above-mentioned microalgae have received widespread attention over the last decades due to the identification of novel molecules and their applications in numerous fields, which will be further explained below.

## 1.2 Biochemistry of Microalgae

Autotrophic microalgae require inorganic C, N, P, and S sources combined with sunlight to photosynthesize organic molecules and generate ATP that will provide energy to the chemical reactions within the metabolic pathways of the growing process increasing their biomass while sustainably generating lipids, proteins, amino acids and carbohydrates (Pignolet *et al.*, 2013).

### 1.2.1 Lipids

Microalgal lipids are versatile in their biochemical structure and composition, existing as neutral lipids (e.g. triacylglycerol, TAG) and polar lipids (e.g. phospho- and glycolipids), forming fatty acid chains ranging from medium (C10-C14) to long (C16-18) or very-long (>C20) and occurring either in unsaturated or saturated forms (Hu *et al.*, 2008; Li *et al.*, 2014). Neutral lipids such as mono-, di- or triacylglicerides are the cells energy sources while polar lipids such as phospholipids are the building blocks of cellular membranes. Thus, it is not surprising that these lipid classes have been long studied due to their chemical composition, molecular dynamics and metabolic role (Heinzelmann et al., 2014).

Chemically, phospholipids are assembled by attaching a polar phosphate head-group to a non-polar hydrocarbon chain, and the classification of these molecules depends mainly on the functional groups linked to the phosphate head. The five major classes are: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylglycerol (PG), each one containing a group of species that differ on the fatty acyl group (Pichot *et al.*, 2013; Yan *et al.*, 2010). The molecular behavior of phospholipids is intrinsically related to their physical properties, mostly known for their cellular role in biological bi-layer membranes as a result of their amphiphilic nature (Bloom *et al.*, 1991).

Metabolically, the synthesis of polar lipids and TAGs can take place either at the endoplasmatic reticulum (ER) membrane or at the chloroplast envelope (Schüler et al., 2017). Microalgae ω-3 PUFA, assembled in the ER membrane, include eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids known for their therapeutically applications in the treatment of inflammatory, cardiovascular and autoimmune diseases, as well as, their essential role in the nervous system in cellular membranes of the brain, fetal development and healthy aging (Dunstan *et al.*, 2007; Schüler *et al.*, 2017; Singh *et al.*, 2005; Swanson *et al.*, 2012). Despite the benefits of these molecules, humans and other vertebrates like

fish lack the metabolic pathways to be able to biosynthesize them and meet the nutritional requirements of these organisms for these biomolecules (Vanthoor-Koopmans *et al.*, 2013). Therefore, there is an urgent need to find sustainable sources for EPA, DHA and other essential PUFA. Structurally, microalgae lipids are present not only as an essential part of the cell and organelle (e.g. chloroplast, thylakoid, mitochondria) membranes in the form of phospholipids, glycerides and glycolipids, but also as an energy and carbon reservoir in the form of TAG, stored in lipid droplets, created as a product of the fatty acid synthesis (Guckert *et al.*, 1990; Radakovits *et al.*, 2010). Microalgae are known to shift from starch to lipid biosynthesis depending on environmental stresses (e.g. nutrient limitation, high light intensity, nitrogen depletion), increasing the concentration of TAG when conditions are unfavorable (Guckert *et al.*, 1990). They do so, at the expense of energy used for growth translating into a biomass productivity decline (Wijffels *et al.*, 2010).

### 1.2.2 Amino acids and Vitamins

Lipid biosynthesis is not the only metabolic pathway undergoing readjustments as a consequence of environmental conditions. In fact, amino acid, protein and vitamin contents are also known to vary between species and strains, depending on environmental parameters, such as pH, temperature, nutrient composition, harvesting stage and photoperiod (Brown, 1991; Juneja *et al.*, 2013).

The amino acid and protein composition of microalgal biomass is extremely rich and versatile and for that reason its potential is not yet fully explored. Cyanobacteria produce a powerful anti-HIV protein (Cyanovirin-N) which acts also as multi-drug resistance reverser, as well as lipopeptides with cytotoxic, antitumor and cholesterol regulation properties, all of them with great impact on human health (Burja *et al.*, 2001; Singh *et al.*, 2005). Moreover, microalgal biomass is also abundant in essential and non-essential amino acids: aspartic acid, serine, glycine, leucine, tyrosine, histidine, arginine and alanine, in different concentrations, depending on the culture medium, abiotic factors and genetics (Ahlgren *et al.*, 1992; Enright *et al.*, 1986). Biochemical composition of these microorganisms also includes vitamins (e.g. biotin, niacin, A, C, E, $B_2$, $B_6$ and $B_{12}$) and minerals, which can represent as much as 30% of the microalgae dry weight composed mainly of Ca, K, Fe, Mg (Batista *et al.*, 2013; Matos *et al.*, 2017).

### 1.2.3 Carbohydrates

Microalgal carbohydrates are the early product of photosynthesis, having either a structural role in cellular membranes or a metabolic purpose as an energy and carbon source for the biosynthesis of more complex molecules (Hecky *et al.*, 1973; Williams *et al.*, 2010). Carbohydrates can be found as monomers of simple sugars (e.g. glucose), disaccharides (e.g. sucrose) or polysaccharides (e.g. starch, chrysolamin), the last being mostly known for their anticancer, antioxidant and immune stimulatory properties (Chen *et al.*, 2011; Koller *et al.*, 2014; Lordan *et al.*, 2011; Matos *et al.*, 2017). Additionally, micro- and macroalgae polysaccharides can interact with a sulfate anion to form sulfated polysaccharides known for their anti-inflammatory and anti-viral bioactivities (Hasui *et al.*, 1995; Matsui *et al.*, 2003; Wijesekara *et al.*, 2011). Depending on their chemical composition and arrangement, microalgal polysaccharides can either be associated in cell walls to provide structure and participate in extracellular communication or as energy molecules (e.g. starch) to fuel the cellular metabolism (Pignolet *et al.*, 2013; Templeton *et al.*, 2012).

### 1.2.4 Pigment composition

These micro-photoautotrophs are provided with pigmentation responsible not only for coloration but mainly for light harvesting, $CO_2$ fixation and protection against excessive illumination by either blocking sunlight or neutralizing reactive oxygen species (ROS) that would produce oxidative damage (Moghadamtousi *et al.*, 2014). There are three major classes of pigments in microalgae: i) Phycobiliproteins, an association of a chromophore called phycobilin that is covalently bound to a protein (Mulders *et al.*, 2014). They are photosynthetic accessory pigments of cyanobacteria and red algae (*Rhodophyta*), among others, playing a role in the phycobilisomes, water-soluble protein complexes responsible for harvesting visible light and transferring excitation energy to the reaction centers for conversion to chemical energy during photosynthesis (Román *et al.*, 2002); ii) Carotenoids, composed of a single long hydrocarbon lipophilic chain made up with eight isoprene units (Mulders *et al.*, 2014; Varela *et al.*, 2015). This pigment class has two main divisions, the non-polar hydrocarbon carotenes (e.g. β-carotene) and the polar xanthophylls (e.g. fucoxanthin, astaxanthin), which are oxygenated byproducts of carotenes (Crupi *et al.*, 2013; Moghadamtousi *et al.*, 2014). Carotenoids function mainly as secondary pigments - although this classification may differ, depending on the pigment composition of each species - in the photosynthesis light harvesting process, and are also

scavengers of free radicals preventing photo-oxidative stress (Gouveia *et al.*, 2010). The antioxidant capacity and the light absorbing properties of these compounds is influenced by the position and number of double blonds within the polyene backbone (Stahl *et al.*, 2003); and finally iii) chlorophylls, often classified as tetrapyrroles due to their molecular arrangement surrounding a magnesium ion with four pyrrole rings forming a large aromatic ring coupled with a hydrocarbon tail (Mulders *et al.*, 2014). They are present in the thylakoids membrane and the differences in their chemical structure are mainly on the aromatic ring, allowing the classification in 3 types: *a*, *b* and *c* (Pignolet *et al.*, 2013). They play a critical role in photosynthesis by selectively absorbing light in the red and blue wavelengths, which gives them the characteristic green color (Hosikian *et al.*, 2010).

All these pigment classes have a high market value due to their multiple applications such as replacing toxic synthetic dyes in food and textile industries (Dufossé *et al.*, 2005); anticancer, antimutagenic and novel drugs with antioxidant properties (Crupi *et al.*, 2013); or as fluorescent dyes bound to antibodies and cellular receptors.

#### 1.2.4.1 Fucoxanthin

Fucoxanthin is an orange-colored carotenoid that has shown a lot of nutraceutical potential revealing to be a promising molecule with anti-cancer, anti-oxidant, anti-obesity (Peng *et al.*, 2011), anti-inflammatory and anti-diabetic bioactivities, as well as being an active reducer of cardiovascular risk factors such as high blood pressure, high cholesterol and chronic inflammation (D'Orazio *et al.*, 2012). This compound has a carbon backbone with an allenic group bond to a 5,6-monoepoxide and an acetyl group on the terminal ring which confers it a higher antioxidant capacity than their metabolites fucoxanthinol and halocynthiaxanthin (Miyashita *et al.*, 2011; Sachindra *et al.*, 2007). The cellular uptake after ingestion is dependent on the lipophilicity of the carotenoid and its interaction with the phospholipid membrane of the intestinal cells (Sugawara *et al.*, 2001). Hence, the bioactivity of fucoxanthin and its metabolites (Fig. 1) relies on the digestibility of fucoxanthin which starts in the gastrointestinal tract by enzymes such as lipases that hydrolyze it or cholesterol esterase that deacetylate it to fucoxanthinol. Fucoxanthinol then travels to the liver where it is dehydrogenated into amarouciaxanthin A *(*Asai *et al., 2004;* Das *et al.*, 2010; Peng *et al.*, 2011). This mammalian metabolic pathway was described in mice after oral ingestion of fucoxanthin and two hours later no presence of it was detected while fucoxanthinol was predominantly present (Asai *et al.,* 2004).

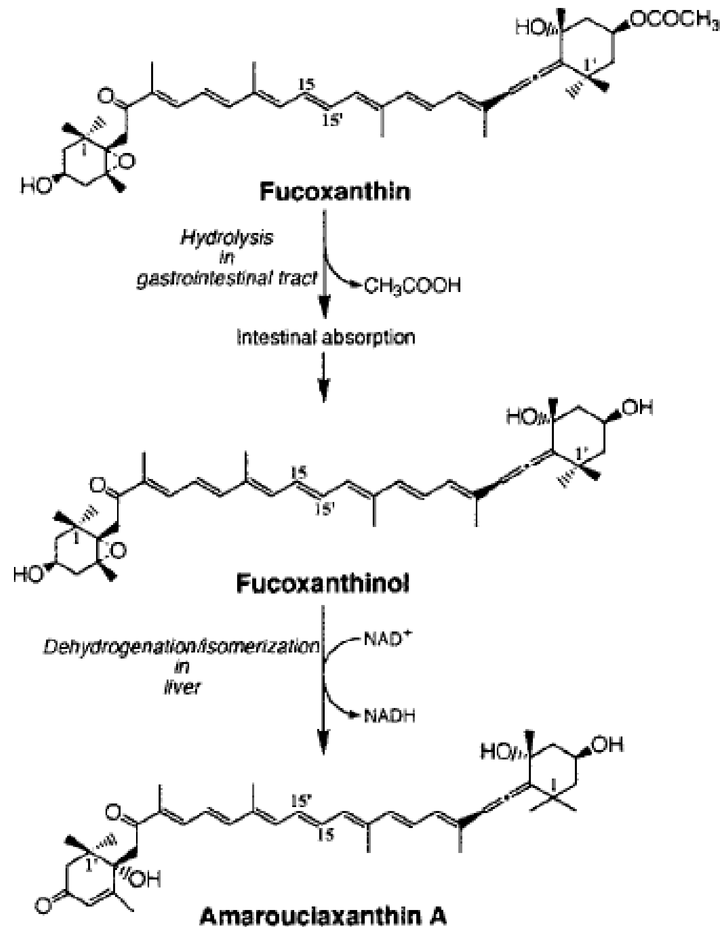


Figure 1 - Metabolic pathway of fucoxanthin during digestion and intestinal absorption in mice.
From Asai *et al.* (2004).

The same pathway was likewise proposed by Miyashita *et al.,* (2011) (Fig. 2), whose work has also established the connection between fucoxanthin and its anti-obesity effect by down-regulating the expression of monocyte chemoattractant protein-1 (MCP-1), which promotes accumulation of macrophages in the adipose tissues leading to inflammatory stress.

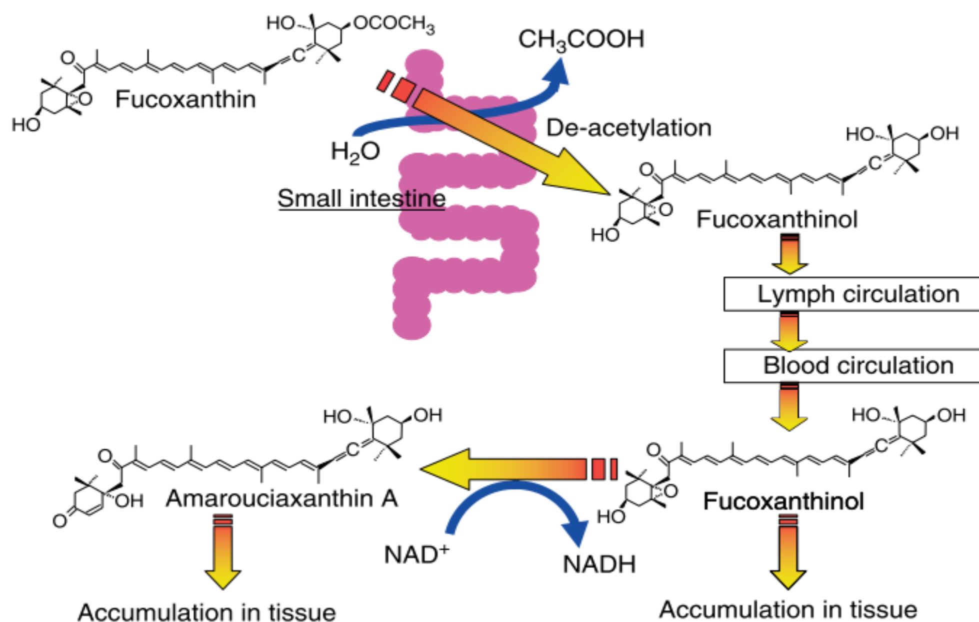


Figure 2 – Metabolic decomposition of fucoxanthin during digestion, intestinal absorption and tissue accumulation in mice. Adapted from Miyashita *et al.,* (2011).

### 1.3 Microalgae Fields of Application

The variability of microalgal biomass composition has attracted a variety of different industries that now look at it as a reliable and clean multipurpose source for: aquaculture and human nutrition (Hariskos *et al.*, 2014; Muller-Feuga, 2000); sustainable production of biodiesel using lipid-rich microalgae (Chisti, 2008; Wijffels *et al.*, 2010); biological $CO_2$ mitigation from either atmosphere or industrial exhaust gases (Sydney *et al.*, 2010; Wang *et al.*, 2008); and pharmaceutical and drug development using carotenoids with anticancer, anti-inflammatory or antioxidant effects ( Moghadamtousi *et al.*, 2014; Peng *et al.*, 2011). For aquaculture or mariculture purposes, different microalgae strains are known to be a suitable feedstock to feed larvae of teleosts, crustaceans and mollusks due to their protein, vitamin and PUFA contents, size, lack of toxicity and high digestibility of the cell walls (Brown, 1991; Spolaore *et al.*, 2006). Additionally, some species such as *I. galbana* contain free fatty acids known for their antibacterial effects against pathogenic microorganisms (e.g. *Vibrio* strains) decreasing the need for antibiotics. The biochemical composition of microalgal biomass is also very desirable for feeding cattle, pigs and poultry (Koller *et al.*, 2014). Although the vast majority of microalgae used as aquaculture and poultry feeds is harmless, toxicity should also play an important role when deciding which species to use, since some strains of

diatoms, blue-green algae and dinoflagellates are known to produce secondary metabolites (e.g. domoic acid, saxitoxin, aplysiatoxin) capable of inducing poisoning, paralysis or destruction of important glutamate receptors in the brain of animals and humans (Shimizu, 2003).

Regarding human consumption, microalgae have been used as an alternative food source for hundreds of years mainly to surpass starvation. In the 16$^{th}$ century, before the Spanish conquest of Mexico, populations used to harvest "Spirulina" from the lake Texcoco, dry it in the sun and use it as food paste (Ciferri, 1983). The same habit, dating hundreds to thousands of years, is known to be present in populations of the Republic of Chad and China that live near lakes and ponds where "Spirulina" and *Nostoc* algae, respectively, were harvested using fine nets with the same purpose of finding another source of protein (Ciferri, 1983; Habib *et al.*, 2008; Spolaore *et al.*, 2006). The struggle to find a food stock that could satisfy the demand felt by these populations made them pioneers in the establishment of a novel food source rich in proteins, lipids and vitamins. Since then, microalgae for human consumption have gained widespread attention, not only because of their higher protein productivities when compared to soybean, rice and/or meat proteins (Kay *et al.*, 2009), but also due to relatively low cost of cultivating them in raceway ponds (Norsker *et al.*, 2011) and their nutrient contents. More recently, the European Union has approved the use of *Chlorella* sp. and *Tetraselmis* sp. for human consumption which represents another step towards the valorization of microalgae as a sustainable novel source of protein.

### 1.3.1 Market Value and Industrial Approaches

Today's market value of microalgae species varies depending on its chemical and nutrient compositions and production costs, increasing when the concentration of compounds such as carotenoids, PUFAs, amino acids and vitamins is higher. The current market value of carotenoids is projected to reach US$ 1.4 Billion this year (D'Alessandro *et al.*, 2016), which encourages industries to adapt production methods and develop a biorefinery model that allows them to extract high-value products and use the residual biomass to produce clean renewable energy such as biodiesel (Draaisma *et al.*, 2013; Hariskos *et al.*, 2014). The market value for microalgal pigments is on the rise, primarily due to the bioactive properties of compounds such as fucoxanthin or astaxanthin, which are known for their anticancer, antioxidant and skin protection properties, and secondarily, due to the biochemistry developments that highlight the metabolic

degradation pathways of these molecules within fish and mammalian organisms, ultimately providing a better understanding of their bioactivities and bioavailability (Asai *et al.*, 2004; Guerin *et al.*, 2003; Miyashita *et al.*, 2011). For these reasons, it is crucial to understand the optimal growth conditions (e.g. light intensity, salinity, nitrogen availability, aeration flow) for the target microalgal strains in order to maximize the intracellular accumulation of these compounds and combine that knowledge with extraction techniques to optimize new industrial procedures.

Micro- and macroalgae have already been compared with results showing that microalgae produce up to fifty times more fucoxanthin (mg/g DW) than macroalgae (Xia *et al.*, 2013), which enhances the need for scalable strategies for the extraction of such compounds. Current methods for carotenoid extraction from freeze-dried biomass include one-phase solvent extractions using ethanol, hexane or acetone with different yields depending on the affinity between fucoxanthin and the solvent chosen (Fig. 3), two-phase liquid systems using polar and non-polar solvents such as water and hexane (Kim *et al.* 2012), or alcohol-salt aqueous two-phase systems which have shown a 70% recovery of fucoxanthin and over 60% recovery of low molecular weight high-value compounds (Gómez-Loredo *et al.*, 2014, 2015).

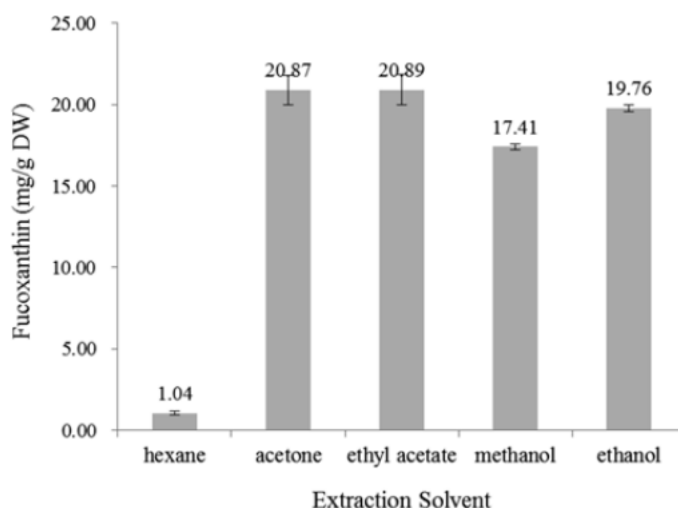


Figure 3- Efficiency of solvent extractions of fucoxanthin from freeze-dried biomass of *Isochrysis aff. galbana.* Extractions were conducted for 1h at room temperature and analyzed with HPLC. From Kim *et al.*, 2012.

In contrast to traditional solvent extractions, techniques such as supercritical $CO_2$ extraction have already proven to be less time-consuming by requiring fewer steps to effectively extract carotenoids from microalgal biomass (Macías-Sánchez *et al.*, 2005). Additionally, the method has already been applied successfully for astaxanthin extraction

from *Chlorela vulgaris* (Mendes *et al.*, 1995), Chlorophyll *a* from *Synechococcus* sp. (Macías-Sánchez *et al.*, 2007) and carotenoids from *Nanochloropsis guaditana* (Macías-Sánchez *et al.*, 2005), revealing to be a less toxic approach in comparison to conventional organic solvent extractions. Furthermore, Gilbert-López *et al.* (2015) successfully demonstrated the efficiency of this technique in extracting fucoxanthin, fucoxanthinol and other fuco-isomers from *I. galbana* biomass, enhancing the biorefinery strategy of downstream processing of biomass by combining supercritical $CO_2$ extraction followed by solvent extractions of residual biomass. The disadvantage of this method relies on its high costs of acquisition and operation which ultimately limits its widespread use in comparison with other cheaper and equally effective methods.

**1.3.2 Emulsions: Liquid Three Phase System (LTPS)**

The Liquid Three Phase System (LTPS) is a liquid-liquid extraction that uses a hexane-water solvent system to create an oil/water emulsion. The stability of the resulting emulsion is greatly enhanced by the presence of amphiphilic compounds such as the phospho- and/or betaine lipids, which are abundant in microalgae. This fact makes the LTPS of great interest in terms of novel techniques for the fractionation of microalgal lipids and the production of extracts rich in high-value compounds such as fucoxanthin and fatty acids.

The production of emulsions has long been used to satisfy our daily demands in a variety of fields either in the petroleum industry by manufacturing crude oil or to supply the food markets with elementary products such as butter, mayonnaise, ice-cream, sauces or milk (Gouveia *et al.*, 2010; Tambe *et al.*, 1994). By definition, they are the result of two immiscible liquids combined together, with one liquid being dispersed in colloidal particles named droplets into the other liquid (Mcclements *et al.*, 2007; Novales *et al.*, 2003). The surrounding liquid is often referred as the "continuous phase" while the droplets are often classified as the "dispersed phase". There are two main types of emulsions: oil droplets dispersed in an aqueous phase, which are classified as oil-in-water emulsions (e.g. milk, ice-cream); and water droplets spread in an oil phase that are classified as water-in-oil emulsions (e.g. butter) (Novales *et al.*, 2003). Therefore, in order to form any type of stable emulsion three conditions must be met: i) the two liquids must be immiscible; ii) agitation must applied to homogenize the mixture; iii) an emulsifier agent of any type must be present (Fig. 4) (Chen *et al.*, 2005).

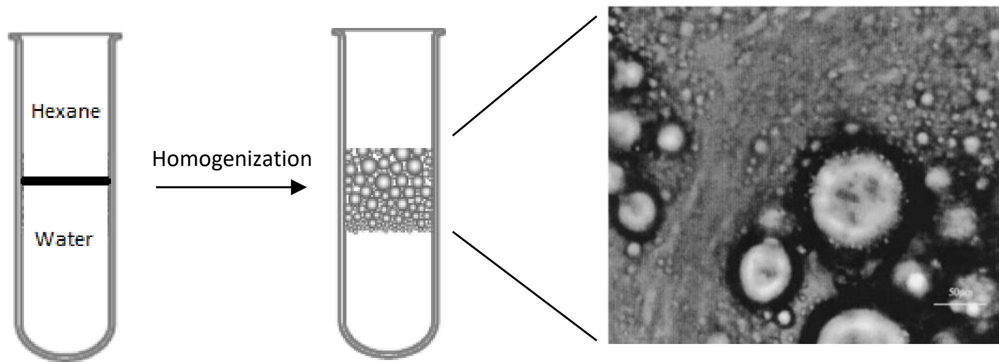


Figure 4- Schematic representation of an oil-water emulsion. The immiscible liquids are homogenized with agitation promoting the formation of colloidal droplets. The emulsifier agent coats the oil droplets enhancing the stabilization of the emulsion. Adapted from McClements *et al.* 2007 and Huang *et al.* 2001.

The complexity of the interactions occurring between dispersed and continuous phases is tremendous due to the various physicochemical properties involved (e.g. size of droplets, charge, particle-fluid interface dynamics, droplet density and viscosity of the continuous phase) acting simultaneous and continuously throughout the process ultimately stabilizing or disturbing it (Gao et al., 2014; Walstra, 1993). The size and interaction of the droplets with the continuous phase are key factors for the overall kinetics of any given emulsion, ultimately revealing to be the cause of different types of instability mechanisms, as shown in figure 5 (McClements *et al.*, 2007).

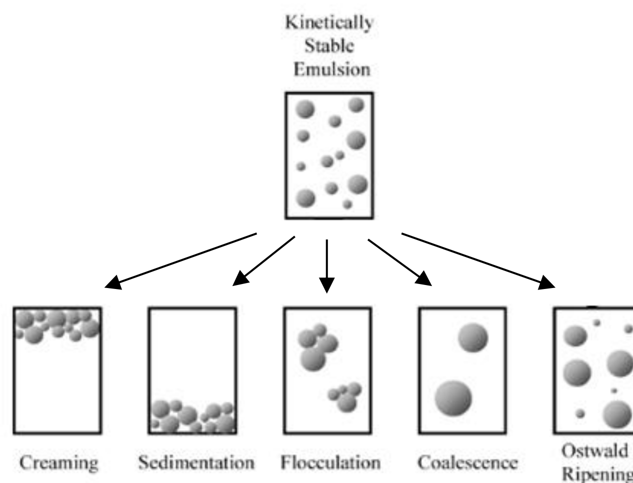


Figure 5- Representation of outcomes for emulsion instability. When colloidal particles have a lower density than the continuous phase they move upwards (creaming) and when they have a higher density, they move downwards (sedimentation). Flocculation is the aggregation of smaller droplets that keep their boundaries intact. By contrast, coalescence is the merging of smaller droplets forming a bigger droplet. The Ostwald Ripening it's the physical interaction of bigger molecules that gradually increase size at the expense of smaller droplets. Adapted from McClements *et al.*, 2007.

Although emulsions are known to be thermodynamically unstable, since the contact of oil and water molecules is unfavorable, one can promote its stability by either using emulsifiers (e.g. phospholipids, proteins), texture modifiers (e.g. polysaccharides) or manipulating physical properties (e.g. temperature) favoring the emulsification (Chen *et al.*, 2005; Dickinson, 2003; Mcclements *et al.*, 2007; Pichot *et al.*, 2013). Emulsifiers act by adsorbing to the droplet creating an external film layer that minimizes any instability mechanism and facilitates the interaction with the interface layer (Chen *et al.*, 2005; Dickinson, 2003). Texture modifiers such as hydrocolloids formed by polysaccharides (e.g. pectin, fenugreek gum, arabic gum) are often used in food emulsions to increase the viscosity of the continuous phase promoting emulsion stability avoiding flocculation and coalescence, as well as, improving the shelf-life of the final product (Dickinson, 2009; Huang *et al.*, 2001). The manipulation and monitoring of temperature throughout the process can greatly increase the outcome of any given emulsion by shifting the effect of parameters such as: viscosity, cohesive force and surface tension of the continuous phase. In fact, these parameters tend to decrease at temperatures greater than 30 °C, critically affecting emulsification (Chen *et al.*, 2005; Joshi *et al.*, 2012). The biochemical profile of microalgae biomass is known to be rich in phospholipids, proteins and polysaccharides thus making it good candidates as emulsifiers.

Starting with the preparation of an ethanolic microalgal extract, the LTPS technique develops a stable emulsion after the dispersion of the extract, containing mostly lipids, small sugars, peptides and pigments, in between both solvents. Due to the presence and amphiphilic nature of phospho- and/or betaine lipids three phases are formed: hexane, colloidal, water. Non-polar molecules reveal great affinity for the organic solvent phase while polar compounds prefer the aqueous solvent. Here, the suitability of LTPS in extracting the major groups of biomolecules including the carotenoid fucoxanthin and the ω-3 PUFA from *I. galbana* biomass is investigated. In addition, a first approach to understand the mechanisms behind the colloidal phase stability are researched by testing different ratios of water and hexane.

*Isochrysis galbana* was selected due to their nutritional composition with regards to DHA and the carotenoid fucoxanthin (Kim *et al.*, 2012; Goméz-Loredo *et al.*, 2014). Understanding the biochemical dynamics of this species and develop extracts rich in fucoxanthin and other high-value molecules in agreement with that knowledge represents one step further to better appreciate the value of microalgal biomass.

## 1.4 *Isochrysis galbana*

*I. galbana* is an unicellular bi-flagellated (Fig. 6) non-coccolith-forming microscopic photosynthetic alga that belongs to the phylum *Haptophyta*, class *Prymnesiophyceae* and order *Isochrysidales* (Kaplan *et al.*, 1986; Parke, 1949; Sánchez *et al.*, 2000; Sorrosa *et al.*, 2005). As a marine microalga, its distribution ranges from European to North American waters crossing the south Iberian coast to the North Atlantic and Irish seas and reaching as far as the Pacific Ocean, from California's coast up to Australia (Ahmed *et al.*, 2014; Bandarra *et al.*, 2003; Moita *et al.*, 1999; Wang *et al.*, 2014). The cellular architecture of this species is versatile not only in terms of morphological shape, but also on an intracellular level, primarily regarding the position, size and profile of its golden-brown chromatophores (Parke, 1949). Furthermore, as they are photosynthetic, they possess a stigma (or eye-spot apparatus), an organelle that plays an important role in sensing light, which helps the cell guiding the flagella to adjust its position towards or away from it (Parke, 1949). Unique to all haptophytes is the haptonema, a linear or arc-shaped microtubular complex present between the flagella with various roles, depending on the species, providing either sensorial guidance, substrate adherence or an appendix for predation in some mixotrophic strains (Green *et al.*, 1977; Thierstein *et al.*, 2004).

The haptonema of *I. galbana* is very rudimental and only visible in electron micrographs. Additionally, its multiplication alongside the flagella are the first signs that the cell is initiating mitosis (Fig. 7) that will culminate in two daughter-cells (Andersen, 2004; Hori *et al.*, 1985). Surprisingly, asexual reproduction via cellular maturation of cysts is not the only form of reproduction for Haptophytes (Parke, 1949). In fact, the life cycle of this microflagellates typically includes haploid and diploid phases, each one capable of independent asexual reproduction (Ramawat *et al.*, 2014).

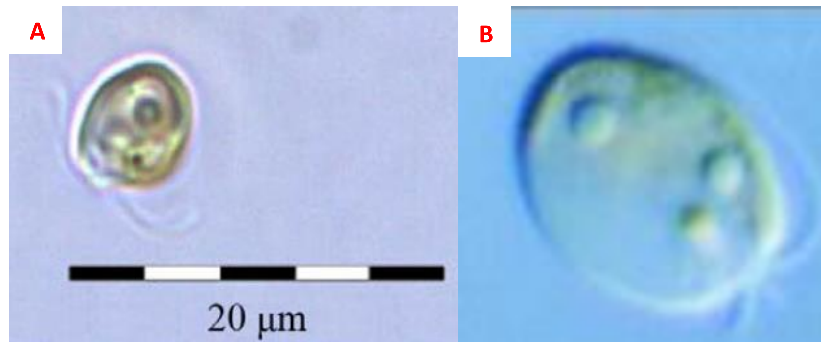


Figure 6- A- *I. galbana* under Olympus S20 microscope (100x). Adapted from Gorgônio *et al.*, (2013). B- *I.galbana* observed with a Nomarski differential interference contrast microscopic. Adapted from Liu *et al.*, 2001. In both images the flagella are visible within the extracellular space surrounding the cells.

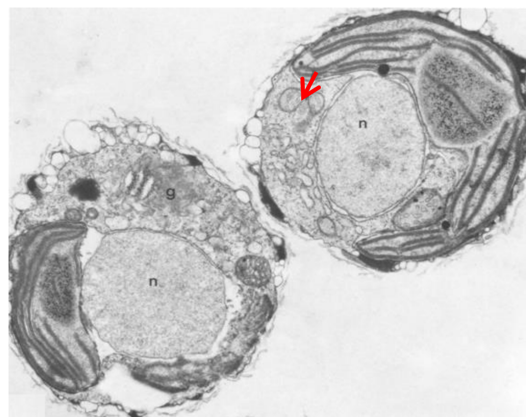


Figure 7- *I. galbana* cells after completing cytokinesis have their edges covered with scales. The red arrow represents the formation of the first flagellum. n= Nucleus, g= Golgi complex. Adapted from Hori *et al.*, (1985).

### 1.4.1 Biochemical Profile

*I. galbana* accumulates lipids (Fig.8), such as TAG, under nitrogen depletion or increased salinities (Breuer *et al.*, 2012; Liu *et al.*, 2001), or synthetize less monounsatured fatty acids (MUFA) under shorter photoperiods or less PUFA under higher temperatures (Bandarra *et al.*, 2003; Zhu *et al.*, 1997). Multi-factor experiments provide an important insight of the fragility and plasticity of these molecular interactions by identifying the biological outcome of any combination of abiotic factors. Biochemically, lipids serve both as structural and fuel molecules and essential, biotechnologically relevant PUFA in this species include mainly EPA and DHA, although other fatty acids are also present in significant amounts, such as myristic, palmitoleic and linoleic acids (Batista *et al.*, 2013).

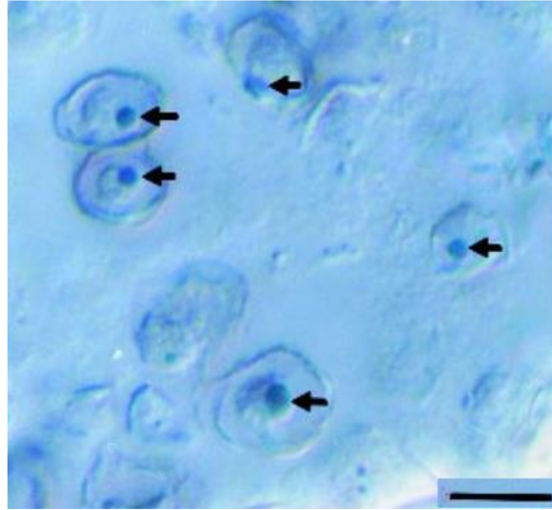

Figure 8 - *I. galbana* cells stained with Sudan Black B dye showing the lipid droplets accumulated intracellularly (arrows). Scale bar = 5 µm. Adapted from Liu et al. (2001).

*I. galbana* has all the essential and non-essential amino-acids with some strains being able to accumulate more histidine, leucine, lysine and methionine. In fact, protein and lipid content of these strains can represent more than 50% of its dry weight (Brown, 1991). Regarding vitamin composition, this species can reveal high values for vitamin PP also known as niacin, vitamin B6 and vitamin C as well as lipophilic vitamins such as A, D, E, and K (Table 1; Roeck-Holtzhauer *et al.*, 1991). Pyridoxine (vitamin B6) serves a very important metabolic pathway in the human organism regarding neurotransmitters and hemoglobin synthesis. Additionally, it is implicated in the prevention of cardiovascular traumas by decreasing homocysteine levels (Thaver *et al.*, 2009). Niacin, is at the genesis of energy for cellular metabolic purposes by being a source for the biosynthesis of $NAD^+$ and NADH which is used in mitochondria for the processes of oxidative phosphorylation and the Krebs cycle, ultimately generating Adenosine Triphosphate (ATP); human deficiencies in this vitamin can culminate in bone marrow leukemia, decreased apoptosis activity and compromised cell cycle regulation (Jacobson *et al.*, 2012).

**Table 1- Vitamin composition of *I.galbana* cultivated in Conway medium at 18 °C**, values from Roeck-Holtzhauer *et al.*, (1991). The human daily intake of vitamins is based either on the Recommended Dietary Allowance (**RDA**) or on the Adequate Intakes (AI*), the values presented are for the life stage group of Females ranging between 31-50 years old, from Trumbo *et al.*, (2001).

| Vitamin | | Species | Daily Intake |
|---|---|---|---|
| | | *I. galbana* (µg/g DW) | **RDA** or AI(*) |
| Hydrophilic | B1 | 462 | **1.1** (mg/d) |
| | B2 | 14 | **1.1** (mg/d) |
| | B6 | 183 | **1.3** (mg/d) |
| | B12 | 89 | 2.4* (µg/d) |
| | C | 772 | **75** (mg/d) |
| | PP | 2690 | **14** (mg/d) |
| Lipophilic | A | 270 | **700** (µg/d) |
| | D | 5 | 5* (µg/d) |
| | E | 117 | **15** (mg/d) |
| | K | 8 | 90* (µg/d) |

In *I. galbana*, carbohydrates (Fig. 9) are mainly constituted of monosaccharides such as glucose, mannose and galactose, which interact to form more complex molecules such as chrysolaminarin a polysaccharide formed by glycosidic bonds of glucose (Gorgônio *et al.*, 2013).

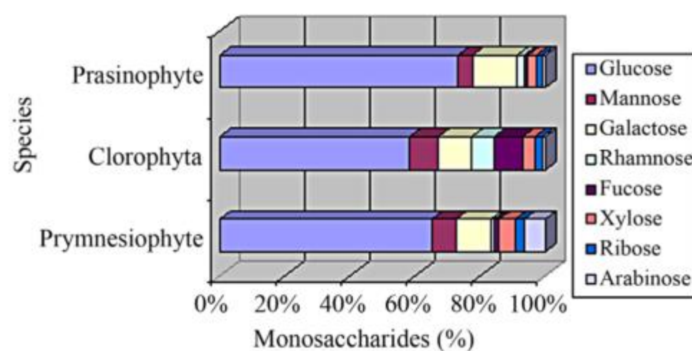


Figure 9 – Comparison of the sugar monomers composition between *Tetraselmis gracilis* (Prasionophyte), *Dunaliella tertiolecta* (Clorophyta) and *Isochrysis galbana* (Prymnesiophyte). From Gorgônio *et al*., 2013.

### 1.4.2 Pigment profile

*I. galbana* is a brown microalga with an intracellular complex pigment composition comprising chlorophyll *a* and *c* as well as *β*-carotene and the polar xanthophylls fucoxanthin, diadinoxanthin and diatoxanthin (Andersen, 2004; Crupi *et al.*, 2013; Takaichi, 2011). Concerning chlorophyll *c*, *I. galbana* contains two sub-types: monovinyl chlorophyll $c_1$ and the divinyl chlorophyll $c_2$, both playing a role in light harvesting (Garrido *et al.*, 1997; Zapata *et al.*, 2000). During photosynthesis, chlorophyll *a*, *c* and fucoxanthin act as primary pigments transferring light energy to the photosynthetic electron transport chain, while diadinoxanthin, diatoxanthin and *β*-carotene are secondary pigments acting as quenchers of excessive energy from chlorophyll *a*, providing photoprotection and preventing the emergence of ROS by releasing the excess energy as heat (Mulders *et al.*, 2014; Takaichi, 2013; Varela *et al.*, 2015; Zapata *et al.*, 2004). *Isochrysis galbana* survival, as any other species, is dependent of the ongoing relationship between its evolutionary heritage and the environment, resulting in metabolic readjustments as a consequence of an extracellular stimulus (e.g. photoperiod, nutrient depletion/repletion). For that reason, the biosynthesis of pigments and isomers along with carotenoid derivatives (e.g. 13Z-, 13’Z-; *all*-E – fucoxanthin) is flexible during the life-cycle of this phototrophs (Crupi *et al.*, 2013; Schüler *et al.*, 2017). *I. galbana* uses fucoxanthin as a primary pigment in the light harvesting complexes of the thylakoid membranes; low light exposure creates a sub-saturating scenario that during acclimation induces carotenoid synthesis to maximize light harvesting, resulting in the intracellular accumulation of fucoxanthin and β-carotene (Gómez-Loredo *et al.*, 2016; Mulders *et al.*, 2014). Additionally, the pigment composition of this species can vary upon strains tested. Ahmed *et al.,* (2014) isolated an Australian strain presenting 3205 µg/g (DW) of a putative violaxanthin and neoxanthin isomer, which is in agreement with the hypothesis developed by Lohr *et al.* (1999) that emphasizes that every algae species with the diadinoxanthyll cycle also possesses the violaxanthin cycle, the latter possibly acting as the biochemical precursor of the former regulated by the photon flux density (Fig. 10). Although working with diatoms, Lohr *et al*., (1999) suggested that when the photosynthetic apparatus is exposed to high light, a conversion of diadinoxanthin to diatoxanthin occurs which is later converted back to diadinoxanthin with low light exposure, leading to the synthesis of fucoxanthin. This theory is supported by Obata *et al.*, (2012) which also highlights the complex pigment dynamics of this species, by concluding that the cell is also capable of decreasing its chlorophyll *a* concentration under

photo-acclimation to high light exposure or during the light phase of photosynthesis, and decreasing it again during the dark phase upon cellular multiplication.

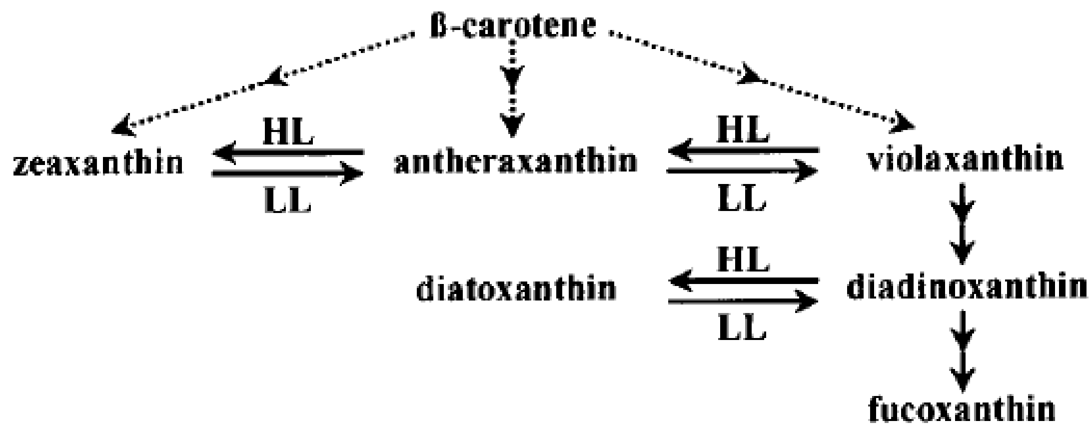


Figure 10 - Hypothetical coupling between the xanthophyll- and the diadinoxanthin- cycles leading to synthesis of fucoxanthin in diatoms as a response of photon flux density. HL = High Light LL= Low Light. Adapter from Lohr *et al.,* 1999.

Overall, one should keep in mind that the yield of all these biomolecules (lipids, amino-acids, vitamins, pigments and proteins) is entirely dependent on the multifactor interaction between the culture growth phase upon harvesting (e.g. lag, exponential or stationary phases), extraction methods used, the genetic variability, culture conditions, and the biomass drying method (Fabregas *et al.*, 1986; Gouveia *et al.*, 2008; Zhu *et al.*, 1997).

Nowadays, markets have turned to microalgae to provide a sustainable source of fucoxanthin, mainly because the current ones (e.g. brown seaweeds) do not accumulate as much of this pigment and require additional steps in extraction protocols to prevent poisoning by iodine, a byproduct of some macroalgae fucoxanthin extractions (Wu *et al.*, 2016).

## 2. Objectives

The aim of this master thesis was to develop and optimize an extraction protocol to produce fucoxanthin-rich fractions and associated biomolecules of interest from the microalga *Isochrysis galbana*. Primarily, the cultures were submitted to a novel technology of environmental modulation, the Algem labscale photobioreactors, to understand the optimal abiotic conditions for maximizing growth rate and infer on the bioaccumulation of these target compounds under different regimes. The carotenoid profile was assessed by diode array-high performance liquid chromatography (HPLC-DAD) and the ω-3 PUFAs were measured using gas chromatography coupled with mass spectrometry (GC-MS). Additionally, a novel liquid-liquid extraction system (LTPS) was tested using the industrially produced biomass of Necton to infer on its efficiency to extract fucoxanthin and DHA. The goal with the present work was to determine if: i) *Isochrysis galbana* could be cultivated in Algem labscale photobioreactors? ii) Possible identification of abiotic parameters leading to accumulation of these target compounds? iii) The suitability of the LTPS system in effectively extracting these molecules?

## 3. Methodology

### 3.1 ALGEM®: Environment Labscale Photobioreactors

At Necton S.A., a Portuguese microalgae producing company placed in Olhão since 1998, ALGEM® labscale photobioreactors (Fig. 11) were used to test the growth response of *I.galbana* to different sets of abiotic factors. These systems simulate environmental parameters in an expedite way, allowing an effective control of temperature, photoperiod, light intensity and mixing, while accurately measuring growth rate by spectrophotometry at 740nm. In order to optimize the growth of this strain and to infer on total amount of fucoxanthin, as well as on fatty acid profile, two weekly trials were performed focusing firstly on the annual variations (seasons) followed by testing a pH range of 7 to 8.5. Nutrients (e.g. Nutribloom® solution produced by Necton) were only added at the beginning of the cultivation period. The media concentration of nitrates was 4 mM.

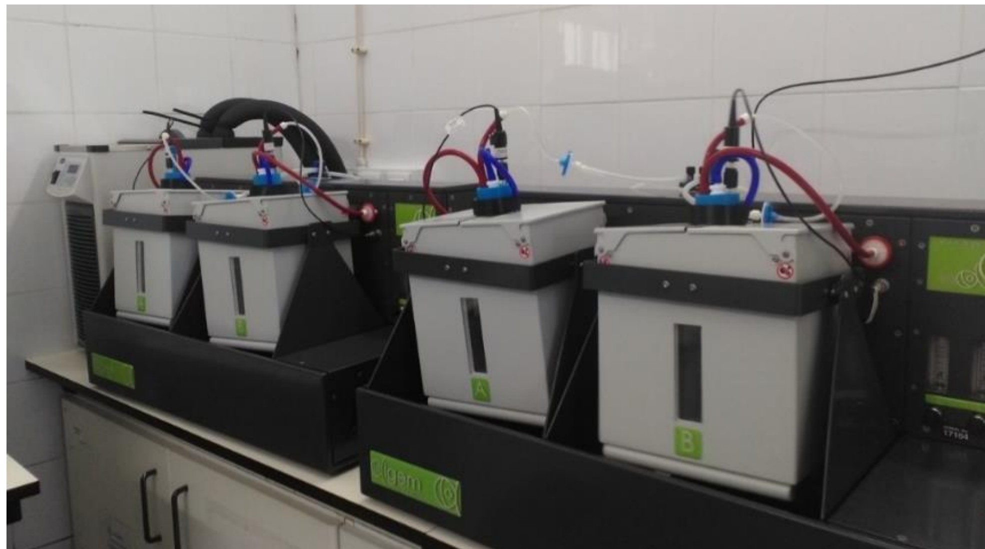


Figure 11- The Algem environment labscale photobioreactors coupled to a dedicated chiller to regulate the temperature inside each chamber.

ALGEM® photobioreactors software contains records of environmental conditions for each season worldwide (Fig.12). In the experiments, the GPS coordinates of Necton's facilities were used, to accurately simulate the seasonal abiotic conditions felt in Belamandil, Olhão. Each chamber was programmed to represent the environmental conditions of each season (Summer, Spring, Autumn, Winter) based on an average of all parameters (e.g. light intensity, temperature, photoperiod) measured in Olhão that are recorded in the database of the ALGEM® software.

The database considered the months from December to February as Winter season; from March to May as Spring season; from June to August as Summer season; and from September to November as Autumn season. During the course of the experiment, the cultures growth was followed spectrophotometrically, on a daily basis. One the second trial,the abiotic conditions used to test the cultures response to the pH range 7-8.5, were the ones that provided a higher growth rate in the first trial; table 2 describes the environmental parameters used in each experiment. In both trials the optical density of the initial inoculum was assessed using a UVmini-1240 spectrophotometer. Afterwards, the inoculum was diluted for a final volume of 2L from which it was equally divided for each chamber, as demonstrated in figure 13. The experiments lasted one week, after which the biomass in each chamber was individually centrifuged and the pellets stored frozen (-20ºC).

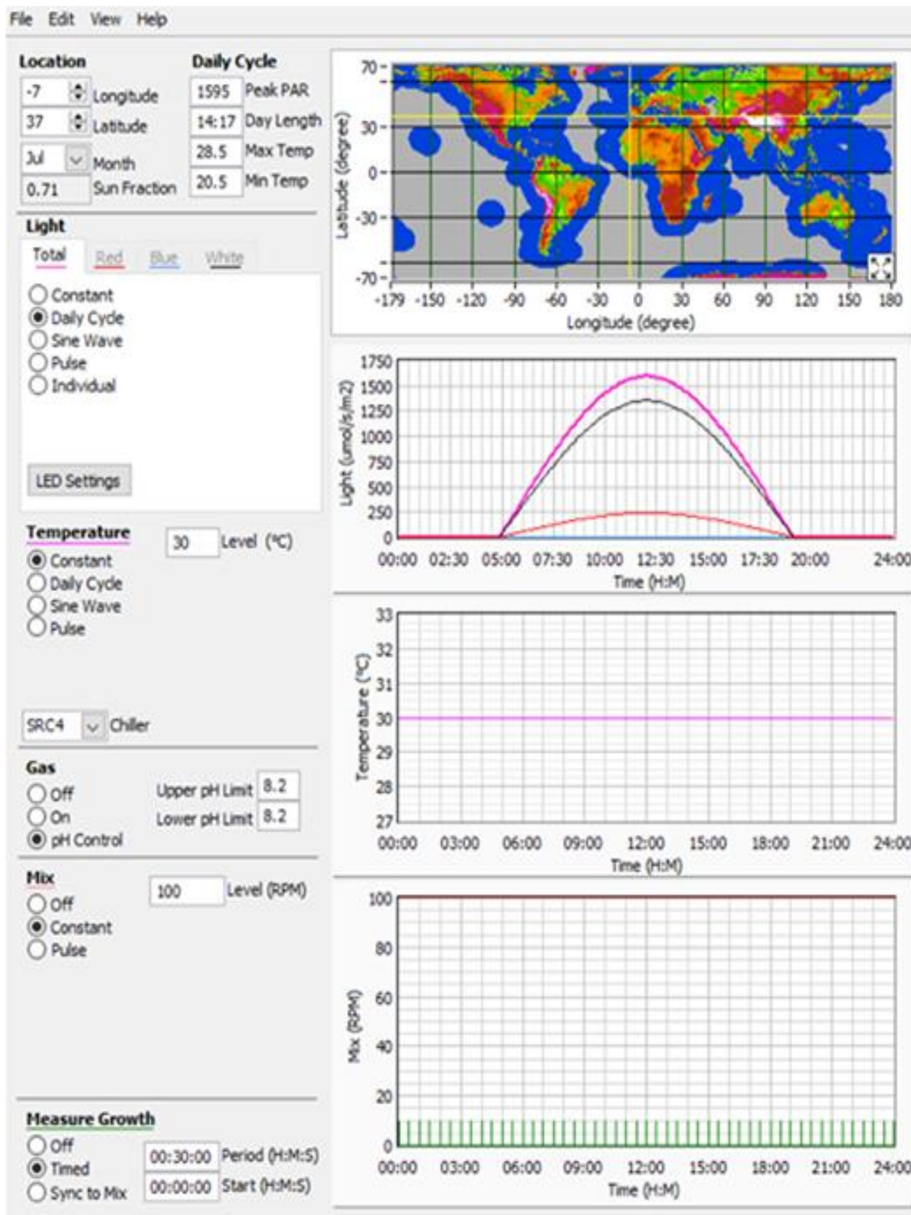


Figure 12 - ALGEM® photobioreactors software. The equipment has a database of climate records from the last 80 years and the interface allows the user to choose a GPS position. The abiotic parameters are then adjusted according to the chosen location. It also gives the possibility to create an abiotic profile by selecting different types of light (e.g. Red, Blue, White or Total), photoperiod and temperature either constant or in daily cycles. The pH control inside each chamber is individually regulated by $CO_2$ injection. Finally, the interface allows the user to assess the growth rate of the culture through a graphic representation of the absorbance (at 740 nm) at a desirable interval of time.

Table 2 – The abiotic conditions determined for the trials conducted in ALGEM® labscale photobioreactors. On the first trial (seasons) each chamber comprehended its unique set of environmental conditions. On the second trial (pH) the environmental profile selected was the one that provided the higher growth rate on the first trial.

| **Trial** | **Seasons** | | | | **pH** | | | |
|---|---|---|---|---|---|---|---|---|
| **Chamber** | **Summer** | **Spring** | **Autumn** | **Winter** | **7** | **7,5** | **8** | **8,5** |
| **Temperature (°C)** | 20.5 - 28.5 | 12.1 - 20.2 | 15.1 - 23.1 | 7.9 - 13.8 | 20.5 - 28.5 | | | |
| **Light Intensity ($\mu$mol $m^{-2}$ $s^{-1}$)** | 0 - 1600 | 0 - 1200 | 0 - 980 | 0 - 650 | 0 - 1600 | | | |
| **Photoperiod (Hours)** | 15 | 13 | 11 | 9 | 15 | | | |
| **pH** | 8 | | | | | | | |
| **Salinity (g/L)** | 33 | | | | 33 | | | |
| **Mixing (RPM)** | 60 | | | | 60 | | | |

Figure 13 – The different stages of preparation for an Algem incubation. A,B – Dilution of Necton's culture and inoculation inside a laminar flow chamber. C,D- Setup of Algem lid which is connected to the $CO_2$ injection system, air entrance and exit, as well as a pH probe. From here, the Erlenmeyers were placed inside each chamber to start the experiment.

### 3.1.1 Pigment Extraction and Analyzes

Pigments extraction and analyses was conducted for the biomass resulting from the Algem® trials, as well as, for the biomass produced outdoors in Necton's photobioreactors (PBR) in order to compare the fucoxanthin content. For this, 20 mL of seawater were added to defrosted biomass and the mixture homogenized for 10 seconds in a vortex homogenizer. From here, a small volume was transferred to new falcon tubes, depending on the overall concentration of each pellet; from the outdoor biomass 0.25 mL were used for the extraction whilst from the Algem® trials 0.5 mL were used. These new falcon tubes were centrifuged at 8000*g* for 5 minutes at 10ºC and the supernatant -salt water- was discarded. Following that, 3mL of acetone and 0.7g of glass beads (mesh 500-750 µm) were added to all tubes that were subsequently submitted to 2 minutes of vortex in order to promote the breaking of cellular walls by the glass beads while the acetone extracted the pigments. During this process, all tubes were kept on ice to freeze the cell walls and potentially increase the extraction yield. Afterwards, the tubes were centrifuged and the supernatant was collected to different vials. This process was repeated 3 times to ensure full pigment extraction. The vials were later dried in nitrogen gas flow (Fig.14A) and all samples were resuspended in 700 µl of methanol HPLC grade and filtered to new vials using 0.25 µm PTFE filters (Fig. 14B).

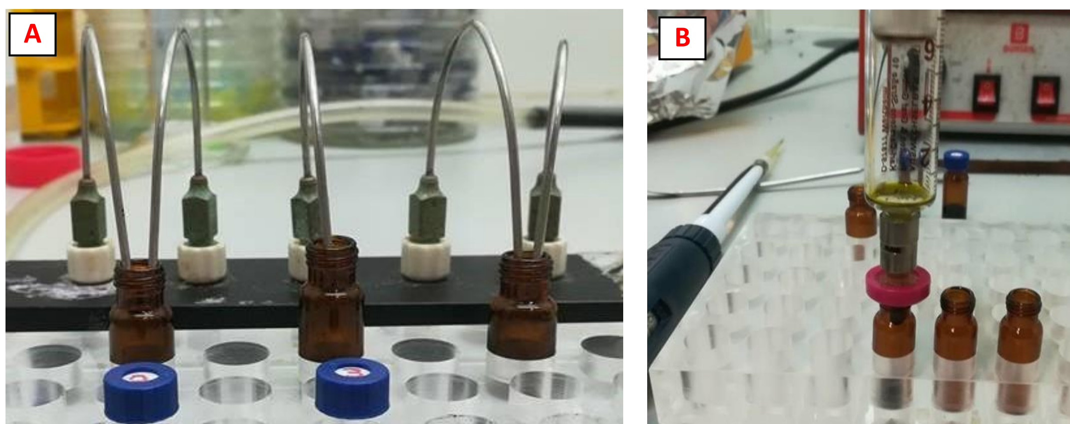


Figure 14 – A: The process of drying the samples using a gentle flow of Nitrogen. B: The resuspension of the samples using a syringe and a PTFE filter.

### 3.1.2 Determination of fucoxanthin by HPLC

The vials containing the recently resuspended and filtered samples were analyzed in a Dionex 580 HPLC System (DIONEX Corporation, United States) equipped with a PDA 100 Photodiode-array detector, P680 Pump, ASI 100 Automated Injector and STH 585 column oven, using a LiChroCART® RP-18 (5µm, 250x4 mm, LiChrospher®) column

and Chromeleon® software. The mobile phase consisted of 9:1 (v/v) acetonitrile:water as solvent A, and, ethyl acetate as solvent B and the gradient program applied was: 0–16 min, 0–60% B; 16–30 min, 60% B; 30–32 min 100% B and 32-35 min change to 100% A at a flow rate of 1 ml/min. The injection volume was 100 µl and the temperature was maintained at 20°C. Fucoxanthin was detected and quantified at 450 nm using a calibration curve made by a serial dilution (400-12.5 µg/mL) from a fucoxanthin standard (Fig. 15).

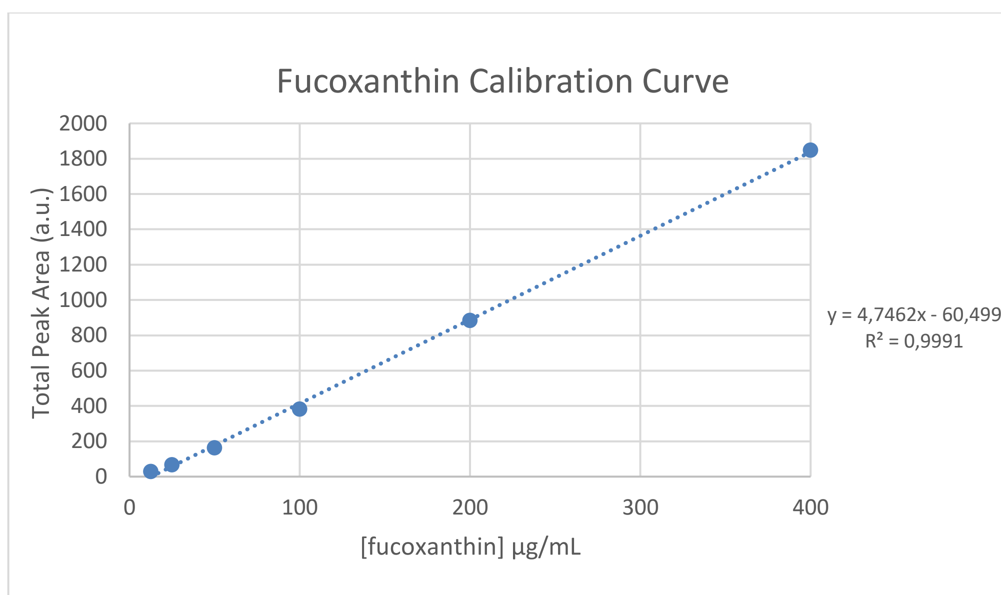


Figure 15 – The calibration curve resulting from the serial dilution of fucoxanthin, measured in HPLC.

To facilitate the evaluation of the extraction efficiency, a spectrophotometric method was developed to estimate the amount of fucoxanthin in the extracts, since spectrophotometry is a cheaper and less time-consuming technique to quantify this pigment. Hence, a calibration between the absorbance of the extracts at 450 nm and the fucoxanthin content of those samples measured by HPLC was performed (Fig. 16). The resulting correlation coefficient was high ($R^2$= 0.96). For this reason, it was decided to quantify the fucoxanthin content of the ethanolic extractions by spectrophotometry.

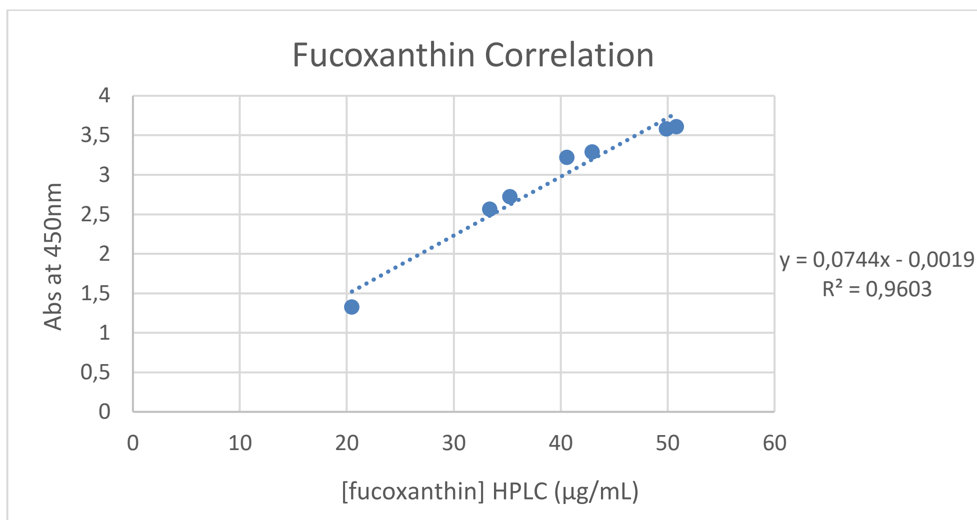


Figure 16 – Correlation between the fucoxanthin present in the samples measured in spectrophotometry at 450 nm and HPLC.

### 3.1.4. Gas Chromatography – Mass Spectrometer

Fatty acid methyl esters (FAME) were produced following a modified protocol from Lepage & Roy (1984). The pellets were resuspended in 1.5 mL of methanol with acetyl chloride (20:1, v/v) and 1 mL of hexane, transferred to derivatization vials and homogenized using an Ultra _Turrax in two cycles of 60 and 30 seconds, always on ice. From here the vessels were placed in a water bath at 70°C for 60 minutes (Fig.17).

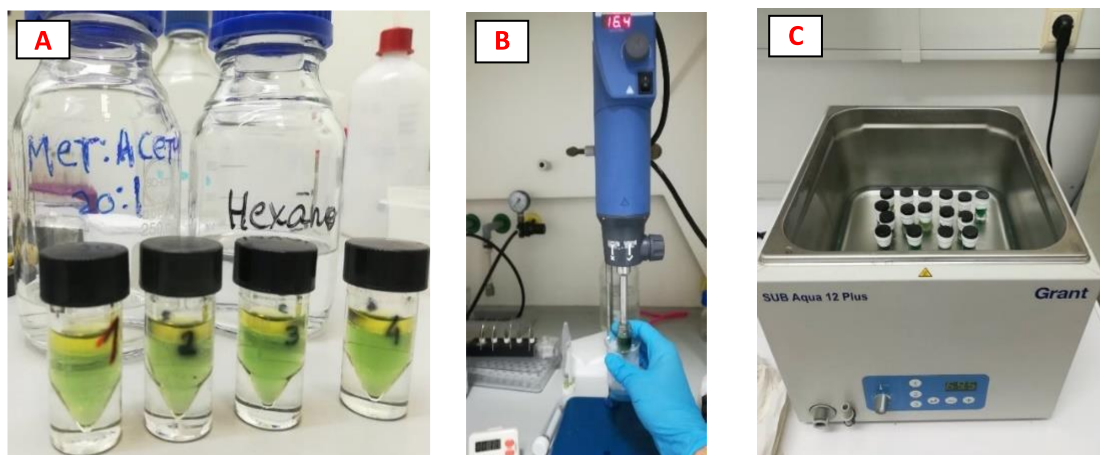


Figure 17 – A: Derivatization vessels with the samples and the solvents. B: The process of breaking the cells using the Ultra turrax while keeping the samples in ice to prevent excess heat and possible degradation of compounds. C: The hot water bath were the samples were placed after being wrapped in Teflon to minimize losses by evaporation.

Later the samples were allowed to rest in ice and the content transferred to centrifuge tubes. To clean the derivatization vials, 1mL of distilled water and 4 mL of hexane were added and transferred back to the centrifuge tubes. Then, the tubes were centrifuged for 5 minutes at 2000*g* and the hexane fraction transferred to new glass tubes. This step was performed twice to minimize FAME losses. Following, anhydrous sodium sulfate ($Na_2SO_4$) was added to remove water residues and the hexane solution was filtered with Whatman 0.25 µm PTFE syringe filters (Fig 18A and B). Afterwards the samples were dried under a gentle nitrogen flow and finally resuspended in 500 µl gas chromatography-grade hexane and transferred to vials after filtration.

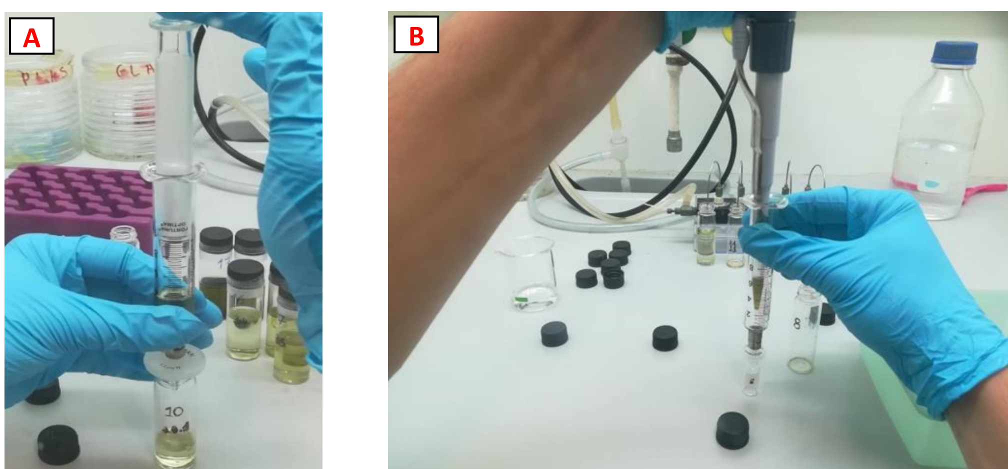


Figure 18 – A: The first filtration of the hexane fraction. B: After a gentle flow of nitrogen to dry the samples, they were resuspended and transferred to chromatography vials.

The FAME profile was assessed using a Bruker GC-MS (Bruker SCION 456/GC, SCION TQ MS, Fig. 19 A,B) equipped with a ZB-5MS capillary column using helium as a carrier gas. The temperature was 60°C (1 min), 30°C $min^{-1}$ to 120°C, 5°C $min^{-1}$ to 250°C, and 20°C $min^{-1}$ to 300°C (2 min). Individual calibration curves were made for each FAME using the standard 37 FAME Mix (Supelco).

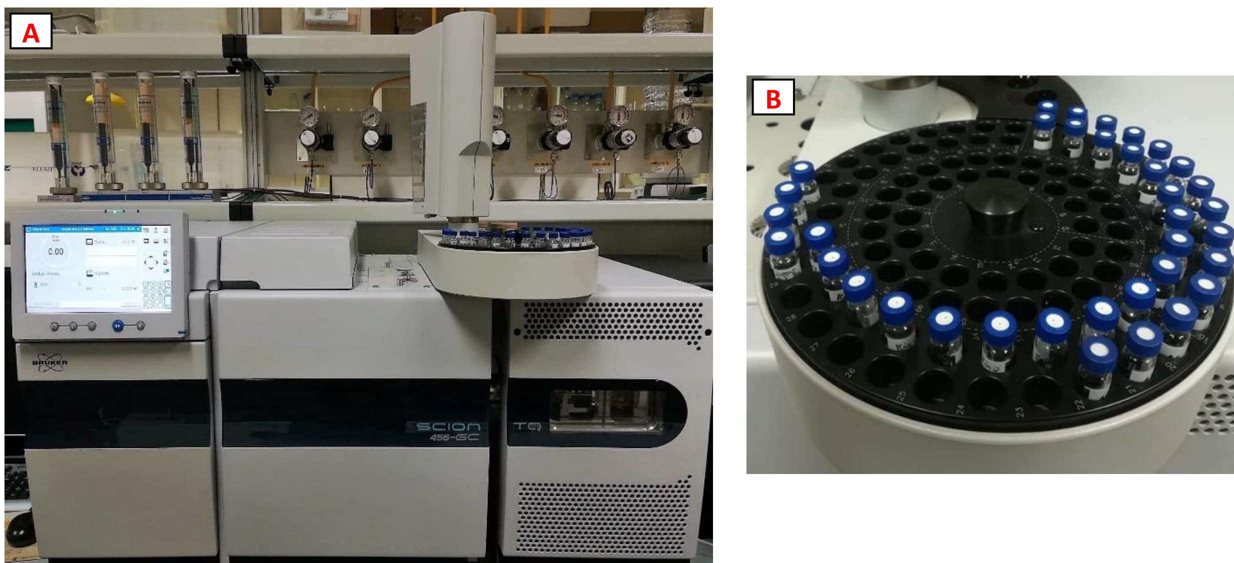


Figure 19 – A,B: The gas chromatography equipment with the coupled auto sampler.

### 3.2 Biomass production: Necton's photobioreactors (PBR)

*Isochrysis galbana* was industrially produced in Necton's tubular photobioreactors (PBR). Figure 20 summarizes the cultivation and harvesting procedures starting with the inocula which were kept in the laboratory under controlled temperature and supplied with aeration enriched with $CO_2$. From here, it is usually transferred to the outside green wall systems ranging from 100 to 800 L. Once the desired optical density was achieved for these systems, the cultures were transferred to the 15 $m^3$ tubular PBR. Throughout this scale-up procedure, the cultures were checked daily for contaminations, using a microscope, and for overall healthiness by evaluating cellular structure and motility. Additionally, nutrients such as industrial iron and Nutribloom® were supplied weekly. The harvest procedure was conducted using an industrial centrifuge and the biomass was packed and stored frozen (-20ºC).

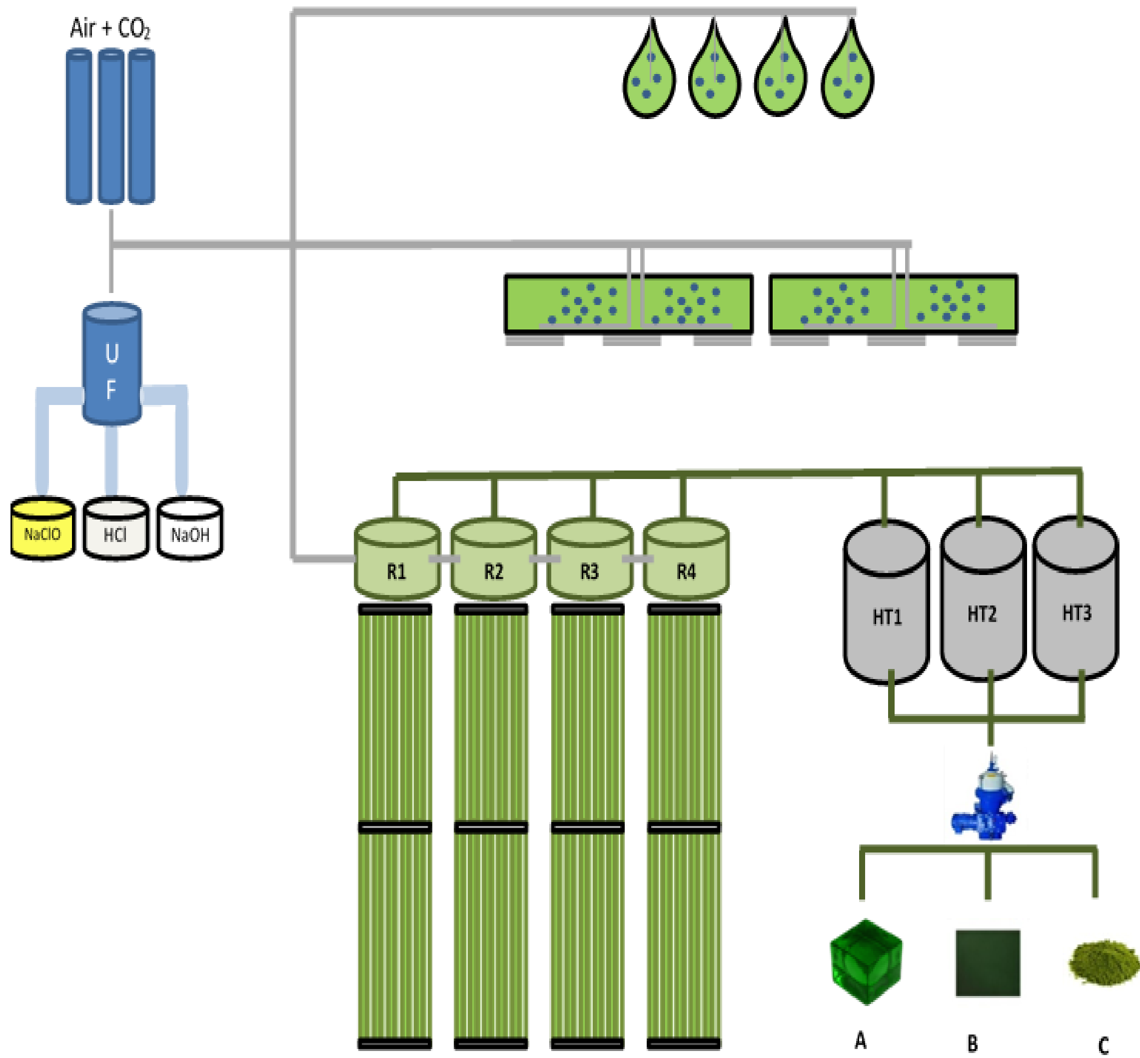


Figure 20– Schematic representation of the industrial process of microalgae production, from the inocula to green wall panels (GWP) and later to tubular photobioreactors (R1-4). The reactors are connected to the harvest tanks (HT1-3), which are in turn connected to the centrifuge. Each reactor and tank has its own pumps and a set of both manual and electronic valves. The aeration system is equipped with a valve and timer that regulates the frequency and duration of $CO_2$ injections. The ultra-filtration (UF) system purifies the water according to industrial standards. After harvesting, the final product (A: GreenFormula®; B: ICE-Concentrated frozen paste; C: Lyophilized biomass) is packed and stored to be sold.

### 3.2.1 Extraction Optimization: Effect of Biomass/Solvent Ratio and Time

In order to optimize the extraction of fucoxanthin from *I. galbana*, the microalgal biomass was submitted to different combinations of solvent/biomass ratios and times of extraction aiming to identify which condition would offer a higher yield of fucoxanthin. Firstly, biomass/solvent ratio trials were assessed by testing 10g of wet biomass (WB) with 10 mL (ratio 1:1), 20 mL (1:2) and 40 mL (1:4) of ethanol (96%) and mixing at room temperature for 2 hours. Subsequently, to identify the most suitable time of extraction the trials were conducted at 1:3 ratio (10g WB : 30 mL ethanol) for 1h, 3h and 4h of mixing at room temperature. Triplicate trials of each condition were performed. To prevent oxidative damage of carotenoids and lipids, butylated hydroxytoluene (BHT) was added to every extract. Furthermore, to minimize pigment degradation by light exposure all extracts were wrapped in aluminum foil while mixing (Fig. 21).

Figure 21 - The extracts mixing in individual stirrers at room temperature. The extracts were wrapped in aluminum foil to prevent pigment degradation.

After extraction, all extracts were filtered twice –in the dark – under vacuum (Fig.22A); a primary filtration was done using a filter paper with mesh size of 20-25 µm followed by a second filtration using a 0.7 µm mesh size filter (Fig.22 B). The result is a purified microalgal extract.

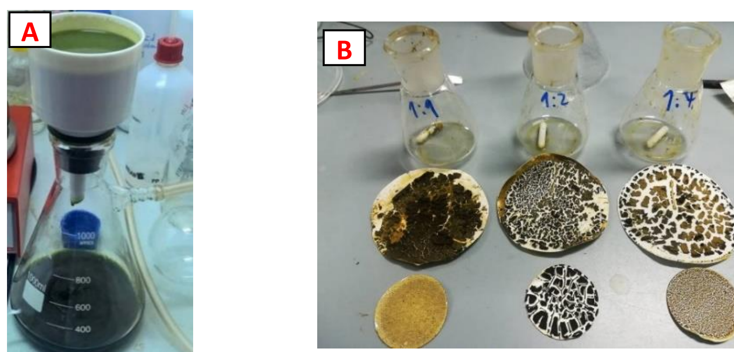


Figure 22 – A: The vacuum filtration system consisting in a funnel coupled with a kitasato connected to a water flow hose. B: The two types of filters used and the biomass recovered.

At this stage, 1mL of each extract was collected to determine the amount of fucoxanthin by spectrophotometry. The extract was then transferred to a pre-weighed round bottom flask and concentrated in a rotary evaporator under controlled temperature (40°C) at reduced pressure (Fig.23 A,B), producing a microalgal oil containing all the desirable compounds.

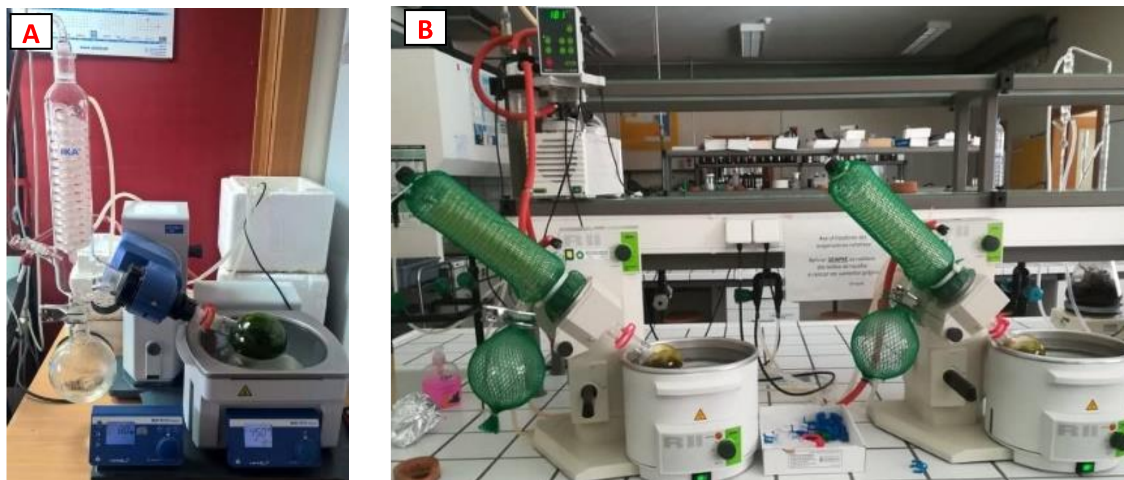


Figure 23 – A,B: The rotary evaporators used for concentrate the extracts.

### 3.2.2. Liquid Three Phase System (LTPS)

Once the best extraction conditions (solvent:biomass ration and time of extraction) were determined, a large amount of extract was prepared to test a Liquid Three Phase System (Fig.24). The main goal was to identify which proportion of solvents (hexane/water, H/W) used in the LTPS method provided a higher amount of fucoxanthin and a higher quantity of colloidal fraction, since this fraction most likely contains higher amounts of DHA, which is expected to be mostly present in phospholipids. All fractions will be later biochemically characterized in terms of pigment composition using High Performance Liquid Chromatography (HPLC) and fatty acid profile by Gas Chromatography coupled with Mass Spectrometry (GC-MS) as described above.

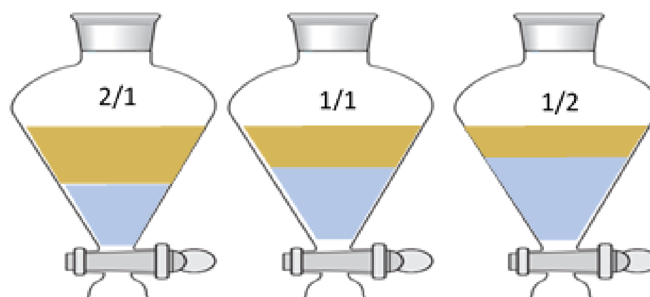


Figure24- A schematic representation of the proportions (Hexane/Water, H/W) on each solvent system inside a separation funnel. The brown color represents hexane and the blue is Water.

The first proportion tested was 1:1 followed by 1:2 and 2:1 (Hexane:Water, H/W), for a final volume of 240 mL in all proportions. For all, the procedure started from a concentrated extract (Fig. 25A) where hexane and water were gradually added to cleanse the round bottom flask walls and the content was gradually poured inside a separation funnel. The flask was vigorously shaken for 30 seconds creating a large emulsion (Fig. 25B,C).

Figure25- A: The concentrated extract after being dried in the rotary evaporator. B: The content of the balloon was cleansed with the solvents and the mixture shaken in the separation funnel. C: The resulting emulsion after being allowed to rest.

Afterwards, the content of the separation funnel was evenly separated to 6 falcon tubes and centrifuged at 10,000 *g* for 15minutes at 25° C. The result is the formation of a thick colloidal layer (stable emulsion) in between both solvents (Fig. 26).

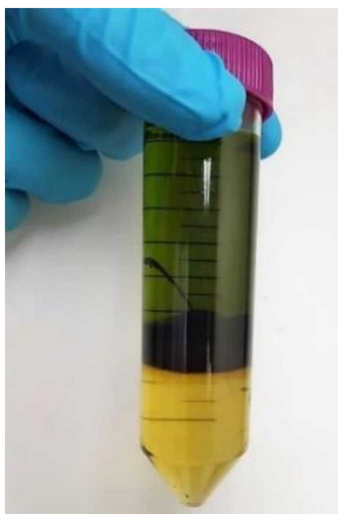

Figure26- The formation of the colloidal layer trapped between hexane and water fractions.

The colloidal layer from all the falcon tubes, was collected by filtration using a filter paper (mesh size: 20-25 μm). A different filter was used for each Falcon tube (Fig.27A,B).

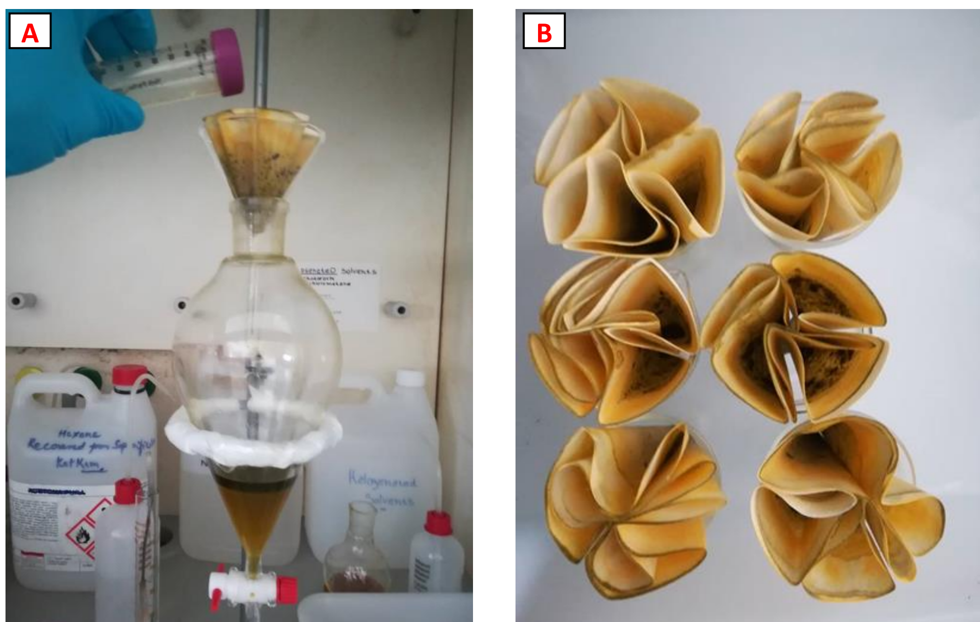


Figure27- A: The technique of collecting the colloidal fraction while keeping the same proportion of solvents. B: The filters containing the colloidal and waiting to dry for the next stage.

This step allowed the solvents to pour gradually inside the separation funnel preserving the original proportion while the colloidal fraction was isolated. The samples for the spectrophotometry and GC-MS were collected at this stage by taking 1 mL of hexane (Fig. 28A) and water (Fig. 28B) from inside the separation funnel to vials.

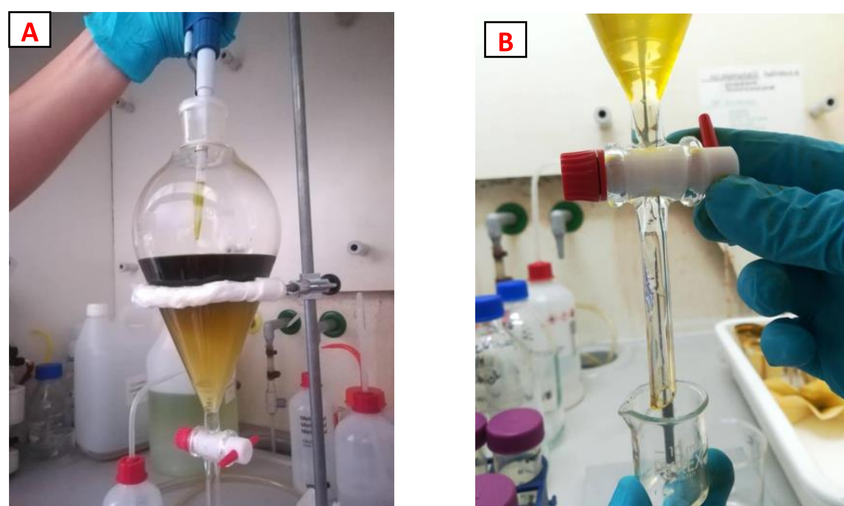


Figure28- A: The hexane samples were collected by pipetting 1 mL from the top of the separation funnel. B: The water samples were collected by pouring a small portion to a goblet.

The filters containing the colloidal fraction were left to dry and the colloidal fraction was recovered using a chloroform:methanol (2:1) solution (Fig. 29 A,B).

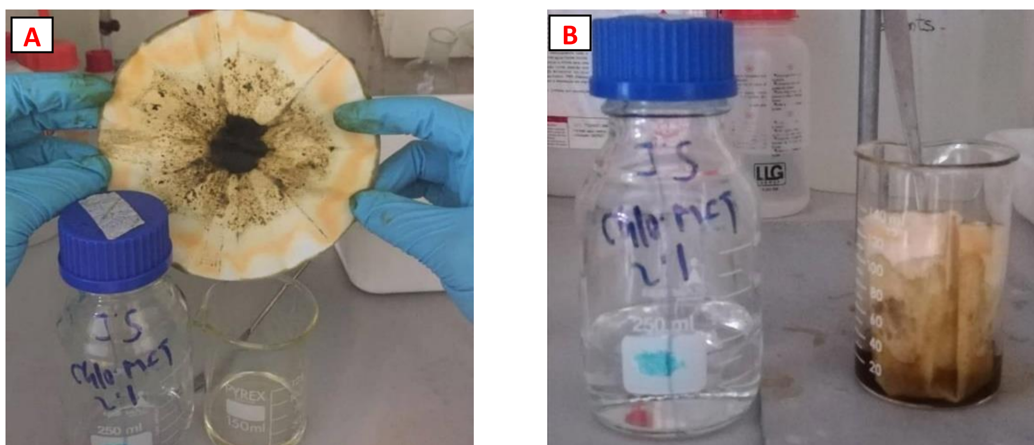


Figure 29- A: The filter containing the dried fraction of colloidal. B: The cleaning process of the filters using a choloform:methanol solution.

Figure 30A shows the different phases obtained after the fractionation of the ethanol extract using the LTPS technique. Each fraction was then dried in a rotary evaporator and the amount of yield fraction determined (Fig. 30B). The process was repeated twice for each proportion tested. The amount of fucoxanthin was assessed in all fractions by spectrophotometry. Additionally, the fatty acid composition was also assessed following the protocol described above.

Figure30- A: All fractions of the LTPS individualized. B: The fractions after concentration in a rotary evaporator.

## 4. Results and Discussion

The ALGEM labscale photobioreactors are a leading-edge technology with an enormous potential for strain improvement. The results here presented show that this equipment may be extremely useful to improve the efficiency in the cultivation of *Isochrysis galbana*. The annual variations trial (Fig. 31) revealed the capacity of *I. galbana* inoculum to withstand temperatures ranging from 15°C in Autumn up to 28°C in Summer and light intensities varying from 980 µmol $m^{-2}$ $s^{-1}$ to 1600 µmol $m^{-2}$ $s^{-1}$. The growth rate was higher in the Summer season followed by Autumn and Spring in comparison with Winter when the abiotic conditions compromised the culture growth. This trial allowed to identify Summer and Autumn seasons as the most promising for the cultivation of *Isochrysis galbana* and proved that its industrial scale-up during Winter is not feasible because it led to a totally culture collapse, most likely due to the induced temperature shock. The results come in agreement with the literature in terms of growth rate as a response of temperatures ranging from 10-35 °C (Renaudl *et al.*, 1995) or 20-25 °C (Kain *et al.*, 1958) with an increased growth at temperatures greater than 20°C. These results are also confirmed by Kaplan *et al.*, (1986) and more recently by Marchetti *et al.*, (2012) whose work identified 27°C and 30°C, respectively, as the optimal temperatures for cultivation of *Isochrysis galbana*. Regarding the light intensity tested in this trial, the values were always higher than the ones reported from these authors which ranged from 80 to 780 µmol $m^{-2}$ $s^{-1}$. This outcome provides a better understanding on the specificity of this strain regarding the identification of the best abiotic conditions for maximizing its growth rate, offering a helpful tool in Necton’s routine by establishing the best season for this strain’s production.

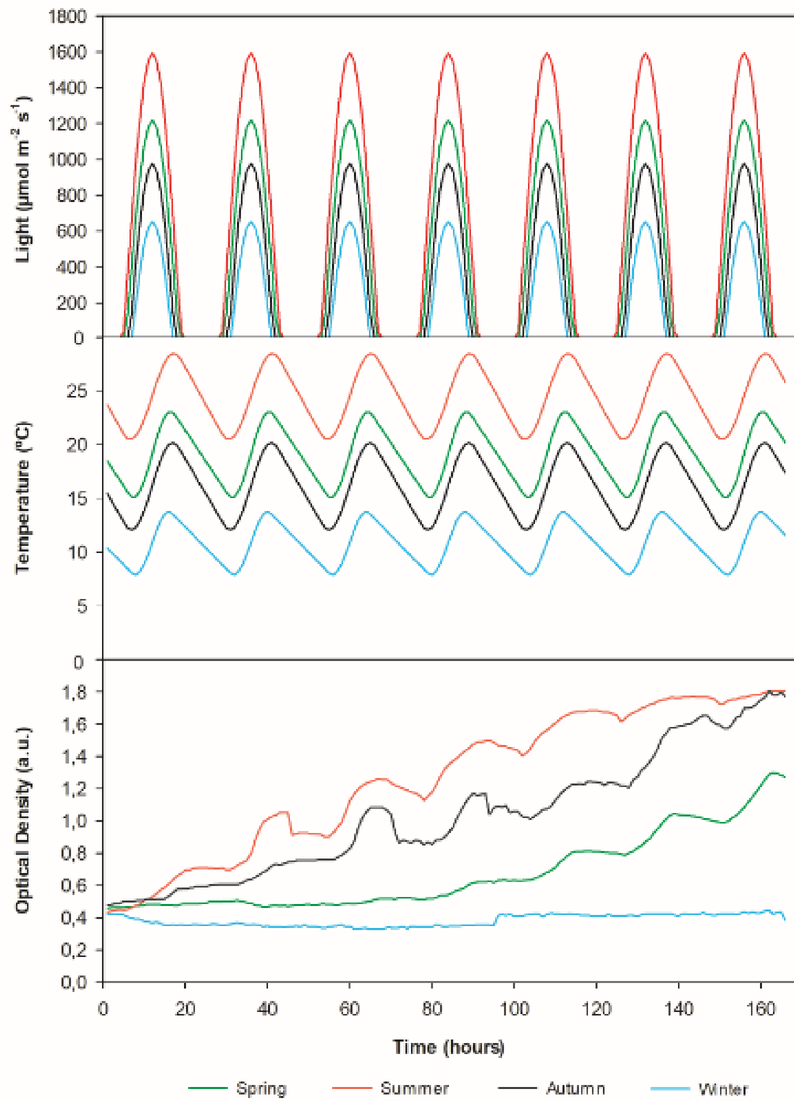


Figure 31 – The response of *Isochrysis galbana* cultures to the seasonal variations felt throughout the year in Olhão, Algarve.

The pH trial (Fig. 32) used the summer conditions for light intensity (1600 µmol $m^{-2}$ $s^{-1}$) and temperature (20.5-28.5°C) while varying the pH between 7 and 8.5. The strain's growth was similar for all pHs' used, revealing that this culture tolerates a relatively wide pH range. This outcome is somehow surprising since some authors experienced either a growth rate decrease (Kain *et al.*, 1958; Kaplan *et al.*, 1986) or a decrease in the photosynthetic efficiency (Ippoliti *et al.*, 2016) and consequent lower growth rate as a result of having a culture medium pH higher than 8. An explanation for this outcome may rely on the combined influence of all the other parameters which were set for providing optimal cultivation conditions, coupled with the predicted differences between strains. In fact, our strain also sustained temperatures far greater than the ones reported in literature

which might indicate that the inoculum itself has already adapted to the abiotic conditions of Algarve.

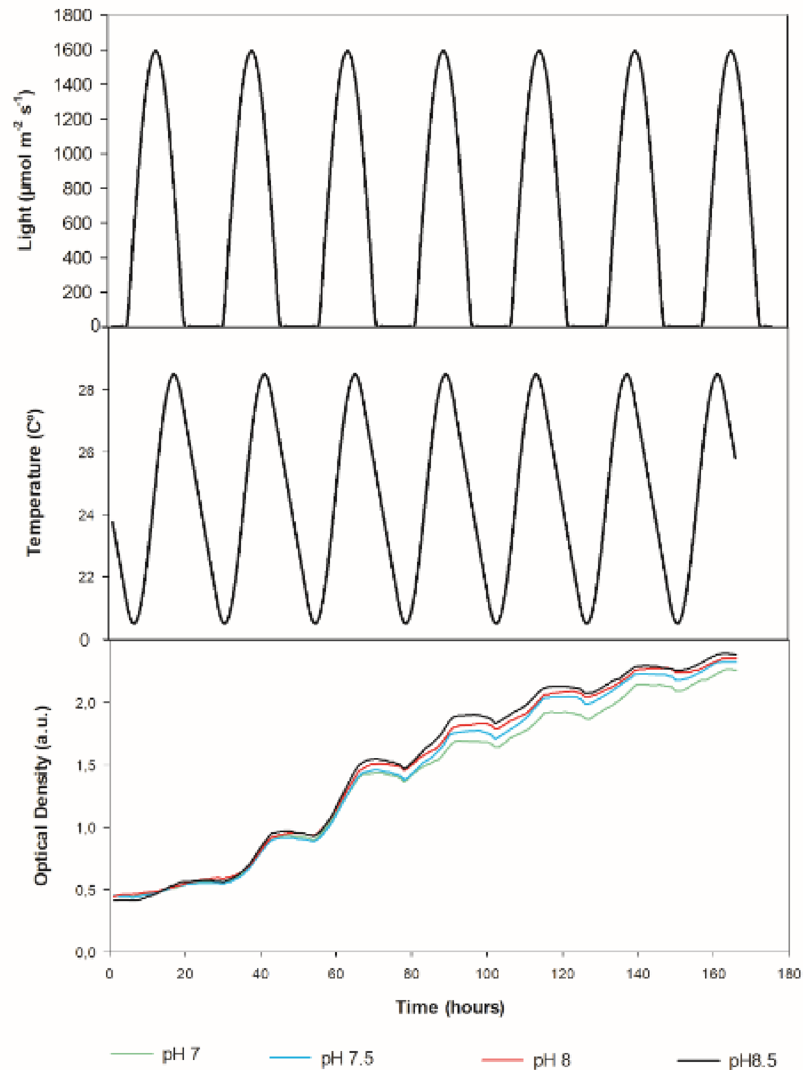


Figure 32 – The *Isochrysis galbana* culture response as a consequence of a pH ranging from 7 to 8,5. Optimal light and temperature were determined from the previous trial.

On an industrial perspective both these graphics provide remarkable information regarding the production and resource managements. The temperature regulation of the tubular PBR is controlled by a probe installed on the reactor which activates a sprinkler system every time the maximum temperature is achieved. By identifying the maximum tolerated temperature for any given culture this system can be adjusted potentially saving hundreds of liters of water per week. On the other hand, the control of the culture pH is done by $CO_2$ injection every time the probe achieves its maximum value. Understanding

the edges of pH tolerance can improve $CO_2$ consumption rate of any industrial production by decreasing its costs.

The impact of annual variations on fucoxanthin content was assessed for all seasons except Winter due to the collapse of the culture (Fig. 33A). The trial was performed at a standard pH 8, which is the one that consumes less $CO_2$. Results revealed that *I. galbana* produces significantly higher amounts of fucoxanthin in Autumn and significantly lower in the Summer season ($p<0.05$). Regarding the pH trial (Fig. 33B), the results present an increase of fucoxanthin at pH 7 and 7.5 (≈ 7.88 mg/g DW) with a 37% reduction at pH 8 up to a 52% reduction at pH 8.5.

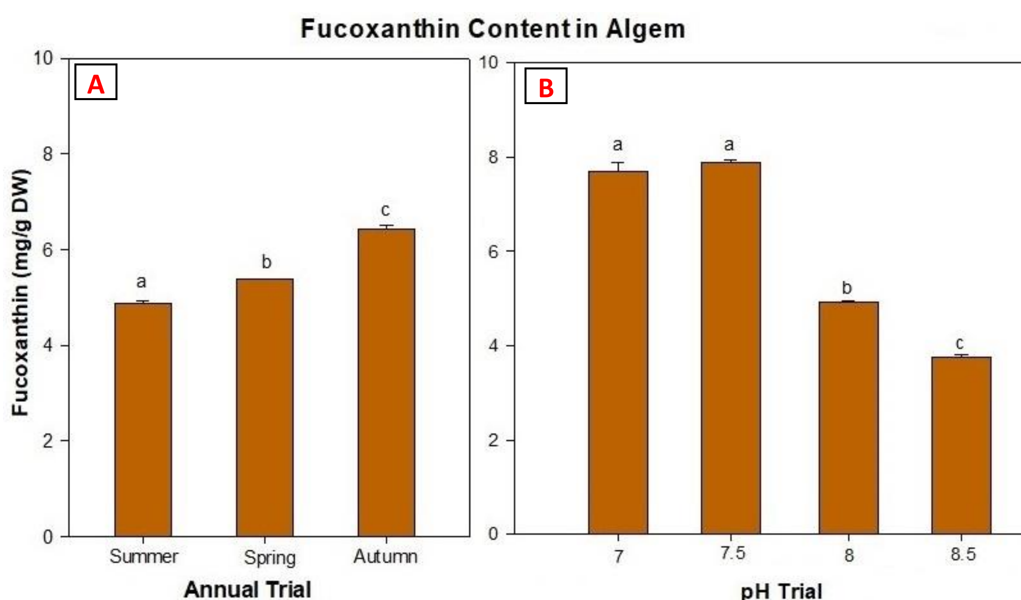


Figure 33 - Quantification of fucoxanthin from the Seasons - Annual Trial (A) and the pH trial (B). Values plotted are the average of two samples from the same trial.

The original biomass produced in Necton's outdoor PBR during summer conditions had a fucoxanthin content of 17.69 ± 1.06 mg/g DW, much higher than those obtained in the ALGEM®. However, these values are not comparable, since the light path is very different between both equipment. Instead, ALGEM® labscale photobioreactors and industrial tubular photobioreactors should be seen as complementary in an industrial symbiose to develop, optimize and scale-up a strain capable of accumulating high-valuable compounds such as fucoxanthin and DHA, or, enhance any other characteristic that fulfills a market need and can be manipulated by shifting abiotic parameters.

The positive influence of low light (15 - 100 µmol $m^{-2}$ $s^{-1}$) on fucoxanthin synthesis is well documented, but surprisingly, in all samples tested the values for fucoxanthin were far higher than the ones reported for such high irradiances (Galasso *et al.*, 2017; Gómez-Loredo *et al.*, 2016; Kim *et al.*, 2012; Mulders *et al.*, 2013; Schüler *et al.*, 2017). In fact, some of these authors reported a drastic reduction of fucoxanthin content when cultures were exposed to light intensities higher than 200 µmol $m^{-2}$ $s^{-1}$, which in theory should be expected considering the photosynthetic role of fucoxanthin. Nonetheless, our results show a significant increase of fucoxanthin as a consequence of decreasing light intensity from 1500 µmol $m^{-2}$ $s^{-1}$ in summer conditions, to 980 µmol $m^{-2}$ $s^{-1}$ in autumn, confirming a cellular behavior similar to the one reported in literature.

Salinity and nutrient depletion represent other stress factors for microalgae cellular membranes with metabolic implications ultimately affecting, either the ratio of total carotenoids to total chlorophylls or, by increasing the accumulation of lipid droplets also known for being a pre-requisite for carotenoid accumulation (Galasso *et al.*, 2017; Lohr *et al.*, 1999; Mulders *et al.*, 2013). The first is generally a result of shifting the regulation of fucoxanthin precursors like the diatoxanthin-diadinoxanthin cycle, which is normally regulated by light intensity, although it has been reported to shift also upon nitrogen or sulfur depletion. In Necton, microalgae are cultivated using Nutribloom® solution, which has been optimized by the company for the cultivation of different microalgae species (e.g. *Nanochloropsis* sp., *Tetracelmis chuii*, *Phaeodactylum tricornutum, Isochrysis galbana*). This might be an advantage in comparison with other culture mediums used such as F/2 or Conway specially depending on the overall N/P ratio (9:1, Nutribloom®) known for playing a crucial role in the regulation of the biosynthesis of lipids and carbohydrates (Batista *et al.*, 2013; Santos-Sánchez *et al.*, 2016).

Nonetheless one should also consider the impact of extraction protocols on the outcome of fucoxanthin concentration, in this case a novel protocol was tested using glass beads and acetone for pigment extraction, which can also be a source of variability when comparing our data with that from the literature. This fact addresses the need to establish a suitable protocol for fucoxanthin extraction, one that allows different research groups to effectively compare their data (Table 3). Additionally, information of fucoxanthin content is scarce and comparisons are impossible when different authors use different protocols and present data in different non-convertible units.

Although there is a general common acceptance of the primary metabolic mechanisms leading to carotenoid genesis, after the work of Lohr et al., (1999), the exact

metabolic pathways within the diatoxanthin-diadinoxanthin cycle of Haptophytes remain to be fully understood (Mulders *et al.*, 2014).

Table 3- The currently available and comparable data of fucoxanthin content in mg/g DW.

| Species/Strain | Pigment | Quantity (mg/g DW) | References |
|---|---|---|---|
| Isochrysis aff. galbana CCMP1324 | Fucoxanthin | 18.2 ± 0.54 | Kim *et al.*, (2012) |
| Isochrysis galbana KMMCC-12 | | 6.0 ± 0.28 | |
| Isochrisys sp. F&M-M36 | | 17 ± 3 | Crupi *et al.,* (2013) |
| Isochrysis galbana | | 9.7 ± 0.58 | Ryckebosch *et al.*, (2013) |
| Isochrysis sp. | | 17.7 ± 1.06 | This thesis |

**Fatty Acid Composition of ALGEM Trials**

The fatty acid profile of Algem samples is presented in Table 4. Results revealed higher bioaccumulation of oleic acid (C18:1) at pHs 7 and 7.5, which drastically decreases at pHs 8 and 8.5. This decrease in oleic acid is accompanied by a sharp increase in DHA (6.8% at pH 7.5 and 16.7% at pH 8.5). These results are very interesting since DHA is one of the most relevant and high-value fatty acids. This evidence, is in agreement with the pathway described by Khozin-goldberg *et al.,* (2011) and Martins *et al.,* (2013) where C18:1 is the major precursor for PUFA synthesis. In fact, the percentage of PUFA is significantly higher in the pH treatments where oleic acid is lower, in comparison with pH 7 and 7.5 where the storage of C18:1 led to a decrease of the overall PUFA content, confirming the reported FA metabolism.

The annual variation trial presents very similar FA profiles when comparing Summer and Spring seasons where the main FA precursors, oleic and linoleic acids, are present in higher concentrations leading to a smaller amount of DHA in comparison with Autumn season. Once again, the Autumn FA profile confirms the described FA metabolism by having the lowest amount of C18:1 and the biggest accumulation of DHA in the annual variations trial. The outcomes of both trials raise the need to a better understanding of the pathway leading to accumulation of oleic acid since they both revealed high percentages for both palmitic (C16:0) and myristic (C14:0) acids which enhances the necessity to comprehend the full spectrum of metabolic synthesis within the

fatty acid dynamics. Cultures from all trials were harvested still in exponential phase, which decreases the variability in the fatty acid profiles and allows a better comparison as they are known to demonstrate different concentrations as a result of harvesting the culture at different cellular growth phases.

Table 4- FAME composition of Algem Trials. Values represent the average of two measurements of each treatment and are displayed as percentages of total fatty acid (TFA) concentration. Only fatty acids above 1% of TFA concentration are displayed.

| Fatty Acid Composition of Algem Trials (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| FA | Common Name | pH | | | | Seasons | | |
| | | 7 | 7.5 | 8 | 8.5 | Summer | Spring | Autumn |
| C14:0 | Myristic acid | 25.9 | 26.3 | 28.8 | 31.2 | 40.1 | 41.8 | 39.7 |
| C16:1 | Palmitoleic acid | 8.5 | 7.9 | 11.4 | 9.0 | 9.2 | 9.1 | 10.4 |
| C16:0 | Palmitic acid | 24.5 | 25.9 | 29.9 | 25.3 | 25.8 | 26.68 | 26.4 |
| C18:3 (n-3) | Linolenic acid | 1.5 | 1.1 | 1.6 | 2.7 | 2.2 | 1.85 | 3.5 |
| C18:2 (n-6) | Linoleic acid | 4.2 | 4.5 | 6.0 | 5.7 | 5.8 | 5.81 | 5.1 |
| C18:1 | Oleic acid | 22.0 | 21.2 | 4.1 | 3.8 | 4.1 | 4.0 | 1.8 |
| C18:0 | Stearic acid | 2.4 | 2.7 | 3.1 | 1.2 | <1 | <1 | <1 |
| C20:4 (n-6) | Arachidonic acid | <1 | <1 | 1.7 | 1.1 | <1 | <1 | <1 |
| C20:5 (n-3) | Eicosapentaenoic acid | 1.6 | 1.5 | 3.0 | 2.2 | <1 | <1 | <1 |
| C22:6 (n-3) | Docosahexaenoic acid | 7.6 | 6.8 | 9.0 | 16.7 | 10.6 | 9.3 | 11.9 |
| | ∑SFA | 54.6 | 56.1 | 63.3 | 58.7 | 67.36 | 69.72 | 67.21 |
| | ∑MUFA | 30.5 | 29.2 | 15.4 | 12.8 | 13.31 | 13.08 | 12.18 |
| | ∑PUFA | 15.6 | 14.8 | 21.2 | 28.5 | 19.33 | 17.20 | 20.61 |

Literature comparisons should have into account the metabolic differences within strains as well as the cultivation conditions with regards to light intensity, temperature, salinity and culture growth phase upon harvesting which ultimately dictates the fatty acid profile of any given microalgae mainly because all the fatty acid synthesis pathways are intrinsically dependent of external stimulus (Batista *et al.*, 2013; Custódio *et al.*, 2014; Renaud *et al.*, 1999; Santos-Sánchez *et al.*, 2016). The results of both Algem trials are in agreement with the values reported by Batista *et al.,* (2013) and Renaud *et al,.* (1999) although the Algem trials were performed at higher salinity and light intensity which can justify the differences in PUFA and SFA. The idea of low light, nutrient repletion and low salinity for fatty acid production in Haptophytes is also supported by Schüler *et al.*, (2017) whose remarkable and extensive work addressed the tremendous variability present in different species by reviewing the most relevant abiotic conditions for fatty acid production.

On a industrial prespective, these trials and the analitical characterization resulting from them represent a valuable step towards a better understanding of the synergy between multiple enviromental variables that can be manipulated and the cellular response promoting the bioaccumulation of high-value compounds such as DHA, resulting in a more desirable microalgae biomass.

### Extraction Optimization

Selecting a solvent for microalgae extractions has been the subject of great debate especially due to the level of selectivity that each solvent system presents and what compounds one wants to extract. Solvent mixtures such as chloroform:methanol tend to extract more polar lipids in comparison with hexane systems in which the extracted non-polar fractions is higher (Ryckebosch *et al.*, 2013). The common disadvantage that both systems share is the high toxicity of the solvents capable of inducing carcinogenic mutations and for this reason they are unsafe for extracting products aiming for human consumption of any kind. For that reason, the food grade solvent chosen for optimizing the extractions was ethanol ($C_2H_5OH$) based not only on the biochemical properties of their hydroxyl group (-OH) which confers it affinity to water and hydrophilic molecules such as polysaccharides, but also, based on the lipophilic behavior of the remaining part of the molecule ($-C_2H_5$). Moreover, it has the ability to reduce the activity of enzymes (e.g. lipases) helping to preserve long chain lipid molecules while extracting them (Kim *et al.*, 2012; Ryckebosch *et al.*, 2012). To prevent degradation of compounds such as lipids and carotenoids due to oxidative damage, 0.03g of butylhydroxytoluene (BHT) were added during the extraction. The results are displayed in figure 34.

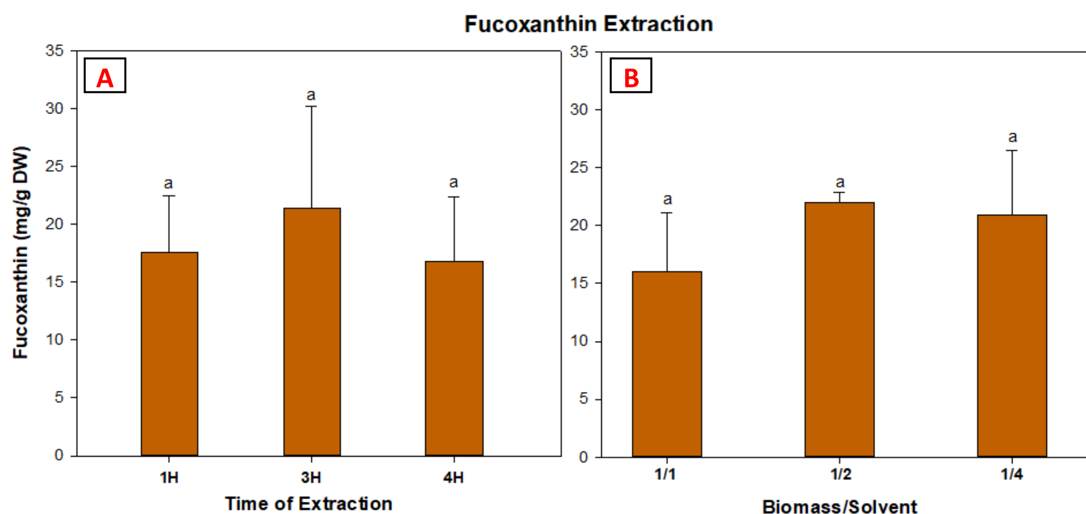


Figure 34 -The effect of Time of Extraction (A) and Biomass/Solvent ratio (B) on fucoxanthin. The values plotted are the average of triplicate trials.

The Biomass/Solvent ratio trial was conducted for 2 hours providing a minimum fucoxanthin content of 16.0 ± 5.12 mg/g DW for a 1/1 ratio and a maximum value of 22.0 ± 0.9 mg/g DW for a 1/2 proportion. Statistical tests were applied showing no significant differences ($p>0.05$) between the different proportions tested. Consequently, the time of extraction trial was conducted assuming a 1/3 solvent biomass ratio and revealed a maximum fucoxanthin content of 21.3 ± 8.8 mg/g DW and a minimal value of 16.8 ± 5.6 mg/g DW. Once again statistical tests were applied showing no significant differences ($p>0.05$) between the times of extraction tested. All these outcomes for fucoxanthin concentration are close to the maximum values or even exceed the ones stated in literature, although one must not forget the variability of extraction protocols regarding the choice of solvent and the parameters here tested: solvent ratio and time of extraction (Crupi *et al.*, 2013; Kim *et al.*, 2012; Ryckebosch *et al.*, 2013).Further experiments should be considered in order to minimize standard deviations and hopefully confirm such results.

Regarding the overall yield of extraction (Fig. 35), the trial 1/1 showed significantly lower extracted fucoxanthin ($p<0.05$) in comparison with the 1/2 and 1/4 proportions where no significant difference was recorded. Additionally, the Time of Extraction had no effect since no significant differences between all the treatments tested were observed.

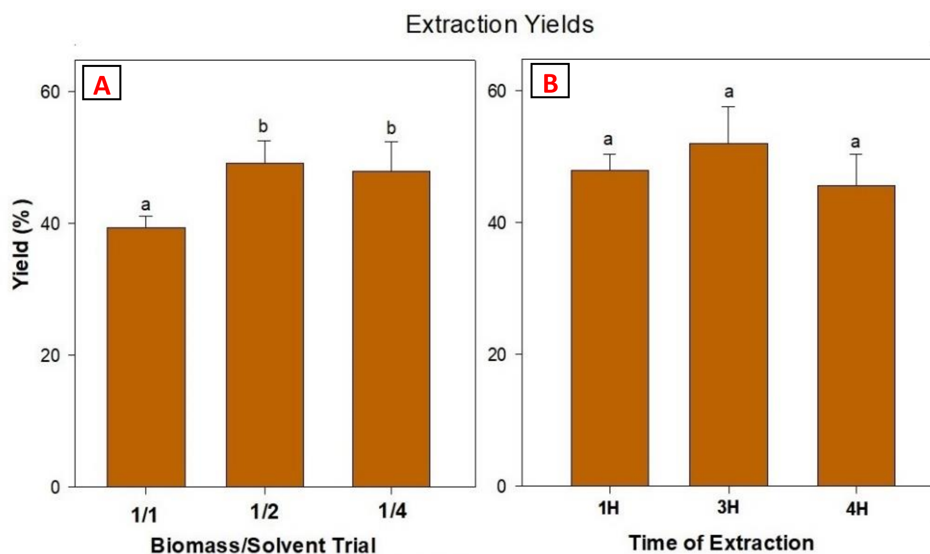


Figure 35 -The effect of biomass/Solvent ratio (A) and Time of Extraction (B) on the overall yield of biomolecules from each extract. The values plotted are the average of triplicate trials. Starting with 10g WB in all extracts, yield was calculated as the recovered percentage of biomolecules.

Therefore, the process of choosing the best extraction procedure was supported on the overall amount of fucoxanthin alongside the percentage of yields of each extraction and the industrial efficiency assuming energy and solvent costs coupled with time of execution. Considering that mixing the extract for longer periods of time would be more energetically expensive as well as using more ethanol, the best solvent ratio chosen was 1/2 for 1-hour mixing. Using this procedure, 6 new ethanolic extracts were prepared to initiate the next stage of the process, a liquid-liquid extraction. The average fucoxanthin concentration in these extracts was 17.7 ± 1.1 mg/g DW determined by spectrophotometry.

**Liquid Three Phase System:**

The LTPS extraction system revealed to be a very promising technique for collecting both fucoxanthin (Fig 36 A,B) and PUFA (Fig 37). Regarding the fucoxanthin content, there is a clear increase in concentration as a consequence of reducing the overall volume of water in the system. The proportion 1/2 (80/160, H/W) was the solvent system with the lowest recovery of fucoxanthin (8.3 mg/g DW). In contrast, the solvent system 2/1 (160/80, H/W) had the highest recovery of fucoxanthin (18.6 mg/g DW) although it was dispersed between the hexane and colloidal fractions. The ratio 1/1 (120/120, H/W) collected an overall amount of fucoxanthin of 9.6 mg/g DW although the majority of it

was present in the colloidal phase. Still, the largest amount of fucoxanthin was present in the colloidal fraction of the 2/1 (H/W) solvent system.

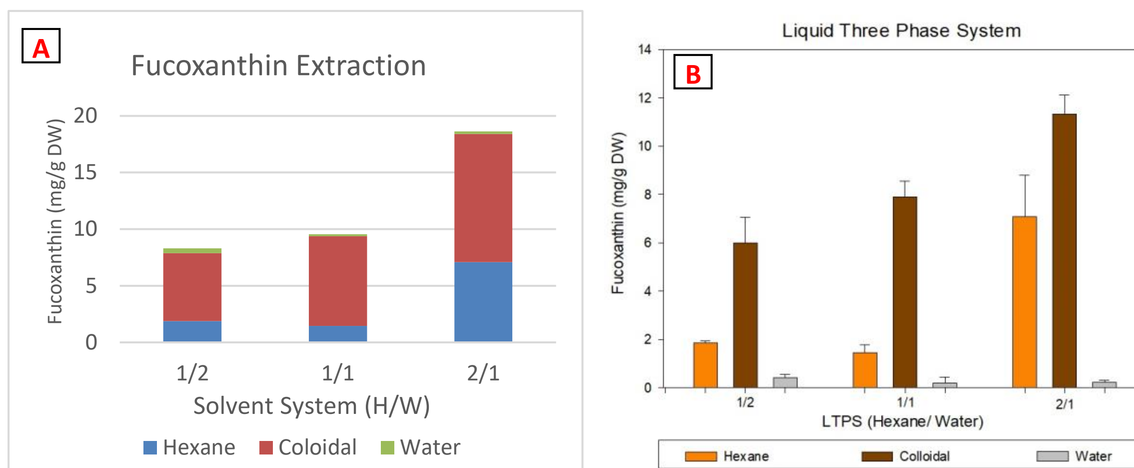


Figure 36 A,B- Distribution of fucoxanthin in each fraction (Hexane, Colloidal, Water) of the different solvent systems.

More trials should be performed in order to gain a better understanding of the dynamics of these solvent systems perhaps using a new proportion in between 1/1 (120/120, H/W) and 2/1 (160/80, H/W), aiming to attenuate the degradation of fucoxanthin felt in solvent system 1/1(120/120, H/W) while recovering more fucoxanthin in the colloidal fraction alone. The role of BHT also needs to be better understood because its solubility is dependent on temperature and the solvent system (Chang *et al.*, 1985; Phipps, 1973; Snipes *et al.*, 1975). The double trials performed for each solvent system (Fig. 36 A,B) clearly demonstrate a trend of increased recovery of fucoxanthin with less amounts of water in the system. Despite that fact, the extraction and distribution of PUFA in the LTPS (Fig. 37) surprisingly revealed that the proportion with highest yield was the 1/2 (80/160, H/W) solvent system, followed by 1/1 and 2/1 systems.

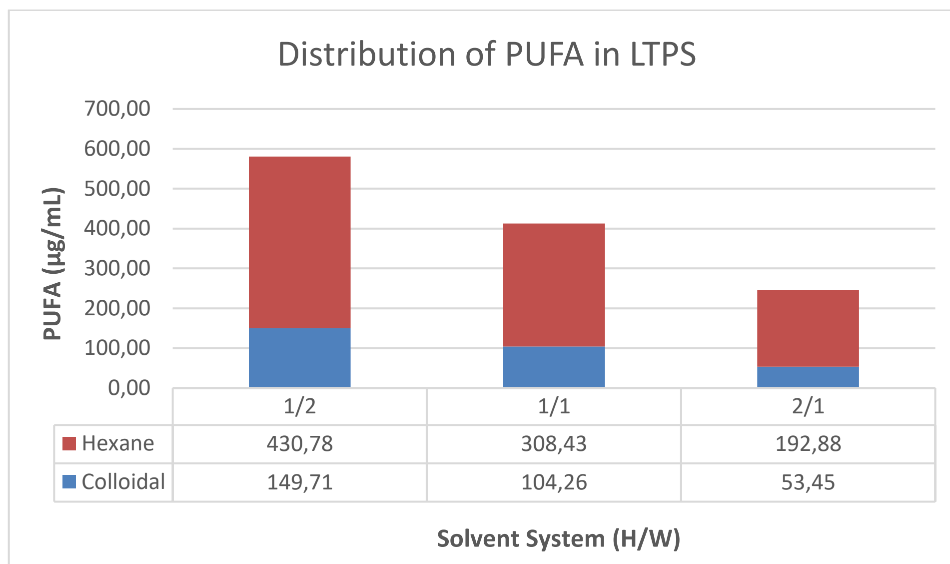


Figure 37 - Recovery of PUFA upon different solvent systems of Hexane (H) and Water (W) in the LTPS.

On an industrially perspective the scale-up and feasibility of this technique is dependent on the amount of chemical engineering processes that must be done in any theoretic industrial scenario to achieve a high enough recovery of valuable compounds (e.g. fucoxanthin and DHA) to be competitive with the current market sources. More chemical steps in the process translates in higher costs of acquisition of equipment as well as higher costs of operating those systems with implications on the overall price for consumers. For this reason, further investigation is required to recover as much fucoxanthin and DHA as possible from the colloidal fraction, hopefully in a single industrial step, using the best solvent proportion.

The physicochemical properties described in the emulsions chapter highlight the individualities of each one of the LTPS solvent systems. The yield of colloidal fractions was higher for the solvent system 1/1 (170 ± 28 mg) followed by 1/2 (130 ± 1 mg) and 2/1 (95 ± 21.2 mg) hence illustrating the variability of molecule retention per fraction as a result of polarity dynamics. By altering the proportion of polar to non-polar solvents it is clear that the molecular degradation as well as its sorting in the colloidal layer are extremely affected to a degree not yet fully understood. The LTPS fraction containing more DHA was hexane (Fig. 38) with the highest amount of DHA being extracted in the system 1/1 (274.2 µg/mL) followed by 1/2 (187.4 µg/mL) and 2/1 (164.1 µg/mL).

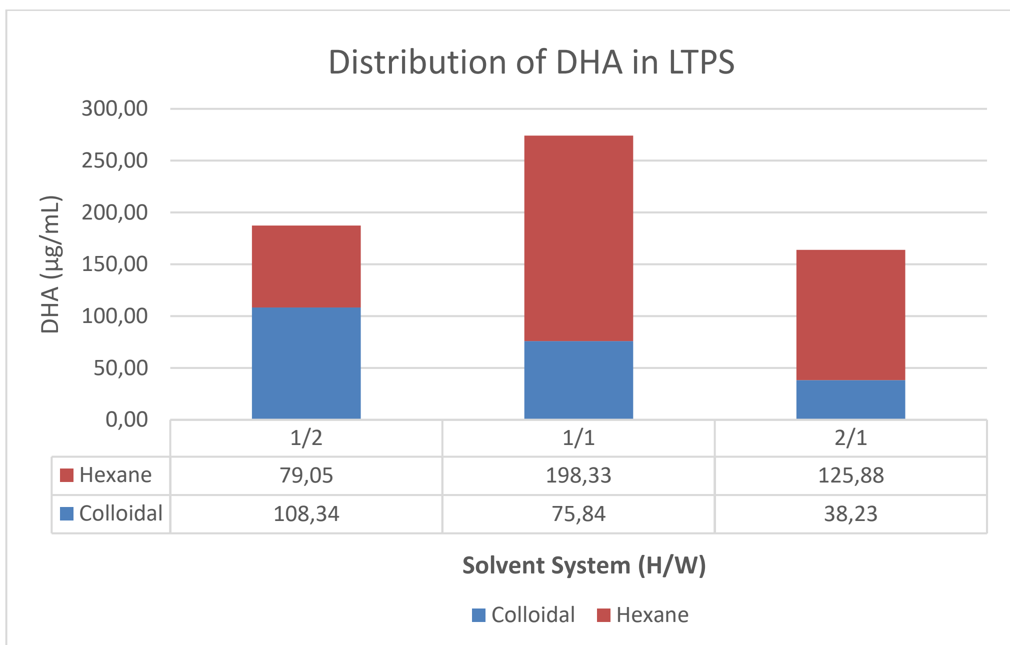


Figure 38 - Distribution of DHA per fraction according to the different solvent systems of Hexane (H) and Water (W) in the LTPS.

This outcome of DHA distribution was not expected as one was predicting more DHA in the colloidal fraction since phospholipids are present there as well. One explanation for this result may be that DHA in *Isochrysis galbana* is more present in TAG rather than in phospholipids, therefore justifying the higher concentration in the non-polar fraction, although one should also keep in mind that *I.galbana* has much more betaine lipids than phospholipids (Cañavate et al., 2017) and its implications in the overall LTPS system are not yet understood. The results of the LTPS proportion trial identified that the highest amount of fucoxanthin extraction was achieved at a 2/1 (160/80, H/W) ratio whilst the majority of DHA was extracted at the 1/1 (160/80, H/W) ratio. Therefore, further tests should be performed to test a new ratio in between both solvent systems aiming to recover more fucoxanthin without significantly compromise the DHA content. The suitability of LTPS for extracting the target components was confirmed and future investigation will focus on determine the best solvent proportion. As one of the goals of the present work was to determine the best solvent system to extract DHA and fucoxanthin in the colloidal fraction, the best ratio here determined was 1/1 (120/120, H/W) because it was the most balanced one in terms of fucoxanthin content, DHA and yield of colloidal fraction.

## 5. Conclusions

ALGEM® labscale photobioreactors are an effective environmental modulation system that can potentially be used to optimize any microalgae strain. The trials identified the Autumn season and pH 7.5 as the best for production of fucoxanthin. Additionally, the LTPS efficacy in extracting of DHA and fucoxanthin was confirmed. The technique needs however to be refined and new trials should be performed aiming to find a solvent system proportion that can extract both molecules without significant losses. The current proportion trials identified 1/1 (H/W) as the best ratio for DHA extraction while the maximum fucoxanthin content was extract using the 2/1 (H/W) solvent system. New trials will be prepared to test this and other conditions known to influence emulsions dynamics. The results revealed that the most promising LTPS solvent system was the 1/1 ratio considering fucoxanthin content coupled with DHA and yield of the colloidal fraction.

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