Universidad de Huelva

Departamento de Ingeniería Química, Química Física y Ciencias de los Materiales

Microalge : source of commercial interest compounds

Memoria para optar al grado de doctora
presentada por:

Elisabeth Bermejo Padilla

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Bajo la dirección de las doctoras:

María Cuaresma Franco

Inés Garbayo Nores

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Tesis Doctoral

MICROALGAE: SOURCE OF COMMERCIAL INTEREST COMPOUNDS

"MICROALGAS: FUENTE DE COMPUESTOS DE INTERÉS COMERCIAL"

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A todas las personas que siempre me han apoyado. Gracias por creer en mí.

A mis *fieles amigos*, Lucky y Turco, por regalarme tanto cariño y buenos momentos.

"Todo el mundo trata de realizar algo grande, sin darse cuenta de que la vida se compone de cosas pequeñas"

Frank Clark

"Reunirse es un comienzo, permanecer juntos es el progreso y trabajar juntos es el éxito"

Henry Ford

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Gracias a todos

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ALA	alpha-lipoic acid
AMD	age-related macular degeneration
APX	ascorbate peroxidase
BSA	bovine serum albumin
C	carbon
Ca	calcium
CAT	catalase
CMM	carbon dioxide concentration mechanisms
Co	cobalt
Cu	copper
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
EPA	eicosapentaenoic acid
FA	fatty acid
FAMEs	fatty acids methyl ester
Fe	iron
FID	flame ionization detector
GC	gas chromatographer
GPX	guaiacol peroxidase
H	hydrogen
HPLC	high pressure liquid chromatography
K	potassium
MAAs	mycosporine-like amino acids
Mg	magnesium
Mn	manganese
Mo	molybdenum
MUFAs	monounsaturated fatty acids
N	nitrogen

Abbreviations

NPK	nitrogen:phosphorus:potassium
O	oxygen
OD	optical density
P	phosphorus
PAM	pulse amplitude modulation
PAR	photosynthetically active radiation (400−700 nm)
PCR	polymerase chain reaction
PSII	photosystem II
PUFAs	polyunsaturated fatty acids
P_v	volumetric productivity
Qy	maximum quantum yield (F_v / F_m)
rcbL	ribulose bisphosphate carboxylase large chain
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
S	sulfur
SFA	saturated fatty acids
TAGs	triacylglycerides (non-polar lipids)
U	enzymatic activity unit
UVA	ultraviolet A (315−400 nm)
UVB	ultraviolet B (280−315 nm)
UVR	ultraviolet radiation (15−400 nm)
v/v	volume/volume
w/v	weight/volume

"Me lo contaron y lo olvidé. Lo ví y lo entendí. Lo hice y lo aprendí"

Confucio

"Invertir en conocimiento produce siempre los mejores intereses"

Benjamín Franklin

Resumen

Las microalgas han despertado en los últimos años un especial interés debido al extraordinario potencial que presentan. Su crecimiento fácil y rápido y la facultad que poseen para convertir la energía solar en biomasa han provocado el desarrollo de diferentes técnicas de cultivo para obtener numerosos y variados productos de interés comercial.

Durante la fotosíntesis, utilizando sólo luz y nutrientes, las microalgas pueden producir metabolitos de alto valor añadido como son los lípidos, proteínas, carotenoides, carbohidratos e hidrocarburos. Las cantidades generadas de estos productos metabólicos están estrechamente ligadas a las condiciones ambientales, en particular, a la cantidad e intensidad de luz, la temperatura, el pH, la salinidad y la disponibilidad de nutrientes. Por tanto, el conjunto de estos factores tiene una gran influencia en la composición bioquímica de las microalgas y, en último término, en la producción de biomasa.

Estos productos de alto valor presentan un amplio abanico de aplicaciones en alimentación, cosmética, industria farmacéutica, así como en la fabricación de biopolímeros. Entre dichos compuestos se encuentran los estudiados en esta Tesis Doctoral, que fueron carotenoides, carbohidratos (exopolisacáridos) e hidrocarburos. Desde un punto de vista comercial, los carotenoides son los pigmentos fotosintéticos más interesantes por su uso como aditivos en la alimentación, especialmente como potenciadores del color. De los carotenoides conocidos, el β-caroteno, la luteína, la zeaxantina y la astaxantina son los más utilizados con propósitos comerciales. Por otro lado, la producción de hidrocarburos y polisacáridos a partir de microalgas, y otras plantas, se está convirtiendo en un campo de interés relacionado con la generación de químicos y polímeros derivados de recursos renovables. Los componentes primarios de los principales polímeros comerciales son derivados de azúcares (poliésteres) e hidrocarburos (poliolefinas), los cuales son producidos por determinadas algas. El interés de estos compuestos radica en el amplio espectro de aplicaciones que pueden tener en la industria, en la gran dependencia energética que existe para obtenerlos a nivel mundial a partir de recursos fósiles que son limitados, así como

por la sostenibilidad de los procesos de producción de químicos y polímeros bio-derivados.

Este trabajo de Tesis Doctoral se basa en el interés que genera la capacidad de algunas microalgas para acumular compuestos de alto valor añadido, como son los ya mencionados carotenoides, carbohidratos e hidrocarburos. Para ello, se estudiaron varias microalgas aisladas del cauce del Río Tinto en Huelva, de las cuales finalmente se seleccionó la identificada como *Coccomyxa onubensis*, y dos razas de otra microalga de un ambiente totalmente distinto llamada *Botryococcus braunii*. Mientras que la primera se caracteriza por vivir en ambientes extremos con altas temperaturas, alta irradiancia, altas concentraciones de metales disueltos, pH ácido y carencia de nutrientes esenciales que le hace aumentar su respuesta metabólica hacia la biosíntesis de moléculas antioxidantes, incluyendo carotenoides (β-caroteno, zeaxantina astaxantina y luteína), *B. braunii* se caracteriza por un lento crecimiento acompañado por la síntesis y acumulación de compuestos de interés como los carbohidratos (exo-polisacáridos) y los hidrocarburos.

En base a estas premisas, se propuso optimizar el crecimiento y la producción de metabolitos de interés en ambas especies mediante el empleo de dos aproximaciones diferenciadas. En el caso de *C. onubensis* se realizó un estudio del efecto que distintos factores abióticos, como son la temperatura y la radiación ultravioleta A y B, producen en el crecimiento y producción de carotenoides. En el caso de *B. braunii* se realizó primero una optimización del medio de cultivo y, posteriormente, con idea de reducir costes y ver si era posible aumentar tanto el crecimiento como la producción de metabolitos, se utilizaron como medios de cultivo fertilizantes comerciales, tanto para la raza productora de exo-polisacáridos (Raza A) como para la productora de hidrocarburos (Raza B).

Fruto de estas aproximaciones ha sido la mejora en las productividades, tanto de biomasa como de los principales compuestos generados por ambas especies de microalgas.

Abstract

Microalgae have generated a special interest in recent years because of the extraordinary potential they present. Their fast and easy growth and the ability they have to convert solar energy into biomass have led to the development of different cultivation techniques in order to obtain numerous and varied products of commercial interest.

During photosynthesis, using only light and nutrients, microalgae can produce high added-value metabolites such as lipids, proteins, carotenoids, carbohydrates and hydrocarbons. The generated amounts of these metabolic products are closely related to environmental conditions, in particular, to the amount and intensity of light, temperature, pH, salinity and nutrient availability. Therefore, all these factors have a great influence on the biochemical composition of microalgae and, eventually, on biomass production.

These high value products present a wide range of applications in food, cosmetics, pharmaceutical industry, as well as in the manufacture of biopolymers. Among these compounds are those studied in this Doctoral Thesis, which were carotenoids, carbohydrates (exopolysaccharides) and hydrocarbons. From a commercial point of view, carotenoids are the most interesting photosynthetic pigments for their use as feed additives, especially as color enhancers. Of all known carotenoids, β-carotene, lutein, zeaxanthin and astaxanthin are most commonly used for commercial purposes. On the other hand, the production of hydrocarbons and polysaccharides from microalgae and other plants is becoming a field of interest related to the generation of chemicals and polymers derived from renewable resources. The primary components of the major commercial polymers are derived from sugars (polyesters) and hydrocarbons (polyolefins), which are produced by certain algae. The interest in these compounds lies on their broad spectrum of application in the industry, in the current strong dependence on the availability of fossil resources, as well as on the sustainability of the production of bio-derived chemicals and polymers.

This Doctoral Thesis is focused on the ability of certain microalgae to accumulate compounds with high added-value, such as the aforementioned

carotenoids, carbohydrates and hydrocarbons. Several microalgae isolated from Rio Tinto (Huelva) were studied, from which the one with the best productivity was selected for further experiments and identified as *Coccomyxa onubensis*. And other two races (A and B) of a microalga from a totally different environment called *Botryococcus braunii* were also used in this Thesis. While *C. onubensis*, is characterized by living in extreme environments with high temperatures, irradiance, dissolved metals, acid pH and lack of essential nutrients, which increases its metabolic response to the biosynthesis of antioxidant molecules, including carotenoids (β-carotene, zeaxanthin, astaxanthin and lutein), *B. braunii* is characterized by its slow growth and, at the same time, by being able to synthesize and accumulate large amounts of compounds as interesting as exo-polysaccharides and hydrocarbons. Thus, in the case of *C. onubensis*, the effect of different factors, such as temperature and ultraviolet radiation A and B, on the growth and production of carotenoids was studied. In the case of *B. braunii*, the optimization of the culture medium was carried out as a first approach, and commercial fertilizers were subsequently used to reduce costs and to see if it was possible to increase growth and carbohydrates and hydrocarbons productivity.

The results obtained in this Thesis have contributed to obtain higher productivities of biomass and metabolites for both microalgal species.

I. INTRODUCTION

1. MICROALGAE.

1.1. Microalgae general characteristics.

Microalgae are a large and diverse group of unicellular, prokaryotic and eukaryotic microorganisms which present different shapes with diameter and length between 1-50 µm. They are found spread across many phyla (Guiry 2012) and are able to grow in fresh or salt water.

These microoganisms play a key role in aquatic ecosystems as they are photosynthetic organisms responsible of 40% of global photosynthesis (Moreno-Garrido 2008) producing half of the atmospheric oxygen on earth.Their cultivation is simple and can be cost-effective as they have the ability to grow autotrophically, using CO_2 and light as sources of energy to convert them into biomass via photosynthesis according to the generic equation:

$$CO_2 + Light + Nutrients \rightarrow O_2 + Biomass$$

The biodiversity of microalgae is enormous, there are more than fifty thousand different types of microalgal species present in oceans and fresh water (lakes, ponds and rivers), and among these species only thirty thousand have been studied (Richmond 2004). Thus, that diversity offers great potential that has yet to be exploited.

Microalgae have been described as rich sources of different compounds of commercial interest (Volk and Furkert 2006) and have gained increasing value as sources of food additives, cosmetics, animal feed, pigments, polysaccharides, fatty acids, and biomass (Hallmann 2007; Borowitzka 2013; Leu and Boussiba 2014). Some of the interesting molecules/compounds come directly from primary metabolism, such as pigments, vitamins, fatty acids and proteins; others are synthesized from secondary metabolism. Bioactive compounds from microalgae can present antiviral, antifungal or antibiotic actions (Amaro et al. 2011).

1.1.1. Microalgae from extreme environments.

Microalgae which can cope with extreme conditions of pH, temperature, pressure or salinity are considered extremophiles (Varshney et al. 2015). These microalgae present some advantages such as the ability to grow under local climatic conditions and exclude potential contaminants, besides the fact that they accumulate certain metabolites which allow them to grow and thrive under the extreme conditions. At the same time extremophiles can be used in biotechnological applications, for instance as a natural source of β-carotene (Varshney et al. 2015). All these characteristics make such kind of microorganisms interesting from a commercial point of view.

Despite that the commercial potential of microalgae has been recognized for over 50 years, the number of microalgal species that are produced on a large scale in a sustainable economic process is limited (Richmond 2004). However, of those few microalgal strains that have reached a stage of being a commercially traded product, two are extremophiles, which are *Dunaliella* (Borowitzka and Huisman 1993) and *Arthrospira* (Silli et al. 2012).

In the first part of Results and Discussion of this Doctoral Thesis, the biotechnological potential of a new microalga isolated from an extreme environment was studied.

1.2. Advantages of using microalgae to obtain bioactive compounds.

Microalgae are an important source of bioactive natural substances, as it is shown in many references in bibliography that describe biological activities and potential health benefits (Chen et al. 2010; Pangestuti and Kim 2011). These high value products have many applications in the cosmetic, food, or pharmaceutical industries (Markou and Nerantzis 2013; Skjanes et al. 2013).

When microalgae are exposed to stress and/or extreme conditions, as changes in temperature, salinity or nutrients, they are able to produce metabolites biologically active which are, sometimes, not produced by other organisms (Plaza et al. 2009; Rodríguez-Meizoso et al. 2010; Herrero et al. 2013). Therefore, the production of the bioactive compounds can be controlled manipulating cultivation

conditions (Singh and Dhar 2011; Singh et al. 2011; Brennan and Owende 2013; Ibáñez and Cifuentes 2013).

Other advantages of microalgae cultivation are related to their chemical composition, taxonomic diversity, and their ability to use solar energy and CO_2 to grow presenting higher growth rates than those for higher plants and thus reducing excess of CO_2 in the atmosphere by biofixation (Priyadarshani and Rath 2012; Pérez-López et al. 2014), which, at the same time, contributes to reduce the greenhouse effect and global warming.

Another advantage is their potential to be cultivated in bioreactors under controlled conditions. Areas and climates unsuitable for agriculture can be used to cultivate microalgae with no competition with food production land. Moreover, nitrogen and phosphorus can be removed from wastewater using microalgae in an efficient solar energy biomass conversion (Priyadarshani and Rath 2012).

It has also been created a biorefinery concept based on microalgae able to offer a broad range of different products with applications in food, pharmaceuticals, medicine and biofuels (Subhadra 2011; Ibáñez and Cifuentes 2013), with benefits that contribute to the economical viability of microalgal production (Markou and Nerantzis 2013).

1.3. Biomolecules of interest from microalgae.

Due to changes in life style and the rising health consciousness of the average population, the demand for nutrient-rich supplements with health benefits (such as supplements rich in proteins and vitamins) has risen. At this respect, microalgae may be widely used as a potential source for the production of several highly valuable bioproducts, since they are a rich source of bioactive compounds such as vitamins, proteins with essential amino acids, polysaccharides, fatty acids, minerals, photosynthetic pigments (carotenoids and chlorophylls), enzymes, and fibers (Mimouni et al. 2012; Matos et al. 2017).

1.3.1. Proteins.

Algae, especially microalgae, are known to be rich in proteins and, therefore, they can be considered as an alternative protein source (Spolaore et al.

2006). Among microalgae, *Arthrospira* is the most known source of single-cell protein with nutraceutical properties (Anupama 2000) and it is considered as "green food" because of its high protein content, equivalent to 60-70% of the total mass according to Belay et al. (1993). Proteins are present in cyanobacteria (blue-green algae) as phycocyanobilins and in certain algae (Rhodophyta) as phycoerythrobilins (Ibáñez et al. 2012). In both, anti-inflammatory, antioxidant, antiviral and antitumor properties have been described (Sekar and Chandramohan 2008). An antibacterial and antifungal activity was also indicated in amino acids extracted from *Chlorococcum humicola* (Bhagavathy et al. 2011).

1.3.2. Carbohydrates.

Carbohydrates, also called saccharides, are molecular compounds made up of three elements: carbon, hydrogen and oxygen. They can be divided into monosaccharides and disaccharides (as glucose and sucrose, respectively) which are relatively small molecules, or into polysaccharides which are very large molecules (such as starch and cellulose).

In microalgae, carbohydrates act as structural components in the cell walls, and as storage components inside the cells. Carbohydrates, as storage compounds, provide the energy needed for the metabolic processes of the organisms and allow, if needed, temporary survival in dark conditions (Geider and La Roche 2002; Raven and Beardall 2004). Among carbohydrates, the polysaccharides are considered as some of the most important products obtained from algae, from an economical point of view (Kraan 2012). The structural and compositional features of microalgal exopolysaccharides can significantly affect the physicochemical properties and biological activities of such compounds (Damonte et al. 2004; Ghosh et al. 2009) Therefore, biochemical functions and structure of polysaccharides have been studied in many papers (Chojnacka et al. 2012; Mohamed et al. 2012; Kadam et al. 2013; Borowitzka 2013).

The polysaccharides produced by microalgae can have anti-inflammatory, antitumor, antibacterial, antioxidative, and antiviral properties with potential applications in pharmaceutical industry (cosmeceuticals, nutraceuticals), medicine, and food. For example, agar, carrageenan, or alginates are commonly

used in food, as stabilizers in pharmaceutical industry (Ibáñez et al. 2012) and as supplements in farm animals (Gupta and Abu-Ghannam 2011).

Polysaccharides extracted from microalgae can be beneficial not only to humans and animals but also to plants as they can stimulate defense responses (Vera et al. 2011) and can act as chelators binding microelement ions, which are important in plant nutrition (Kaplan et al. 1987).

Little research has been done, so far, regarding the usage of microalgal polysaccharides as biopolymers to produce bioplastics and fibers, despite the fact that it would reduce the current dependency on fossil resources from which most of the industrial materials are made. However, an interesting approach was recently carried out in an European Project, in which it was proposed to use the exo-polysaccharides produced by the microalga *Botryococcus braunii* for their conversion in polyesters and further production of polymer fibers ("SPLASH", Project reference nº. 311956, funded by the 7th Framework Programme). The selection of *B. braunii* was based on the large amount of polysaccharides that such microalga produces (Díaz and Atehortúa 2014) and its composition, which is mainly based on galactose (Allard and Casadevall 1990); since it is known that sugars (as galactose) can be converted into ethanol by fermentation, and after that into bio-ethylene which is one of the most common plastics (Mohsenzadeh et al. 2017).

1.3.3. Lipids**.**

Microalgae contain interesting lipids that can be extracted and are a valuable source of commercial biomolecules, including Polyunsaturated Fatty Acids (PUFAs) (Priyadarshani and Rath 2012).

The lipid fraction consists primarily of Fatty Acids (FA), defined as carboxylic acids with a long aliphatic chain, which can be either saturated or unsaturated. Microalgal biomass is rich in PUFAS as, for example, omega-3 fatty acids -eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-, and omega-6 fatty acids -γ-linolenic acid and arachidonic acid-. PUFAs are essential for the human body and its health (Ibáñez et al. 2012) due to their antibacterial,

antifungal, and antioxidative properties. They are widely used as nutritional supplements, pharmaceutical, and functional food (Borowitzka 2013).

Sterols are another group of lipids extracted from algal biomass. Their composition has been studied by Volkman (2003) and Cardozo et al. (2007). Phytosterols from algae have application in several fields as pharmaceuticals, nutraceuticals, nutrition and cosmetics (Francavilla et al. 2012).

Other promising area for the utilization of algal lipids is biodiesel production (Brentner et al. 2011) as algae have the potential to reduce dependence on petrofuels and to reduce gas emissions. The current bottleneck for the large-scale production of biodiesel from biomass of microalgae is the high cost of the cultivation techniques and lipid extraction operations that makes its production not profitable for the diesel market.

1.3.4. Antioxidants**.**

Currently, there is a need to search for new antioxidants for food and medicine components as the use of synthetic ones generates many health problems of toxicity (Vadlapudi 2012). Having that into account, microalgae must be considered as a source of secondary metabolites with antioxidant properties, such as for example, carotenoids.

1.3.4.1. Carotenoids**.**

Carotenoids are the most diverse and wide spread pigments found in nature, which have reaching special interest due to their biotechnological applications and, more importantly, their potential beneficial effects on human health, food processing, pharmacy and cosmetics (Stahl and Sies 2003; Jaswir et al. 2011). In recent years, several reviews have been published summarizing the main applications of carotenoids (Gong and Bassi 2016; Sathasivam and Ki 2018). Carotenoids are lipophilic compounds which usually have a yellow, orange or red colour, and can be synthesized de novo by photosynthetic organisms and some bacteria, archaea and fungi (Gouveia et al. 2008, Vilchez et al. 2011, Forján et al. 2015). They are biologically active compounds whose main functions consist

of light absorption -to perform photosynthesis- and photoprotection -to preserve the photosynthetic apparatus from photodamage- (Vilchez et al. 2011). Besides, they present antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory, and antitumor properties (Martín et al. 1999; Jyonouchi 2000; Salminen et al. 2008). The study of the beneficial effects of carotenoids in the healthcare and cosmetics industries is lately reaching a rising interest, as their antioxidant and anti-inflammatory responses are important functions for human health (Woodside et al. 2015). In food, carotenoids serve as precursors of aroma compounds and as natural antioxidants that may help to extend the shelf-life of food (Rodriguez-Amaya 2015).

Current interest in carotenoids obtained from algae results from a market that demands new and natural colorants that can be applied to many products. In this sense, nowadays algal carotenoids are used as food pigments (in dairy products, beverages...), as feed additives (in aquaculture), as components of cosmetics, and pharmaceuticals (Spolaore et al. 2006; Christaki et al. 2013).

For all these reasons, the production of carotenoids is considered as an important business opportunity for the healthcare and cosmetic industries in the future. The main carotenoids that are currently used commercially in the global market are β-carotene, astaxanthin, lutein, canthaxanthin, zeaxanthin and fucoxanthin (Sathasivam and Ki 2018).

1.3.4.1.1. β-carotene.

First product commercially produced from a microalga was β-carotene obtained from *Dunaliella salina* (Borowitzka 2013). The structure of this carotenoid was deduced by Karrer et al. in 1930 and, as for all carotenoids, such structure is basically made up of isoprene units (Figure 1). In nature, β-carotene is the most abundant an efficient precursor (inactive form) of vitamin A via the action of β-carotene 15, 15'-monooxygenase. Then vitamin A is assimilated or further converted into retinoids, therefore it does not cause hypervitaminosis. Being highly conjugated, it is deeply coloured, and as a hydrocarbon lacking functional groups, it is very lipophilic.

As a source of vitamin A, it plays an important role in human health and it is also an excellent antioxidant (Grune et al. 2010).

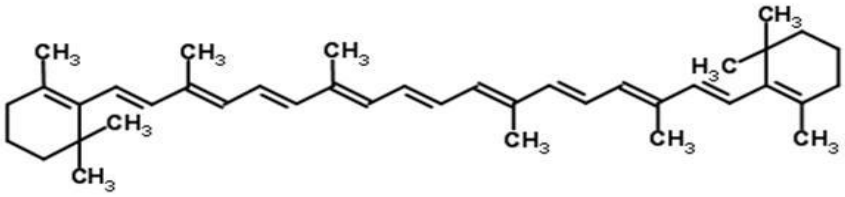


Figure 1. Molecular structure of β-carotene.

1.3.4.1.2. Astaxanthin**.**

Astaxanthin is a secondary carotenoid 11 times more potent than β-carotene. This compound occurs naturally in a wide variety of living organisms including microalgae, fungi, plants, seafood, and some birds such as flamingos and quail; it gives salmon, shrimp and lobster their distinctive colouration (Fasset and Coombes 2012). From the nutritional point of view, astaxanthin is considered as the most powerful antioxidant in nature, serving the role of a highly efficient scavenger of free radicals build up within the human body (Naguib 2000; Koller 2014). Nonetheless, it must be consumed in the diet since it cannot be synthesized de novo by the majority of the animals (Riccioni et al. 2011).

With the aim of obtaing astaxanthin at large scale for commercial applications, its biotechnological production from different sources has been thoroughly studied (Schmidt et al. 2011; Ambati et al. 2014). Thus, astaxanthin products are used for commercial applications in different dosage forms, as tablets, capsules, syrups, oils, soft gels, creams, biomass and granulated powder. Astaxanthin patent applications are available in food, feed and nutraceutical which are currently the major market driver for the pigment (David and Melchior 2009; Honda and Takahashi 2010).

1.3.4.1.3. Lutein**.**

Lutein is a carotenoid belonging to the group of xanthophylls, whose most typical feature, as well as in other carotenoids, is the long conjugated double-bond chain, as it can be seen in Figure 2. Such molecular structure determines the photochemical properties and chemical reactivity that give the basic biological functions of carotenoids, such as antioxidant activities (Britton 1995). It is synthesized only by plants and microalgae and, like other xanthophylls, is found in high quantities in green leafy vegetables such as spinach, kale and yellow carrots.

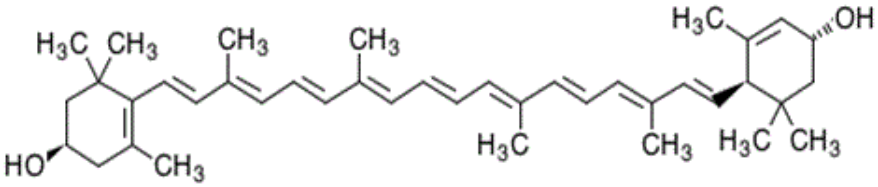


Figure 2. Molecular structure of lutein.

Lutein has been widely applied in the food industry. Besides, it is one of the two major components of the macular pigments of the retina and can be found in high amounts in human serum (Bone et al. 2003). It is also able to filter high-energy blue light (Alves-Rodrigues and Shao 2004), but the most notable bioactivity of lutein is its antioxidant property, which makes it a class of important nutrients in human health. Previous studies reported that the dietary intake of lutein effectively prevented the occurrence of early atherosclerosis (Dwyer et al. 2001) and that there was a direct relationship between carotenoid intake and a decreased risk of age-related macular degeneration (AMD), the leading cause of blindness (Seddon et al. 1994).

1.3.4.1.4. Zeaxanthin**.**

Zeaxanthin is also a carotenoid belonging to the group of xanthophylls (like lutein and violaxanthin), which is synthesized by plants and some microorganisms, being the pigment that gives paprika and other plants and microbes (such as marine bacteria) their characteristic colour (Prabhu et al. 2014). It is also one of the two major components of the macular pigments of the retina

(the other one is lutein, as mentioned before). Zeaxanthin exhibits no vitamin A activity, however, as lutein, it plays a critical role in the prevention of AMD (Tanaka et al. 2012). Due to its high-value and demand in the nutraceutical market, several methods have been used to produce it at large scale (Li et al. 2015).

1.3.4.1.5. Violaxanthin**.**

Violaxanthin is a natural, orange coloured, xanthophyll pigment which can be found in a variety of brown algae and plants. Hager and Yamamoto in the 60s and 70s established the basic biochemical characteristics of the violaxanthin-cycle (Hager 1969; Yamamoto et al. 1971), in which violaxanthin is reversibly converted to zeaxanthin via the intermediate antheraxanthin (Figure 3). Violaxanthin and zeaxanthin interconversions are mainly due to the activity of the enzyme violaxanthin de-epoxidase, mechanism that has been directly correlated with dissipation of excess excitation energy (Demmig-Adams et al. 1989). Thus, this pigment plays an important role in the light harvesting complexes of higher plants and green algae (Sun et al. 1998). Violaxanthin biological role as abscisic acid precursor has also been described so far from leaves (Li and Walton 1990) and fruits (Neuman et al. 2014). Despite the fact that the role of this carotenoid has already been described in vegetables, not too much is known about its potential benefits on human health.

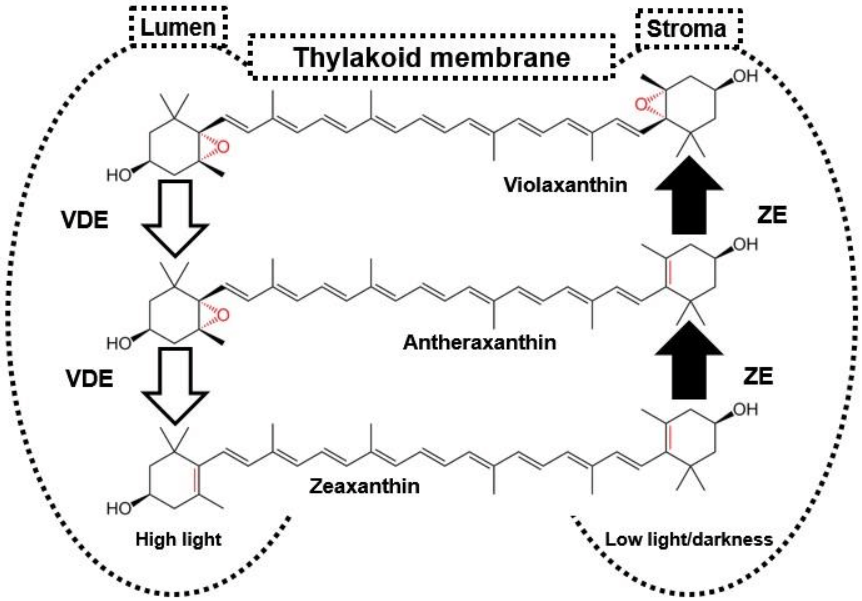


Figure 3. Scheme of the violaxanthin cycle. VDE (violaxanthin de-epoxidase); ZE (zeaxanthin epoxidase).

1.3.4.1.6. Canthaxathin.

Canthaxanthin is a secondary carotenoid pigment with an orange-red colour which is considered, as the antheraxanthin, to be a stronger antioxidant than primary carotenoids (Jin et al. 2006). It is produced at the end of the growth phase in several green algae and also in blue-green algae instead of, or in addition to, primary carotenoids. It has also been found in bacteria, crustacean, birds and various species of fish (Tanaka et al. 2012). Canthanxanthin presents industrial interest because of its widespread applications in nutraceutical, cosmetic, food and feed industries (Veiga-Crespo et al. 2005). Due to the fact that it is an interesting compound to be commercialized in the market, its biosynthesis has been studied extensively so far (Nasrabadi and Razavi 2010; Hojjati et al. 2014).

1.3.4.1.7. Fucoxanthin.

Fucoxanthin is one of the most abundant carotenoids, which can be found as an accessory pigment in the chloroplasts of brown algae, phytoplankton, brown seaweeds and diatoms, giving them a brown or olive-green colour (Wright and Jeffrey 1987; Peng et al. 2011). Two of the main microalgal producers of fucoxanthin are *Phaeodactylum tricornutum* and *Isochrysis galbana* (Kim et al. 2012a, b). It contributes to more than 10% of the estimated total production of carotenoids in nature, especially in the marine environment (Tanaka et al. 2012). From a structural point of view, fucoxanthin has an unusual double allylic carbon and two hydroxyl groups (Figure 4), which is thought to be related with its high energy transfer efficiency (>80%) (Kajikawa et al. 2012). Like other carotenoids, it is a good antioxidant due to its singlet oxygen-quenching activity, being also able to work as an effective radical scavenger (Yan et al. 1999). Fucoxanthin does not exhibit toxicity and mutagenicity under experimental conditions and it may have the ability to increase circulating cholesterol levels in rodents as a common feature (Tanaka et al. 2012).

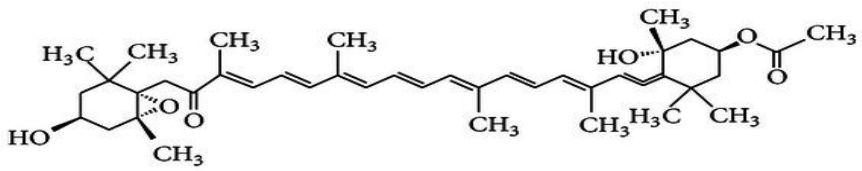


Figure 4. Molecular structure of fucoxanthin.

1.3.5. Hydrocarbons.

Hydrocarbons are organic compounds composed of hydrogen and carbon and are one of the most important energy resources (Timmis and Qin 2010). The structure, hydrogen to carbon ratio, and the length of a particular hydrocarbon determine its properties. In general, small linear hydrocarbons will be gases while medium sized linear hydrocarbons will be liquids. Branched hydrocarbons of intermediate size tend to be waxes with low melting points. Long hydrocarbons tend to be semi-solid or solid. Unsaturated hydrocarbons are more likely to be solid than their saturated counterparts as are cyclic hydrocarbons.

Microalgae can produce biofuel precursors, among which are the hydrocarbons (long chain alka(e)nes), that can be directly utilized as fuels or as additives in petroleum fuels. The interest in using hydrocarbons from microalgae to generate biofuel is because they produce such compounds at a faster rate than agricultural oleaginous crops and do not compete for arable land (Wijffels and Barbosa 2010). Those fatty hydrocarbons are mainly accumulated by some bacteria (Schirmer et al. 2010), a unicellular green microalga *Pseudochoricystis ellipsoidea* (Satoh et al. 2010) and a colonial green microalga *Botryococcus braunii* (Yoshida et al. 2012). Specifically *B. braunii* is able to produce hydrocarbons up to 75% of its dry weight, presenting the advantage of being able to excret them outside the cell (Lupi et al. 1994; Volova et al. 1998; Kalacheva et al. 2002; Weisset al. 2012), which allows the development of strategies for in situ extraction such as “milking” without impairing cell viability (Moheimani et al. 2013).

Since concern over the persistence of petrochemical plastics in the environment has led to the production of biologically derived polymers (Babel and Steinbüchel 2001), these hydrocarbons produced by microorganisms can be used to replace fossil-based hydrocarbons and therefore they have a wide range of potential applications in the chemical and biopolymer industry.

1.4. Effects of different abiotic factors on metabolites production.

Under abiotic stress, microalgae undergo strong metabolic changes in their physiology and biochemistry for survival. Microalgae exposed to nutrient starvation (Simoniato et al. 2013; Chokshi et al. 2017), excess of salt (Pancha et al. 2015), UVA (ultraviolet A) light (Salguero et al. 2005; Srinivas and Ochs 2012) or metal toxicity (Soto et al. 2011) show response by increasing their intracellular concentration of lipids, carotene, and antioxidant enzymes. Hence, they could be used in different biotechnological applications: as source of biofuels, bioproducts, food supplements, and in agriculture (Rothschild and Mancinelli 2001; Varshney et al. 2015; Forján et al. 2015). However, if the stress is too intense and/or persistent, the microalgal growth ceases and leads to apoptosis (Jahnke 1999; Giorgos and Elias 2013; Forján et al. 2015). Metabolism of microalgae and synthesis of compounds of interest can be influenced by the conditions of cultivation. It has been described that pH, temperature, luminescence, salinity and

medium constituents have a strong effect on microalgal metabolites synthesis as well as on biomass production (Morais et al. 2015).

In this Doctoral Thesis, different environmental and nutritional conditions have been performed during the cultivation of two species of microalgae (*Coccomyxa onubensis* and *Botryococcus braunii*) in order to enhance their productivity and the accumulation of certain molecules of commercial interest, such as carotenoids, lipids, carbohydrates and hydrocarbons.

1.4.1. Temperature.

Temperature is the most important factor that influences the production of biomass, proteins, lipids and phenolic compounds, all this due to its influence on the enzymatic reactions (Fox 1996). The optimum growth temperature for phytoplankton is between 20 ºC and 25 ºC, although this range can vary depending on the composition of the medium and the species. Most commonly cultivated species tolerate temperatures between 16 ºC and 30 ºC. Temperatures lower than 16 ºC slow down the growth of microalgae, while those that exceed 35 ºC are lethal for many species (Butterwick et al. 2005). Nonetheless, it has been observed that some microalgae like *Synechococcus leopoliensis* should be cultivated at 35 ºC and pH 8 to obtain the best production of bioactive compounds (Noaman 2004).

Several research studies stablish a trend between the lipid profile in microalgae and temperature. As example, polar lipid content increases when the temperature decreases, whereas a temperature increase leads to a higher accumulation of non-polar lipids (TAG) (Renaud et al. 2002). A significant increase in the Alpha-Lipoic Acid (ALA) and Docosahexaenoic Acid (DHA) content has been observed in *Isochrysis galbana* with a decrease in temperature from 25 ºC to 10 ºC, (Zhu et al. 1997). Low temperature in growing conditions influences fatty acids profile composed mainly of PUFAs (EPA and DHA). This indicates that those lipids could be important for the microalga to survive under unfavorable conditions. Increasing production of unsaturated fatty acids such as PUFAs, which maintain membrane fluidity at low temperatures, is a mechanism for the algae to adapt to such temperatures. Further, temperature is also known to

influence carotenoids content. In a study with *Dunaliella salina* it was reported that temperature strongly affects the β-carotene accumulation (Wu et al. 2016).

1.4.2. Light.

Light is an important factor that influences the biochemical composition of biomass and photosynthesis (Chrismadha and Borowitzka 1994). Remarkable changes in the biomass and biomolecules profile of microalgae have been reported when stressed with various light intensities. It has been described a reduction in the total lipids content of the microalga *Pavlova lutheri* due to an increase of light intensity (Guedes et al. 2010). A higher light energy overcomes the need for a high chloroplastidial activity and, consequently, as lipids are major components of chloroplasts, a decrease in the content of such biomolecules is observed. An increase of neutral lipid content (TAG) with a simultaneous decrease of polar lipids has been observed in many species such as *Chlorella sp.* (He et al. 2015), *Scenedesmus obliquus* (Breuer et al. 2013), *Nannochloropsis gaditana* (Mitra et al. 2015a) and *Monoraphidium sp.* (He et al. 2015) with high light intensity. Nevertheless, the accumulation of TAG varies for the different species (He et al. 2015; Breuer et al. 2013). Although light irradiance acts as a stimulant in the production of microalgal biodiesel, PUFAs (especially EPA and/or DHA) content of microalgae is inversely related to light irradiation. This is demonstrated with several research studies, including *Nannochloropsis sp.* (Sukenik et al. 1989), *Nannochloropsis salina* (Van Wagenen et al. 2012) and *Nannochloropsis gaditana* (Mitra et al. 2015a). The explanation may be due to the fact that the Reactive Oxygen Species (ROS) formed under unfavourable conditions attenuate the activity of PUFAs. The photosynthetic potential of algae decreases with high irradiance and it could indicate that less thylakoid membranes are required (Sukenik et al. 1989, 2009).

Another microalgal response due to light stress is the induction of carotenoids synthesis in order to protect the cells from photodamage. Depending on the kind of light and on the microalgal strain, the mechanism of induction may follow different metabolic pathways. As example, it has been reported that sudden exposure of *Chlamydomonas reinhardtii* to high light intensity led to the induction of the xanthophylls cycle (Couso et al. 2012).

On the other hand, it has been also reported in a study carried out by Madhyastha et al. (2009) that the application of blue light in the cultivation of the microalga *Spirulina fussiformis*, enhanced its antioxidant capacity through a phenomenon where the microalgae cells alter the sequence of amino acids with cysteine repeats, which may be considered as a non-enzymatic antioxidant mechanism.

1.4.3. UV-radiation.

UV-radiation (UVR) has been reported to affect growth, photosynthesis, biochemical composition, nutrient uptake, reproduction and many other physiological processes as well as causes damage to DNA of microalgae which inhabit terrestrial and aquatic ecosystems (Hughes 2006; Häder 2006; Wong et al. 2007). In that sense, to be able to cope with potencially harmful UVR, some microalgae have developed strategies like synthesizing or accumulating a series of photoprotective compounds such as mycosporine-like amino acids (MAAs) or antioxidant compounds that directly, or indirectly, absorb the energy of the solar radiation (Oren and Gunde-Cimerman, 2007; Huang and Cheung 2011).

In eukaryotic microalgae, UV-radiation (UVR) induces the carotenoid synthesis, especially β-carotene and lutein (Mulders et al. 2014; Rao et al. 2007; Gu et al. 2012; Fernández-Sevilla et al. 2010). And, in general, UVR induces the accumulation of high value-added products in mesophilic (Srinivas and Ochs 2012; Balan and Suraishkumar 2014; Wong et al. 2007; Huang and Cheung 2011) and extremophilic microalgae (Salguero et al. 2005; Jahnke 1999; Wong et al. 2007). Therefore, it must be said that biomass nutritional value and microalgal growth are influenced by UVA and UVB radiation.

Regarding microalgal fatty acids accumulation, it has been reported that exposure to UVA radiation significantly increased Polyunsaturated Fatty Acids (PUFAs) content in *Nannochloropsis oculata* (Srinivas and Ochs 2012) and in *Phaeocystis Antarctica,* being in the last case also increased under low UVB radiation (Skerratt et al. 1998). On the other hand, the combined effect of UVA and UVB increased Monounsaturated Fatty Acids (MUFAs) content in *Chaetoceros mualleri* (Liang et al. 2006). In the membrane repair mechanism of

cells, PUFAs participate actively. Therefore, abiotic stress conditions which lead to the formation of Reactive Oxygen Species (ROS) and lipid peroxidation are generally related to an elevated PUFAs content.

Other compounds influenced by UVR are carbohydrates and carotenoids. In a study carried out by Ganapathy et al. (2017) with *Chlorella vulgaris*, the results demonstrated that small doses (time of exposure and intensity) of UVB radiation stimulated the synthesis of extracellular carbohydrates, whereas carotenoids content increased even being subjected to high intensity but during short periods of time. The authors suggested that this increase in UV-absorbing compounds may occur as a way to counteract the negative effect of UVB radiation on the microalgal growth. However, certain microalgae, as *Dunaliella bardawil,* are able to accumulate carotenoids without compromising its growth when subjected to modulated UVA radiation (Mogedas et al. 2009).

1.4.4. pH.

pH control is essential for an effective absorption of the culture medium components and for helping to prevent contamination by some microorganisms, including other microalgal species. It is also worth noting that pH is particularly relevant in microalgal cultures because it interferes with CO_2 availability (which is essential for photosynthesis), since the relative concentration of CO_2 and the pH of the medium are closely linked. Thereby, as pH increases carbonate concentration increases and bicarbonate and molecular CO_2 decrease, and vice-versa. However, at neutral pH values, any CO_2 consumed by photosynthesis can be replenished from a large pool of bicarbonate, since that is the predominant carbon source at neutral pH. In acidic media, microalgae do not have such amount of bicarbonate so carbon availability is a limiting factor for them. Thus, some microalgae have adapted to this low CO_2 condition through the development of CO_2-concentration mechanisms (CCM) (Badger et at. 2000; Giordano et al. 2005).

Regarding the effect of this factor on carotenoids accumulation, Fernández-Sevilla et al. (2010) reported that, in general, the maximum lutein productivity is achieved at the optimum pH for biomass productivity. In the case of microalgal lipids composition, pH fluctuations in culture medium can alter the content of total

fatty acids (measured as PUFAs and EPA) as it was observed in *Pinguiococcus pyrenoidosus* at a pH value of 7 (Sang et al. 2012). Another example is *Chlorella sp*, where a decrease in the membrane lipid content with simultaneous accumulation of TAGs was observed to be directly related to alkaline pH stress (Guckert and Cooksey 1990).

1.4.5. Nutrients.

Microalgae require macronutrients such as C, N, O, H, P, Ca, Mg, S, and K for their growth. The micronutrients usually required are Fe, Mn, Cu, Mo, and Co and some species need low concentrations of vitamins in the culture medium (Guillard 1975). Manipulating cultivation conditions and the presence or absence of nutrients, the biosynthesis of specific compounds can be stimulated. As example *Chlorella* changed its biomass composition (in terms of proteins and lipids content) varying its cultivation conditions (Richmond 1990). Other example can be found in *Synechococcus leopoliensis,* in which higher concentrations of antimicrobial agents were found using leucine combined with citrate or acetate (Noaman 2004). Also a culture medium supplemented with vinasse resulted in an increase in protein yield from *Spirulina platensis* (Coca et al. 2015). On the other hand, the production of astaxanthin in *Chlorella zofingiensis* increased when it was cultivated under low nitrate and high glucose concentrations (Alonso et al. 2000; Ip and Chen 2005).

Microalgal growth and production of molecules of interest are closely related to the nutrient content in the culture media. In that sense, if a nutrient is limited or omitted from the cultivation medium, the microalgal cells change their metabolism resulting in the alteration of their biomass composition. In a study carried out by Alonso et al. (2000), an accumulation of saturated and unsaturated fatty acids in *Phaeodactylum tricornutum* cultures was described when the nitrogen source was reduced. In the majority of the cases, the alteration of the biomass composition is related to the accumulation of carbohydrates or lipids. Thereby, after Spoehr and Milner demonstrated that *Chlorella pyrenoidosa* cultivated under nitrogen starvation accumulated 80% higher lipid content than the typical cultures (Spoehr and Milner 1949), nutrient starvation (especially nitrogen

and/or phosphorus) has been considered as the most efficient approach to enhance lipid content in microalgae (Chen et al. 2011; Zhu et al. 2016).

1.4.5.1. Nitrogen stress conditions.

Nitrogen stress is the most applied nutrient stress factor towards the optimization of biomolecules production in microalgae. Under nitrogen starvation, the flow of the photosynthetically fixed carbon is turned from the protein synthesis metabolic pathway to the lipid or carbohydrate synthesis pathway (Hu 2004), resulting to an accumulation either of carbohydrates or lipids. It has been observed that the required nitrogen starvation period for carbohydrates accumulation is shorter than that for lipids accumulation, which might be due to the fact that carbohydrates are first synthesized to reserve energy and then, if the stress is prolonged, lipids are produced as long-term storage mechanism (Siaut et al. 2011). Studies related to carbohydrates accumulation reported that in nitrogen starved cultures of *Chlorella vulgaris* carbohydrates accumulated up to 38-41% (Brányiková et al. 2011; Dragone et al. 2011), above 35% in *Tetraselmis subcordiformis* (Ji et al. 2011) while in *Spirulina maxima* carbohydrates accumulated 60-70% (De Philippis et al. 1992) and in *Spirulina platensis* 55-65% (Sassano et al. 2010). On the other hand, accumulation of lipids has been detected in cultures of *Scenedesmus sp.* (Pancha et al. 2015), *Chlorella sp.*, (Praveenkumar et al. 2012) and *Chlorella vulgaris* (Yeh and Chang, 2011), subjected, as mentioned before, to nitrogen starvation. Moreover, it has been reported under nitrate stress an increase of EPA in *Phaeodactylum tricornutum* (Yongmanitchai and Ward 1991). However, there is a negative correlation between PUFAs production and nitrogen stress, possibly due to the fact that when algal cells are exposed to nitrogen limiting conditions, cells escalated TAGs synthesis at the expense of polar lipids (mainly PUFAs) (Cohen 1999).

1.4.5.2. Phosphorus stress conditions.

Phosphorus is involved in process of energy transfer of cells, phospholipid biosynthesis and membrane development. Phosphorus in low concentration is taken-up at high rate and is stored as polyphosphate in amounts considerably

higher than the immediate metabolic needs, which is known as luxury uptake (Powell et al. 2009). It is known that carbohydrates start to accumulate when the intracellular phosphorus drops below a threshold limitation level (Cade-Menun and Paytan 2010) and when minimum intracellular phosphorus is reached then the carbohydrates production increases to its maximum values (Markou et al. 2012a). There are some studies which claim that carbohydrates are more significantly affected by the phosphorus starvation and their accumulation is more notable than that of lipids (Guerrini et al. 2000; Dean et al. 2008), whereas in contrast, Ji et al. (2011) reported that in cultures of *Tetraselmis subcordiformis*, phosphorus starvation had no effect on the carbohydrates accumulation. Considering lipids accumulation, Reitan et al. (1994) have described increased accumulation of TAGs in some microalgae species due to phosphorus limitation, and even 6-fold increase in the TAGs percentage was observed in *Monodus subterraneus*, when subjected to phosphorus limiting stress condition (Khozin-Goldberg and Cohen 2006). On the other hand, in phosphorus starved cells of *Chlorella kessleri* high levels of unsaturated fatty acids were observed (El-Sheek and Rady 1995).

1.4.5.3. Sulfur.

Sulfur is an element that is mainly found in proteins, since it is the main source for the biosynthesis of the amino acids cysteine and methionine. But it is also found in lipids and in other chemical structures involved in different functions, such as regulation of redox processes, antioxidant function or resistance to metals (Grossman 2000). In a study carried out by Brányicová et al. (2011), it was demonstrated that sulfur deprivation was a better factor than nitrogen or phosphorus deprivation to produce starch accumulation in *Chlorella vulgaris* cultures, which was able to maintain a content of 60% of such compound even after 32 hours of deprivation (stationary phase). Furthermore, it has been found that total fatty acids content in *Chlamydomonas reinhardtii* was doubled after exposure to sulfur limitation (Matthew et al. 2009). Carotenoids production is also affected by sulfur limitation. It has been reported that in *Dunaliella salina* the levels of β-carotene in sulfur-limited cultures were approximately a 20% higher as compared with the levels in sulfur sufficient cultures (Giordano et al. 2000).

1.4.6. Carbon source.

The ability of microalgae to fix carbon and the changes that occur in their fatty acid profile when cultures are stressed with organic and inorganic carbon sources is gaining interest. An increase in the saturated fatty acids (SFA) content on *Monoraphidium minutum* grown with glucose, fructose and sodium acetate was studied by Patidar et al. (2014). Also changes in the FA profile of *Chlorella variabilis* ATCC 12198 stressed with sodium hydrogen carbonate at different pH were observed (Patidar et al. 2016). On the other hand, using glucose as carbon source has been reported to increase biofuel potential of *Scenedesmus sp*. (Pancha et al. 2015).

Regarding carbohydrates production, the carbon dioxide concentration affects its accumulation in microalgae. Decrease of carbon dioxide concentration results to an increase of the carbohydrate content in the algal biomass (Thyssen et al. 2001). This is due to the presence of carbon dioxide concentrating mechanisms (CCM) in certain strains, enabling microalgae to acquire and concentrate inorganic carbon from the extracellular environment. The CCM are induced under low carbon dioxide conditions and cause efficient carbon dioxide utilization during photosynthesis (Izumo et al. 2007).

1.4.7. Micronutrients.

Micronutrients, or trace elements, such as iron, molybdenum and manganese can play critical roles in a variety of metabolic pathways involving utilization of light, nitrogen, phosphorus, and CO_2 (Raven 1988; Raven 1990). Among trace elements, iron is essential for photosynthetic electron transport, respiratory electron transport, nitrate and nitrite reduction, and detoxification of reactive oxygen species (Maldonado and Price 1996; Sunda and Huntsman 1997; Sunda and Huntsman 2004). Mojaat et al. (2008) demonstrated that the addition of iron to the *Dunaliella salina* culture medium stimulated β-carotene production.

Molybdenum is coupled with iron in the enzymes for nitrate reduction, and its deficiency diminishes the nitrate uptake mechanism and interferes with lipid synthesis (Carvalho et al. 2006).

Manganese is another important component in algal photosynthesis and it is also present in enzymes to remove toxic superoxide radicals to sustain algal growth (Peers and Price 2004). It has been reported that manganese ($MnCl_2$) enhanced the capacity to accumulate inorganic minerals and catalysed protein synthesis in *Spirulina platensis* (Chernicova et al. 2006).

1.4.8. Culture media optimization and fertilizers usage.

Culture media optimization is an important factor to take into account in microalgae cultivation due to the current high cost associated with their biomass and metabolites production. Previous research studies showed that in the development of microalgal products, one of the major tasks is to select a suitable culture medium (Monserrat et al. 1993; Gong and Chen 1997), so that the quality and quantity of biomass can be enhanced. The choice of such medium mainly depends on factors as its chemical composition (Borowitzka 2005) or the desired major metabolite accumulation. However, to obtain biochemical compounds with high added value at a commercial level, it is necessary to produce biomass on a large scale (Liu and Hu 2013), which implies a high demand of optimum analytical grade reagents and a considerable consumption of time due to the preparation of such media. All this also has the disadvantage of raising the production costs of microalgae cultures (Molina et al. 2003; Borowizka 2005).

With the purpose of reducing costs and even reaching better productivity results, some studies have been carried out with agricultural fertilizers as culture media for different species of microalgae (Fábregas et al. 1987; Kanlis et al. 2004; Nayak et al. 2016; Silva-Benavides 2016). In this sense, it has been recognized that the replacement of chemical compounds by such fertilizers must be a way to make microalgae cultivation even easier and cheaper (Scardoelli-Truzzi and Sipaúba-Tavares 2017).

1.5. Microalgae biomass and their added-value molecules in the market.

The interest in microalgae has increased as a result of the need for additional food supplies, energy resources and various raw materials. As a result, market size of functional food derived from microalgae has increased 5-fold since

the beginning of the century, and now its development is relatively mature (Vicentini et al. 2016). It may be pointed out that microalgal biotechnology has emerged due to the great diversity of the products that can be developed from its biomass, which is currently used both as dried whole algae and for the extraction of high-value foods supplements (such carotenoids and omega 3 fatty acids). Gantar and Svircev (2008) suggested employing the microalgal biomass as a source of biomolecules of interest instead of using the whole biomass, in order to be accepted by consumers, since its fishy taste, as well as a powdery consistency, are some of the inconveniences of the incorporation of microalgal biomass into traditional food products.

Most microalgae currently used on the market belong to the genus *Arthrospira, Chlorella*, and *Dunaliella* (Brennan and Owende 2010). *Arthrospira* is a cyanobacteria that presents a significant protein content (60% to 70% of total dry weight) and an excellent nutritive value that is also based on the high content of iron and essential unsaturated fatty acids, which makes interesting its consumption. Moreover, it is also one of the richest natural sources of vitamin B12 (Doshi et al. 2010). Regarding the human health, it is known that this microalga has various beneficial effects: antihypertensive, prevention of renal failure or improvement of the growth of beneficial intestinal *Lactobacillus* bacteria (Beheshtipour et al. 2013). A close substitute of *Arthrospira* is the green microalgae *Chlorella,* which is considered a super food based on its high protein content. Its biochemical composition shows that it contains 11-58% protein, 12-28% carbohydrate and 2-46% lipids (Zhu et al. 2014). This microalga also contains an important substance called β-1,3-glucan, which is a free radical scavenger that could work as an active immunostimulator, an antioxidant, and a reducer of the lipid level in the blood (Iwamoto 2004). Other health-promoting effects have been identified (efficacy on gastric ulcers, wounds, and constipation) so *Chlorella* can also be administered as a food supplement (Spolaore et al. 2006; Mata et al. 2010; Sastre 2012). Another potential microalga is *Dunaliella,* which is mainly exploited to obtaine β-carotene. The content of such pigment produced by *Dunaliella* can reach up to 14% of its dry weight (Tafreshi and Shariati 2009). Under stress conditions of growth, such as high salinity, temperature or irradiance, the cells accumulate high amounts of carotenoids and change their color from green to orange (Sastre 2012; Tran et al. 2014).

Regarding the production of *Arthrospira* and *Chlorella,* it is known that both species have the largest production worldwide (12.000 and 5.000 tons of dry weight/year respectively), with estimated market values, in 2017, of about $360 and $220 Millions/year, respectively (García et al. 2017). *Arthrospira* production is located especially in Asia and USA, whereas *Chlorella* is mainly produced in Asia, although both could be produced in a small number of countries with warm climates (Milledge 2012).

There are also many countries (Japan, USA, China, Thailand, Germany and France) which have started to market functional foods containing microalgae and cyanobacteria under different forms (Figure 5), for instance, capsules and tablets. Microalgae can also be incorporated into pasta, bread, yogurt, soft drinks, snack foods, candy bars, or chewing-gum (Pulz and Gross 2004; Mohamed et al. 2013).

Figure 5. Some commercially available products containing *Spirulina* (*Arthrospira's* commercial name) (a, e, f) and *Chlorella* (b, c, d).

Some microalgae are also commonly used in cosmetics (Borowitzka 2013). The extracts of these microalgae are added in regenerating creams for face and body lotions. They are also used in sun protection creams, shampoos and hair masks. An example of that is an extract of *Chlorella vulgaris* which stimulates the

synthesis of skin collagen and which is already found in the market (Dermochlorella, Codif, St. Malo, France).

Despite all the advantages of microalgae cultivation, as they offer a unique opportunity to produce biofuel, food, feed, cosmetics, biofertilizer, bioplastic and pharmaceuticals, the primary microalgal products will not be economically viable unless the costs for processing are lower. Considering that microalgae are still not a well-studied life form from a biotechnological point of view, further research should be carried out to be able to maximize all their benefits.

1.6. Studied microalgae.

In this Doctoral Thesis the effects of certain cultivation conditions over the metabolites productivity of the acidophile microalga *Coccomyxa onubensis* and two strains belonging to the species *Botryococcus braunii* (race A and race B) were studied. Both, *Coccomyxa* as well as *Botryococcus*, belong to the class *Trebouxiophyceae*, being phylogenetically close, as it is shown in Figure 6. In a study carried out by Fang et al. (2015), it was suggested that the transcriptome of a strain of *B. braunii* race A was closely related to that of *Coccomyxa* specie.

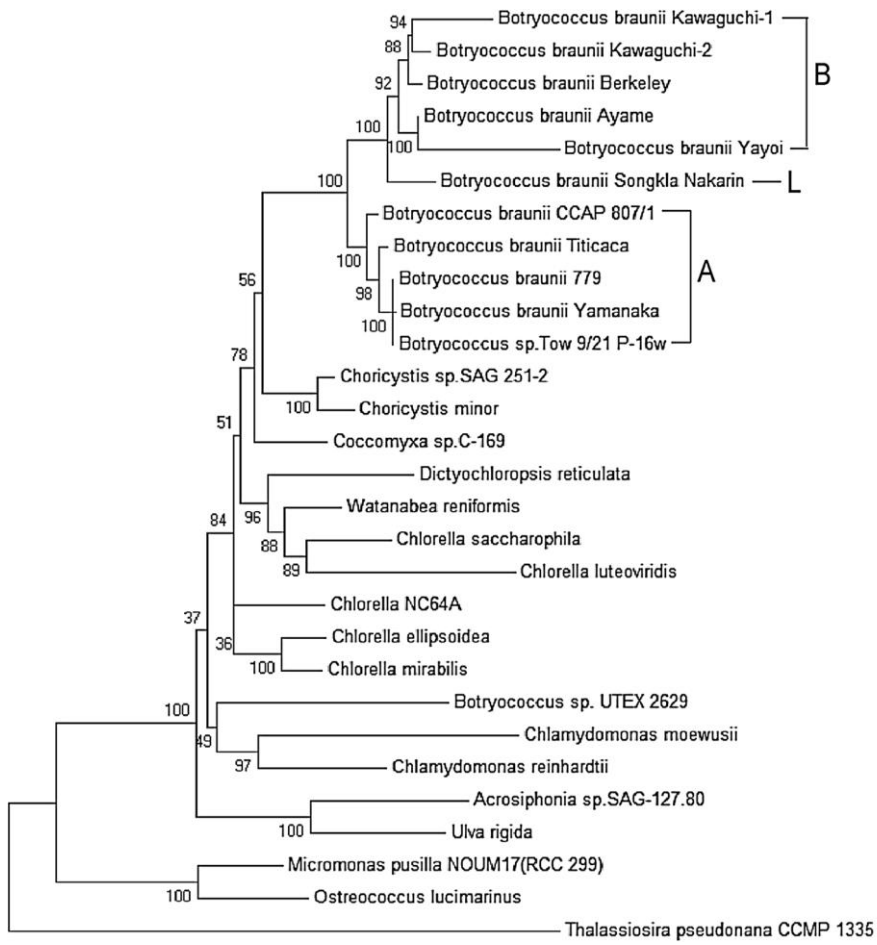


Figure 6. Phylogenetic tree, based on the 18S rDNA sequence, showing the relationship between *Botryococcus* and *Coccomyxa* (Fang et al. 2015).

1.6.1. *Coccomyxa onubensis*.

Coccomyxa onubensis is a novel eukaryotic green microalga isolated from an acidic environment, which is already known to accumulate carotenoids when it is grown mixotrophically with urea (Casal et al. 2011) or in response to metal stress (Vaquero et al. 2012). Under nutrient starvation, an increase in the total lipid content, including changes in the Fatty Acids Methyl Ester (FAMEs) content profile was observed (Ruíz-Domínguez et al. 2015).

The biochemical composition of *C. onubensis* was determined by Navarro et al. (2016) which showed to contain a high protein content of 44.60% and moderate content of carbohydrates (24.8%) and fiber (15.73%). Navarro et al. (2016), also carried out an experiment to test the effect of this biomass on rats that were fed with it, and it could be demonstrated that this microalga may be good as a food supplement for laboratory animals and may also serve as a nutraceutical in functional foods.

In Part I of Results and Discussion of this Thesis, it is assayed the potential of *C. onubensis* to produce antioxidant molecules, such as carotenoids and, in some cases, lipids as well as its antioxidant enzymatic response, when it is subjected to stress conditions. In this sense, two new factors were studied: high temperature and UV-radiation (A or B).

1.6.2. *Botryococcus braunii.*

Botryococcus braunii is a green colony-forming microalga which has the almost unique capacity to synthesise, accumulate and excrete large amounts of long chain hydrocarbons and/or interesting groups of polysaccharides which can be further converted into bio-chemicals (Banerjee et al. 2002). In the last two decades, these characteristics have increasingly attracted the attention of researchers in attempts to exploit *B. braunii* as renewable source of products (Li and Qin 2005).

This microalga can be classified into three races (A, B and L), generally depending on the type of hydrocarbon that it synthesizes (Table 1). Race A produces odd-chain fatty hydrocarbons (alkadiene, alkatriene); race B produces polyunsaturated and branched triterpenes called Botryococcenes and methylated squalene, and race L produces tetraterpenoids hydrocarbons from isoprene, namely lycopadiene (Yoshida et al. 2012).

In Part II of Results and Discussion of this Thesis, experiments were carried out with a *B. braunii* race A (carbohydrates producer), and another *B. braunii* race B (hydrocarbons producer) in order to enhance its productivity.

Table 1. Distinctive features of *B. braunii* Races A, B and L, modified by Banerjee et al. (2002).

	Race A	Race B	Race L
Nature of hydrocarbons	C_{25} – C_{31} odd numbered n-alkadienes/trienes	Botryococcenes (triterpenes) C_nH_{2n}, n=30-37	Lycopadienes (tetra-terpene) $C_{40}H_{78}$
Colony color in stationary phase	Pale yellow or green	Orange-reddish or orange-brownish due to accumulation of carotenoids	
Long chain alkenyl phenols	Present	Absent	
Nature of biopolymers	Very long aliphatic chains cross-linked by ether bridges and bearing fatty esters		Tetraterpenoid cross-linked by ether bridges

Regarding the polysaccharides, its composition and the amount produced by *Botryococcus* also depends on the race, ranging from 0.250 $g \cdot L^{-1}$ (races A and B) to 1 $g \cdot L^{-1}$ (race L) (Allard and Casadevall 1990).

Considering the extreme variability between the different *Botryococcus* races and strains, and their slow growth, working with *B. braunii* still presents important challenges (Banerjee et al. 2002). The main reasons for their slow growth and low productivities are related to the composition of the culture media (nutrient limitation/starvation) and the morphology of the colonies, where cells are tightly connected together not allowing a fast diffusion of nutrients (Li and Qin 2005). Furthermore, different species of *Botryococcus braunii* seem to have different growth requisites and necessitate a specific fine tune of the media. In fact, differences in nutrients composition and concentration result in different biomass densities and morphologies, and affect the growth rate and the accumulation of intracellular compounds (Yang et al. 2004; Metzger and Largeau 2005).

In this Thesis, it is attempted to establish an optimum culture medium for each *B. braunii*'s race (A and B), which leads to the best productivity values of both, biomass as well as carbohydrates and hydrocarbons (depending on the race).

2. THESIS OUTLINE.

This Doctoral Thesis aimed at obtaining high productivities of different metabolites of commercial interest, such as carotenoids pigments, polyssacharides and hydrocarbons, from three strains of microalgae, one isolated from an extreme acidic environment and other two from the genus *Botryococcus.* To achieve this, the dissertation was organized as follows:

Chapter 1 provides information about the isolation and characterization of new species of extremophile microalgae from Río Tinto. Our research group has experience in the isolation of microalgae species from the same river (Cuaresma et al. 2006; Garbayo et al. 2012) which produce important amounts of lutein, carotenoid of high commercial value. In this respect, it was expected that the species used in this first part of the Thesis might also produce pigments of commercial value. From all the isolated microalgae, the most promising from the point of view of biomass and carotenoids production was eventually molecularly identified to carry out its optimization in the next Chapter.

Chapter 2 presents experimental results about the effects of some abiotic stress factors, such as high temperature and UV-radiation, on *C. onubensis* cultivation. For that, the influence of a range of temperatures, ranging from 25 ºC to 40 ºC, and the exposure to UVA and UVB radiation was assessed in the microalgal growth and carotenoids and lipids production was assessed. Besides, the antioxidant enzymatic response of catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) was also studied.

Chapter 3 includes, on one hand, a literature review about the different culture media in which *B. braunii* can be cultivated. That information was used to carry out the culture medium optimization in well-plates by means of an experimental design in which 46 new culture media were defined. Finally, a validation of the results was carried out in Roux flasks in order to finally select the new media which might allow to achieve higher productivities (biomass and metabolites) for each race.

Chapter 4 evaluated the use of commercial fertilizers (NPKs) to grow both *B. braunii* races in order to see if productivity could be further improved and to reduce the production costs. First, the evaluation of different nitrogen sources was carried out. NPKs were selected accordingly and evaluated. Once the best NPK was selected, its concentration was also evaluated at different levels in order to see if productivities could be further improved.

Finally, **Chapter 5** is a general discussion about the main findings of this Doctoral Thesis as well as it gives insight on future research prospects. Therefore, the role of some of the main abiotic factors in *C. onubensis* and *B. braunii* growth and product productivity was considered and conclusions were drawn in weather such factors were needed to improve those parameters.

II. MATERIALS AND METHODS

1. MICROALGAL STRAINS USED

1.1. Microalgal species.

In this Doctoral Thesis three strains of microalgae, belonging to the class *Trebouxiophyceae*, were used for the experimentation; one strain belonging to the genus *Coccomyxa* and two other strains belonging to the genus *Botryococcus* (race A and race B).

1.1.1. *Coccomyxa onubensis.*

The experimentation described in Part I of this Thesis has been carried out with the extremophilic microalga *Coccomyxa onubensis*, isolated by our research group (Biotechnology of Algae Group, BIO-214) in the northern part of the riverbed of the Río Tinto, in Huelva (south-west of Spain). The Río Tinto rises at the north of the town of Nerva in Huelva and flows, after 92 Km, into the Atlantic Ocean. This area is characterized by the presence of a pyritic belt of submarine hydrothermal origin, which has generated intense mining activity since ancient times (González-Toril et al. 2003).

The Río Tinto, natural habitat of *C. onubensis,* is characterized as an extreme environment, with acidic pH values (≤ 2.5) and high concentrations of heavy metals in solution, especially iron, manganese, copper and aluminum, as well as sulfate and nitrate anions. Ferric ion is one of the most abundant ions and, because it remains in solution at low pH, it is the one that gives the river its characteristic red colour. This river is a heterogeneous ecosystem in which multiple microorganisms proliferate, both prokaryotes and eukaryotes, which form interdependent but at the same time connected ecosystems. Along the river course the channel is wide and the river flows forming a shallow sheet of water that allows the penetration of light to the bottom, facilitating the development of primary photosynthetic producers, mainly as biofilms (López-Archilla 2005), the main suppliers, in terms of biomass, of organic matter to the system. However, the extreme conditions of the river are, in large part, produced and maintained by chemolithotroph prokaryote organisms. In addition to primary producers, fungi, protists and heterotrophic bacteria act as consumers and decomposers (López-Archilla et al. 2001, López-Archilla 2005).

Within the community of microalgae existing in the river, species have been identified as belonging to the classes *Bacillariophyta*, *Euglenophyta* (*Euglena Mutabilis* which represents 32% of the total algae of the Río Tinto), *Chlorophyta* (mainly of the genera *Chlamydomonas* and *Chlorella*) and *Rhodophyta* (López-Archilla et al. 2001). More specifically, *Coccomyxa onubensis*, microalgae used in this Thesis and isolated by our research group from Río Tinto belongs to the *Eukaryota* domain, *Chlorophyta* division, *Trebouxiophyceae* class and the *Coccomyxaceae* family.

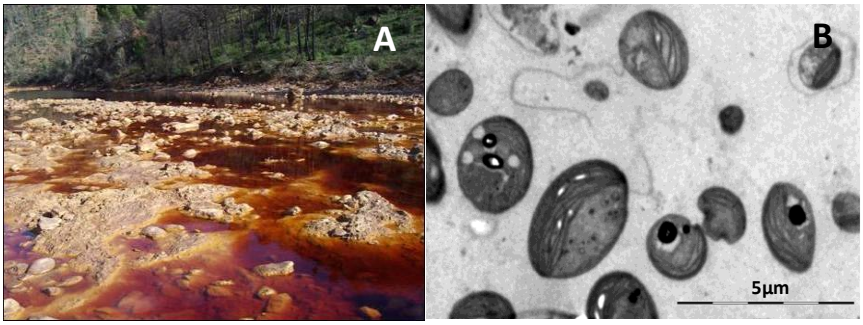


Figure 7. A) Picture of Río Tinto showing the striking reddish color of its waters. B) Transmission electron microscopy of *C. onubensis* cells. Scale bar is present.

1.1.2. *Botryococcus braunii.*

The experiments described in Part II of this Thesis have been carried out using two strains of the microalgal species *Botryococcus braunii*, obtained from the Culture Collection of Autotrophic Organisms of Trebon (Czech Republic), and the laboratory of Chemistry Bio-Organic and Organic Physics of the National Higher School of Chemistry of Paris (France). *B. braunii* is a unicellular microalga of the *Chlorophyta* division, widely distributed, which can be found in freshwater, brackish, saline lakes, reservoirs and ponds (Dayananda et al. 2007), forming colonies.

As it has already been detailed in the Introduction chapter, depending on the characteristic hydrocarbons produced, *B. braunii* can be classified into three different races designated A, B and L.

In this Part II of the Thesis, different experiments were carried out with a microalga of the race A, producer of carbohydrates, and another of the race B, producer of hydrocarbons (Figure 8).

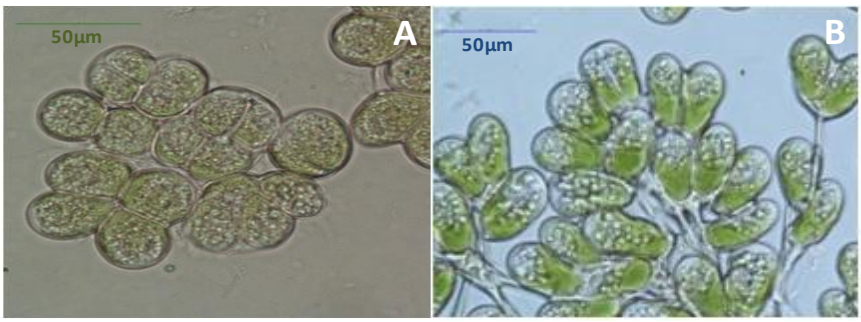


Figure 8. Pictures of **A)** *B. braunii* race A, and **B)** *B. braunii* race B, both observed under the optical microscope (100x). Scale bar is present.

From the taxonomic point of view, the microalga *B. braunii* is included in the *Eukaryota* domain, *Chlorophyta* division, *Trebouxiophyceae* class and *Botryococcaceae* family.

2. ISOLATION AND MOLECULAR IDENTIFICATION OF MICROALGAE.

2.1. Sampling and isolation procedure

Sample collection was carried out in different points of the northern part of the Río Tinto's riverbed, specifically in the area of Nerva. To do this, samples of water were collected in Falcon tubes from areas where the shallow waters allowed the formation of green biofilm at the margins of the river. The different Falcon tubes were previously coded according to the sampling site. All the codes started with an "N" for sampling in the Nerva area, followed by a number designating the exact collection point.

Isolation of microalgae was carried out under sterile conditions in the laboratory, inoculating for it 0.5 mL of the different liquid samples collected in petri dishes previously prepared with solid culture medium K9 (see section 3.1.1.1) and supplemented with 5% agar. The petri dishes were incubated in a culture room at 25 ºC and with continuous PAR light supply, 100 µmoles of photons·m^2·s^{-1}, until

the growth of photosynthetic microorganisms was observed. By repetitive streaking on agar plates isolated colonies were obtained. Transfer of colonies from solid medium to liquid medium was carried out in a laminar flow cabinet and with the help of a sterile seeding handle. Initially, the colonies were transferred to a volume of 1 mL of K9 liquid culture medium in a 2 mL eppendorf tube, keeping sufficient headspace to favors the exchange of gases between the culture medium with microalgae cells, and the empty space above the liquid medium. Subsequently, scale-up was carried out until a final working volume of 500 mL was achieved to maintain the different algal strains in erlenmeyers.

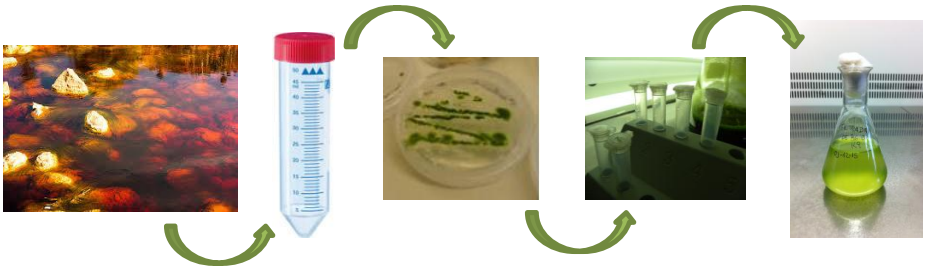


Figure 9. Scheme of the microalgae isolation process carried out.

2.2. Molecular identification.

DNA of the selected microalgae colony was extracted using the Plant DNA Kit (Omega Biotek). From the extracted DNA, the 18 rDNA sequence was partialy amplified by using the primers NS1F (GTA GTC ATA TGC TTG TCT C) and NS2R (GGC TGC TGG CAC CAG ACT TGC) (White et al. 1990). The PCR was performed in a thermocycler CFX96 Real-Time System (BIO-RAD, California) and consisted of 35 cycles in which the following steps were taken: denaturation at 95 °C for 10 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 30 seconds, preceded by 5 minutes at 95 °C for initial denaturation, and followed by 10 minutes at 72 °C for final extension. The PCR fragment obtained was cleaned before sequencing using the E.Z.N.A. Cycle-Pure kit (Omega BioTek). Sequencing was performed using ABI PRISM 377 (Applied Biosystems, Perkin-Elmer). DNA sequences obtained were processed with BioEdit software (Ibis Biosciences, Abbott Laboratories, USA). Sequence analysis was performed

with BlastN program in the GenBank database (National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov). This method based on the query cover and the identified percentage.

2.3. Cell staining.

In order to visualize and to morphologically differentiate the cells of each microalga isolate, two different cell-staining protocols were used: the hematoxylin-eosin stain and the hematoxylin-picrofuchsin stain. Both differ in the more intense cell coloration obtained with the second one.

Cell staining protocol started with the fixation of the samples. To do that, the following protocol was carried out: extension of a culture drop over a previously degreased glass slide, drying in a stove at 37 °C, fixation by covering it with methanol for 3 minutes, and second drying step in the stove at 37 °C until methanol is evaporated.

Once the samples were fixated, the staining protocol was applied. The glass slides with the samples already fixated were washed with distilled water to remove any remaining methanol and the extensions were subsequently covered with hematoxylin for 5-6 minutes. The excess dye was washed with water and the extensions were covered with eosin for 4-5 minutes or with picrofuchsin for 10 minutes, according to the desired staining. Finally, the slides were washed with distilled water to remove the excess dye and allowed to dry. The samples were visualised under an optical microscope.

3. CULTIVATION CONDITIONS.

3.1. Microalgal culture media.

3.1.1. Composition of the standard culture media for the different microalgal strains maintenance.

3.1.1.1. Culture medium for *Coccomyxa onubensis.*

Medium K9, developed by Silverman and Lundgren (1959), was used as culture medium for *C. onubensis*. Some modifications were done in order to

resemble the chemical composition and pH of the natural environment from which the microalgae was isolated, the Río Tinto. The composition per liter of K9 medium is presented in Table 2.

To prepare the Hutner's trace solution (Hutner et al. 1950) 10 g of EDTA (free acid) were dissolved in 250 mL of distilled water, facilitating its dissolution with concentrated KOH. Apart from that, the compounds mentioned in Table 2 (Hutner's Trace elements solution section) were added in the specified table order to 550 mL of distilled water maintained at 100 ºC for their dissolution. Subsequently, both solutions were mixed, heated until 100 ºC, and then allowed to cool down to 90 °C. At this time, the pH of the solution was adjusted to a value of 6.8 using a 20% (w/v) solution of KOH while keeping the temperature above 70 ºC. Finally, the total volume was adjusted to one liter with distilled water and the solution was left to settle in darkness at 4-6 °C for 48 hours prior use.

Table 2. Chemical reagents used in the preparation of one liter of K9 medium.

Component	**Volume** (mL)	**Amount** (g)
K_2SO_4		3.95
KCl		0.10
$K_2HPO_4 \cdot 3H_2O$		0.50
$MgCl_2 \cdot 6H_2O$		0.41
KNO_3		2.29
$CaCl_2 \cdot 2H_2O$		0.01
Hutner's trace elements solution	5	-
Hutner's trace elements solution *(composition per liter)*		
H_3BO_3		2.28
$ZnSO_4 \cdot 7H_2O$		4.40
$MnCl_2 \cdot 4H_2O$		1.02
$FeSO_4 \cdot 7H_2O$		1.00
$CoCl_2 \cdot 6H_2O$		0.32
$CuSO_4 \cdot 5H_2O$		0.32
$Mo_7O_{24}(NH_4)_6 \cdot 4H_2O$		0.22

Once the K9 culture medium was prepared, it was adjusted to pH 2.5 with the addition of diluted H_2SO_4 or HCl at 10% (v/v).

3.1.1.2. Culture medium for *Botryococcus braunii.*

Both *B. braunii* races (A and B) were cultivated, for maintenance, using a modification of the Chu 13 medium (Gouveia et al. 2017) proposed by Largeau et al. (1980) for *Botryococcus*. The composition per liter of the Chu 13 medium is presented in Table 3.

Table 3. Chemical reagents used in the preparation of one liter of modified Chu13 medium (Gouveia et al. 2017).

Compound	**Volume** (mL)	**Amount** (g)
$CaCl_2 \cdot 2H_2O$		0.11
$MgSO_4 \cdot 7H_2O$		0.20
KH_2PO_4		0.10
KNO_3		0.40
Na_2O_4Se		0.01
Fe NaEDTA		0.02
Micronutrients solution (x10)	1	-
Micronutrients solution		
(composition per liter)		
$CuSO_4 \cdot 5H_2O$		0.01
$ZnSO_4 \cdot 7H_2O$		0.02
$CoSO_4 \cdot 7H_2O$		0.01
$MnCl_2 \cdot 4H_2O$		0.18
$Na_2MoO_4 \cdot 2H_2O$		0.01
H_3BO_3		0.29
HSO_4	1	-

In order to avoid problems of precipitation, stock solutions of 200 mL were prepared (Table 4). All the stocks except Sol. 3 as well as the micronutrients solution were added before autoclaving the medium. Sol. 3 was independently

autoclaved and added to the final media into the laminar flow cabinet once the medium was sterile. The latter was made to avoid phosphates precipitation in the medium after the autoclaving.

Table 4. Stock solutions used in the preparation of one liter of modified Chu13 medium (Gouveia et al. 2017).

	Stock solution	Amount ($g \cdot 200mL^{-1}$)	Volume per liter of medium ($ml \cdot L^{-1}$)
Sol.1	**$CaCl_2 \cdot 2H_2O$**	10.80	2
Sol.2	**$MgSO_4 \cdot 7H_2O$**	20.00	2
Sol.3	**KH_2PO_4**	10.48	2
Sol.4	**KNO_3**	40.00	2
Sol.5	**Na_2O_4Se**	1.88	1
Sol.6	**Fe NaEDTA**	2.00	2

3.1.2. Different culture media used during the specific experimentation.

3.1.2.1. Culture medium optimization for *Botryococcus braunii* races A and B.

Chapter 3 of Results and Discussion presents data related to the optimization of the culture media for both *B. braunii* strains. The culture medium Chu13 (Gouveia et al. 2017) (section 3.1.1.2.) underwent different modifications in the concentration of some nutrients with the aim of optimizing the growth and production of the relevant metabolites on the part of *B. braunii*.

To do this, a literature review was first carried out in order to identify the composition of the culture media in which *B. braunii* is normally grown. The calculation of the identified media molar composition and the comparison with conventional culture media used to grow other microalgae species allowed the identification of possible nutrients limitations in the reference medium. Once the main nutrients were identified, an experimental 3 factorial design was performed using the Design Expert software with the aim of defining new culture media to be compared to the reference media.

3.1.2.2. Cultivation of *Botryococcus braunii* races A and B with fertilizers.

Chapter 4 of Results and Discussion presents data related to the cultivation of both races of *B. braunii* in culture medium prepared with different commercial fertilizers (Agroliq NPKs, Agralia Fertilizers, S.L), whose codes and composition are shown in Table 5. The objective of these assays was to minimise production costs without compromising the productivity of biomass and relevant metabolites (carbohydrates or hydrocarbons according to the race).

Table 5. Code and composition of the different commercial fertilizers used for the optimization of *B. braunii* cultivation.

NPK	N $mol \cdot L^{-1}$	P $mol \cdot L^{-1}$	N / P	K $mol \cdot L^{-1}$	Nitrogen source	Other nutrients
12-6-4	5.54	0.50	11.02	1.01	9% urea, 3%ammonium	S (0.45 M)
4-10-10	2.66	0.84	3.15	2.55	4% ammonium	
18-6-6	8.01	0.52	15.28	1.58	15% urea, 2.1% ammonium	
2.4-4.8-6	1.08	0.38	2.84	1.44	1.6% nitrate, 0.8% ammonium	
8-6-6	3.63	0.49	7.35	1.49	6% urea, 2% ammonium	

In order to avoid nutritional deficiencies related to the presence of calcium and magnesium, elements that naturally can be found in the tap water which would be used to prepare culture media at large scale, the various media prepared with fertilizers were supplemented with these elements at a concentration equal to that of the control medium of each *B. braunii* race. Likewise, the cultures were supplemented with a solution of commercial micronutrients (Agralia AG Complex, Agralia Fertilizers, S.L) depending on the concentration of iron present in the control medium. The composition of this micronutrient solution is presented in Table 6.

Table 6. Composition of AG Complex micronutrients solution used for the cultivation of *B.braunii* in media prepared with fertilizers.

Micronutrients	% w/v	mol·L^{-1}
Boron	0.40	0.37
Copper	0.20	0.03
Iron	5.10	0.91
Manganese	2.60	0.47
Molybdenum	0.10	0.01
Zinc	0.60	0.09

The use of fertilizers was independently evaluated for each strain of *B. braunii*. The different reference media to be compared were chosen according to the result of the media optimization experiments (Chapter 3).

Prior to the experiment with the commercial fertlizers, the preferred nitrogen source of the *Botryococcus* strains was evaluated (ammonium, nitrate, urea). For that, the chemical reagent used to supply the nitrogen was accordingly modified while keeping the final molar nitrogen concentration. Once the appropriate nitrogen source was determined, fertilizers whose nitrogen source was adequate were used to prepare the new media. The amount of fertilizer used was adjusted based on the nitrogen concentration of their respective reference media but, in order to avoid precipitation and to save nutrients, it was decided to use only one-third and one-half of the original N content, depending on the fertilizer. Finally, once the fertilizer with the best productivities for both strains was determined, different concentrations of such fertilizer were evaluated to see if the productivities could be further improved.

3.2. Microalgal cultivation conditions.

3.2.1. Standard cultivation conditions.

Reference cultures of microalgae in liquid medium were cultivated under photoautotrophic conditions in a culture room at 25 ºC, using erlenmeyer flasks of different volumes unless otherwise indicated. The cultivation flasks containing the different medium were previously prepared as follow: flasks were closed with

hydrophobic cotton coated with gauze; the cotton was crossed, for subsequent aeration of the culture, by a hollow glass rod with an air filter of 0.2 µm pore size placed at the top end of the rod; the flasks containing the medium were sterilized in an autoclave for 20 minutes, at a temperature of 126 °C and a pressure higher than one atmosphere; and, finally, the flasks were inoculated with the corresponding microalgae inside a laminar flow cabinet to preserve the sterility.

The inorganic carbon source was supplied by bubbling CO_2-enriched air through the hollow glass rod immersed in the medium with the cellular suspension. The CO_2 source consisted of a mixture of air and CO_2 at 2.5% (v/v). These conditions of cultivation are the so-called "standard" in this Thesis text.

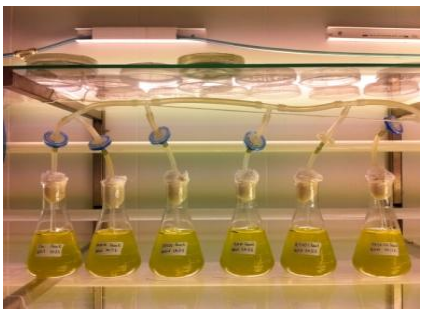


Figure 10. Cultivation of *B.braunii* race B under photoautotrophic conditions.

Finally, the light source used to provide light energy to the cultures were white light fluorescent tubes (Philips 30W/33) unless otherwise indicated and they were placed in the side panels of the culture room. The distance of the different cultivation flasks to the lamps was regulated in order to control the incoming light intensity. Under standard conditions this intensity is 100 µmoles of photons·m^2·s^{-1} of PAR light on the surface of the culture flasks.

3.2.2. Cultivation of *Coccomyxa onubensis* under ultraviolet light irradiance.

Chapter 2 of Results and Discussion presents results related to the cultivation of *C. onubensis* under ultraviolet light irradiance. Different cultures were incubated under PAR + ultraviolet-A (UVA) and PAR + ultraviolet-B (UVB) radiation conditions in order to analyze the influence on the growth and production

of carotenoids in *C. onubensis.* Philips TLD 15W/05 and Philips TL 20W/01 lamps were used respectively to supply UVA and UVB radiation, and they were placed on independent panels whose distance to the cultures was also regulated according to the desired UV intensity. The different irradiances used in this study, were 140 µmoles of photons $m^2 \cdot s^{-1}$ PAR light + 37 µmoles of photons$\cdot m^2 \cdot s^{-1}$ of UVA light and 140 µmoles of photons$\cdot m^2 \cdot s^{-1}$ PAR light + 0.67 µmoles of photons$\cdot m^2 \cdot s^{-1}$ of UVB light.

As previously commented the cultures were incubated under standard conditions inside a culture room and supplemented with CO_2-enriched air (see section 3.2.1.).

3.2.3. Cultivation of *Botryococcus braunii* in 24-well plates during the media optimization assays.

Chapter 3 of Results and Discussion presents results related to the media optimization for both *B. braunii* races. Once the new media were defined, they were axenically assessed in 24-well plates incubated at 25 ºC in an environment enriched with CO_2, with an incident light of 100 µmol of photons$\cdot m^2 \cdot s^{-1}$ and mechanical agitation by rotation in an orbital shaker (OL30-he, OVAN, Spain), as shown in Figure 11.

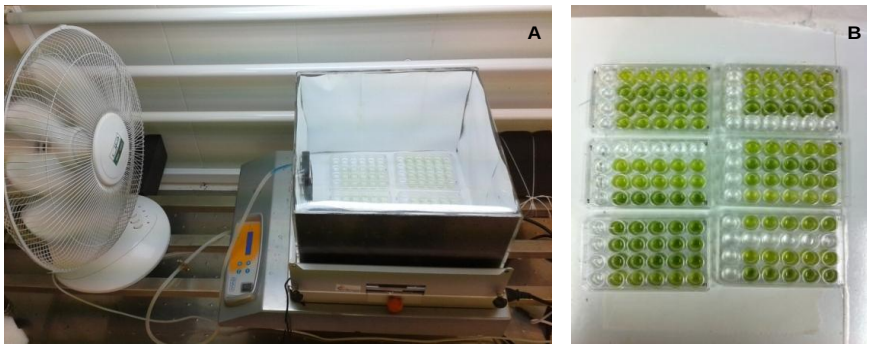


Figure 11. (A) Experimental set-up used for the cultivation in 24-well plates; top illumination provided by white light fluorescent tubes (not shown in the picture), external fan to remove the excess of heat, silicone tube to provide the CO_2 enriched air, which is distributed inside the transparent incubator by another small fan. (B) 24 –well plates inoculated with *B.braunii* race A after 168 h of incubation (each row represents different replicates of the same medium).

Prior to the medium optimization experiment, both races of *B. braunii* were adapted to the new culture media and the experimental conditions by incubation in 24-well plates for one week. After that time, the biomass grown in each culture medium was collected independently, centrifuged and used to inoculate new plates for the real optimization experiment. The different races of algae were inoculated with a low cellular density (OD 750 nm) of 0.1 and 0.2 for *B. braunii* race A and race B respectively, so as not to produce photo-limitation in the microplates. To avoid contamination problems, sterile microplates were used and different culture media were sterilized by filtration (pore size syringe filter of 0.2 µm, Labware, USA). All manipulation was carried out in a laminar flow cabinet (Telstar PV-100, Terrassa, Spain) under sterile conditions. Cell growth was monitored daily for one week by measurement of absorbance at 680 nm and 750 nm in a micro-well plate reader (ELX 800, Biotek, Bad Friedrichshall, Germany).

Figure 12. Well-plate reader used to measure optical density of the different *B. braunii* cultures at 680 nm and 750 nm.

4. ANALYTICAL DETERMINATIONS.

4.1. Physical determinations.

4.1.1. Incident irradiance determination.

The irradiance to which the cultures were exposed was measured with a brand-name radiometer HD 9021 (Delta OHM, Italy) equipped with the corresponding probes for measurements of photosynthetically active radiation (PAR) and ultraviolet light (LP9021 PAR, LP9021 UVA and LP9021 UVB).

4.1.2. Determination of cellular biomass concentration.

4.1.2.1. Optical density determination.

Cellular concentracion was indirectly evaluated by measuring spectrophotometrically the optical density of the cultures at 680 and 750 nm in an UV/Visible spectrophotometer (Evolution 201, Thermo Fisher Scientific, United States). Samples were previously diluted, if necessary, to obtain absorbance values between 0.2 and 0.8. Prior to the sample analysis, the culture medium used in each experimentation was used as blank to be substracted from the samples.

Optical density data at 750 nm, wavelength at which the photosynthetic pigments do not interfere (Griffiths et al. 2011), allows to identify the different growth phases of the different cultures by plotting it against the time. The quick recognition of the culture growth stages makes possible to take immediate decisions on the harvesting, sampling or dilution of the different cultures. On the other hand, the absorbance ratio 680/750 estimates the chlorophyll content of the different cultures.

4.1.2.2. Dry weight determination.

Filter-Lab glass microfiber (Ø 47 mm, pore size of 0.7 µm or 2.7 µm depending on the microalgal strain) were used for microalgae dry weight determination. Prior to the filtration of the cell suspension, the filters were washed with filtered Milli-Q water and dried in an oven (Memmert, Germany) at 100 °C for at least 16 hours. After cooling down inside a dessicator the filters were weighed in a precision balance (Sartorius CP225D, Germany).

The wet biomass was obtained by low pressure filtration, by means of a vacuum pump, of a known volume of the culture, chosen according to its cellular density. This volume was previously diluted in 50 mL of Milli-Q water and once the filtration of the cellular suspension was carried out, the filters were washed twice with the same volume of Milli-Q water, in order to eliminate the salts of the medium that could be retained. The filters were then dried in an oven at 100 °C for at least 16 hours and then placed in a desiccator until they were cooled down to room temperature. Finally, the filters were weighed on the precision balance.

The dry weight is equivalent to the cellular biomass present in the filtered volume ($V_{filtered}$) and is calculated by the difference between the weight of the dry filters after the filtration of the cell suspension ($W_{filter\ +\ cells}$) and their initial weight (W_{filter}).

$$\text{Dry weight} = \frac{W_{filter+cells} - W_{filter}}{V_{filtered}}$$

4.1.2.3. Maximal specific growth rate calculation.

The maximal specific growth rate was calculated by regression from the linear portion of the linear phase of the growth curve, expressed as the Napierian logarithm (Ln) of the difference between the optical density at a time "t" and its initial value ($C_t - C_0$) versus time. Optical density values, as well as dry weight data, can be used interchangeably for the calculation.

$$\mu = \ln \frac{(C_t - C_0)}{\Delta t}$$

4.1.2.4. Productivity calculation.

Biomass productivities were calculated as the increase in dry weight in a given culture volume and for a specific period of time in the linear phase of the microalgae growth.

Similarly, the specific productivity of each relevant metabolite produced by the different microalgae was calculated as the increase in the quantity of this product in a given culture volume, over a given period of time.

$$P_v = \frac{(C_t - C_{t-x})}{\Delta_{t(t,t-x)}}$$

4.2. Chemical determinations performed for the quantification of cellular components.

4.2.1. Extraction and determination of photosynthetic pigments content.

The extraction and determination of the total content of photosynthetic pigments (chlorophylls a and b, and carotenoids) of the different microalgae cultures was carried out by means of a modification of the method proposed by Liechtenthaler (1987). The extraction conditions are specified below.

4.2.1.1. Photosynthetic pigments extraction.

The extraction of pigments was carried out by taking an adequate sample volume to ensure enough biomass (normally 1 mL) in 15 mL falcon tubes. After sample centrifugation at 3,000 g for 5 minutes (Centrifuge 5702, Eppendorf) the supernatant was removed. The biomass pellet was resuspended in an adequate volume of methanol, adjusted according to the amount of pigments expected in the sample (normally 5 mL), and the tubes were placed into an ultrasonic bath (Bandelin Sonorex) for 5 minutes to cause cellular disaggregation. Subsequently, sample tubes were vortexed (VelpScientifica) and then incubated in a dry bath (FB15103, Fisher Scientific) at 60 °C for 20 minutes. The samples were then introduced into an ice bath (0 °C) for 10 minutes and finally centrifuged again at 3,000 g for 5 minutes. The extraction was finished once a completely white pellet was obtained, free of pigments.

4.2.1.2. Spectrophotometric determination of total photosynthetic pigments.

The spectrophotometric determination of the total chlorophylls and carotenoids was performed by measuring the absorbance in the supernatant obtained after extraction at 470, 652 and 665 nm in the UV/Vis spectrophotometer (Evolution 201, Thermo Fisher Scientific, United States) according to the method described by Lichtenthaler (1987). The concentrations of chlorophyll a and b present in the extracts were calculated using the following extinction coefficients at 665 nm and 652 nm: 74.46 and 18.3 $ml \cdot mg^{-1} \cdot cm^{-1}$, respectively. Modified

Arnon´s equations (Lichtenthaler 1987) were used to calculate the chlorophyll a and b content, as well as total carotenoids:

$$Chl_b(\mu g \cdot ml^{-1}) = (34.09 \cdot A_{652} - 15.28 \cdot A_{665}) \cdot dilution\ factor$$

$$Chl_a(\mu g \cdot ml^{-1}) = (16.72 \cdot A_{665} - 9.16 \cdot A_{652}) \cdot dilution\ factor$$

$$Chl_{total}\ (\mu g \cdot ml^{-1}) = Chl_a + Chl_b$$

$$Car_{total}\ (\mu g \cdot ml^{-1}) = \frac{dilution\ factor \cdot 1000 \cdot A_{470} - 1.63 \cdot Chl_a - 104.96 \cdot Chl_b}{221}$$

4.2.1.3. Separation, identification and quantification of specific carotenoids by means of High-performance liquid chromatography (HPLC) in cell extracts.

For the identification and quantification of specific carotenoids, the different methanolic extracts were analyzed by high-performance liquid chromatography (HPLC). After the extraction phase (section 4.2.1.1.), the different supernatants were filtered using a syringe and a 0.45 µm pore size filter (Millipore). The filtered extracts were kept in eppendorf tubes at -4 ºC until the HPLC analysis.

For the subsequent separation and quantification of the main specific carotenoids, a HPLC equipment was used (Figure 13) (Merck-Hitachi L-7100), provided with an UV-Visible detector model L-7420 of the same trademark and a RP18 column (LichroCart RP18, Merck) (5 µm, size 250 x 4 mm). The wavelength of the detector was adjusted to 450 nm.

Acetonitrile:water (9:1) and pure ethyl acetate were used as mobile phases. Before each use, the column had to be balanced with the acetonitrile:water mixture. For the elution of each sample, a modification of the gradient described by Young et al. (1997) was used, which is shown in Table 7.

All the reagents used were HPLC grade, belonging to the PANREAC trading brand.

Table 7. Elution gradient used in the chromatographic separation and quantification of carotenoids. Used method was a modification of the one described by Young et al. (1997).

Time (min)	**Ethyl acetate** (%)	**Acetonitrile : water (9:1)** (%)
0	0	100
10	50	50
15	60	40
20	65	35
25	76	24
32	100	0

The identification and quantification of specific carotenoids was based on the comparison of the retention times of the different peaks with the commercial standards of known concentration (between 0.7 and 1.5 $\mu g \cdot mL^{-1}$) (DHL Water and Environment, Denmark). All standards (chlorophylls a and b, violaxanthin, zeaxanthin, lutein and β-carotene) had purity higher than 99% and were diluted in 100% methanol. From the chromatograms obtained for each standard solution, the appropriate conversion factors were calculated for the quantification of the pigments, dividing the concentration ($\mu g \cdot mL^{-1}$) of the standard between the corresponding peak area (μV).

Figure 13. High-performance liquid chromatography equipment (HPLC) used for the separation and quantification of specific carotenoids.

4.2.2. Total lipids content determination.

4.2.2.1. Extraction of total lipids.

The extraction of total lipids from microalgae was carried out using an automatic Soxhlet (DET-GRAS N, J.P. Selecta) which is shown in Figure 14. The organic solvent used for the extraction was a chloroform:methanol (2:1 v/v) mixture as described by Bligh and Dyer (1959), which ensures the entrainment of the polar and neutral lipid compounds when it gets in contact with the microalgal cells.

During the experimentation, culture samples of approximately 0.5 g of dry biomass were necessary. To do this, the volume taken was adjusted in each case to the existing cellular concentration and that volume was centrifuged. The obtained pellet was washed with distilled water to remove any remaining salt.

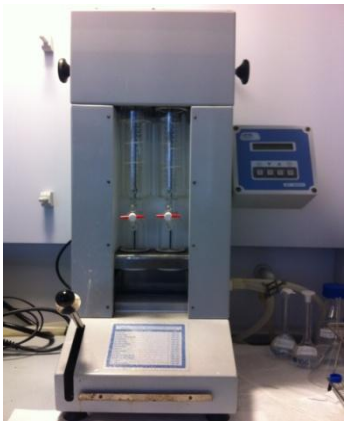


Figure 14. Multi-point grease extractor or automatic soxhlet.

Prior to the lipids extraction, the samples were pretreated in order to break the cells and thus facilitate the extraction. The wet pellet was freeze dried (FD8512, Ilshin) and the moisture percentage of the biomass was then determined. The dry pellet was again hydrated with chloroform:methanol (2:1 v/v), well mixed by intense vortex agitation and finally broken by sonication (Vibra-Cell 75041, BioBlockScientific, Francia) during 5 minutes at 750 W, by pulses of 30 seconds and stops of 59 seconds, in order to facilitate the extraction of total lipids.

Once the pellets were pretreated, they were introduced into the soxhlet cellulose cartridges (ref. 1177-4043, Fisherbrand) and the extraction lasted approximately 3 hours according to the following stages: Boiling 70 minutes, Rinsing 90 minutes, Recovery 15 minutes and Pre-drying 15 minutes. The vials where the oil was collected were dried in an oven for 24 hours at 80 ºC. Differential weight between the empty vial and the vials containing the dry oil allowed to calculate the amount of lipids extracted. The amount of lipids contained per gram of biomass was calculated as follows:

$$Lipids\ (g \cdot g\ dry\ weight^{-1}) = \frac{Weight_{vial+lipids} - Weight_{empty\ vial}}{Weight_{dry\ biomass\ used}}$$

4.2.2.2. Transesterification of total lipids.

The analysis of fatty acids by gas chromatography requires them to be volatile at the working temperatures used in the chromatographer. To do this, it is necessary to transform the fatty acids, esterified or free, in their respective methyl esters through a reaction of esterification with alcohol. This procedure, called transesterification, was performed following the modified method described by various authors (Gouveia and Oliveira 2009; Ota et al. 2009; Widjaja et al. 2009; Halim et al. 2011; Prabakaran and Ravindran 2011).

The transesterification was acidic and approximately 10 mg of the oil residue (triglycerides), previously extracted by Soxhlet, was combined with an alcohol containing a catalyst (methanol enriched at 5% v/v in H_2SO_4) to produce glycerol and alkyl esters of fatty acids. The transesterification reaction was carried out at 70 °C during 3 hours and in continuous agitation by a shaker incubator (model MaxQ 4450, ThermoScientific) to favour the conversion. Subsequently, the samples were refrigerated at 4 ºC and distilled water was added to wash them. Finally, hexane was added, which is the matrix that drags all those methylated fatty acids obtained in the transesterification reaction. The hexane solution was preserved at -20 ºC for further analysis by gas chromotography.

4.2.2.3. Separation and quantification of fatty acid methyl esters (FAMEs) by gas chromatography (GC-FID).

For the later separation and quantification of the main microalgae fatty acids, a gas chromatographer (6890N, Agilent) was used. It included an automatic sampler (7683 B, Agilent) and a flame ionization detector (FID), with a capillary column (Omegawax 320, Supelco) 30 m long, 0.32 mm wide and 0.25 µm internal diameter. The carrier gas was He and the flow rate selected was of 20 $mL \cdot min^{-1}$.

Table 8 shows the temperature gradient of the gas chromatographer (GC) used to obtain defined peaks in the resulting chromatograms. A FID detector temperature of 260 °C, Split injector mode and injection volume of 1 µL were used.

The identification and quantification of the specific fatty acids present in each sample was based on the comparison of their retention times with those of a mix of commercial standards with known concentration (Supelco 37 FAME Mix, ref CRM47885). The used fatty acids standard had a purity from 98.7 to 99.9% and was diluted in hexane as well as the tested samples. The appropriate conversion factors for the fatty acids quantification were calculated from the resulting chromatogram of the standards.

Table 8. Temperature gradient used in the fatty acids determination.

Oven ramp	**Slope** (ºC·min^{-1})	**Temperature** (ºC)	**Retention time** (min)	**Runtime** (min)
0	---	90	0	0
1	10	140	0	5
2	5	165	2	12
3	1	190	2	39
4	5	220	15	60

4.2.3. Proteins determination in microalgal cells.

Proteins extraction was carried out in approximately 1 mL of culture broth, depending on the cell density. Culture samples were centrifuged at 13,000 g (MiniSpin centrifuge, Eppendorf) and supernatant was discarded. The biomass

pellet was placed in a liquid nitrogen bath for at least 2 minutes and the frozen pellet was then disrupted by sonication (Vibra-Cell 75041, BioBlockScientific, Francia) with a total of 5 pulses of 10 seconds and stops of 59 seconds. After defrosting the pellet, it was resuspended in 1 mL of phosphate buffer and the appropriate dilutions were performed to result in a crude extract solution with an adequate protein content to be measured (between 2 and 8 µg).

The protein content was spectrophotometrically determined according to the Bradford's Colorimetric Method (Bradford 1976). The proteins contained in the crude extract interact with the Bradford reagent resulting in a coloured complex, whose maximum of absorption is at 595 nm. The Bradford reagent calibration curve was performed with a solution of bovine serum albumin (BSA, Sigma) at a concentration of 0.02 $mg \cdot mL^{-1}$. To determine the protein content, a volume of 0.2 mL of Bradford reagent (Bio-Rad Protein Assay) was added to 0.8 mL of the crude extract solution and the colored complex was allowed to be formed after gently shaking. Absorbance was measured at 595 nm after 15 minutes.

To carry out the calibration, the exact amount of protein in the BSA solution was determined spectrophotometrically at 280 nm, using the extinction coefficient at such wavelength (ε = 0.69 $mL \cdot mg^{-1} \cdot cm^{-1}$), according to the equation:

$$A = \varepsilon \cdot C$$

in which “A” is the measured absorbance, “ε” is extinction coefficient at 280 nm and “C” is the exact concentration of protein in the BSA solution. Thereby, the concentration of proteins in the analysed samples was determined by interpolation of their absorbances at 595 nm in the calibration curve previously obtained with the BSA in a range of concentration from 1 to 10 $\mu g \cdot mL^{-1}$.

4.2.4. Carbohydrates content determination.

Carbohydrates content was analyzed according to a colorimetric method based on the Dubois principle (Dubois et al. 1956). In order to have reproducible data a minimum of 3 to 10 mg of dry biomass was needed (freeze dried or fresh). Primarly, an acid hydrolysis was carried out by adding 2.5 M HCl in a proportion of 0.5 mL of HCl per mg of biomass, and its subsequent incubation at 100 °C in a water bath during 1.5 hours. The neutralisation of the samples was carried out

with 2.5 M NaOH in the same proportion as the acid. After that, phenol and H_2SO_4 were added to the samples and incubation in a water bath at 35 °C during 30 minutes was carried out, as shown in Figure 15. This last step resulted in an orange-yellow colour which could be measured spectrophotometrically at 483 nm (Evolution 201, Thermo Fisher Scientific, United States).

The method requires the prior preparation of a calibration curve with glucose at concentrations located in the range of 0 – 0.1 $mg \cdot mL^{-1}$.

Figure 15. Water-bath used for samples incubation during carbohydrates extraction protocol.

4.2.5. Hydrocarbons content determination.

4.2.5.1. Extraction of hydrocarbons.

Hydrocarbons in *B. braunii* race B were extracted using organic solvents (methanol:dichloromethane) according to the methodology describes by Folch et al. (1957) and Bligh and Dyer (1959). It was necessary 1 mL of sample with a minimum biomass concentration of 0.5 $g \cdot L^{-1}$. Once the solvents were added to the samples, these were incubated in an incanter (Rotator SB3, Stuart, UK) for 4 hours. After that time, it was added NaCl in order to obtain two phases. The phase which contained the hydrocarbons (bottom phase) was carefully transferred to another glass tube and dried with nitrogen gas. Subsequently the residue obtained was resuspended in 1 mL of hexane for its analysis by a gas chromatographer.

4.2.5.2. Quantification of hydrocarbons by gas chromatography (GC-FID).

For the quantification of hydrocarbons obtained from the extraction described in the previous section (4.2.5.1), a gas chromatographer was used (Agilent 7890A, FID detector, United States). Squalene was used as analytical standard for hydrocarbons quantification (SUPELCO 442785, C30 analytical standard, USA). It included an automatic sampler (7683 B) and a FID detector, with a fused silica capillary column (Restek Rxi-5ms) 30 m long, 0.25 mm wide and 0.25 µm internal diameter. The carrier gas was He and the flame was obtained with a mixture of H_2: Air (40:400 mL·min^{-1}).

Figure 16. Gas chromatography equipment used for hydrocarbons quantification.

Table 9 shows the temperature gradient of the gas chromatographer (GC) used to obtain defined peaks in the resulting chromatograms. A FID detector temperature of 350 °C, Splitless injector mode and injection volume of 1 µL were used.

Table 9. Temperature gradient used for the hydrocarbons determination.

Oven ramp	**Slope** (ºC·min^{-1})	**Temperature** (ºC)	**Retention time** (min)	**Runtime** (min)
0	---	80	0	0
1	15	245	0	11
2	5	350	10	42

In order to quantify the hydrocarbons present in each sample, a calibration line with a squalene pattern was previously made at concentrations between 50 and 850 ppm. From the equation resulting from the calibration and the area obtained in the chromatograms for each of the samples, the concentration of hydrocarbons could be calculated.

4.3. Biological activities determination.

4.3.1. Cell viability determination.

4.3.1.1. Photosynthetic activity determination.

The photosynthetic activity is determined by following the evolution of the O_2 produced by microalgal samples exposed to a high luminous intensity. At the same time, the respiratory activity is determined in darkness as the evolution of the consumption of O_2. The oxygen that is accumulated during the photosynthetic process, or the oxygen consumed in respiration, is detected by a Clark type oxygen electrode (Oxygraph Hansatech Instruments, United Kingdom) (Figure 17). To do this, it is necessary to consider the oxygen solubility coefficient, this being 0.2594 µmol·mL^{-1} at 25 ºC. The measuring cell is equipped with a Pt cathode and an Ag/AgCl anode immersed in a saturated KCl solution. Upon applying the potential, the existing oxygen in the cell is reduced by generating an electrical current proportional to the amount of O_2.

The reactions that take place are as follows:

$$O_2 + 4\,H^+ + 4\,e^- \rightarrow 2\,H_2O \quad (\text{cathode})$$

$$4\,Ag \rightarrow 4\,Ag^+ + 4\,e^- \quad (\text{anode})$$

After introducing 1 mL of the culture broth into the reaction chamber the evolution of oxygen concentration at 25 ºC is followed in time under saturating white light conditions (650 µmol photons·m^{-2}·s^{-1}) for its photosynthetic production, and under darkness conditions for its consumption by endogenous respiration. The kinetics of oxygen detachment obtained in the light period is the net result of the simultaneous operation of photosynthesis and cellular respiration. Thus, the gradient obtained during the period of light (net production of O_2) must be added

to the value of the gradient obtained during the period of darkness (consumption of O_2), resulting in the total photosynthetic activity of the microalgae.

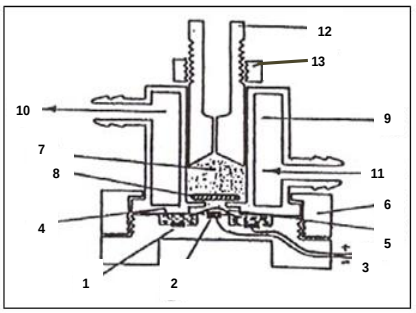


Figure 17. Diagram of an oxygen electrode Clark type. 1) Silver ring anode. **2)** Platinum cathode. **3)** Wires to control box. **4)** Potassium chloride solution. **5)** Teflon membrane. **6)** Adjusting screw. **7)** Reaction chamber. **8)** Magnetic rod. **9)** Thermostated chamber. **10)** Output to the water-bath. **11)** Input to the water-bath. **12)** Closing plunger. **13)** Fixing closing plunger.

4.3.1.2. Maximum photosynthetic efficiency of Photosystem II determination.

The maximum photosynthetic efficiency of Photosystem II (PSII) or Maximum quantum yield (Qy) can be used as an indirect indicator of cell viability (Maxwell and Johnson 2000). The production of fluorescence by chlorophyll is one of the forms of dissipation of excess light energy by the chloroplast, this being a useful tool for evaluating the photochemical potential of PSII and also to monitor possible cellular stress. Thus, changes in the maximal photosynthetic efficiency often precede changes in the cellular content of chlorophylls. Therefore, the variation of fluorescence can be observed much more in advance to visual-type chlorotic symptoms, being a non-destructive, non-invasive and rapid technique (Masojídek et al. 2010), which allows repeated measurements on the same sample.

The maximum quantum yield (F_v/F_m) was determined using a PAM device (Pulse Amplitude Modulation, model AquaPEN AP100; Photon System Instruments, Czech Republic) in accordance with Cuaresma et al. 2012. The determinations were carried out by placing an aliquot of the culture broth in the cuvette of the PAM and keeping it in darkness for 15 minutes, to oxidize the reaction centres of the photosystems and to obtain the level of basal fluorescence (F_0). Subsequently, a pulse of saturated light of 1,500 µmol photons.m^{-2}.s^{-1} (50%

of the total intensity) is emitted, which quickly saturates the reaction centers of the photosystems and induces the emission of maximum fluorescence (F_m). The variable fluorescence of dark-adapted sample (F_v) can be determined from the difference between the above parameters (F_m-F_0). From these data, the value of F_v/F_m was calculated according to Cosgrove and Borowitzka (2011) as:

$$\left({}^{F_v}/_{F_m}\right) = \frac{(F_m - F_0)}{F_m}$$

always given as a fraction of the unit (Schreiber et al. 2004).

4.3.2. Determination of enzymatic activities of the microalgal antioxidant system.

4.3.2.1. Crude extracts preparation.

Cells of *C. onubensis* cultures in the linear growth phase were collected in pre-weighed centrifuge tubes. Subsequently, samples were centrifuged at 3,500 g in a Thermo Scientific Heraeus Biofuge Stratos centrifuge for 4 minutes and supernatant was discarded. To remove remaining residues of the culture medium two successive washing steps with distilled water and subsequent centrifugation were carried out. The resulting pellet was used immediately or stored at -20 ºC until use. The cellular wet weight (cww) in grams was calculated as the difference between the weight of the tube containing the pellet and the empty tube.

Cellular disruption was carried out by vibrations in a Bühler homogenizer (Vibrogen-Zellmühle 7400 Tübingen) (Figure 18). To do this, a potassium phosphate buffer solution was added to the wet pellet in a proportion of 4 mL of buffer per gram of cellular wet weight, together with glass beads of 0.3 mm diameter in a proportion of 90 mL per 20 grams of cellular wet weight. The resulting mixture was subjected to high velocity vibration in the Bühler by means of one cycle of 5 pulses of 1 minute and 30 seconds of rest; the latter is to avoid the overheating of the proteins and their consequent denaturalization. After the cycle the resulting emulsion was then filtered through 4 layers of nylon gauze to separate the glass beads, and the collected supernatant was centrifuged at

31,200 g for 25 minutes at 4 ºC obtaining the crude extract that could be stored at -20 ºC until analysis.

Figure 18-. Bühler homogenizer used in the preparation of crude extracts from cultures of *C.onubensis*.

4.3.2.2. Catalase:

The catalase enzyme (CAT) catalyses the decomposition of hydrogen peroxide into oxygen and water. The activity of the enzyme was measured in a Clark type oxygen electrode, by following the kinetics of emergence of oxygen at 25 ºC, according to a modification of the method described by Halbach (1977). The reaction mixture had a volume of 2 mL, and it consisted in: 1 mL of 100 mM phosphate buffer at pH 7, 500 µL of crude extract and 500 µL of a solution of H_2O_2 40 mM, which initiates the reaction. An activity unit (U) represents the amount of enzyme that catalyses the production of 1 µmol of O_2 per minute.

4.3.2.3. Ascorbate peroxidase.

The enzyme ascorbate peroxidase (APX) catalyses the decomposition of hydrogen peroxide in the presence of ascorbic acid to produce ascorbate monohydrate and water:

$$H_2O_2 + 2\,ASC \rightarrow 2\,H_2O + 2\,MDHA\,(APX)$$

The ascorbate peroxidase was determined spectrophotometrically by following the disappearance of reduced ascorbate at 290 nm (ε = 2.8 $mm^{-1} \cdot cm^{1}$) and at 25 ºC using the method described by Asada (1984) and modified by Amako et al. (1994). The reaction mixture had a volume of 1 mL, and it contained:

500 µL of 100 mM phosphate buffer at pH 7, 100 µL of 10 mM EDTA; 10 µL of 24 mM reduced ascorbate; 50 µL of 0.1 M H_2O_2, 100 µL of crude extract and 240 µL of milli-Q water. An activity unit (U) represents the amount of enzyme that catalyses the disappearance of 1 µmol of H_2O_2 per minute.

4.3.2.4. Guaiacol peroxidase.

The peroxidase enzyme catalyses the oxidation of certain hydrogen-giving compounds such as phenols (Guaiac, pyrogallol) and aromatic amines (fenilendiamina) by means of peroxides (H_2O_2). The most commonly used oxidisable substrate is guaiacol, which is oxidized to a coloured tetraguaiacol complex in the presence of peroxidase.

$$2\,H_2O_2 \xrightarrow{peroxidase} 2\,H_2O + O^{-2}$$

$$O^{-2} + guaiacol\ (colorless) \rightarrow tetraguaiacol\ (brown)$$

The activity of this enzyme is determined spectrophotometrically at 470 nm. The reaction mixture had a volume of 1 mL, and it contained: 850 µL of 0.5 M phosphate buffer at pH 7.8; 50 µL of H_2O_2 at 5%; 50 µL of guaiacol at 5%, and 50 µL of crude extract. An activity unit (U) represents the amount of enzyme that catalyses the disappearance of 1 µmol of H_2O_2 per minute.

5. STATISTICAL ANALYSIS.

Unless otherwise indicated, the results presented during the whole Thesis are the average value of three replicates in each experiment and they were expressed as means with ± standard deviation (SD). Mean values are presented in the corresponding figures with error bars as standard deviation.

In addition, in the specific case of the experiments carried out in well-plates for the culture medium optimization of *Botryococcus braunii* races A and B, so as to evaluate the differences among Reference medium and the new culture media, a one-way analysis of variance (ANOVA) was used. If ANOVA were significant,

comparisons among means were followed by a post-hoc using Fisher's Least Significant Difference (LSD) multiple comparison tests, with a confidence level of 0.05. The mean variability was indicated by the standard deviation and was analyzed statistically using the R project software version 3.2.3 (R Development Core Team, 2015). Boxplot were plotted to check outliers and to show differences among each new culture medium and the Reference one.

III. RESULTS AND DISCUSSION

PART I

Acidophile microalgae and accumulation of metabolites of interest in *Coccomyxa onubensis* under stress conditions

Chapter 1

ISOLATION, GROWTH CHARACTERIZATION, SELECTION AND IDENTIFICATION OF MICROALGAE FROM RÍO TINTO IN HUELVA

1. ISOLATION, GROWTH CHARACTERIZATION, SELECTION AND IDENTIFICATION OF MICROALGAE FROM RÍO TINTO IN HUELVA.

In this first Chapter of Results and Discussion, it was carried out the isolation and growth characterization of different microalgae samples obtained from the northern part of the Río Tinto riverbed in Huelva, by members of our research group. According to the growth characteristics of each microalgae sample, it was selected the best candidate to be further characterized according to its biotechnological potential.

A key challenge in microalgae cultivation is to find strains that not only produce important biomass or commercial biomolecules amounts, but also grow well under industrially relevant outdoor conditions. In view of this, extremophilic microalgae should be taken into account as they are able to survive in a variety of extreme environments where competitors are poorly developed and, at the same time, they are able to produce interesting value-added compounds. In Part I of this Thesis, carotenoids were chosen as the main molecules to be studied due to their antioxidant property, which can protect cells from reactive radicals, prevent lipid peroxidation, and promote the stability and functionality of the photosynthetic apparatus (Grossman et al. 2004). Therefore, once all the studied microalgae isolated from Río Tinto where characterized in terms of growth and carotenoids production, the final candidate was selected and molecularly identified.

1.1. Sampling, isolation and growth of different microalgae collected from Río Tinto.

1.1.1. Selection of the sampling points.

Nerva area, small town at the North of the Río Tinto, was selected for the microalgae sampling for being a place characterized by an intense mining activity. Río Tinto river flows over this area and the characteristics of the substrate, together with the mining activity, causes its water to present a large amount of heavy metals that, in principle, makes life difficult. This fact, added to the high irradiance and the acidic pH of its water, led us to think that the microorganisms which were able to survive in such an inhospitable environment must present characteristics worthy of being studied (Garbayo et al. 2012). For that reason,

microalgae living in this acidic environment were isolated, and their biotechnological potential as antioxidant producers was studied.

Based on this consideration, 12 random sampling points were stablished in thea area of Nerva, which are shown in Figure 1.1.

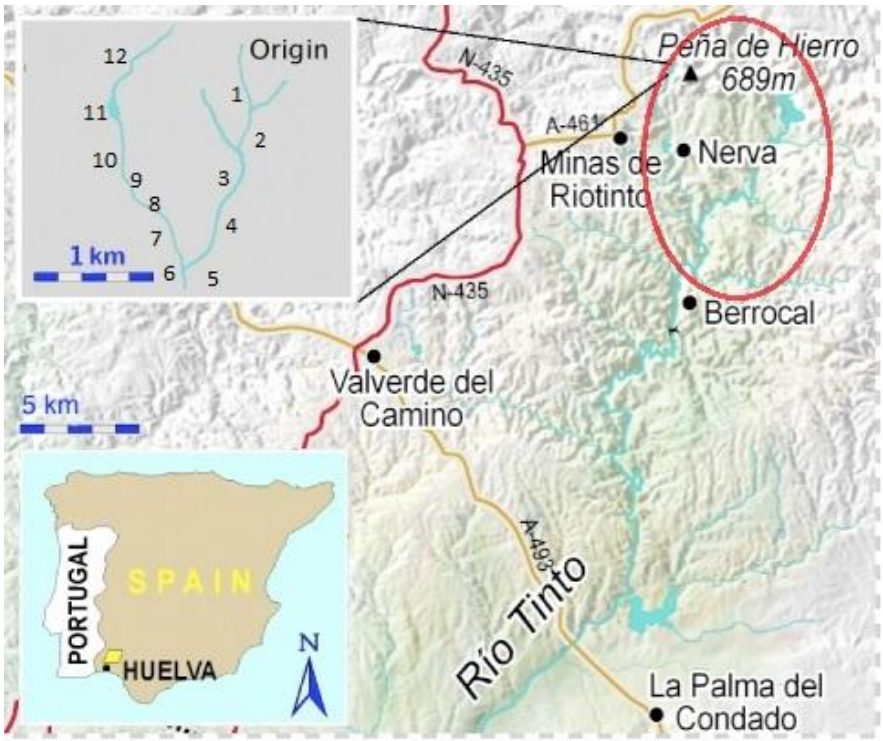


Figure 1.1. Map of the northern riverbed of Río Tinto in which the 12 random sampling points selected are shown.

1.1.2. Isolation of microalgae collected from Río Tinto samples.

It is known that to achieve a successful isolation, understanding and mimicking the naturally occurring environmental conditions is often the main step to be considered (Andersen and Kawachi 2005). Taking that into account, the different samples were cultivated in the acid K9 culture medium, as it was described in section 2.1. of Materials and Methods.

In total, 12 different samples were taken from the riverbed of the Río Tinto, specifically from the area closest to the edges where the growth of microalgae

could be observed (Figure 1.2.). From the different samples some monoclonal colonies could be isolated by repetitive inoculation in agar plates. Finally, 5 samples could be cultivated in K9 liquid culture medium, which were initially named with the same code as the Falcon tube from which they came, and that was N1, N2, N6, N8 and N9.

Figure 1.2. Picture of Río Tinto in which it can be observed the accumulation of microalgae in the right edge of its riverbed.

As long as the cultures were growing in liquid K9 medium, there were no visual differences in color between cultures and it was difficult to discern if the samples were different. In this sense, it was decided to use different staining methods in order to allow a better differentiation under the optical microscope. Cell staining was used as a first approximation to the microalgae characterization in addition to the study of their growth behaviour and production of carotenoids.

1.1.3. Cell staining and observation under the optical microscope.

In order to visualize by optical microscopy the morphological characteristics of the cells from each culture of microalgae and to make an initial assessment of them, two different stains were used for each of the five samples, a hematoxylin-eosin stain and another hematoxylin-picrofuchsin, following the protocols described in section 2.3. of Materials and Methods.

On one hand, the combination of hematoxylin and eosin was chosen because it is the most widely used stain for the observation of biological samples since it combines two dyes that bind to cellular components according to pH (Fisher et al. 2008). Hematoxylin stains acidic structures in blue-purple tone and eosin the basic ones in pink tones, thus, the staining of practically all types of structures is achieved.

On the other hand picrofuchsin, a dye that is part of the contrast staining technique of Van Gieson (acid fuchsin) was used (Van Gieson 1889). It presents reddish color (similar to that of eosin) which, in contrast, also stains the basic structures. As it gives a more intense color, it was tested as a contrast stain against hematoxylin, which stained the cells with a pale coloration.

Figures 1.3. and 1.4. show pictures of the five isolated microalgae obtained by optical microscopy after staining.

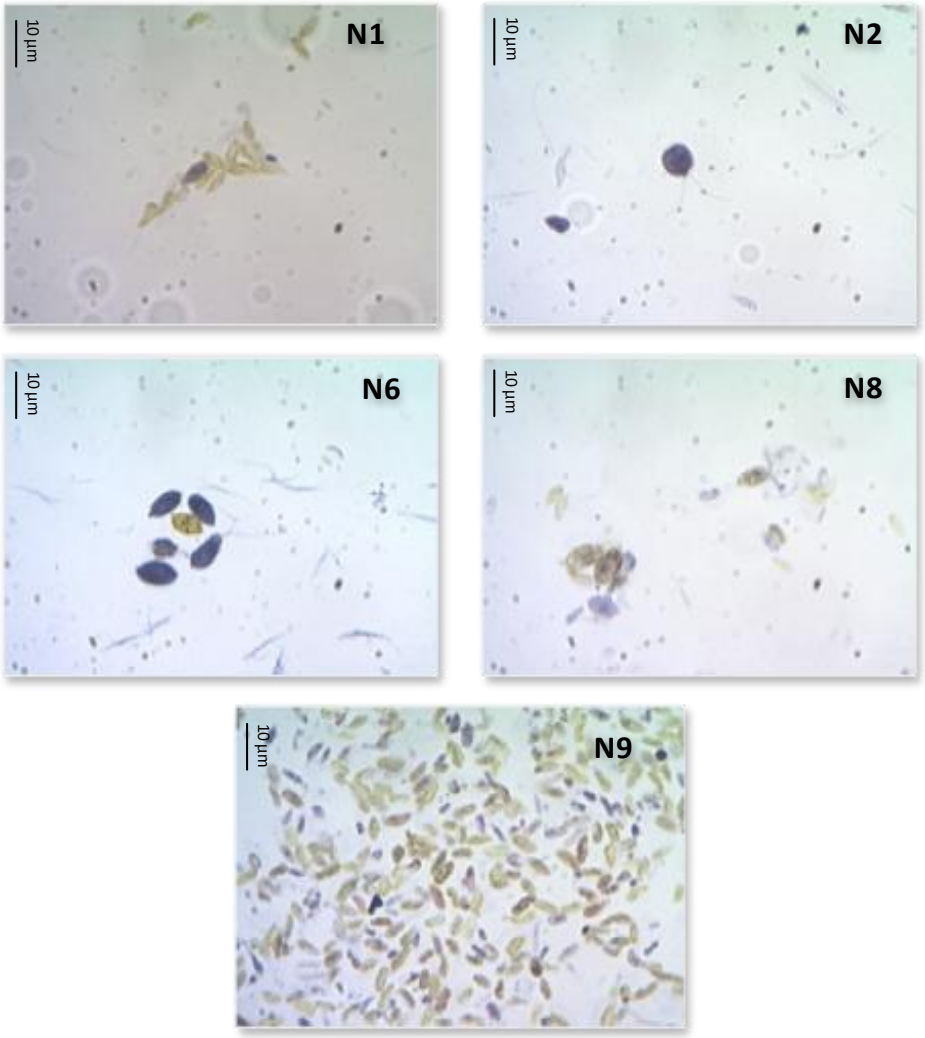


Figure 1.3. Pictures under the optical microscope (1000x) of the isolated species from Río Tinto river (N1, N2 N6, N8 and N9) stained with hematoxylin-eosin. Staining was made as described in Materials and Methods.

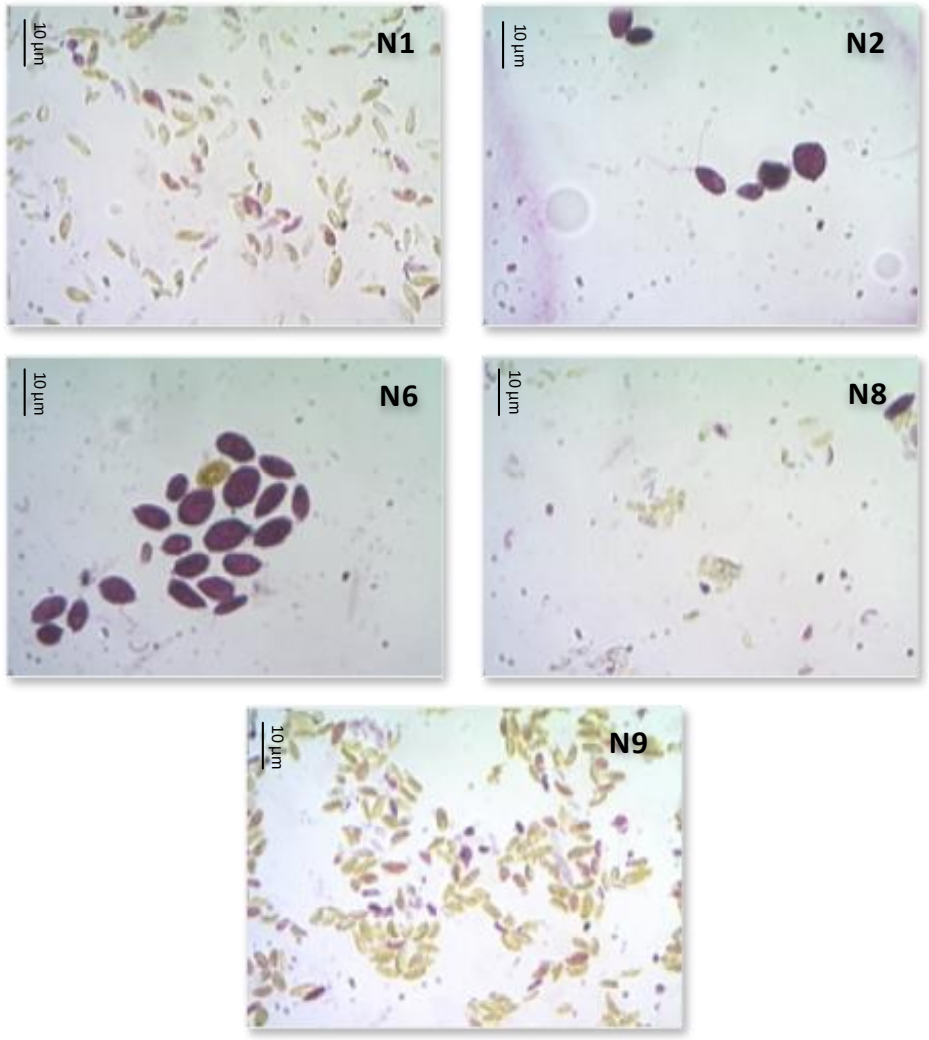


Figure 1.4. Pictures to the optical microscope (1000x) of the isolated species from Río Tinto river (N1, N2 N6, N8 and N9) stained with picrofuchsin-eosin. Staining was made as described in Materials and Methods.

From these pictures, it could be observed that: N1, N8 and N9 were presented as unicellular microalgae with a similar oval shape and a cell size of about 5 µm. N2 and N6 had a similar unicellular rounded shape. They presented two flagella and a cell size of approximately 10 µm.

Due to the lack of specific dyes for microalgae, those commonly used in cellular biology were used, which did not lead to the expected results. In this sense, it was not possible to differentiate the organelles inside the cells, although the cell staining allowed, in principle, to morphologically differentiate at least two microalgae cellular forms. Thus, in order to obtain further information, it was decided to characterize the growth pattern of every culture.

1.1.4. Growth characterization of the different microalgae.

To carry out the characterization of the cultures, the different microalgae were cultivated in K9 medium in erlenmeyers flasks of 500 mL, at a pH of 2.5. Growth was daily monitored by determining the biomass dry weight content of the cultures. On the other hand, chlorophyll accumulation and cell viability of every culture were assessed.

Figure 1.5. shows the evolution of biomass dry weight measured as grams of dry biomass per liter of culture broth. It can be observed how all cultures followed the same profile, being noted that, since the 148 hours of incubation, N6, N8 and N9 cultures reached the stationary phase while N1 and N2 continued growing. This could indicate that, although N2 and N6 visually seemed to be the same organism, the different behavior in growth must be a sign that they were different microalgae.

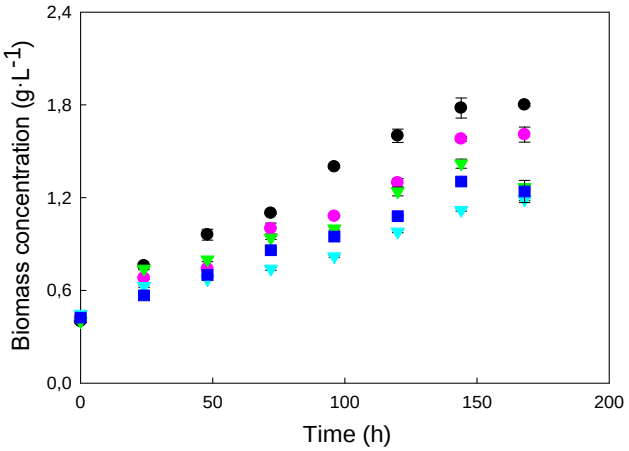


Figure 1.5. Evolution of biomass dry weight of the 5 isolated microalgae from Río Tinto river cultivated under standard conditions in K9 medium. Evolution of different microalgae is represented as: (●) N1, (●) N2, (▼) N6, (▼) N8, (■) N9. Error bars show standard deviation of replicates.

From the data of the evolution of dry weight for each culture, in its linear growth phase, maximum specific growth rates were calculated, as described in Materials and Methods. Despite the advantage of acidophilic microalgae to selectively grow in extreme media at low pH, it has been reported that growth rates are lower than the so-called “common” microalgae (Pulz and Gross 2004).

In Figure 1.6., it can be observed that the microalga N1 presented a growth rate higher than the rest of the cultures, being almost 2-fold higher than N8. Nonetheless, such value (0.31 d^{-1}) is low when compared to non-extremophilic microalgae growth rates (Chia et al. 2013), but it is consistent with those determined to other acidophile ones (Langner et al. 2009; Casal et al. 2011), which can present values of 0.34 d^{-1}.

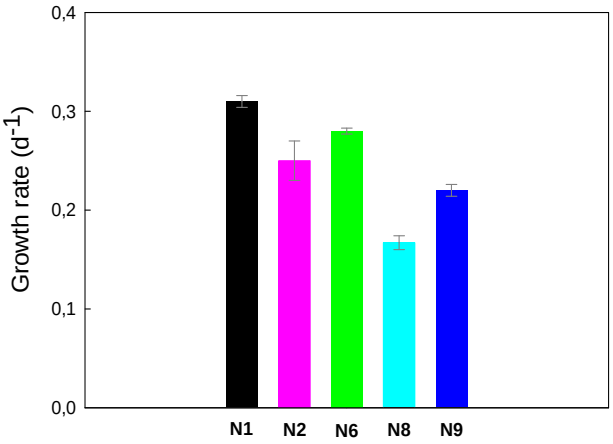


Figure 1.6. Maximum specific growth rate of the 5 isolated microalgae from Río Tinto river, cultivated under standard conditions in K9 culture medium. Microalgae cell cultures were incubated in erlenmeyers flasks for 168 hours. Error bars show standard deviation of replicates.

Since chlorophyll plays a central role in photosynthesis by absorbing and transferring light energy (Li and Chen 2015) the accumulation of such pigment was also daily monitored. In Figure 1.7b can be observed that all cultures presented a similar trend in their chlorophyll intracellular content. However, the culture called N1 was the one which accumulated the highest concentration of this pigment (between 1.2 and 1.4-fold higher than the rest of the cultures) after 96 hours of cultivation, dropping from that moment until values similar to the rest of the cultures. Regarding the chlorophyll content per volume of culture broth (Figure 1.7a) the same pattern was observed highlighting that, once again, N1 was the one which accumulated the highest content of chlorophyll, but in this case during the whole process.

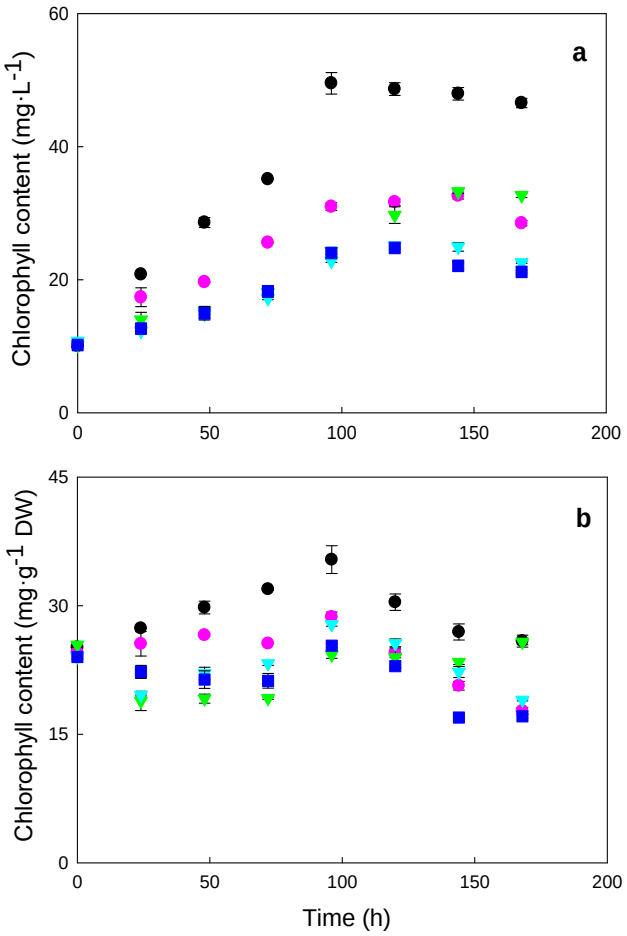


Figure 1.7. Evolution of chlorophyll content as milligrams per liter of culture broth (a) and as milligrams per gram of biomass dry weight (b) of the 5 isolated microalgae from Río Tinto river, cultivated under standard conditions in K9 medium. (●) N1, (●) N2, (▼) N6, (▼) N8, (■) N9. Error bars show standard deviation of replicates.

In order to evaluate the physiological status of the cultures, a useful parameter is the photosynthetic activity of PSII. It was determined as the oxygen production in mmol O_2·mg Chl^{-1}·h^{-1}. Figure 1.8. shows that cultures N1, N2 and N6 followed similar tendencies with values above the rest of the cultures during the first 120 hours, which is consistent with the results of growth rates. However, despite they showed similar trends, N1 culture started to present higher photosynthetic activity after 48 hours of cultivation, which was in accordance with

the intracellular chlorophyll content results. The low medium pH may force acidic environment microalgae to expend energy in order to maintain neutral pH into the cytosol, using appropriate biochemical systems that withstand the proton gradient across the plasma membrane (Gross 2000). The energy demand of such systems could partly justify a remarkable activity of PSII.

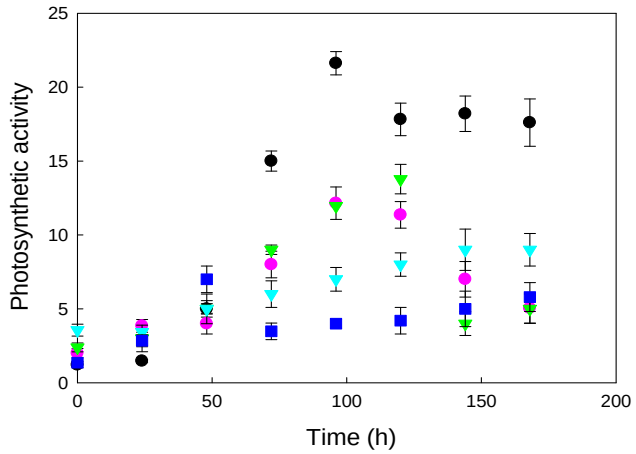


Figure 1.8. Evolution of Photosynthetic activity measured as light-dependent oxygen production (mmol O_2·mg Chl^{-1}·h^{-1}), of the 5 isolated microalgae from Río Tinto river, cultivated under standard conditions in K9 medium. Evolution of different microalgae is represented as: (●) N1, (●) N2, (▼) N6, (▼) N8, (■) N9. Error bars show standard deviation of replicates.

Since it is known that environmental factors such as irradiance, pH, nutrients, temperature and salinity influence the photosynthetic activity and consequently biomass productivity (Costache et al. 2013), understanding the effect of culture conditions on the microalgal photosynthetic activity could be a useful tool for designing and modeling photobioreactors to predict biomass productivity and optimize the operating conditions (Yun and Park 2003; Brindley et al. 2010; Fernández et al. 2012).

1.1.5. Characterization of carotenoids.

The final microalgal selection best suited to the objectives of this Thesis was based on the content of high added value carotenoids, such as lutein and β-carotene. As explained in the Introduction, carotenoids are one of the molecules

produced by microalgae that, currently, present more applications at a commercial level, particularly in colorants, food, pharmaceutical and cosmetic markets. In this regard, the production of these compounds has become one of the major interest activities in the field of microalgal biotechnology in recent years (Ambati et al. 2018).

Figure 1.9a shows the total carotenoids content per volume of culture broth of the 5 microalgae isolates. Once again, it can be observed that all cultures followed the same tendency except N1 which since the 48 hours of cultivation started to present higher amount of total carotenoids, until the end of the cultivation. However, taking into account the intracellular content (Figure 1.9b), no important differences in their trends were observed although N1 was also the one which reached the maximum content after 72 hours of cultivation (between a 6.2 and 27.2% higher).

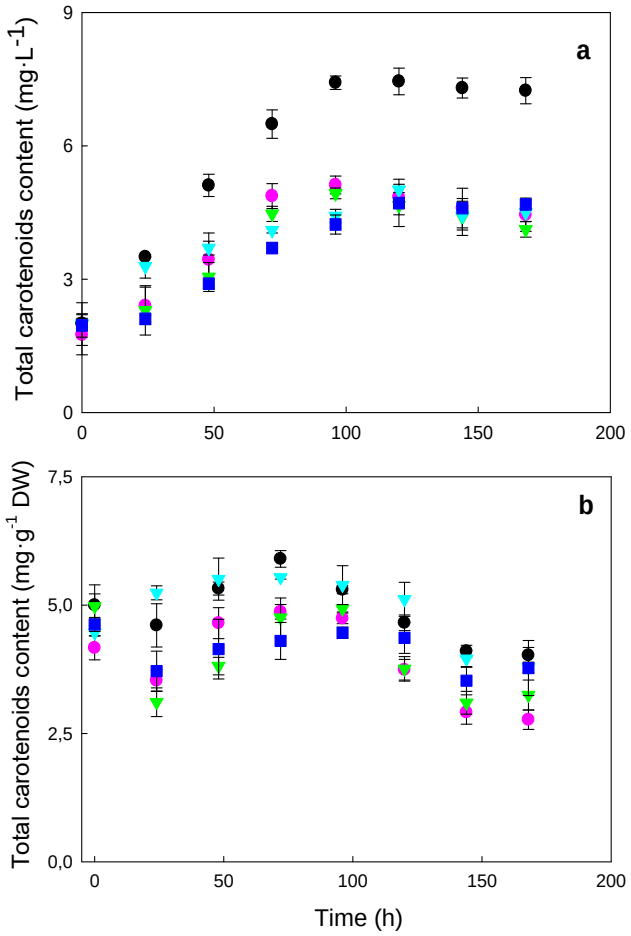


Figure 1.9. Evolution of total carotenoids content as milligrams per liter of culture broth (a) and as milligrams per gram of biomass dry weight (b), of the 5 isolated microalgae from Río Tinto river, cultivated under standard conditions in K9 medium. Microalgae cell cultures were incubated in erlenmeyers flasks for 168 hours. (●) N1, (●) N2, (▼) N6, (▼) N8, (■) N9. Error bars show standard deviation of replicates.

Figure 1.10. represents lutein and β-carotene content of microalgae extracts obtained in organic solvent (methanol) after 72 hours of cultivation. The pigments were extracted by means of procedures that are described in section 4.2.1.1. of Materials and Methods. The separation, identification and quantification of the most abundant pigments were done using liquid chromatography techniques (HPLC).

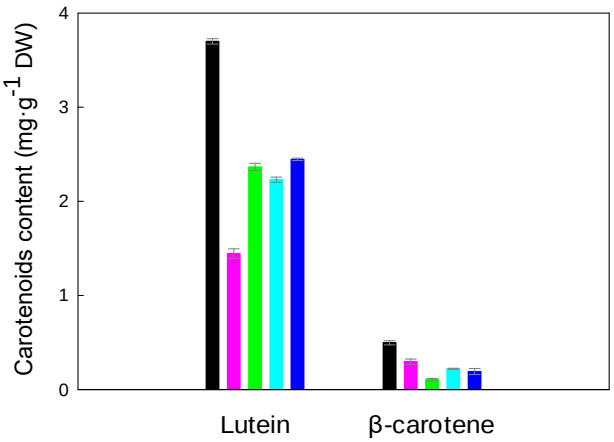


Figure 1.10. Lutein and β-carotene intracellular content after 72 hours of cultivation, measured as milligrams per gram of biomass dry weight, of 5 isolated microalgae from Río Tinto river cultivated under standard conditions in K9 medium. N1 (■), N2 (■), N6 (■), N8 (■), N9 (■). Error bars show standard deviation of replicates.

Both, lutein and β-carotene content were higher for N1, being the difference in lutein more remarkable (between 1.4 and 2.5-fold higher) compared to the rest of the cultures than the obtained with β-carotene. Lutein has recently gained attention as an additive in food industry and especially as a powerful antioxidant (Sun et al. 2016). Average intracellular lutein content of N1 is around 3.8 $mg \cdot g^{-1}$ of dry weight, which is within the range of the most promising lutein-producing microalgae species (Fernández-Sevilla et al. 2010).

Biomass and carotenoids (lutein and β-carotene) volumetric productivities were determined at 72 hours of cultivation for every microalgae culture (Table 1.1.), since the maximum carotenoids content was produced at that moment (Figure 1.9.). As it can be observed, higher volumetric productivity values (biomass and carotenoids) were reached with N1 cultures. Extremophile microalgae have been reported not to have very high biomass productivities (Pulz and Gross 2004; Tittel et al. 2005). Nonetheless, N1 seemed to present a quite reasonable value, considering that it is a microalga from an acidic environment and that other microalgae isolated from extreme environments present productivities lower than 170 $mg \cdot L^{-1} \cdot d^{-1}$ (Onay et al. 2014).

Table 1.1. Biomass, lutein and β-carotene volumetric productivities (P_V) of 5 isolated microalgae from Río Tinto river, expressed as milligrams per liter of culture broth per day, after 72 hours of cultivation.

Microalga	Biomass P_V ($mg \cdot L^{-1} \cdot d^{-1}$)	Lutein P_V ($mg \cdot L^{-1} \cdot d^{-1}$)	β-carotene P_V ($mg \cdot L^{-1} \cdot d^{-1}$)
N1	183±0.42	1.21±0.09	0.21±0.01
N2	160±0.12	1.08±0.05	0.13±0.01
N6	180±0.25	0.71±0.04	0.19±0.02
N8	97±0.10	1.05±0.05	0.12±0.01
N9	147±0.85	1.01±0.07	0.17±0.01

1.2. Selection of microalga of interest and molecular identification.

Based on the results obtained from the characterization of the different microalgae, it was decided to select N1 as it presented the most promising data at a production level of both, biomass and carotenoids, and finally, it was carried out its molecular identification by members of our research group.

The identification was based on DNA coding for ribosomal RNA, since it is the most frequently used marker in studies of phylogeny and taxonomy of this type of microorganisms. Specifically, DNA homologies corresponding to the 18S subunit of ribosomal RNA were analyzed. For the identification of the selected microalga, it was used the analysis of chloroplastic DNA homologies corresponding to the large subunit of ribulose biphosphate carboxylase (Rubisco, rcbL gene), a gene used routinely in plant phylogenetic analyzes and from which a large database of strains is available (Novis et al. 2009).

Table 1.2. shows the results obtained from the identification analysis. As it can be seen, it was reached 100% of “query coverage” or similarity with the results obtained from the database (http://www.ncbi.nlm.nih.gov), as well as 100% of “max score” or maximum alignment. Therefore, such results indicated that the microalga initially called N1, was 100% identified as belonging to the genus *Coccomyxa onubensis*.

Table 1.2. Results obtained from the molecular identification of N1 culture and subsequently comparison with GenBank database sequences (http://www.ncbi.nlm.nih.gov).

	Description	Query coverage (%)	Max score (%)	Accesion number
N1	*Coccomyxa onubensis*	100	100	HE617183.1

On the other hand, the ribulose-bisphosphate carboxylase (rbcL) gen sequence for N1 was determined as:

ACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTAC
CTTGCTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCGTAAATCCC
GACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCGACCGGACTCTGTCC
GACTCGCGGTGAATCATGATAACTCCACGGATCGCATGGCCTCGTGCCGGC
GATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGACGGTAAGGTATTGGCT
TACCGTGGTGGTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGGG
AGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTA
CCCAATCTTGACACAAGGAGGTAGTGACAATAAATAACAATACCGGGGTTTTT
CAACTCTGGTAATTGGAATGAGTACAATCTAAACCCCTTAACGAGGATCAATT
GGAGGGCAAGTC

This microalga had already been isolated and reported for the first time as an uncultured microalga in 2012 by Garbayo et al. (2012), although its complete coding sequence was recently determined and deposited in GenBank as HE617183 by Fuentes et al. (2016). The last mentioned authors also compared *C. onubensis* with representative of all *Coccomyxa* species previously defined by Darienko et al. (2015), and showed with a phylogenetic tree (Figure 1.11.) that such microalga belonged to a new specie.

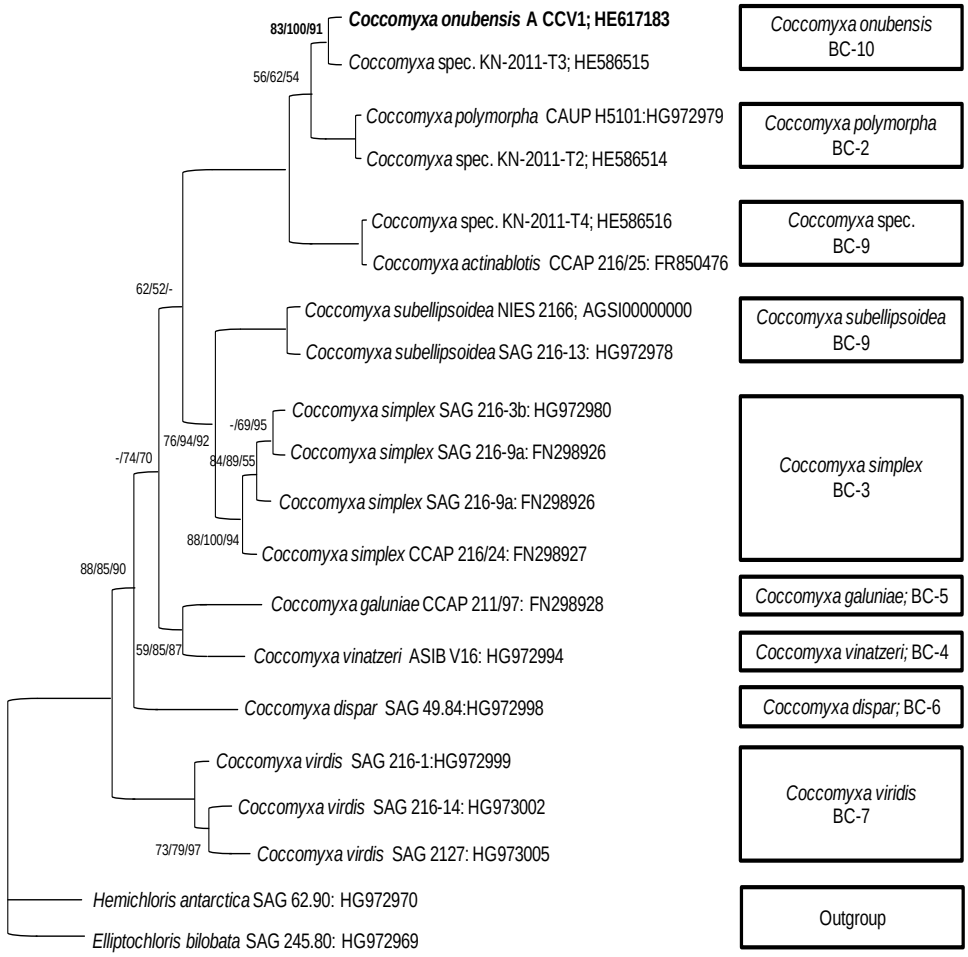


Figure 1.11. Maximum likelihood tree based on 18S, 5.8S, and ITS2 rRNA gene sequences of the genus *Coccomyxa* (Fuentes et al. 2016).

The identification of the selected microalga as *C. onubensis* allowed to review its potential on carotenoids production in order to carry out experiments to further improve their productivity.

1.3. Main advantages of cultivating microalgae from extreme environments.

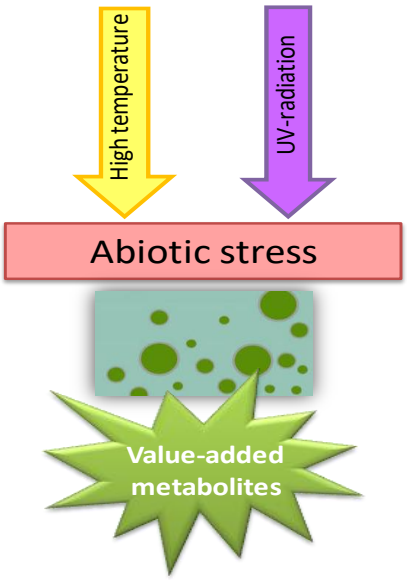
It could be concluded from the results obtained in this Chapter of the Thesis that microorganisms living in extreme environments develop special mechanisms as the expression of some typical antioxidant response, which make them to be able to cope with such conditions. In this regard, one of the most remarkable physiological responses in this kind of microalgae, in terms of commercial applications, is the large accumulation of lutein, as it has been already reported in a previous work where *C. onubesis* was shown to naturally accumulate a high constitutive pool of lutein (Garbayo et al. 2012).

On the other hand, production of acidophilic or acid-tolerant microalgae meets the advantage of selective growth in acidic culture medium, which avoids competition from most microorganisms which are non-adapted to that condition.

In addition to the above, taking into account both biomass and carotenoids (lutein and β-carotene) productivities, it was decided to choose N1 for further experiments, which was eventually identified as *Coccomyxa onubensis.* Thus, in order to enhance the production of carotenoids with a commercial interest, in Chapter 2 of Results and Discussion of this Thesis some of the main abiotic factors that stimulate their accumulation were studied.

Chapter 2

GROWTH AND VALUE-ADDED PRODUCTS PRODUCTION OF *Coccomyxa onubensis* UNDER STRESS CONDITIONS



Part of the work presented in this Chapter has been published as:
Bermejo, E., Ruíz-Domínguez, M.C., Cuaresma, M., Vaquero, I., Ramos-Merchante, A., Vega, J.M., Vílchez, C., Garbayo, I. (2018). Production of lutein, and polyunsaturated fatty acids by the acidophilic eukaryotic microalga *Coccomyxa onubensis* under abiotic stress by salt or ultraviolet light. Journal of Bioscience and Bioengineering, 125(6): 669-675. https://doi.org/10.1016/j.jbiosc.2017.12.025

2. GROWTH AND VALUE-ADDED PRODUCTS PRODUCTION OF *Coccomyxa onubensis* UNDER STRESS CONDITIONS.

The production of metabolites of commercial interest and algal biomass can be enhanced under environmental stress conditions (Mata et al. 2010; Mulders et al. 2014; González et al. 2015). One of these stress factors is the temperature. As mentioned in the Introduction Chapter, temperature is a very sensitive factor for microalgal growth in general, and more specifically for metabolic activities in microalgal cells. Added to this, the temperature also stimulates the reactions involved in the carotenogenic route of microalgae as part of the response to oxidative stress (Bhosale 2004).

Other factors, as the intensity and quality of the light, are also fundamental in the development of the photosynthetic organisms. Light effects on the biochemical composition of photosynthetic algae are greatly influenced by photoadaptation and photoinhibition processes. In these processes, algae cells show important changes in their cellular composition, often with alterations in their ultrastructure and physiological behaviour, in order to increase the efficiency of photosynthesis and growth (Benavente-Valdés et al. 2016).

Taking into account that the studied microalgae, *C. onubensis*, was isolated from an area subjected to high temperatures, it was hypothesized that the cells would have developed mechanisms to counteract the state of oxidative stress induced by their exposure to them (López-Archilla et al. 2004). Besides that, temperature is also an easy-control factor in the practical operation of microalgae cultivation. Regarding light, *C. onubensis* grows very shallow in the Río Tinto water, where the CO_2 from the atmosphere is easily available. In this location the acidophilic microalga is exposed to very high PAR light intensities, as well as to UV-radiation, having been adapted to grow in the presence of such conditions. In general, UV-radiation has to be considered unfavorable to living matter and different protecting strategies have been developed to cope with these impacts. However, the modes of action of UVA and UVB differ. Exposure to shorter UVB wavelengths results in a higher degree of DNA damage, higher levels of oxidative stress, and greater expression of cell cycle genes than exposure to UVA (Lesser 2006).

Considering the effect that temperature and light could have in the microalgae growth, as well as in the production of interesting metabolites, it was decided to evaluate the response of *C. onubensis* when exposed to a temperature range from 25 ºC to 40 ºC. Under such conditions, growth and carotenoids production were studied. Subsequently, it was decided to evaluate the influence of UVA and UVB radiations on *C. onubensis* growth and carotenoids and lipids production. And, finally, it was decided to focus on the impact of UVA and UVB radiations on the microalgal antioxidant enzymatic response.

2.1. Temperature effects on *C. onubensis* cultivation.

In this part of the Thesis, the effect of different temperatures on the growth of *C. onubensis* was studied in order to analyze whether the culture viability and the accumulation of carotenoids may have undergone changes.

2.1.1. Influence of temperature on *C. onubensis* growth.

In the following experiments, the effect of the temperature on growth and cell viability of *C. onubensis* was studied. For that, the microalgal cultures were incubated under a temperature range between 25 ºC and 40 ºC. A culture of *C. onubensis* incubated at 25 ºC was used as control, and the rest of the cultures were incubated in heated water baths in order to maintain the temperature at 30, 35 and 40 ºC.

Aliquots of each culture were daily taken during 96 hours to determine specific growth parameters such as optical density at 750 nm (as a measure of cell density), dry weight, growth rate, chlorophyll content and total carotenoids content. Likewise, to know the state of the Photosystem II (PSII), its maximum photosynthetic efficiency (F_v/F_m) was determined, as an indicator of cell viability.

In Figure 2.1. the evolution of cell density, measured as absorbance at 750 nm, and biomass content of the different cultures during the period of the experiment, are shown. As it can be observed, in both cases all cultures followed the same trend.

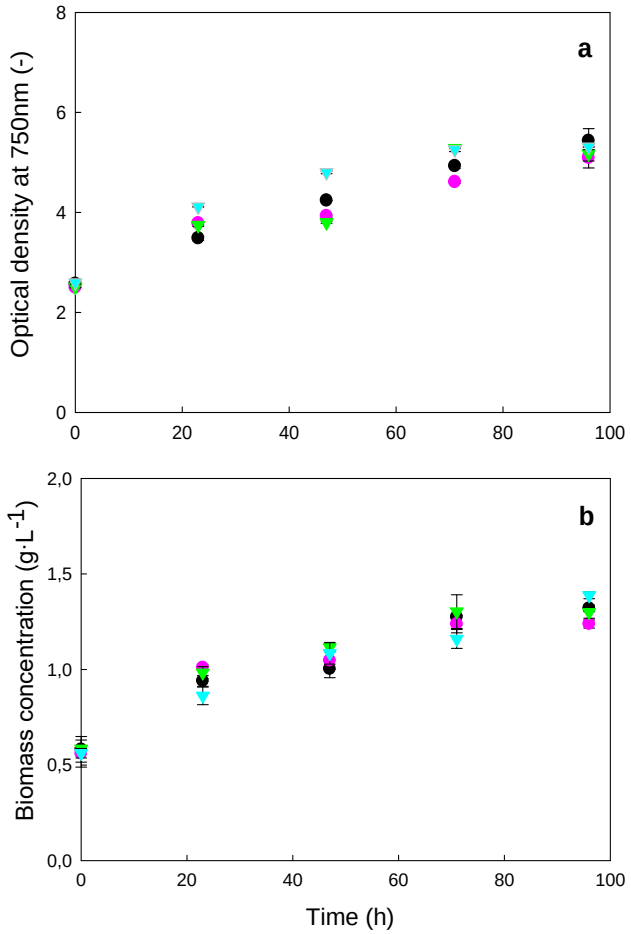


Figure 2.1. Evolution of cell density expressed as optical density at 750 nm (a) and biomass dry weight expressed as grams per liter of culture broth (b) in *C.onubensis* cultures incubated under different temperatures. (●) 25 ºC (control); (●) 30 ºC; (▼) 35 ºC; (▼) 40 ºC. Error bars show standard deviation of replicates.

Nevertheless, while cell density was not affected by the high temperatures, the chlorophyll content showed differences (Figure 2.2.). In both cases, per volume of culture broth (Figure 2.2a) and per biomass dry weight (Figure 2.2b), the cultures incubated at 30 ºC presented a similar trend compared to the control, although its chlorophyll production was 10% lower at the end of the experiment. On the other hand, the cultures incubated at 35 ºC and 40 ºC presented a higher amount per biomass of such pigment in the first 48 hours although it dropped

thereafter to values 40.3% and 58.8% lower than the control, respectively, at the end of the experiment (Figure 2.2b). This cellular decrease in chlorophyll content may indicate deficiencies in the light-collecting protein complexes, which imply defects in the number of complexes, in the chlorophyll molecules or in their photochemical capacity (Melis 2007). The last can be evidenced by the analysis of the maximum photosythetic efficiency of Photosystem II (Figure 2.3.), which was also measured during the whole experiment.

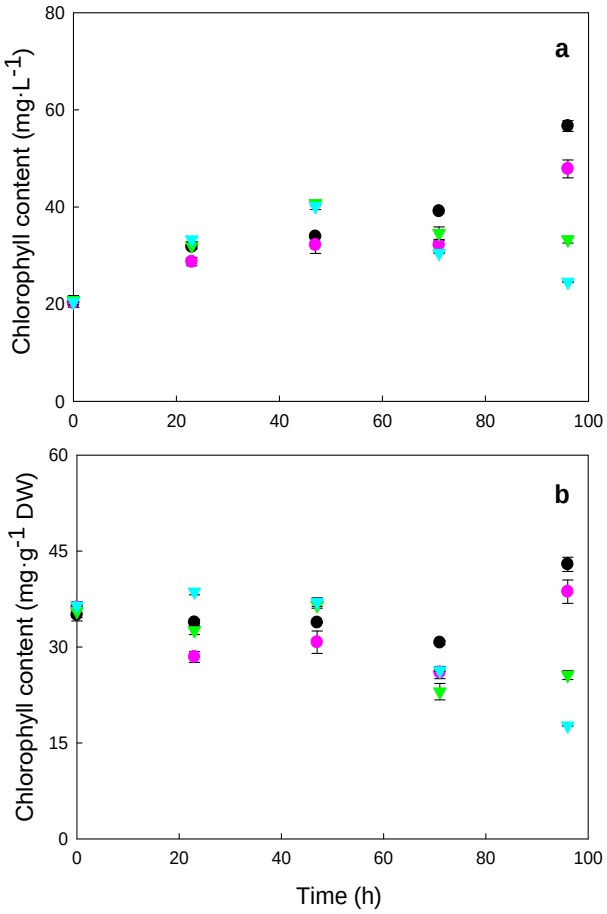


Figure 2.2. Evolution of chlorophyll content, expressed as milligrams per volume of culture broth (a) and as milligrams per gram of biomass dry weight (b), in *C. onubensis* cultures cultures incubated under different temperatures. (●) 25 ºC (control); (●) 30 ºC; (▼) 35 ºC; (▼) 40 ºC. Error bars show standard deviation of replicates.

The maximum photosynthetic efficiency of PSII gives relevant information about the photosynthetic capacity and cell viability of the cultures, being a useful parameter to evaluate the effect that the temperature causes on the photosynthetic activity of microalgae (Zhang and Liu 2015). As it can be seen in Figure 2.3., cultures incubated at 30 ºC and 35 ºC showed a similar trend to that of the control along almost all the experiment, showing a slight decrease at the end of the cultivation period. However, the culture incubated at 40 ºC showed, from the 24 hours of cultivation to the end, a decrease in the photosynthetic yield, being lower than the rest of the cultures in the last 48 hours. It is known that under stress situations by high temperatures there is an impact on the structure and the activity of proteins, including those that are part of the PSII reaction centers (Béchet et al. 2017), thus decreasing the PSII efficiency, which agrees with the results obtained during the experiment.

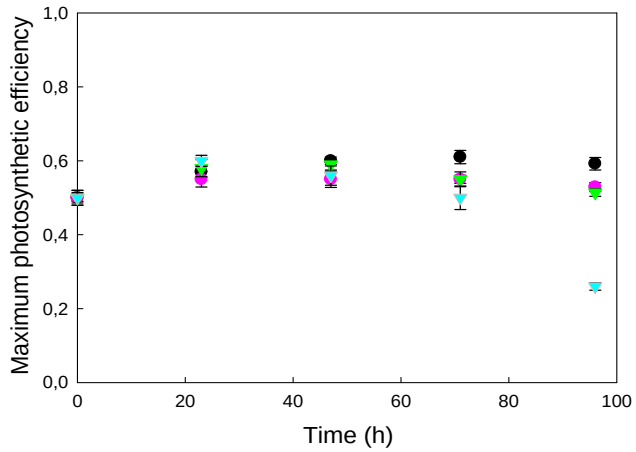


Figure 2.3. Evolution of maximum photosynthetic activity of PSII (F_v/F_m) in *C. onubensis* cultures incubated under different temperatures. (●) 25 ºC (control); (●) 30 ºC; (▼) 35 ºC; (▼) 40 ºC. Error bars show standard deviation of replicates.

Figure 2.4. shows the maximum specific growth rates calculated from the kinetics of the cell density in the different cultures. In general, each microalga can stand a range of growth temperature in which its growth rate increases as does the temperature until an optimum value is reached, and from which growth starts to decrease (Forján et al. 2015). In the case of *C. onubensis* it was observed that the growth rates of the different cultures were similar during the time in which the

experiment was carried out. However, in Figure 2.3., it can be seen that at the end of the experiment cell viability considerably decreased for the cultures incubated at 40 ºC, so it could be said that this temperature is acceptable for the microalga growth in periods of less than 96 hours of cultivation.

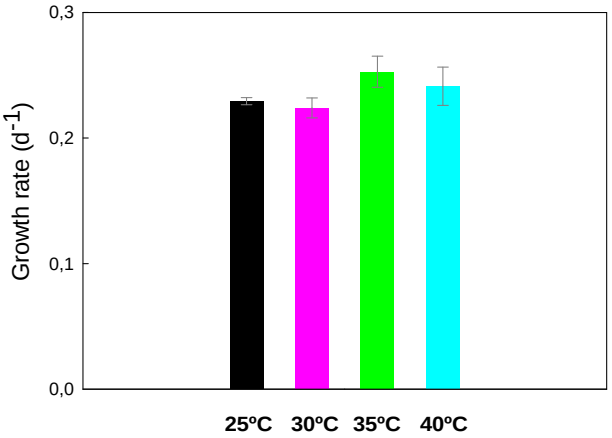


Figure 2.4. Maximum specific growth rate of *C. onubensis* cultures incubated under different temperatures: 25 ºC (control) 30 ºC, 35 ºC and 40 ºC. The rates were calculated as described in Materials and Methods. Error bars show standard deviation of replicates.

Biomass volumetric productivity for the different cultures after reaching the stationary phase (72 hours) was calculated (Table 2.1.), highlighting that the culture incubated at 35 ºC resulted in 1.3-fold higher biomass productivity than the control one (25 ºC).

Table 2.1. Biomass volumetric productivities (P_V) of *C. onubensis* expressed as milligrams per liter of culture broth per day, after 72 hours of cultivation at different temperatures.

Culture broth Temperature	**Biomass P_V** ($mg \cdot L^{-1} \cdot d^{-1}$)
Control (25 ºC)	233±0.03
30 ºC	229±0.01
35 ºC	310±0.05
40 ºC	202±0.02

From the results obtained in this section (2.1.1.), 35 ºC must be considered as the most suitable temperature to enhance *C. onubensis* growth and thus, its biomass productivity during the time the experiment lasted (96 hours).

2.1.2. Influence of temperature increase on carotenoids production of *C. onubensis*.

Temperature has an important role in the formation of carotenoids. The accumulation of these compounds in microalgae can be stimulated by the temperature due to the increase of the oxidative stress of the cells, which results in the cellular response. The formation of active oxygen radicals or the enhancement of the biosynthetic enzymatic activity are cellular mechanisms to cope with such oxidative stress (Juneja et al. 2013).

As a continuation of what has already been described in section 2.1.1. of this Chapter, in which the effect of temperature on the growth of *C. onubensis* was studied, it has also been investigated the effect of this factor on the content of total and specific carotenoids in this microalga. For its determination, samples of each one of the cultures, incubated at 25 ºC, 30 ºC, 35 ºC and 40 ºC, were taken at different points of the growth curve.

Figure 2.5. shows the evolution of total carotenoids content per unit of volume (a) and biomass (b), in *C. onubensis* cultures grown at temperatures between 25 ºC and 40 ºC. In both cases, all cultures followed the same trend during the whole experiment, accumulating a higher amount of carotenoids at the end, which coincides with the beginning of the stationary phase. However, at that moment, the cellular carotenoid content for the culture incubated at 40 ºC dropped to a value 58.6% lower than the control.

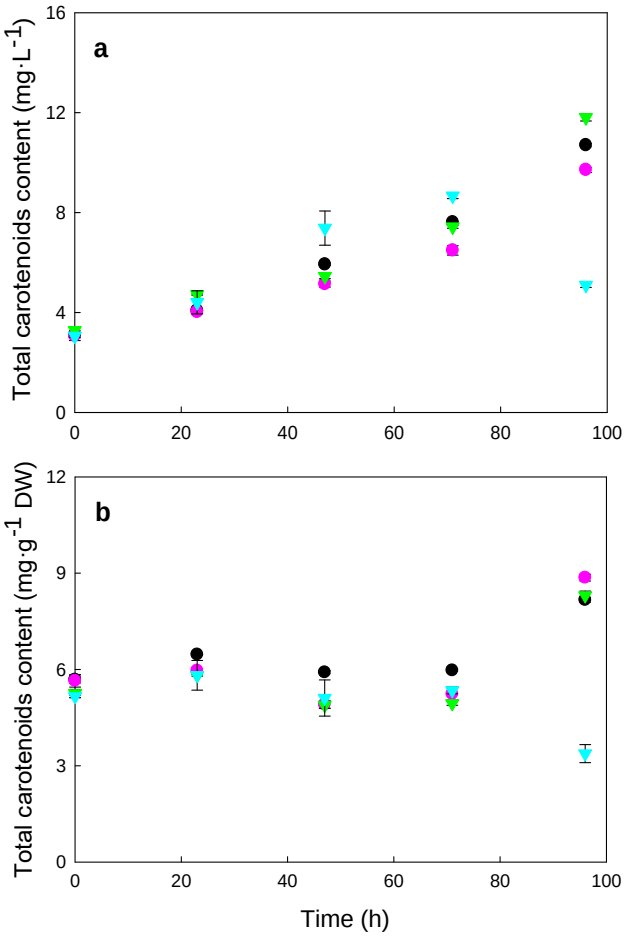


Figure 2.5. Evolution of total carotenoids content, expressed as milligrams per volume of culture broth (a) and as milligrams per gram of biomass dry weight (b), in *C. onubensis* cultures cultures incubated under different temperatures. (●) 25 ºC (control); (●) 30 ºC; (▼) 35 ºC; (▼) 40 ºC. Error bars show standard deviation of replicates.

In order to evaluate the effect of the temperature on the quantitative composition of the main carotenoids present in *C. onubensis,* cellular content of lutein and β-carotene was determined in the cultures. Figure 2.6. shows the results corresponding to the analyzed samples at different points of the experiment. As can be observed, cultures incubated at 35 ºC and 40 ºC showed higher accumulation of both carotenoids after 48 hours of cultivation, as a response to the adaptation to such conditions. However, values in cultures

incubated at 35 ºC kept higher until the end, whereas in cultures incubated at 40 ºC it dropped. This decrease in lutein and β-carotene content (47.6 and 47.5% lower than the control respectively) at that temperature might be due to the fact that cells were not able to cope with the oxidative stress to which they were subjected, as already observed in the photosynthetic efficiency of PSII (Figure 2.3.).

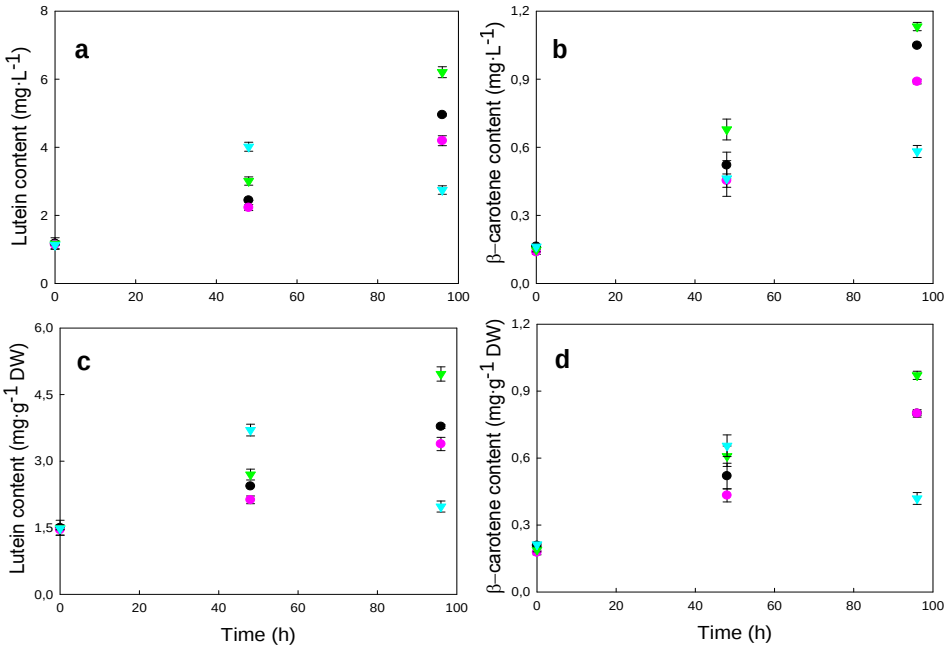


Figure 2.6. Lutein and β-carotene content expressed as milligrams per volume of culture broth (a, b) and as milligrams per gram of biomass (c, d), obtained in *C. onubensis* cultures incubated under different temperatures. Carotenoids were determined by HPLC as decribed in Materials and Methods. (●) 25 ºC (control); (●) 30 ºC; (▼) 35 ºC; (▼) 40 ºC. Error bars show standard deviation of replicates.

From the obtained data, volumetric productivity of lutein and β-carotene, were calculated (Table 2.2.). It can be observed that cultures incubated at 35 ºC obtained the highest lutein as well as β-carotene productivity values respect to the control, besides biomass productivity as already mentioned.

Table 2.2. Biomass, lutein and β-carotene volumetric productivities (P_V) of *C. onubensis* expressed as milligrams per liter of culture broth per day, at the end of the cultivation at different temperatures.

Culture broth Temperature	**Lutein P_V** ($mg \cdot L^{-1} \cdot d^{-1}$)	**β-carotene P_V** ($mg \cdot L^{-1} \cdot d^{-1}$)
Control (25 ºC)	0.95±0.02	0.22±0.03
30 ºC	0.76±0.01	0.19±0.01
35 ºC	1.26±0.04	0.25±0.05
40 ºC	0.40±0.02	0.10±0.01

Despite the obtained results in cultures at 35 ºC of temperature, productivities were not tremendously improved. Previous studies, as the one carried out by Pissal and Lele (2005), revealed a five-fold increase (from 1.65 to 8.28 $pg \cdot cell^{-1}$) in β-carotene concentration of *D. salina* when increasing the temperature from 25 ºC to 35 ºC under high light exposures. The results obtained in our experiment revealed that *C. onubensis* did not overexpress the accumulation of lutein neither β-carotene under the conditions assessed. It might be an indicator of the cellular acclimatation of the microalgae to the high temperature conditions in its natural habitat. Therefore, in order to further increase the production of carotenoids, cultivation of *C. onubensis* should be carried out at high temperature in combination with other stress factors.

2.2. UVA and UVB radiation effects on *C. onubensis* cultivation.

In this section of the Thesis, the effect of the UVA and UVB light exposure on the growth of *C. onubensis* was studied, to subsequently analyze whether the culture viability and the accumulation of carotenoids and lipids under such conditions could be compatible processes.

2.2.1. Influence of UVA and UVB radiation on *C. onubensis* growth.

To study the effect of UVA and UVB radiation on the microalga growth, cultures were incubated under continuous PAR light of 140 µmol photons$\cdot m^{-2} \cdot s^{-1}$. Moreover, one of these cultures was used as control and the other two were illuminated with UV light (one of them with UVA radiation of 37 µmol photons·

$m^{-2} \cdot s^{-1}$ and the other with UVB radiation of 0.67 µmol photons$\cdot m^{-2} \cdot s^{-1}$, both in addition to PAR light).

During five days of experiment samples of the different cultures were taken in replicate to determine the evolution of specific growth parameters such as optical density at 750 nm (as cell density measure), dry weight, and chlorophyll content. Likewise, a parameter such as the maximum photosynthetic efficiency of Photosysthem II (PSII), cell viability indicator, was determined.

Figure 2.7. shows the evolution of the optical density (measured as absorbance at 750 nm) and biomass concentration (measured as biomass dry weight) throughout the time the experiment lasted. As it can be seen, both parameters showed similar trends between PAR and PAR+UVA cultures, whereas on the other hand, growth in cultures subjected to UVB radiation started to drop after 48 hours of cultivation until the end of the process, being completely inhibited at that moment (Data not shown at 96 hours in Figure 2.7b). This behavior in cultures subjected to UVB, must indicate that this level of radiation is detrimental to the viability of the cells.

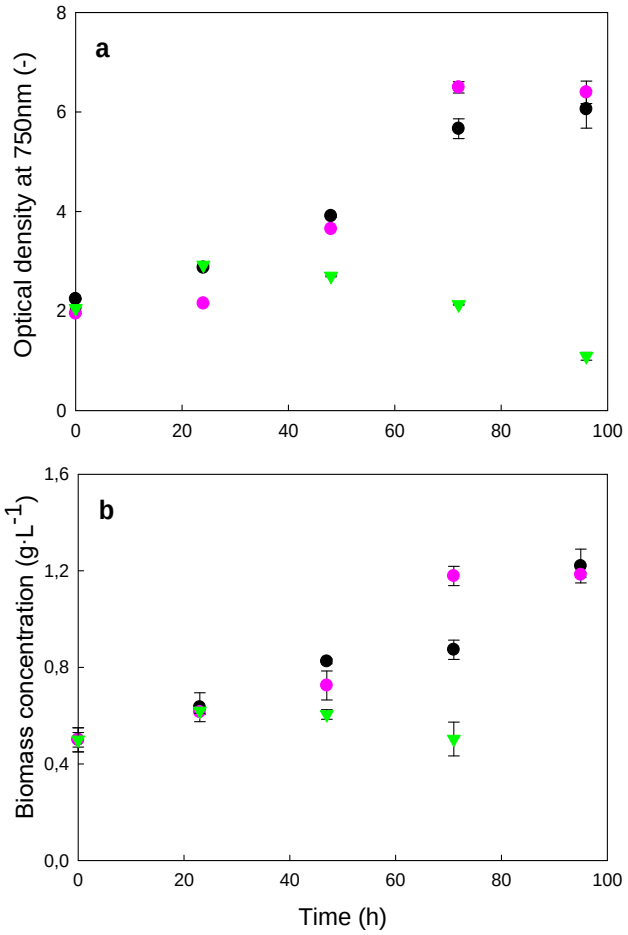


Figure 2.7. Evolution of chlorophyll content, expressed as milligrams per volume of culture broth (a) and as milligrams per gram of biomass dry weight (b), in *C. onubensis* cultures incubated under UVA and UVB radiation. (●) PAR (control); (●) PAR+UVA; (▼) PAR+UVB. Error bars show standard deviation of replicates.

There are studies which show that the continuous microalgal exposure to UVB radiation produces the cessation of growth due to the alterations caused in the photosynthetic process, among which are the loss in the efficiency of the electronic transport of the PSII and the increase in the formation of free radicals (White and Jahnke 2002).

Regarding the chlorophyll accumulation, it can be observed in Figure 2.8. that, despite the fact that in the first 24 hours there was an increase in chlorophyll values of the cultures irradiated with UVB, from that moment, and as expected, lower chlorophyll content was obtained in those cultures until biomass collapsed (cell death) at 96 hours. The cultures irradiated with UVA followed a similar trend to the control in both measures, per volume of culture broth (Figure 2.8a) and per biomass dry weight (Figure 2.8b), being observed an increase in the content of chlorophyll after the first 48 hours, and reaching its highest value (10% higher chlorophyll value than the control) after 96 hours of cultivation.

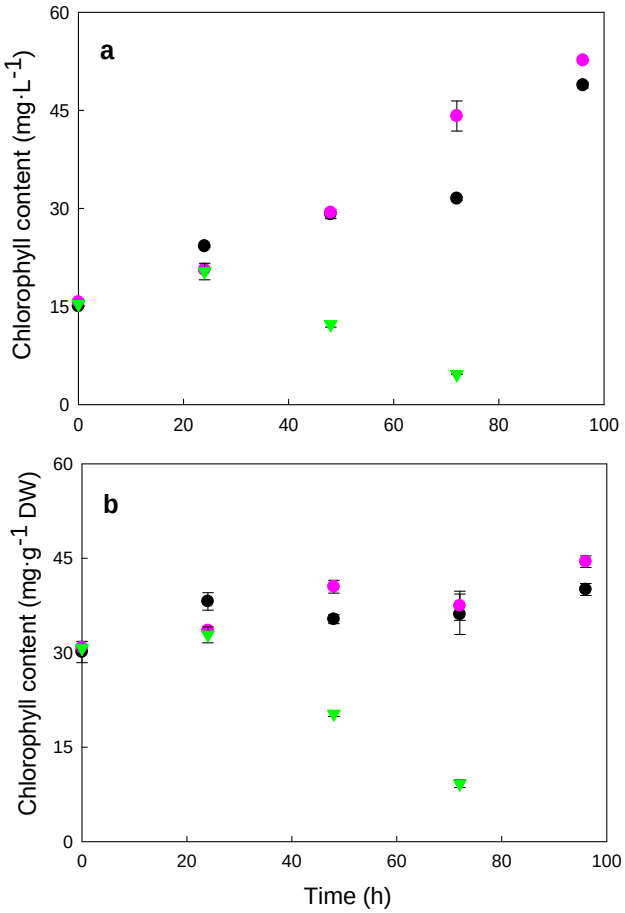


Figure 2.8. Evolution of chlorophyll content, expressed as milligrams per volume of culture broth (a) and as milligrams per gram of biomass dry weight (b), in *C. onubensis* cultures cultures incubated under UVA and UVB radiation. (●) PAR (control); (●) PAR+UVA; (▼) PAR+UVB. Error bars show standard deviation of replicates.

As in the temperature experiment, the viability of the cultures was assessed by means of the maximum photosynthetic activity of PSII. As observed in Figure 2.9., a maximum photosynthetic efficiency of PSII, similar to that for the control, was obtained in the cultures incubated with UVA radiation. In the case of the culture subjected to UVB, a drop in photosynthetic efficiency was observed from the beginning of the experiment until a value of zero was reached coinciding with the death of the culture. This behavior is consistent with the results obtained for chlorophyll content and cell density, in which no growth occurred after the first 24 hours, and which may indicate, once again, that this level of radiation is detrimental to cell viability.

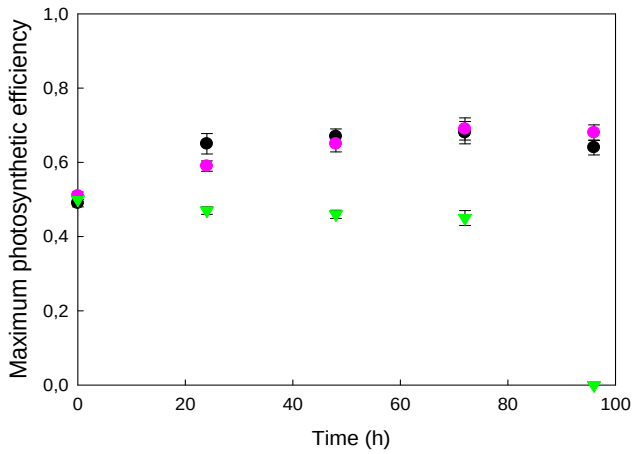


Figure 2.9. Evolution of maximum photosynthetic efficiency of PSII (F_v/F_m) in *C. onubensis* cultures incubated under UVA and UVB radiation. (●) PAR (control); (●) PAR+UVA; (▼) PAR+UVB. Error bars show standard deviation of replicates.

It has been reported that both UVA and UVB radiation affect the photosynthetic processes of marine phytoplankton, causing a decrease in chlorophyll synthesis which, at the same time, causes a reduction in the energy captured and an increase in the content of carotenoids pigments active in energy dissipation and neutralization of oxidant species (Xue et al. 2005; Raja et al. 2007). As the obtained trend of chlorophyll production was similar to that described by such studies, it was hypothesized that the carotenoids production in the cultures subjected to UVA and UVB radiation would be higher than in the control.

Results obtained in this section (2.2.1.) showed that UVB radiation had a negative effect on *C. onubensis* gowth, and to see the difference between each culture, maximum specific gowth rates were calculated from the optical density kinetics at 750 nm (Figure 2.10.). As it can be observed, the lowest growth rate obtained corresponded to the cultures incubated with UVB radiation, in which the growth was almost completely inhibited (0.01 d^{-1}). Nonetheless, cultures irradiated with UVA presented a growth rate of 0.4 d^{-1}, representing a value 33.3% higher than that obtained for the control culture (0.3 d^{-1}).

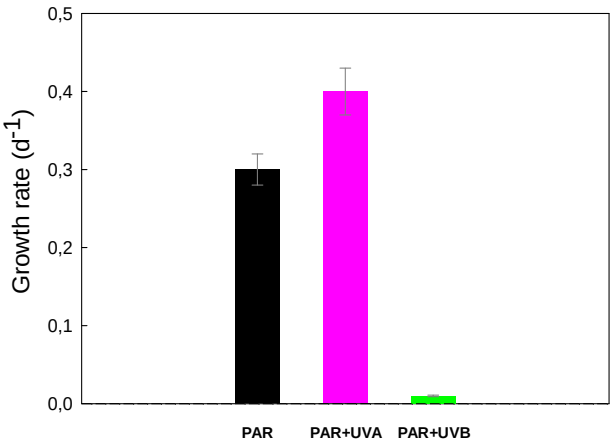


Figure 2.10. Maximum specific growth rate of *C. onubensis* cultures incubated under UVA and UVB radiations Microalga cell cultures were incubated under different radiations: PAR (Control); PAR+UVA; PAR+UVB. The rates were calculated as described in Materials and Methods. Error bars show standard deviation of replicates.

Biomass volumetric productivity for the different cultures after 72 hours of cultivation was also calculated (Table 2.3.). As it was expected from the growth rates results, when microalgal cultures were illuminated with PAR light supplemented with UVB radiation a significant inhibition in biomass productivity was also observed, being the value obtained 99% lower than the value obtained for the control. On the other hand, contrary to what might be expected, biomass productivity was improved in cultures irradiated with UVA, being obtained a value 1.8-fold higher than in the case of the control.

Table 2.3. Biomass volumetric productivities (P_V) of *C. onubensis* expressed as milligrams per liter of culture broth per day, after 72 hours of cultivation under PAR, PAR+UVA and PAR+UVB radiations.

Radiation	**Biomass P_V** ($mg \cdot L^{-1} \cdot d^{-1}$)
Control (PAR)	123.8±0.11
PAR+UVA	226.3±0.11
PAR+UVB	1.2±0.03

These biomass productivity results suggest that *C. onubensis* must have a strong resistance against UVA radiation, since its growth was not compromised by such radiation exposure, at least at that intensity (37 µmoles photons $\cdot m^{-2} \cdot s^{-1}$) and during the period of time the experiment lasted.

2.2.2. Influence of UVA and UVB radiation on carotenoids production of *C. onubensis*.

Many commercially useful carotenoids absorb in the spectrum of UVA radiation (λ = 320-400 nm) (Tsukida et al. 1982), being the accumulation of such compounds one of the molecular adaptation mechanisms in microalgae as response to the oxidative stress induced by UVA rays (Janhke 1999; Wu et al. 2005). Like UVA radiation, UVB (λ = 280-315 nm) produces oxidative stress in microalgae (Ghetti et al. 1998) but also generates irreversible damage in organisms, ranging from burns of varying degrees (Afaq et al. 2005) to DNA mutations (Sethow 1974).

Although there are many references in the literature about how UVA radiation stimulates the production of carotenoids (Salguero et al. 2005; Mogedas et al. 2009; Huang and Cheung 2011), there is limited information about the effect of UVB over carotenoids synthesis. And sometimes the information is even contradictory; UVB radiation stimulates the synthesis of terpenoids in microalgae (Dholer 1998), while other studies find no effect on their accumulation (Jahnke 1999).

In this section of the Thesis, the effect of UVA and UVB radiation on the accumulation of total and specific carotenoids was studied in order to discern the

possible role that both oxidative radiations could have in the productivity of this microalga, and thus address the possibility of a biocompatible production of carotenoids by *C. onubensis* using UVA and/or UVB radiation as a tool to increase the productivity of these compounds. For that purpose, samples of each culture were specifically taken for its analysis during the whole experiment.

Figure 2.11. shows the total carotenoids content in *C. onubensis* cultures incubated under PAR, PAR+UVA and PAR+UVB radiation for 96 hours. As it can be seen (Figure 2.11b), during the first 24 hours of incubation no variation was observed in the cellular content of total carotenoids for the three conditions. After 48 hours, an increase (47% respects to the control) in the content of intracellular carotenoids in cultures irradiated with UVA began to be noticed, which remained above the control until the end of the experiment, reaching values 1.2-fold higher than the control culture. Cultures under UVB radiation followed a trend, in the content of intracellular carotenoids, similar to the control until its death at 96 hours of incubation, which was a sign of the harmful effect caused by prolonged exposure to UVB radiation.

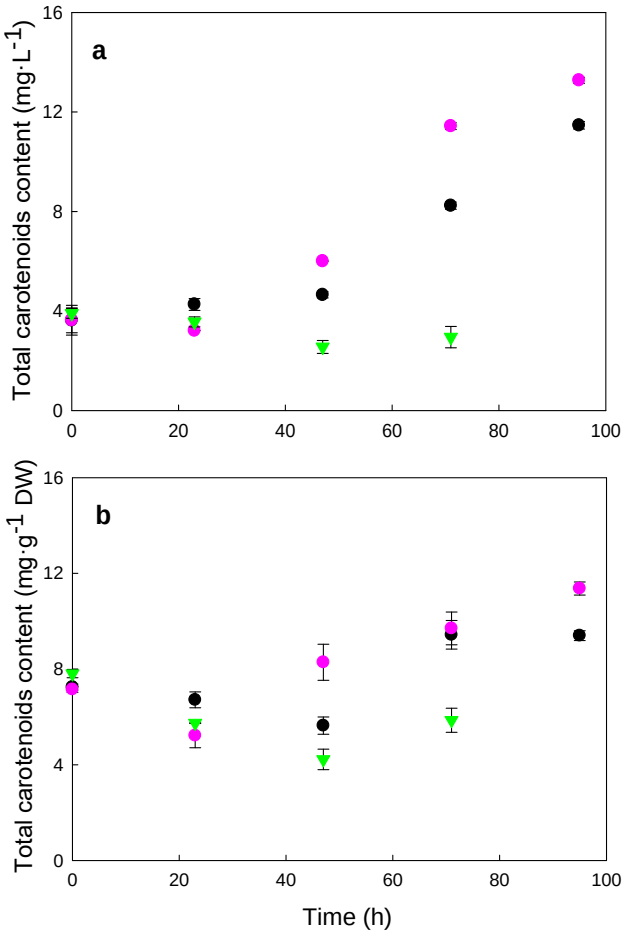


Figure 2.11. Evolution of total carotenoids content expressed as milligrams per volume of culture broth (a) and as milligrams per gram of biomass dry weight (b), in *C. onubensis* cultures incubated under UVA and UVB radiation. (●) PAR (control); (●) PAR+UVA; (▼) PAR+UVB. Error bars show standard deviation of replicates.

Regarding the effect of UV-radiation on the specific carotenoids of *C. onubensis*, lutein was the most abundant carotenoid followed by β-carotene (Figure 2.12.). However, whereas this microalga is considered as one of the potential sources of lutein (Garbayo et al. 2012), for β-carotene production there are other species of microalgae, such as *D. salina,* that accumulate it in much higher quantities (Hejazi and Wijffels 2003).

Figure 2.12. shows lutein and β-carotene content, obtained at 0, 48, 72 and 96 hours of incubation for the different conditions of UVA and UVB radiation. In a general view, the obtained results indicated that moderate UVA radiation produced a stimulating effect in the intracellular accumulation of both carotenoids (Figures 2.12c and 2.12d) (1.4 and 1.3-fold higher than the control, respectively, at the end of the experiment), which took place, mainly, during the first 48 hours of incubation under such radiation. On the other hand, cultures incubated under UVB radiation presented a trend similar to that for the control until the cell death occurred at the end of the experiment (no measures at 96 hours).

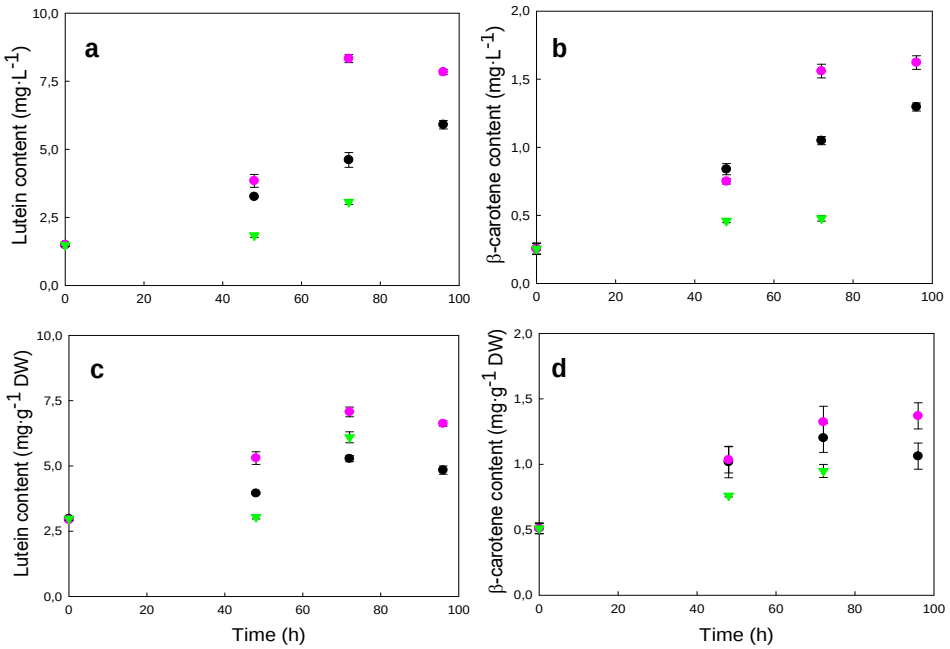


Figure 2.12. Lutein and β-carotene content expressed as milligrams per volume of culture broth (a, b) and as milligrams per gram of biomass (c, d), obtained in *C. onubensis* cultures incubated under different radiations. Carotenoids were determined by HPLC as decribed in Materials and Methods. (●) PAR (control); (●) PAR+UVA; (▼) PAR+UVB. Error bars show standard deviation of replicates.

Regarding the lutein and β-carotene volumetric productivity, maximum values were reached at 72 hours of cultivation (Table 2.4.). As it can be seen, cultures subjected to UVA radiation got the best productivity values for both, lutein and β-carotene, becoming a 119.2% and 65.4%, respectively, higher than the control.

Table 2.4. Biomass, lutein and β-carotene volumetric productivities (P_V) of *C. onubensis* expressed as milligrams per liter of culture broth per day, after 72 hours of cultivation under different radiations.

Radiation	Lutein P_V ($mg \cdot L^{-1} \cdot d^{-1}$)	β-carotene P_V ($mg \cdot L^{-1} \cdot d^{-1}$)
Control (PAR)	1.04±0.06	0.26±0.05
PAR+UVA	2.28±0.03	0.43±0.02
PAR+UVB	0.53±0.02	0.07±0.01

In conclusion, illumination with UVA radiation stimulated carotenoids accumulation in *C. onubensis*, which became already evident in the first 48 hours of cultivation (Figure 2.11.). This is consistent with a greater involvement of such molecules and antioxidant mechanisms in processes of neutralization of free radicals, more abundant as the intensities of UVA radiation increase (Miller et al. 1996; Shao et al. 2008). Thus, obtained results suggested that this type of radiation can be used as a suitable oxidative factor to induce the biocompatible production of carotenoids in *C. onubensis*, specifically of lutein and β-carotene, while preserving the cell viability.

Regarding UVB radiation, the results revealed that, for this microalga, the exposure to this type of radiation was harmful to its development. Biomass was not able to neither produce enough antioxidant molecules nor activate mechanisms that could help to dissipate the excess energy of such radiation and, therefore, the death of the culture occurred between 72 and 96 hours of incubation. This coincides with the results obtained in other studies, in which the exposure to UVB radiation damaged the ultrastructure of nuclear or chloroplast DNA, causing mutations or even cell death (Holzinger and Lütz 2006; Buma et al. 2006).

2.2.3. Influence of UVA and UVB radiation on total lipids and fatty acids production of *C. onubensis*.

In general, when microalgae are subjected to abiotic stress, there is an intracellular accumulation of lipids (Forján et al. 2015; Srivastava et al. 2017). Under unfavorable conditions, many species of eukaryotic microalgae modify their lipid biosynthetic pathway towards the formation and accumulation of fatty acids

(FAs), which are mainly used as energy storage compounds (Alyabyev et al. 2007). One of these factors is UV-radiation, which is known to induce metabolic changes in microalgae that alters the biomass composition and increases the lipids fraction (Guihéneuf et al. 2010; Khozin-Goldberg et al. 2011). Considering that, it was decided to assess the effect that UVA and UVB radiations could have on the accumulation of total lipids as well as fatty acids in *C. onubensis*. To do that, samples of each culture were punctually taken (at 0, 24 and 72 hours) during the experiment to determine the content of totals lipids and, at the same time, to analyze the amount of the main fatty acids produced by this microalga.

Figure 2.13. shows the results from the total lipids analysis. If the content per volume of culture broth is the focus (Figure 2.13a), it can be observed that UVB culture followed a similar trend to the control, maintaining the lipids content more or less constant after 24 hours of cultivation, whereas the one subjected to UVA presented a continuous accumulation of lipids during the whole process. Nevertheless, as it can be seen in Figure 2.13b, the lipid intracellular content in the cultures subjected to UVA and UVB radiation was higher than that in the control at 72 hours of experiment. And more specifically, whereas UVA culture followed a similar trend to the control, in which lipids reached a maximum at 24 hours of cultivation, the UVB culture showed a continuous increase reaching a final lipid content 94% higher than the control. This lipid accumulation might be triggered by ROS production and oxidative stress under such UV-radiation conditions.

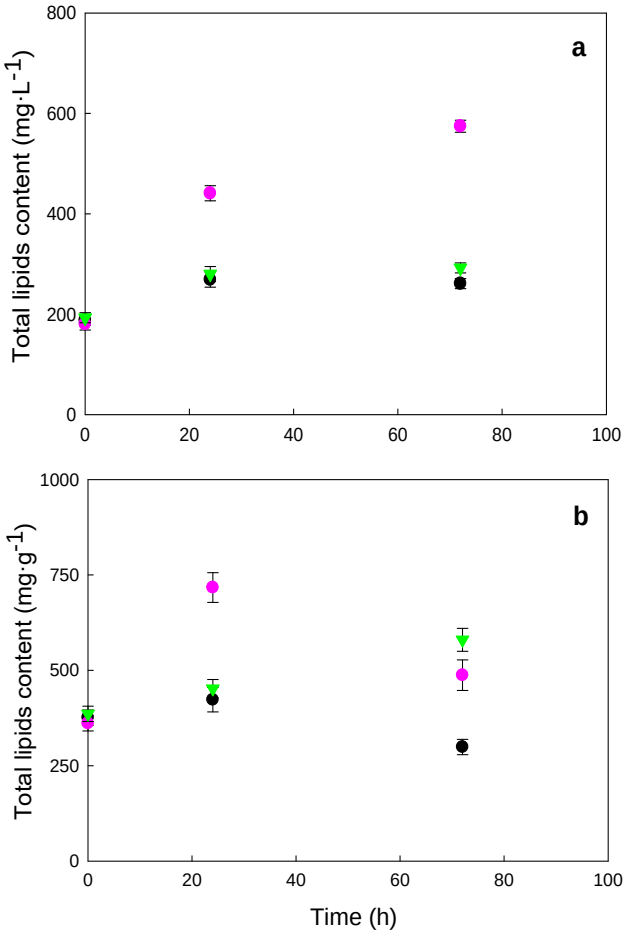


Figure 2.13. Evolution of total lipids content expressed as milligrams per volume of culture broth (a) and as milligrams per gram of biomass dry weight (b), in *C. onubensis* cultures incubated under different radiations. (●) PAR (control); (●) PAR+UVA; (▼) PAR+UVB. Error bars show standard deviation of replicates.

Regarding the fatty acids content, it was determined which fraction of the accumulated total lipids corresponded to such molecules. Figure 2.14. shows the content, expressed as milligrams per gram of dry weight, of the main fatty acids produced by *C. onubensis* after 72 hours of cultivation. It should be highlighted that UVA radiation strongly increased the palmitic acid (C16:0) content by 2.4-fold and oleic acid (C18:1) content by 4.7-fold respect to the control culture. Furthermore, another thing to highlight would be the fact that UVB radiation

induced a considerably higher accumulation of palmitic acid than PUFAs, which is interesting from the point of view of the desired final product.

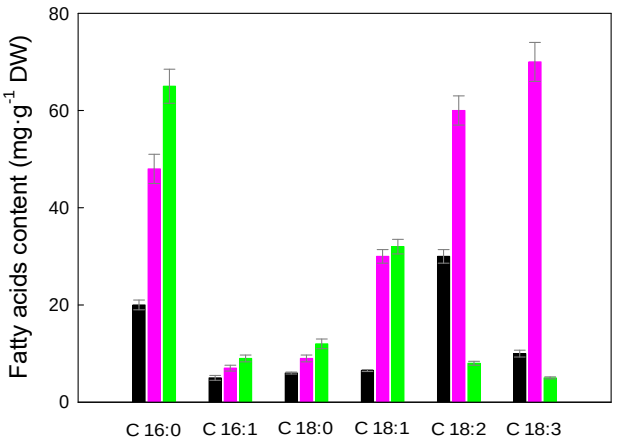


Figure 2.14. Evolution of the main fatty acids content expressed as milligrams per gram of biomass dry weight, in *C. onubensis* cultures incubated under different radiations after 72 hours of cultivation. (■) PAR (control); (■) PAR+UVA; (■) PAR+UVB. Error bars show standard deviation of replicates.

According to the obtained results, a study carried out by Srinivas and Ochs (Srinivas and Ochs 2012), demonstrated that the exposure to UVA radiation mainly increased PUFAs content in *Nannochloropsis oculata,* whereas it has been also reported that in *Tetraselmis sp*., UVB radiation resulted in an overall increase in the Saturated Fatty Acids (SFAs) and Mono-Unsaturated Fatty Acids (MUFAs) content, with a decrease in Polyunsaturated Fatty Acids (PUFAs) (Goes et al. 1994). This behavior is due to the fact that under unfavorable environmental or stress conditions for growth, many algae react to the oxidative stress modifying their fatty acids biosynthetic pathways towards the formation and accumulation of storage lipids (SFAs and MUFAs) which has a more active role in the stress response, in addition to functioning as carbon and energy storage under such conditions (Hu et al. 2008).

Total lipids and fatty acids volumetric productivities of the different cultures were also calculated after 72 hours of cultivation (Table 2.5.). It was observed that in both cases, lipid productivity values improved respect to the control when the

cultures were grown under UVA or UVB radiation, reaching the highest values with UVA (5.4 and 1.1-fold higher, respectively).

Table 2.5. Total lipids and fatty acids volumetric productivities (P_V) of *C. onubensis* expressed as milligrams per liter of culture broth per day, after 72 hours of cultivation under different radiations.

Radiation	Total lipids P_V ($mg \cdot L^{-1} \cdot d^{-1}$)	Fatty acids P_V ($mg \cdot L^{-1} \cdot d^{-1}$)
Control (PAR)	24.33±0.75	1.42±0.05
PAR+UVA	131.33±0.90	1.57±0.07
PAR+UVB	33.10±0.53	1.45±0.02

Results obtained in this section of the Thesis (2.2.3.) are in accordance with studies found in the literature (Guihéneuf et al. 2010; Khozin-Goldberg et al. 2011) where it is indicated that illumination with PAR+UVA radiation can be considered a suitable tool to produce a significant amount of microalgal biomass enriched with FAMEs and with a high content of PUFAs.

2.2.4. Enzymatic antioxidant response of *C. onubensis* to UVA and UVB radiation.

Ultraviolet radiation exposure, either UVA or UVB, has harmful effects on the cellular metabolism of living organisms, stimulating the accumulation of reactive oxygen species (ROS). The antioxidant response to the excess of irradiance, including ultraviolet radiation, has been extensively investigated in plants and macroalgae, but little information is available in microalgae (Janknegt et al. 2009).

To determine if the ultraviolet radiation, A and/or B, generates oxidative stress in *C. onubensis*, enzymatic activity of the antioxidant system of the microalga was measured when it was independently subjected to UVA and UVB radiation. The so-called antioxidant enzymes are proteins that catalyze the detoxification of ROS. To avoid the damage generated by the presence of ROS molecules, microorganisms have developed enzymatic and non-enzymatic defense mechanisms (Mallick and Mohn 2000; Schützendübel and Polle 2002). Non-enzymatic antioxidant mechanisms include low molecular weight compounds

such as glutathione, phenolic compounds and ascorbate, while enzymatic defenses include superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT) and a variety of peroxidases, such as ascorbate peroxidase (APX) or guaiacol peroxidase (GPX). These enzymes, with the help of reduced substrates, such as glutathione or ascorbate, participate in the reduction of such oxidant radicals in the cell.

In this section of the Thesis, the activity of the enzymes ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (GPX) were studied, taking samples for this purpose at 0, 24, 48 and 72 hours of incubation under the previously mentioned experimental conditions.

One of the ways to remove ROS such as H_2O_2 is by action of the antioxidant enzyme catalase (CAT), the only enzyme that degrades H_2O_2 without consuming reducing power. Therefore, catalase is an energy-efficient mechanism for the cell to remove hydrogen peroxide (Mallick and Mohn 2000). Figure 2.15. represents the relative variation of catalase activity that participates in the detoxification of H_2O_2 in *C. onubensis* cultures incubated independently under UVA and UVB radiation. The figure shows the variation of the CAT activity respect to the same enzymatic activity of the control cultures at time zero. It can be observed a decrease in the activity of the enzyme exposed to UVA radiation in the first 24 hours, followed by a recovery and progressive increase in the activity reaching values 50% higher than those for the control at the beginning of the experiment. In the case of exposure to UVB radiation, although a considerable increase in activity was observed after 24 hours, from then CAT activity progressively decreased reaching, at the end of the experiment, values 11% lower than those for the control at time zero. This response, as for the other enzymatic activities measured, shows that the incubation in the presence of ultraviolet radiation triggers situations that produce oxidative stress, with the consequent increase in the activity of enzymes which participate in antioxidant defense reactions (Ledford and Niyogi 2005), and experimentally ratifies the existing relationship between ultraviolet radiation A or B and the generation of oxidative stress.

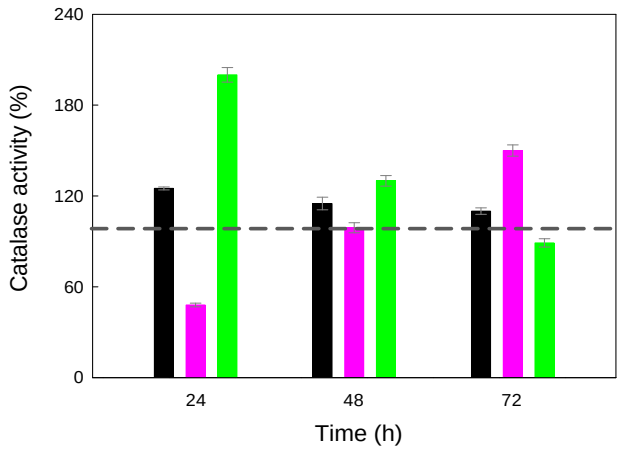


Figure 2.15. Relative increase in catalase activity (CAT) of *C. onubensis* incubated under UVA and UVB radiation independently. ( ) PAR (control); ( ) PAR+UVA; ( ) PAR+UVB. Enzymatic activity measurement was carried out in crude extracts obtained from the control and UV cultures, as described in Materials and Methods. The dotted line indicates 100% of activity (13.06±1.01 $U \cdot mg^{-1}$ proteins), which is the one presented by the microalga at the beginning of the experiment in the control culture (time 0h). The unit of measurement of the activity is ($U \cdot mg^{-1}$ prot). Error bars show standard deviation of replicates.

Figure 2.16. shows the relative increase of ascorbate peroxidase activity (APX) of *C. onubensis* incubated under UVA and UVB radiation. Ascorbate is the most important biological reducer of H_2O_2. The enzyme ascorbate peroxidase oxidizes ascorbate molecules regenerating reducing power, NADPH, which is used to recover the reduced glutathione by the action of the enzyme glutathione reductase on oxidizing glutathione (Noctor and Foyer 1998). At 24 hours of cultivation under PAR radiation (control culture), it can be seen, as with the CAT enzyme, an increase in APX activity 15% higher than that obtained at the beginning in the crude extracts of the control, although it was followed by a progressive decrease until the end of the experiment. Under UVA radiation, there was an increase in the activity of this antioxidant enzyme respect to the crude extracts of the control culture, reaching an APX activity value 42% higher after 72 hours of incubation. However, the highest values were reached with UVB, highlighting the fact that, after 24 hours of cultivation under such radiation, APX activity increased a 250% respect to the control at the beginning of the experiment (time 0 hours).

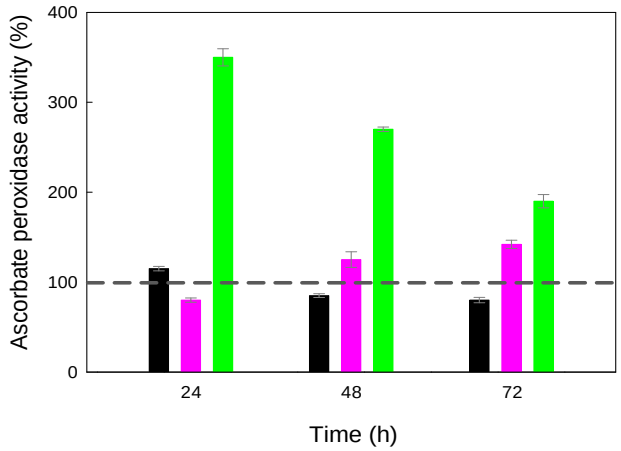


Figure 2.16. Relative increase in ascorbate peroxidase activity (APX) of *C. onubensis* incubated under UVA and UVB radiation independently. ( ) PAR (control); ( ) PAR+UVA; ( ) PAR+UVB. Enzymatic activity measurement was carried out in crude extracts obtained from the control and UV cultures, as described in Materials and Methods. The dotted line indicates 100% of activity (3.76±0.09 $U \cdot mg^{-1}$ proteins), which is the one presented by the microalga at the beginning of the experiment in the control culture (time 0h). The unit of measurement of the activity is ($U \cdot mg^{-1}$ prot). Error bars show standard deviation of replicates.

Figure 2.17. shows the antioxidant activity of guaiacol peroxidase enzyme in *C. onubensis* cultures exposed to UVA and UVB radiation for 72 hours. As all peroxidase enzymes, guaicol is able to control the cellular concentrations of superoxide radicals (O_2^{*-}) and hydrogen peroxide (H_2O_2), thereby preventing the formation of reactive radicals (Zhekisheva et al. 2002). It can be observed that ultraviolet radiation produces an increase in the activity of this antioxidant enzyme with respect to the values of the crude extracts of the control culture. Regarding the ultraviolet radiation, there are no relevant differences in the variations of enzymatic activity in the cultures radiated with UVA or UVB, highlighting the increase observed at 24 hours, which might indicate, once again, an increase in ROS in *C. onubensis* metabolism, which enhance the oxidative state of the microalga.

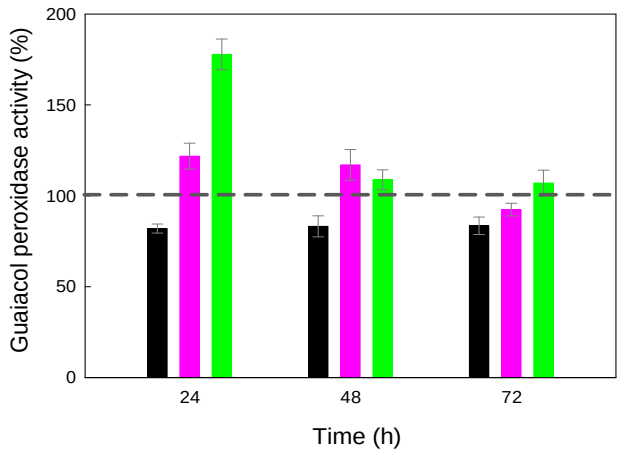


Figure 2.17. Relative increase in guaiacol activity (GPX) of *C. onubensis* incubated under UVA and UVB radiation independently. ( ) PAR (control); ( ) PAR+UVA; ( ) PAR+UVB. Enzymatic activity measurement was carried out in crude extracts obtained from the control and UV cultures, as described in Materials and Methods. The dotted line indicates 100% of activity (1.32±0.01 $U \cdot mg^{-1}$ proteins), which is the one presented by the microalga at the beginning of the experiment in the control culture (time 0h). The unit of measurement of the activity is ($U \cdot mg^{-1}$ prot). Error bars show standard deviation of replicates.

Finally, to get an overview of changes in the activity of the three mentioned enzymes (CAT, APX and GPX) depending on if they were cultivated under UVA or UVB radiations, Figure 2.18. shows the percentage of activity, after 72 hours of cultivation, of each enzyme respect to the activity of their respective controls at that time. As it can be seen in the figure, CAT activity is more influenced by UVA radiation, producing an increase of around a 56% higher than UVB. On the other hand, the enzymes APX and GPX were more influenced by UVB radiation, showing 1.8 and 2.6-fold higher activity than with UVA, respectively. It must be also highlighted that, in general, APX was much more activated by UV-radiation than the other two studied enzymes, and as it is known that APX is presented in high concentrations in chloroplasts (Noctor and Foyer 1998), it might be considered that UV-radiation acts directly on such type of organelles.

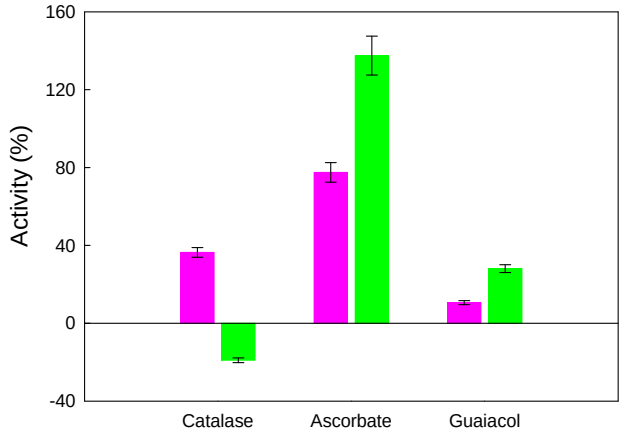


Figure 2.18. Influence of UVA ( ) and UVB ( ) radiation in Catalase (CAT), Ascorbate peroxidase (APX) and Guaiacol peroxidase (GPX) antioxidant activity respect to the control (zero value) in *C. onubensis* after 72 hours of cultivation. Enzymatic activity measurement was carried out in crude extracts obtained from the control and UV cultures, as described in Materials and Methods.

The antioxidant capacity of microalgae extracts is a reference parameter to know its oxidative stability in case of food or chemical use, however, there are hardly any studies in the literature that relate stress conditions to the oxidative response that is generated by the accumulation of carotenoids and/or fatty acids in microalgal cultures (Garbayo et al. 2008). In this sense, and as a conclusion, it should be noted from the experiments carried out in this section (2.2.4.) that the determination of CAT, APX and GPX activities in *C. onubensis* shows its usefulness to identify situations of cellular oxidative stress and, accordingly, to take decisions over the cultivation process.

2.3. Effects of using stress conditions on *C. onubensis* cultivation.

According to the results obtained in this Chapter 2 of the Thesis, cultivation of *C. onubensis* under different stress conditions, as higher temperature or UV-radiation, produced interesting effects, having been concluded that:

1. Increasing temperature in *C. onubensis* cultures, from 25 ºC to 35 ºC progressively improved biomass and carotenoids productivities until a

temperature value (40 ºC) from which the microalgae could not mantain its cell viability.

2. Carotenoids productivity value at 35 ºC was higher than that for the control but not greatly improved. Therefore, in order to further increase the production of carotenoids, it was considered that *C. onubensis* should be cultivated at such temperature in combination with other stress factors.

3. *C. onubensis* cultivation under UVA radiation greatly improved its biomass and carotenoids productivities. However, cultures subjected to UVB radiation were not able to deal with the cellular damaged produced by such radiation and thus, their biomass and carotenoids productivies were negatively affected.

4. UVA radiation also produced a highly positive effect in *C. onubensis* lipids productivity, whereas UVB radiation only presented a slight improvement respect to the control.

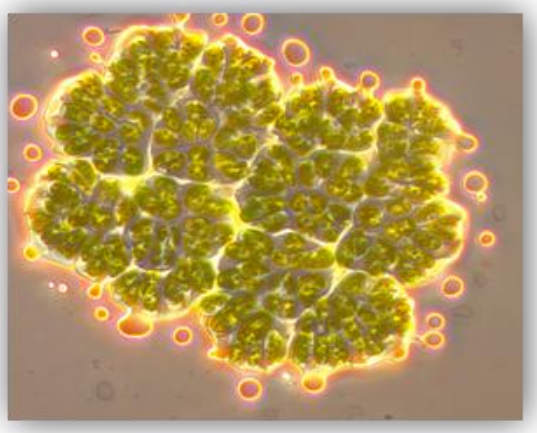
5. UV-radiation enhaced the antioxidant enzymatic capacity of CAT, APX and GPX enzymes in *C. onubensis* cultures. UVA radiation had more influence in CAT activity, whereas APX and GPX activities were mainly influenced by UVB radiation.

PART II

Biomass and metabolites productivity enhancement for two *Botryococcus braunii* races

Chapter 3

OPTIMIZATION OF GROWTH AND CARBOHYDRATES AND HYDROCARBONS PRODUCTION OF TWO *Botryococcus braunii* RACES



The work presented in this Chapter will be submitted as:
Bermejo, E., Muñoz, A., Ramos-Merchante, A., Vílchez, C., Garbayo, I., Cuaresma, M. Optimized media for growth and carbohydrates and hydrocarbons production in two *Botryococcus braunii* strains.

3. OPTIMIZATION OF GROWTH AND CARBOHYDRATES AND HYDROCARBONS PRODUCTION OF TWO *Botryococcus braunii* RACES.

In the first chapter of Results and Discussion Part II, it was carried out the culture medium optimization for two races of the microalgal species *Botryococcus braunii* with the aim of improving growth and product productivity, as these are key parameters to enable feasible microalgal industrial production.

As it has been already mentioned in the Introduction Chapter, *B. braunii* is a microalga which has the almost unique capacity of accumulate and excrete large amounts of long chain hydrocarbons and interesting groups of polysaccharides, which can be convertible into bio-chemicals. Nevertheless, working with this microorganism presents a number of challenges considering the extreme variability between the different races and strains and their slow growth and productivities (Banerjee et al. 2002). In this sense, it has been proposed that the composition of the most commonly used culture media and the morphology of the colonies, which not allow a fast diffusion of nutrients (Li and Qin 2005), might be the limiting factors.

Although medium and operational conditions optimization can be considered the first step leading to microalgal growth and productivity enhancement, there are not many studies regarding the culture medium optimization of *B. braunii*, which together with its slow growth make quite limited the industrial use of this microalga. In this sense, it was proposed to optimize the culture medium composition for two *B. braunii* strains -race A and B- in terms of growth and carbohydrates (exopolysaccharides) and hydrocarbons productivity respectively. This medium optimization was focus, at the same time, on selenium removal from the micronutrients source. Although selenium might play a role in biomass growth and product formation (Harrison et al. 1988; Wheeler et al. 1982; Araie and Shiraiwa 2009; Yoshimura et al. 2013), it has not been proved for *B. braunii.* Besides, considering that the chemical form in which Se is present in the commonly used media is toxic, it is preferred to avoid its use, especially when medium-large scale cultivation is performed.

In this work, different phases were identified for the media optimization. First, a thorough literature review was performed, in which the different media used for *B. braunii* cultivation were identified. The media composition was

analysed and it allowed to define critical micro- and macronutrients which needed to be optimized. Secondly, an experimental design was carried out in microwell plates in order to evaluate the modified media and to pre-select the best candidates. Finally, validation of pre-selected media was carried out in Roux flasks in order to select the final optimized media for each race.

3.1. Literature review and experimental design.

During the literature research 26 different culture media were identified for *B. braunii* (Ambati et al. 2010; Cheng et al. 2013; Dayananda et al. 2005, 2007; Fernandes et al. 1991; Furuhashi et al. 2013; Ge et al. 2011; Kawachi et al. 2012; Kojima and Zhang 1999; Largeau et al. 1980; Lee et al. 1998; Li and Qing 2005; Liu et al. 2013; Lupi et al. 1994; Qin 2005; Rao et al. 2007; Ruangsomboom 2012; Sakamoto et al. 2012; Shimamura et al. 2012; Song et al. 2012; Tanoi et al. 2011; Weiss et al. 2012; Yang et al. 2004; Yoshimura et al. 2013). The molar composition of the 26 culture media was compared to the composition of other general culture media normally used to grow green algae: BBM, BG-11, F2, K9, M8a and Seuoka between others (Bischoff and Bold1963; Sueoka et al. 1967; Stanier et al. 1971; Guillard 1975; Cuaresma et al. 2009; Ruíz-Domínguez et al. 2015). In total, 38 culture media were analysed and the importance of each nutrient was assessed in terms of relative presence and average concentration. Modified Chu 13 (Gouveia et al. 2017) was used as Reference medium in this study to compare the performance of the new media. Comparison of Reference media nutrients with the calculated average concentrations of the other 38 media allowed the identification of macro- and micronutrients to be optimized. In order to simplify the comparison, 2 groups of media were used: culture media commonly used to grow microalgae and culture media commonly used to grow *Botryococcus* strains. A Box Plot was done in order to visually summarize, compare and identify outlier data during the comparison of the general culture media and the media used to cultivate *Botryococcus*.

As it can be seen in Figure 3.1., the main macronutrients which clearly differed between both groups were potassium, sodium, nitrogen and phosphorus. In general, the *Botryococcus* media showed much lower concentrations of such macronutrients.

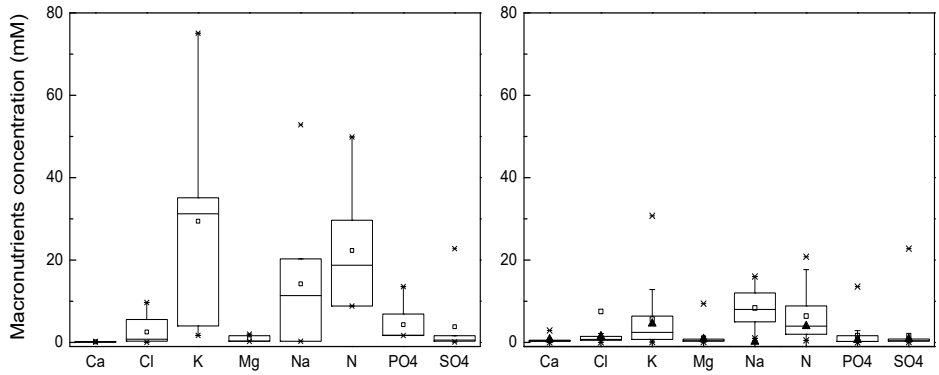


Figure 3.1. Box plot representing the macronutrients composition of the culture media commonly used to grow algae (a) and the media used to grow *Botryococcus* (b). Values corresponding to the Reference medium used in this work (Chu 13, Gouveia et al. 2017) are presented with black triangles in the right plot. Box represents 25-75% percentiles, range bar represents 1 and 99% percentiles, (□) mean values and (*) beyond these bars represent outliers.

Regarding the micronutrients, main differences were found in the content of barium, EDTA, iron, molybdenum, selenium and zinc (Figure 3.2.). Besides, in this case, the media used to cultivate *Botryococcus* presented other trace elements which were never present in the other media group -coloured in red in the box chart-, as aluminium, bromine, chromium, iodine and selenium between others. As well as with macronutrients, most of the trace elements were present in minor quantities in the culture media used for *Botryococcus*.

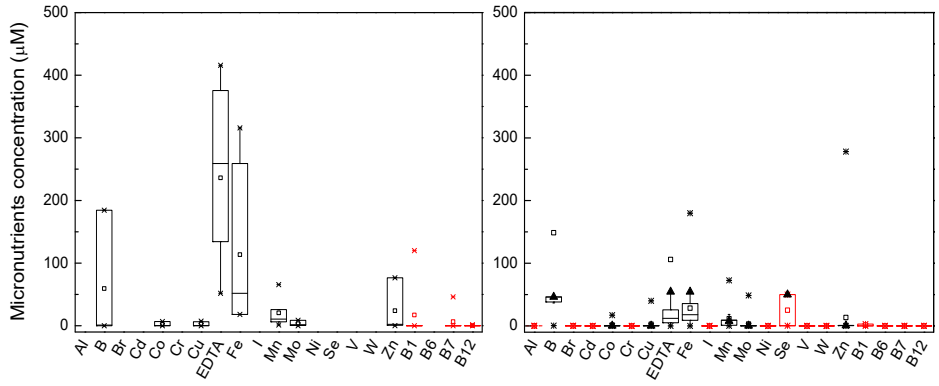


Figure 3.2. Box chart representing the micronutrients composition of the culture media commonly used to grow algae (a) and the media used to grow *Botryococcus* (b). Values corresponding to the Reference medium used in this work (Chu 13, Gouveia et al. 2017) are presented with black triangles in the right plot. Elements which were scarcely present in the different media are coloured in red. Box represents 25-75% percentiles, range bar represents 1 and 99% percentiles, (□) mean values and (*) beyond these bars represent outliers.

As it was already mentioned in the Introduction Chapter, nitrogen and phosphorus are essential elements for all life forms. After carbon, nitrogen is considered the most important nutrient for biomass development and it has an important role in regulating photosynthesis (Smith 1979). The nitrogen content of the biomass can range from 1% to more than 10% and nitrogen limitation can be also used as a tool to promote accumulation of organic carbon compounds such as polysaccharides and certain oils (polyunsaturated fatty acids) (Becker 1994). Phosphorus is an essential component of nucleic acids and many intermediary metabolites, such as sugar phosphates and adenosine phosphates, which are an integral part of the metabolism of all life forms (Correll 1998). Iron is one of the most important elements required by most microalgae because ferric ion is involved in fundamental enzymatic reactions, photochemistry in photosystem II, nitrogen consumption and chlorophyll synthesis in the algal cells (Hutchins and Bruland 1998; Kolber et al. 1994). Deficient or excessive iron can generally reduce the photosynthetic efficiency of microalgae (Geider and La Roche 1994; Kolber et al. 1994). And finally, molybdenum is a very scarce element playing key roles in the metabolism of nitrogen and sulphur, in hormone biosynthesis and act as important enzyme-cofactor (Jacobs and Lind 1977; Llamas et al. 2011; Tejada-Jiménez et al. 2011). According to the biological importance of the elements commented above, and considering the deviations between the two groups of media, it was decided to optimize the concentration of the mentioned elements -N, P, Fe and Mo- in the Reference culture medium of *Botryococcus* (Chu 13, Gouveia et al. 2017).

Apart from that, Reference medium also contained selenium. Selenium is toxic and, therefore, it becomes a problem when medium/large volumes of culture media needs to be handled. Even under laboratory conditions it implies strict regulations in terms of waste management and personal safety. Moreover, the role of Se in *Botryococcus* metabolism is not clear. Only few authors mention its role as growth enhancer for other microalgae, but even in these studies the Se concentrations used are much lower than the one in the Reference medium (Araie and Shiraiwa 2009; Harrison et al. 1988; Wheeler et al. 1982; Yoshimura et al. 2013). Other studies with selenium are focussed on its bioaccumulation and biotransformation to Se-aminoacids with the aim to produce Se-enriched microalgae biomass with value in the food/feed/health-care industry (Gómez-

Jacinto et al. 2012; Kouba et al. 2014; Gojkovic et al. 2015). Altogether seems to indicate that selenium might not be essential for *Botryococcus* growth and it was decided to, at least, reduce its concentration in the Reference medium. Table 3.1. shows the averaged values found for the different element concentrations in the different media groups. As previously commented, nitrogen, phosphorus, iron and molybdenum were found to be the nutrients with the highest deviation compared to the averaged values.

Table 3.1. Descriptive statistic of selected nutrients for the different media groups. Mean value is shown with its standard error. General media corresponds to the culture media used to grow other microalgal species; *B. braunii* media corresponds to culture media commonly used to grow *B. braunii*, and Reference medium corresponds to the concentration in Chu13 medium (Gouveia et al. 2017), used as reference media in this study. Finally, proposed study range is the concentration range tested in this study.

Nutrients	General media			*B. braunii* media			Reference medium	Proposed study range
	Mean	Median	Mode	Mean	Median	Mode		
N, mM	13.97±4.25	8.82	3.60	6.24±1.31	3.78	2.00	4.0	4 - 40
P, mM	2.54±1.15	1.68	0.09	0.76±0.39	0.23	0.23	0.6	0.6 - 6
Fe, µM	72.52±30.22	20.81	17.98	29.33±8.12	17.85	17.85	54.5	10 - 70
Mo, µM	3.74±1.59	1.80	8.71	3.09±2.41	0.21	0.21	0.25	0.2 - 2
Se, µM	-	-	-	0.32*	0.32*	-	50.0	0 - 50

*Only one media presented Selenium in its composition.

Based on the media composition of the different groups a range of concentration was defined to be tested for each nutrient (Table 3.1) and a 3-level factorial design (Design Expert software) was used to prepare 46 different new media accordingly (Table 3.2).

Table 3.2. Composition of new media defined by the experimental design. Green lines indicate the new media without selenium in its composition.

New media Code	Nitrate (mM)	Phosphate (mM)	Iron (µM)	Molybdenum (µM)	Selenium (µM)
M1	22	3.3	40	0.2	50
M2	22	3.3	40	1	25
M3	22	3.3	70	1	50
M4	4	3.3	40	0.2	25
M5	40	3.3	10	1	25
M6	4	3.3	40	1	50
M7	40	3.3	70	1	25
M8	22	3.3	70	2	25

M9	40	3.3	40	1	0
M10	4	3.3	10	1	25
M11	22	3.3	10	1	0
M12	40	0.6	40	1	25
M13	40	6	40	1	25
M14	22	6	40	2	25
M15	22	6	70	1	25
M16	22	3.3	10	2	25
M17	22	3.3	40	1	25
M18	22	6	40	0.2	25
M19	22	3.3	10	0.2	25
M20	22	0.6	70	1	25
M21	4	3.3	40	1	0
M22	22	3.3	70	0.2	25
M23	4	0.6	40	1	25
M24	22	0.6	40	1	50
M25	4	3.3	70	1	25
M26	22	3.3	40	1	25
M27	22	3.3	40	1	25
M28	22	6	40	1	0
M29	4	3.3	40	2	25
M30	40	3.3	40	1	50
M31	22	3.3	40	0.2	0
M32	22	0.6	40	1	0
M33	22	3.3	10	1	50
M34	4	6	40	1	25
M35	22	3.3	70	1	0
M36	22	3.3	40	1	25
M37	40	3.3	40	0.2	25
M38	22	0.6	40	0.2	25
M39	22	3.3	40	2	0
M40	22	3.3	40	2	50
M41	40	3.3	40	2	25
M42	22	3.3	40	1	25
M43	22	0.6	10	1	25
M44	22	0.6	40	2	25
M45	22	6	40	1	50
M46	22	6	10	1	25

Nitrogen and phosphorus were decided to be evaluated up to 10-fold the Reference medium concentration. The final purpose of this part of the Thesis was to enhance productivity and therefore, these nutrients must be in excess to avoid any limitation, especially in case of growing *B. braunii* at medium-large scale. Iron was proposed to be evaluated at an intermediate range, considering it can be limiting at low concentrations but also harmful at high concentrations; molybdenum was decided to be increased due to the low concentration in the Reference medium, and selenium was proposed to be reduced and/or removed due to its toxicity.

3.2. Optimization and pre-selection of potential media in microwell-plates.

The 46 new media were evaluated in 24-well plates, as described in Materials and Methods section. Microwell plates allow for a fast and reliable evaluation of different media in a short period of time. As it can be observed in Figure 3.3., each plate row contained a different medium inoculated in 5 replicates (the first well contained only the culture media as blank for the optical density analysis). In order to avoid that the results could be influenced by the different experimental conditions that could be experienced in each well-plate, each plate contained a Reference culture (Chu 13, Gouveia et al. 2017) used as a control.

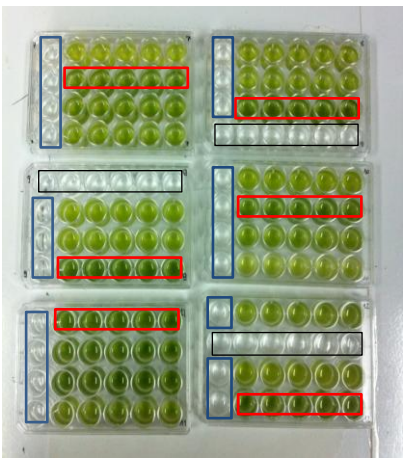


Figure 3.3. Six microwell plates which represent one of the three experiments carried out for each race of *B. braunii.* First well of every row (blue box) contained the different culture medium as blank. Red boxes indicate the position of the Reference medium in each plate. Black boxes indicate rows which contain only water to complete the plates. The rest of the rows represent the new culture media.

Growth and photosynthetic efficiency (measured as maximum Quantum yield of Photosystem II) of each medium was daily monitored and dry weight biomass, as well as the final product content for both strains, were determined at the end of the experiment. Based on that, it was calculated the final product productivity (carbohydrates and hydrocarbons) which allowed the pre-selection of several potential media to be further tested. The biomass productivity and the absence of selenium were also considered as important criteria for the final selection.

3.2.1. Effect of new media composition on biomass production.

Biomass concentration followed a different pattern for both *B. braunii* races. Figure 3.4. shows, as example, the growth performance of some of the new media (M1-M4) and the Reference medium. As it can be seen, *B. braunii* race A reached a higher cell density during the same experimental time compared to race B, which indicates that race A presents a higher growth rate. And comparing the growth of each race in the new media with the Reference medium, it can be said that whereas for *B. braunii* race B there is no remarkable difference for many of the media, for *B. braunii* race A it can be observed that most of the new media presented a slightly higher growth since the beginning of the linear growth phase. It was also confirmed by the calculated values of maximum specific growth rate (Figure 3.5) when compared to the Reference medium.

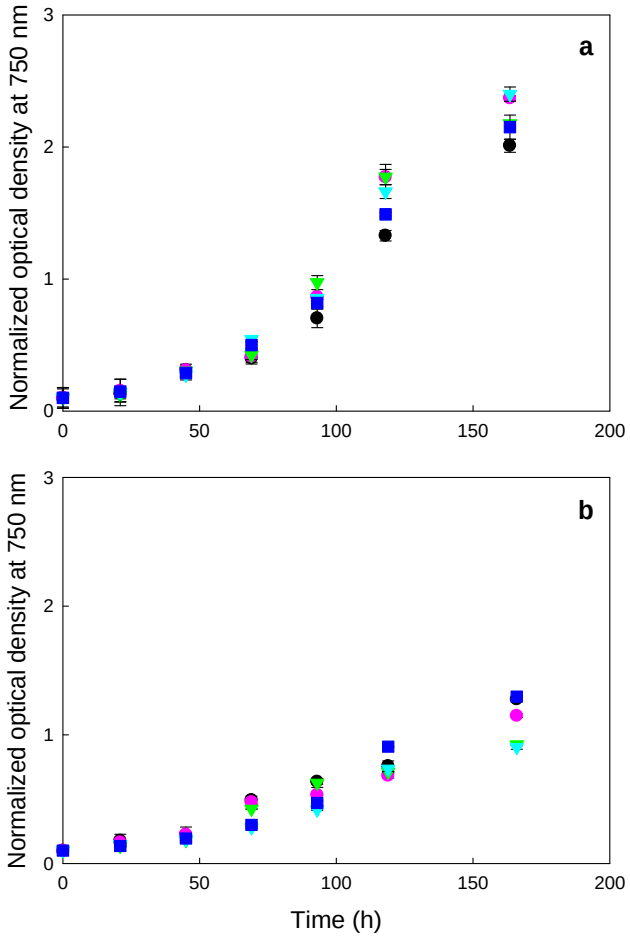


Figure 3.4. Example of cell density evolution of *B. braunii* race A (a) and *B. braunii* race B (b), measured as normalized optical density at 750nm ($Ln(N/N_0)$), with different media (M1-M4) cultivated in 24-well plates. (●) Reference medium; (●) M1; (▼) M2; (▼) M3 and (■) M4. Error bars show standard deviation of replicates.

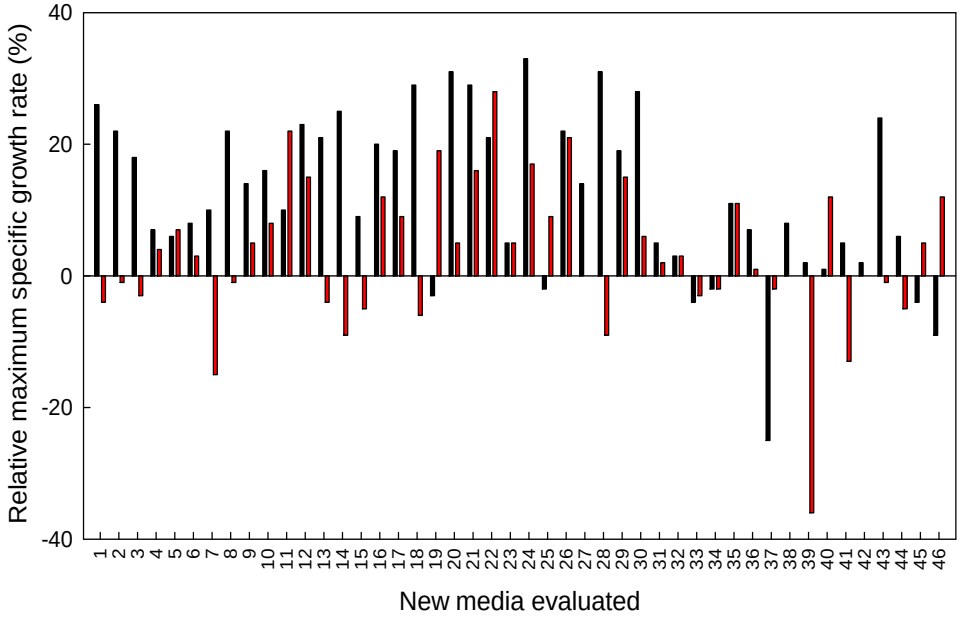


Figure 3.5. Variation of maximum specific growth rate for both *B. braunii* races, calculated as the difference between the values obtained for the 46 new media and the Reference medium. X-axis interception represents the Reference medium efficiency (zero value). Black bars represent data for *B. braunii* race A and red bars for *B. braunii* race B.

The analysis of the biomass productivity and product content at the end of the experiment, when the different cultures entered the stationary phase of growth, allowed to compare all the new media with their corresponding Reference cultures.

A statistical analysis carried out in order to assess if there was dispersion between every new culture medium and its respective Reference culture data, showed that there were significant differences (ANOVA, <0.05). Thus, Post hoc tests (LSD) were carried out, being shown the resulted data in Figures 3.6. and 3.8. The different cultures are represented in box plots and plotted in decreasing order according to the average values obtained from each one.

Figure 3.6. shows the biomass volumetric productivity data of *B. braunii* race A and race B, respectively. In order to avoid the influence of the experimental cultivation conditions, the results are grouped according to the three independent experiments in which the different culture media were evaluated. Each experiment had its own Reference cultures.

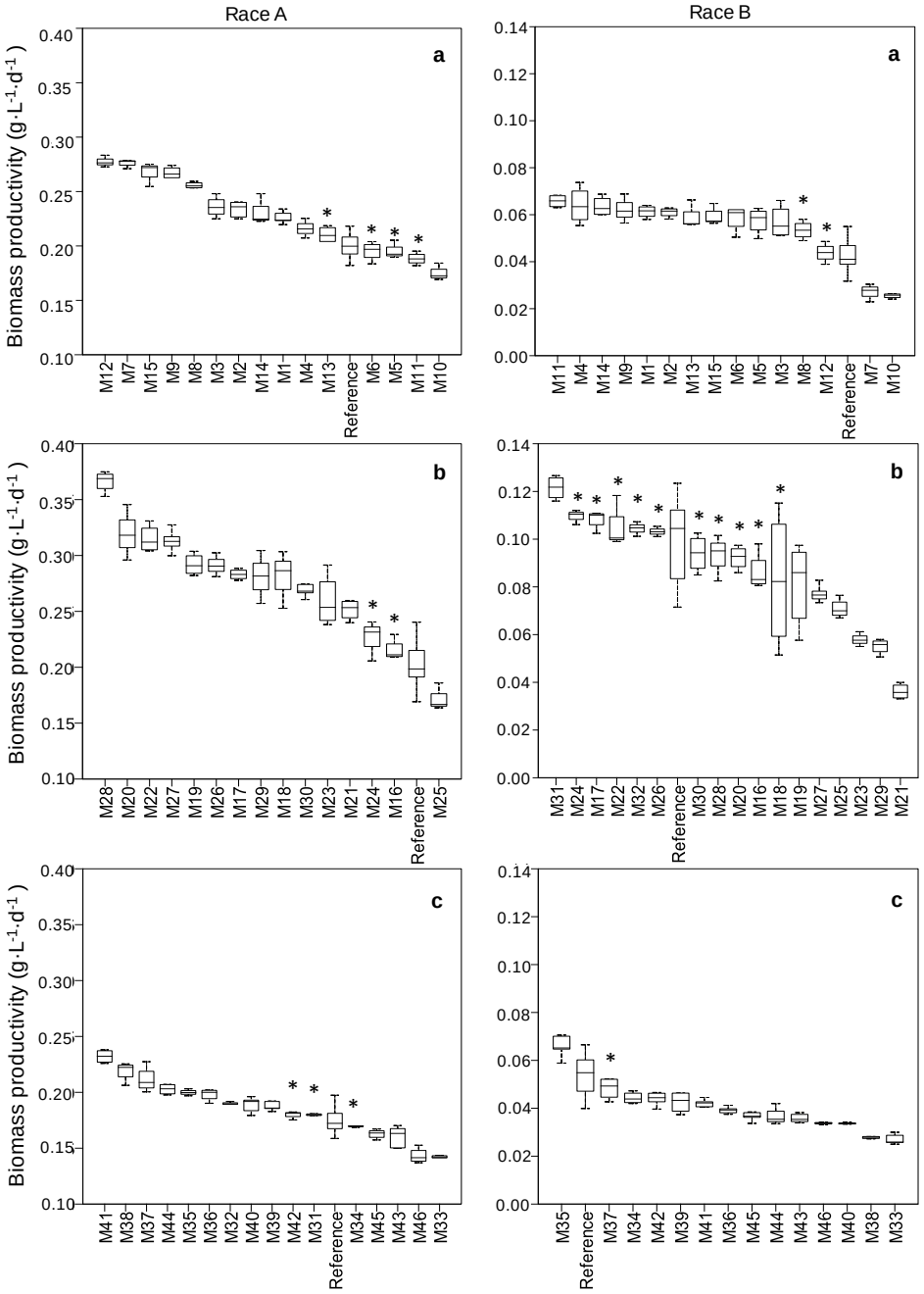


Figure 3.6. Box plots of biomass productivity data, expressed as grams of biomass produced per liter of culture broth and per day ($g \cdot L^{-1} d^{-1}$) of each culture media in experiment 1 (a), experiment 2 (b) and experiment 3 (c), for races A (left colum) and B (right colum), at the end of the experiment. (*) means non-significant differences among Reference medium (Reference) and new culture media (M). Significant differences were set at $p<0.05$ by using Fisher's Least Significant Difference (LSD) test.

Despite of the dispersion of the data for some of the new culture media, related to the difficulties to perform accurate sampling in the well plates with a colony forming microalga, a clear trend can be observed for each experiment. As it can be seen in Figure 3.6., many of the new media (78% for *B. braunii* race A and 46% for *B. braunii* race B) showed better biomass productivities compared to their corresponding Reference media, which might indicate that the higher availability of nutrients as nitrogen, but also as iron and molybdenum, which are essential in nitrogen assimilation, could partially enhance the *B. braunii* biomass productivities (Raven 1988). It is also known that nitrogen and iron are involved in photosynthetic reactions (Kolber et al. 1994; Negi and Barry et al. 2015) so, these data were in accordance with the photosynthetic efficiency data obtained for each new culture medium, in which most of them resulted in higher efficiencies (Figure 3.7.). The fact that *B. braunii* race A showed better maximum photosynthetic efficiency values for the majority of the new media respect to the Reference medium might be a sign that such race is more sensitive than the race B to the presence, in enough quantities, of nutrients involve in the photosynthetic route, as could be nitrogen or iron.

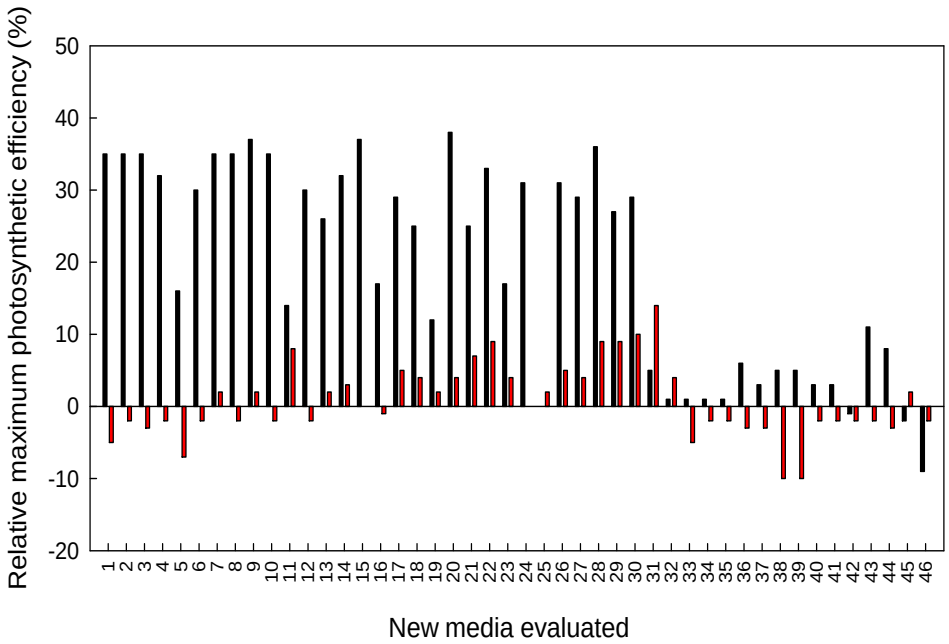


Figure 3.7. Variation of maximum photosynthetic efficiency of PSII (F_v/F_m) for both *B. braunii* races, calculated as the difference between the values obtained for the 46 new media and the Reference medium. X-axis interception represents the Reference medium efficiency (zero value). Black bars represent data for *B. braunii* race A and red bars for *B. braunii* race B.

3.2.2. Effect of new media composition on product accumulation and media pre-selection.

In order to reinforce the potential of *B. braunii* as interesting metabolite producer, the carbohydrates and hydrocarbons productivities were calculated at the end of the experiment (Figure 3.8.). Carbohydrates productivity was only improved for some of the new culture media, whereas many of them presented higher hydrocarbons productivity. However, such improvement was not very pronounced. In general, it was due to the lower cellular carbohydrates and hydrocarbons content found in most of the new media (data not shown). These results are consistent since biomass synthesis and metabolites biosynthesis compete for photosynthetic assimilation of inorganic carbon. And as biomolecules accumulation and/or excretion have been proposed as a stress response of living cells (Markou et al. 2012b; Cheng et al. 2017), the higher availability of nutrients in most of the new media might result in lower carbohydrates and hydrocarbons content, which might compromise the final productivity of these products.

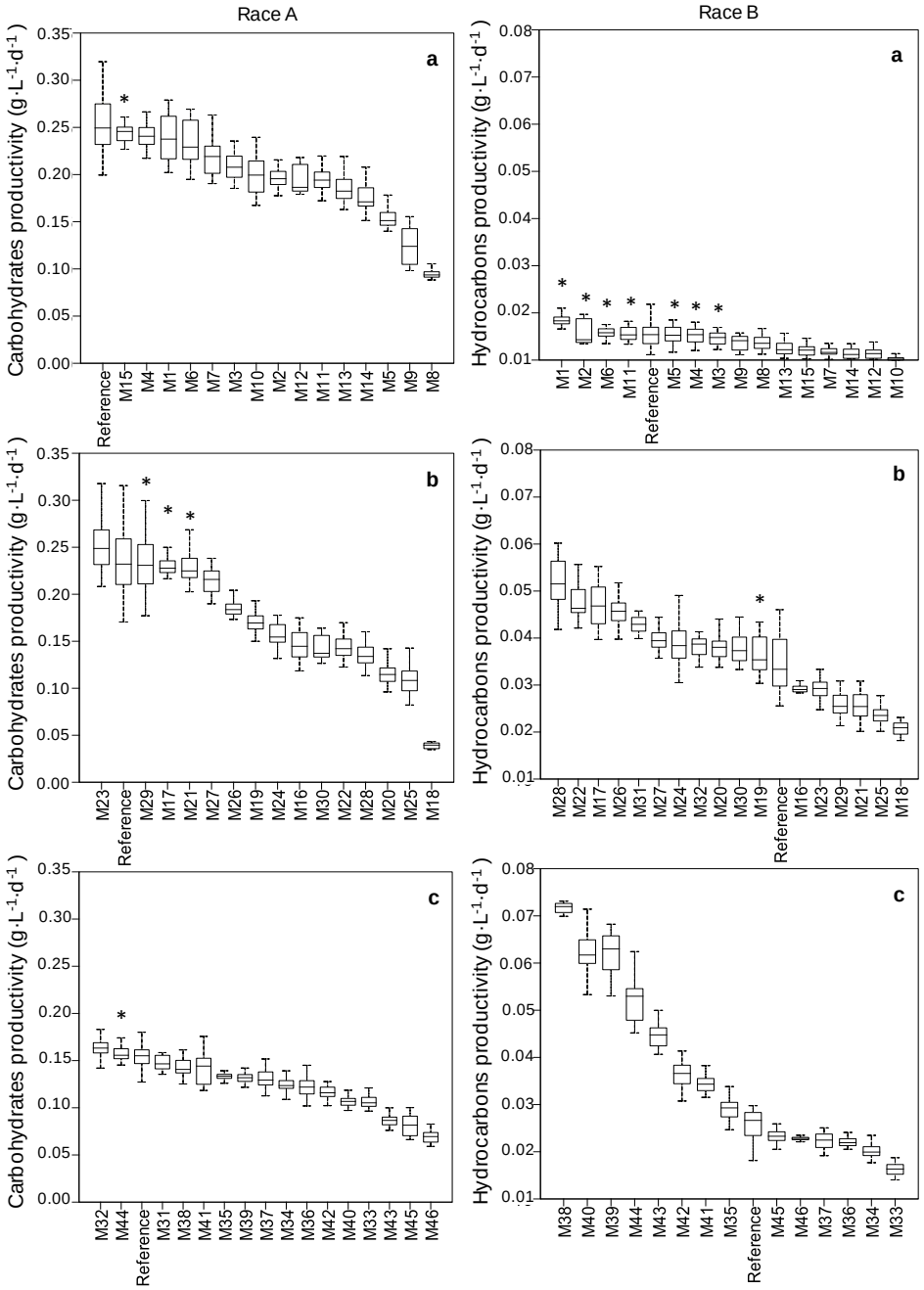


Figure 3.8. Box plots of carbohydrates and hydrocarbons productivity data, expressed as grams of biomass produced per liter of culture broth and per day ($g \cdot L^{-1} d^{-1}$) of each culture media in experiment 1 (a), experiment 2 (b) and experiment 3 (c), for races A (left colum) and B (right colum), at the end of the experiment. (*) means non-significant differences among Reference medium (Reference) and new culture media (M). Significant differences were set at $p<0.05$ by using Fisher's Least Significant Difference (LSD) test.

Since microwell-plates experiments do not allow intermediate sampling, due to culture volume limitations, it was decided to include an intermediate validation phase in Roux flasks before the final selection of media is carried out. That intermediate phase also permitted to gain insight into product accumulation during the experimental time. For that purpose, a pre-selection of potential media was done based on the product productivity, but also bearing in mind the biomass productivity and the cell viability (measured as maximum photosynthetic efficiency of PSII) obtained during the cultivation in microwell-plates (Table 3.3.). Nonetheless, the absence of selenium was also taken into account in the pre-selection.

Table 3.3. Biomass productivity (Biomass P_v), total carbohydrates productivity (CH P_v) and hydrocarbons productivity (HC P_v) of *B. braunii* race A and B respectively, expressed as grams per liter of culture broth per day, for every assayed medium at the end of the experiment in the micro-well plates. Shaded results indicate higher/similar productivities than the ones for the Reference medium.

Experiment	Culture Media	*B. braunii* race A		*B. braunii* race B	
		Biomass P_v $g \cdot L^{-1} \cdot d^{-1}$	CH P_v $g \cdot L^{-1} \cdot d^{-1}$	Biomass P_v $mg \cdot L^{-1} \cdot d^{-1}$	HC P_v $mg \cdot L^{-1} \cdot d^{-1}$
E1	**Ref. 1**	**0.200±0.010**	**0.250±0.022**	**43±6.00**	**15.23±1.93**
	M1	0.226±0.006	0.238±0.024	59±5.63	18.40±1.08
	M2	0.234±0.007	0.197±0.010	61±2.00	16.10±2.57
	M3	0.236±0.010	0.209±0.014	57±7.00	14.57±1.42
	M4	0.216±0.009	0.240±0.012	64±8.00	15.14±1.76
	M5	0.195±0.007	0.155±0.011	58±6.00	15.31±1.79
	M6	0.195±0.009	0.234±0.023	59±6.00	16.49±2.07
	M7	0.276±0.003	0.219±0.020	27±3.00	11.75±0.87
	M8	0.256±0.003	0.095±0.004	53±4.00	13.69±1.52
	M9	0.267±0.006	0.124±0.020	62±5.00	13.75±1.58
	M10	0.175±0.007	0,199±0.019	26±2.00	10.16±0.62
	M11	0.188±0.006	0.195±0.017	66±3.00	15.51±1.48
	M12	0.277±0.005	0.195±0.015	44±4.00	11.29±1.24
	M13	0.210±0.007	0.188±0.018	59±6.00	12.56±1.63
	M14	0.232±0.014	0.174±0.015	64±4.00	11.47±1.15
	M15	0.267±0.011	0.249±0.015	59±4.00	12.12±1.17
E2	**Ref. 2**	**0.202±0.020**	**0.240±0.031**	**99±16.00**	**35.58±5.79**
	M16	0.215±0.010	0.145±0.016	86±8.00	28.48±1.61
	M17	0.283±0.005	0.230±0.009	109±5.00	47.01±4.53
	M18	0.281±0.026	0.039±0.003	107±8.00	20.75±1.46
	M19	0.292±0.010	0.170±0.010	59±7.35	36.32±4.19

	M20	0.320±0.025	0.115±0.011	92±5.00	38.07±2.70
	M21	0.252±0.009	0.229±0.018	36±3.00	25.50±2.90
	M22	0.315±0.012	0.143±0.012	106±11.00	47.64±4.49
	M23	0.259±0.024	0.254±0.029	58±3.00	29.06±2.27
	M24	0.226±0.018	0.155±0.014	110±11.00	38.78±4.28
	M25	0.172±0.012	0.108±0.014	71±4.00	23.61±1.80
	M26	0.291±0.011	0.189±0.013	103±6.33	45.77±2.94
	M27	0.313±0.010	0.214±0.013	77±4.00	39.66±2.18
	M28	0.366±0.010	0.136±0.011	93±10.00	51.79±5.89
	M29	0.281±0.024	0.233±0.031	55±3.00	25.89±2.54
	M30	0.269±0.006	0.143±0.013	94±8.00	37.83±3.39
	M31	---	---	122±5.00	42.89±4,39
	M32	---	---	104±3.00	38.15±2.30
E3	**Ref. 3**	**0.174±0.010**	**0.147±0.013**	**54±8.00**	**25.55±3.72**
	M31	0.180±0.001	0.146±0.008	---	**---**
	M32	0.190±0.007	0.163±0.009	---	**---**
	M33	0.145±0.006	0.107±0.007	27±2.15	16.28±1.26
	M34	0.170±0.007	0.124±0.007	44±2.35	20.18±1.34
	M35	0.203±0.008	0.135±0.006	66±5.00	29.12±2.28
	M36	0.201±0.008	0.122±0.009	39±1.47	22.20±1.14
	M37	0.211±0.011	0.132±0.012	48±5.02	22.28±1.74
	M38	0.219±0.009	0.143±0.009	28±0.50	69.48±4.65
	M39	0.188±0.004	0.132±0.005	43±5.00	61.58±4.83
	M40	0.189±0.007	0.107±0.005	34±3.00	62.92±5.06
	M41	0.232±0.006	0.142±0.017	42±2.00	34.38±1.70
	M42	0.180±0.003	0.117±0.007	44±2.86	36.45±2.80
	M43	0.160±0.010	0.086±0.006	36±2.00	44.63±2.61
	M44	0.206±0.009	0.157±0.007	37±4.00	52.06±4.58
	M45	0.163±0.004	0.081±0.011	37±1.90	23.24±1.38
	M46	0.143±0.007	0.069±0.006	34±0.50	22.23±1.25

For *B. braunii* race A the best media that showed either higher or non-significant difference in carbohydrates productivity compared to the Reference medium from each well-plates experiment were:

- E1: M15
- E2: M23, M29, M17, M21
- E3: M32, M44

From these 7 media, 3 were selected to be further validated in a new independent experiment in Roux flasks. The final selection was M15, M21 and M44. M15 was selected as the best media from experiment 1 although it contained selenium. From experiment 2, it was decided to select M21 because it did not contain selenium. Finally, from experiment 3, M32 did not contain selenium. However, M44 was selected due to the fact that M32 only presented a slightly higher carbohydrates productivity (3.7% higher) than M44, whereas M44 showed 8-fold higher photosynthetic efficiency (Figure 3.7.) and better biomass productivity (7.8% higher) compared to the Reference medium. As this study also had the purpose of increasing growth, it was decided to select M44 to see if the higher availability of nutrients could result in higher productivities, which might be preferred in a cultivation strategy of 2-phases: growth and stress.

For *B. braunii* race B, many of the new media resulted in improved hydrocarbons productivities and, therefore, the best 4 media from each well-plates experiment were selected:

- E1: M1, M2, M6, M11

- E2: M28, M22, M17, M26

- E3: M38, M40, M39, M44

From all these media, 4 were selected to be further validated in a new independent experiment in Roux flasks. The final selection was M11, M22, M28 and M35. From the experiment 1, M11 was selected due to the lack of Se and its higher biomass productivity respect to the other three media as well as respect to the Reference one. From experiment 2, M28 and M22 were selected as the best two media. M28 due to the absence of selenium, and M22 was selected in order to assess the behaviour of this strain when it was grown in a medium with an intermediate amount of such compound (25 µM). Finally, M39, from experiment 3, did not contain Se, however it was decided to select M35 due to the fact that it was the only medium in this experiment that presented both, higher biomass and hydrocarbons productivity respect to the Reference medium and, besides, it did not contain Se.

In general, for both strains, the highest productivities were mainly related to the presence of higher concentration of nutrients in the new media, which seems

to indicate a certain degree of nutrients limitation in the Reference medium (Chu 13, Gouveia et al. 2017), as it has already been reported for similar Chu media (Shivandappa et al. 2016). Moreover, most of the new media resulted in higher photosynthetic activities for both strains (Figure 3.7.). All of this can be explained by the fact that when nutrients like nitrogen or iron are not limited, a higher synthesis of photosynthetic pigments in the cells occurs. It leads to an increased rate of photosynthesis which focuses mainly on biomass production (Geider and La Roche 1994; Díaz Bayona and Atehortúa Garcés 2014).

3.3. Validation of pre-selected media in Roux flasks.

Validation of pre-selected media was decided to be carried out in Roux flasks because, compared to the well-plates, they have a larger volume (800 mL), and, compared to normal Erlenmeyer flasks, their flat shape allows a better light penetration in the culture.

Performance of pre-selected media was compared to Reference medium (Chu 13, Gouveia et al. 2017) in terms of growth and product accumulation. Roux flasks were placed inside a culture room at 25 ºC illuminated with 100 µmol photons·m^{-2}·s^{-1} and aerated with 2.5%$_v$ CO_2-enriched air. Prior to the final validation experiment, biomass was acclimated to the different media and the cultivation conditions for 3 weeks (until stationary phase was reached). After that period, biomass was partially harvested and re-inoculated in duplicate with fresh media at an initial optical density of 0.1 (OD at 750 nm). Daily analysis included optical density, pH and photosynthetic efficiency (measured as maximum efficiency of Photosystem II). Dry weight and product content was punctually analysed along the growth curve, and, according to it, the product productivities were calculated. It allowed getting insight into product formation along the cultivation cycle.

3.3.1. Validation of pre-selected media in Roux flasks for *B. braunii* race A.

Adaptation period to the new media (M15, M21 and M44) allowed to get, in the final experiment, a short lag phase of around 50 hours after which linear growth was observed until 240 hours of cultivation (Figure 3.9.). At that time

growth started to slow down and stationary phase was reached for all the media except for M44. Even after two weeks of cultivation the stationary phase was not reached for M44, resulting in the highest biomass concentration (3.8 $g \cdot L^{-1}$). On the other hand, M15 showed a different pattern compared to the rest of the cultures, which resulted in the lowest final biomass concentration (1.6 $g \cdot L^{-1}$), being similar to the biomass content obtained in other studies with cultures media used for *B. braunii* (Díaz Bayona and Artehortúa Garcés 2014).

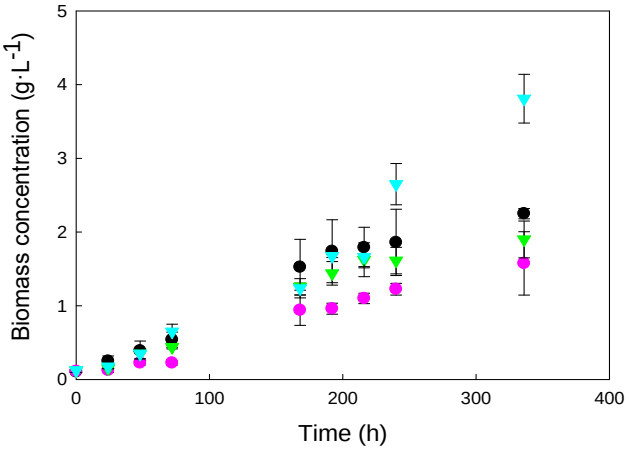


Figure 3.9. Biomass concentration evolution of *B. braunii* race A, expressed as biomass dry weight in grams per liter, during the cultivation in the different media in Roux flasks. (●) Reference medium; (●) M15; (▼) M21; (▼) M44. Error bars show standard deviation of replicates.

Visual observation of cultures revealed differences in color (Figure 3.10.). M44 started to show a more intense green colour after 72 hours of cultivation. An indirect indicator of the cellular chlorophyll content is the ratio between the optical densities at 680 nm and 750 nm. M44 showed an increasing ratio almost until the end of the experiment (Figure 3.11a) which could explain the difference in color observed.

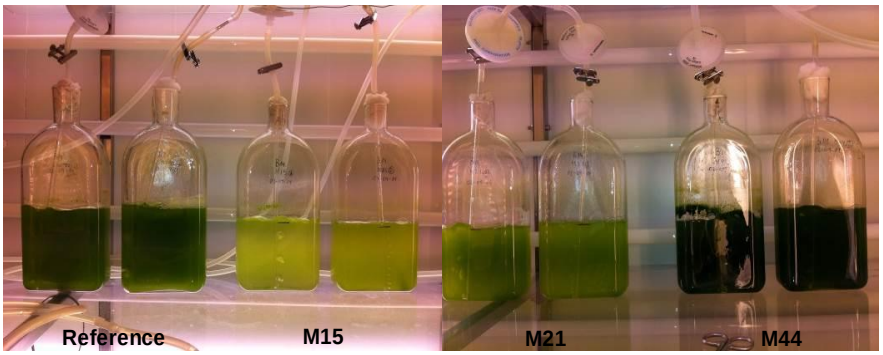


Figure 3.10. Picture of different cultures of *B. braunii* race A cultivated in Roux flasks at the end of the experiment. From the left to the right (cultures are in duplicate): Reference medium, M15, M21 and M44.

Moreover, the higher chlorophyll content also implies a certain degree of photo-limitation and, until a certain extent, a better photosynthetic efficiency. This was confirmed by the analysis of maximum quantum yield or maximum photosynthetic activity of Photosystem II, which was also higher in M44 until the end of the experiment (Figure 3.11b).

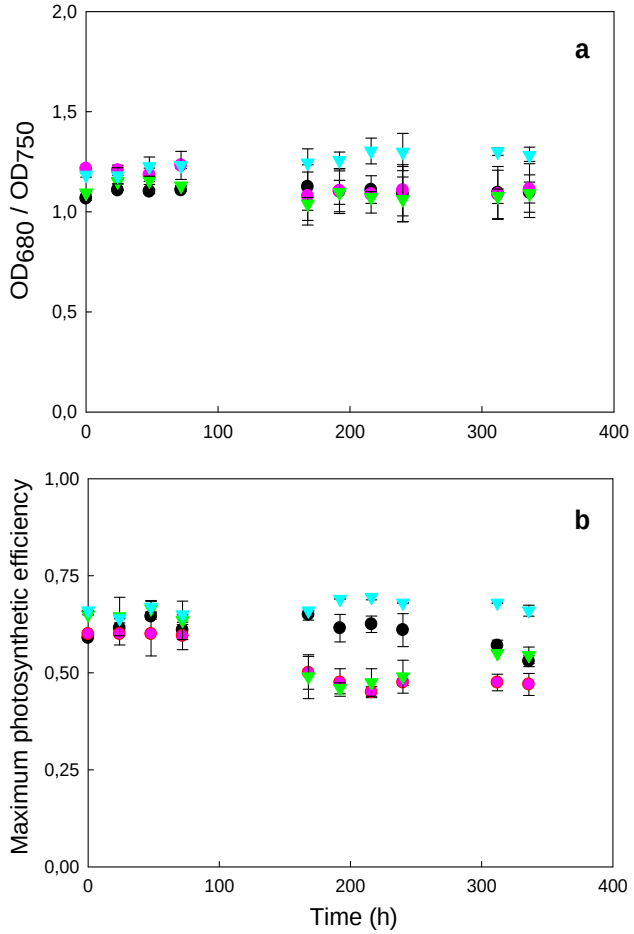


Figure 3.11. Relative cellular chlorophyll content (a) expressed as the ratio between optical densities at 680 nm and 750 nm, and maximum photosynthetic efficiency of PSII (F_v/F_m) (b), during the cultivation in the different media in Roux flasks. (●) Reference medium; (●) M15; (▼) M21; (▼) M44. Error bars show standard deviation of replicates.

Thus, in terms of photosynthetic efficiency M44 was also a promising media. On the other hand, M15 showed the palest colour. Moreover, the culture broth in M21 as well as in M44 was very viscous since the beginning of the cultivation. Such viscosity might be a sign of the accumulation of carbohydrates in those two cultures since it has been confirmed that the viscosity of cultures correlated well with their total sugar, and thus exopolysaccharides (EPS) content (Mancuso Nichols et al. 2009).

3.3.1.1. Carbohydrates accumulation in *B. braunii* race A cultivated in different media in Roux flasks.

Carbohydrates content was punctually analysed during the entire cultivation and it was expressed as milligrams per liter of culture broth (Figure 3.12a) and as milligrams per gram of biomass (Figure 3.12b). The high initial intracellular carbohydrates content (time zero) was related to the previous adaptation experiment carried out during 3 weeks. After that adaptation period, biomass was harvested and inoculated in fresh media. Biomass was suddenly transferred to nutrients-replete media and more light was available. Therefore their metabolism got adapted and carbohydrates content started to decrease. As a future recommendation, it could be interesting to assess the effect of a longer cultivation time and to evaluate differences between the effect of first exposure to media and adapted biomass on final total carbohydrates content.

Regarding the carbohydrates accumulation per volume of culture broth, it can be observed in Figure 3.12a that once cultures entered the stationary phase, after 240 hours, the carbohydrates content remained more or less constant until the end of the experiment, with the exception of M44 which showed 1.4-fold higher final carbohydrates content compared to Reference medium.

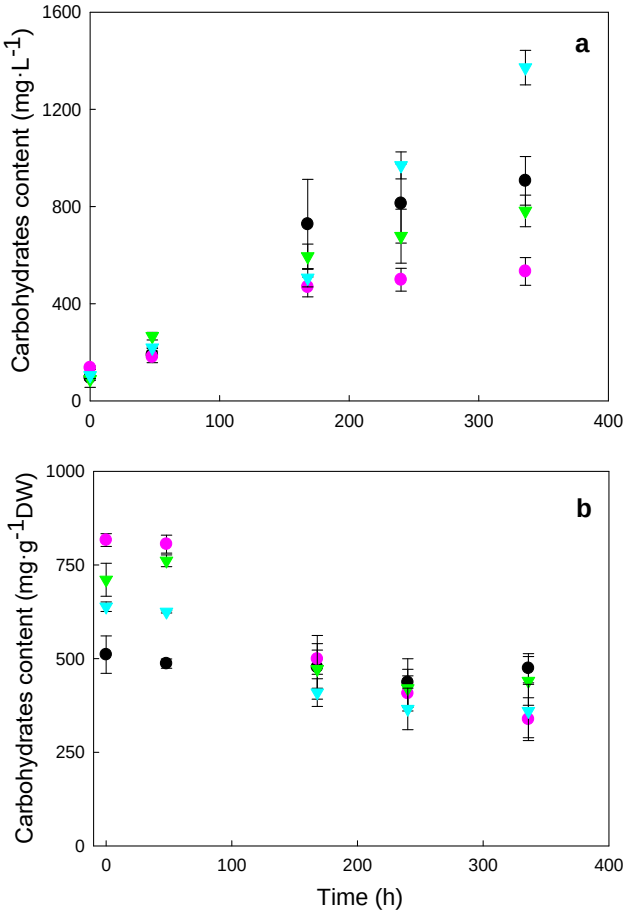


Figure 3.12. Total carbohydrates content of *B. braunii* race A, expressed as (a) milligrams per liter of culture broth and (b) milligrams per gram of dry weight, during the cultivation in the different media in Roux flasks. (●) Reference medium; (●) M15; (▼) M21; (▼) M44. Error bars show standard deviation of replicates.

The lower total carbohydrates content observed for the rest of the media, compared to the Reference, was related to the lack of over-expression/activation of carbohydrates accumulating mechanisms during the experimental time.

Calculation of biomass and carbohydrates productivities was done based on dry weight data and total carbohydrates content per volume of culture broth. As it can be seen in Table 3.4., M44 was the only medium that resulted in higher biomass and carbohydrates productivities compared to the Reference medium.

Table 3.4. Biomass and total carbohydrates productivity of *B. braunii* race A, expressed as grams per liter of culture broth per day, after 240 hours of cultivation.

Culture media	Biomass P_V ($g \cdot L^{-1} \cdot d^{-1}$)	Carbohydrates P_V ($g \cdot L^{-1} \cdot d^{-1}$)
Reference	**0.233± 0.014**	**0.176± 0.022**
M15	0.100± 0.006	0.050± 0.004
M21	0.149± 0.018	0.101± 0.017
M44	0.253± 0.027	0.245± 0.027

According to the obtained results, M44 seems to be a promising media for *B. Braunii* race A cultivation. However, one of the objectives of media optimization was to avoid the presence of selenium in the media, and M44 only reduced the Se content to 25 µM. In this sense, Media 21 should be considered as an interesting media due to the absence of Se, although the resulting productivities were lower than the Reference media. For that reason, it was decided to perform a second validation with M21 and M44. In that second validation selenium was removed from M44 and nitrogen content of M21 was increased to see if productivities could be further increased.

3.3.1.2. Modification of pre-selected media in Roux flasks for *B. braunii* race A.

As it was already commented in the previous section (3.3.1.1.), in order to further increase productivities, it was decided to perform another experiment in which selenium was removed from M44 (M44m) and nitrogen content of M21 was increased (M21m) (Table 3.5.).

Table 3.5. Modified composition of the best 2 media for *B. Braunii* race A.

Culture media	N (mM)	P (mM)	Se (µM)	Fe (µM)	Mo (µM)
Reference	4	0.60	50	55	0.25
M21m	(4) → **22**	3.30	0	40	1
M44m	22	0.60	(25) → **0**	40	1

Figure 3.13. shows biomass evolution with the modified media. Biomass was pre-adapted to the new media during 2 weeks and cultivation was done in duplicate as in previous experiment. As it can be seen, lag phase was hardly present and linear growth phase was maintained until around 240 hours of cultivation (same trend as observed before). M44m showed, again, the highest biomass concentration at the end of the cultivation time (3.7 $g \cdot L^{-1}$) and no stationary phase seemed to be reached until then.

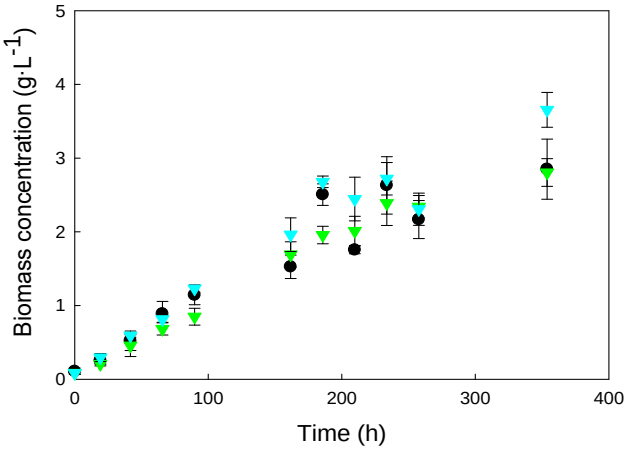


Figure 3.13. Evolution of biomass concentration, of *B. braunii* race A, expressed as biomass dry weight in grams per liter, during the cultivation in the different modified media in Roux flasks. (●) Reference medium; (▼) M21m; (▼) M44m. Error bars show standard deviation of replicates.

Visual observation also revealed a darker green colour in M44m at the end of the cultivation (Figure 3.14.). Differences observed after inoculation (Figure 3.14a) are explained by the adaptation phase in which biomass was cultivated in the selected media for 2 weeks.

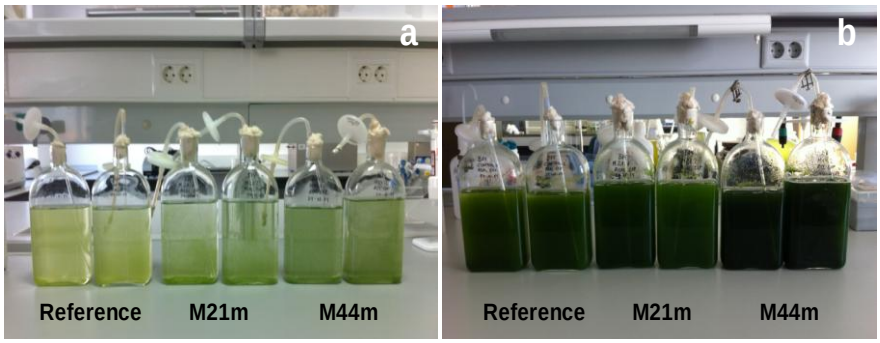


Figure 3.14. Picture of different cultures of *B. braunii* race A cultivated in Roux flasks at the beginning (a) and at the end of the experiment (b). From the left to the right (cultures are in duplicate): Reference medium, M21m and M44m.

Compared to the previous experiment, the final biomass concentration in M44m was slightly lower which could be related to the absence of selenium in the modified media. In this sense, Se could play a role in biomass growth as already mentioned, although the effect was almost despicable. Biomass concentration at the end of the experiment for Reference medium was 2.85 $g \cdot L^{-1}$, slightly higher than the biomass observed previously, which could be explained by the larger experimental time. On the other hand, the increase in nitrogen content of M21m hardly affected growth during the experimental time and results were comparable to Reference medium, as in previous experiment.

Carbohydrates content is shown in Figure 3.15. The high initial intracellular carbohydrates content (time zero) (Figure 3.15b), as commented before, was related to the previous adaptation phase carried out. As soon as biomass was exposed to nutrient-replete conditions, after inoculation, the carbohydrates content started to decrease. Maximal intracellular carbohydrates content was found around 250 hours of cultivation, when the cultures entered the stationary phase, which is in accordance to previous studies in which it has been reported that carbohydrates concentration starts to increase at that moment (Moreno et al. 1998; Banerjee et al. 2002).

At that point, M44m showed the highest content expressed as percentage of dry biomass (56%). After that point carbohydrates content started to decrease and final values were similar for the different media.

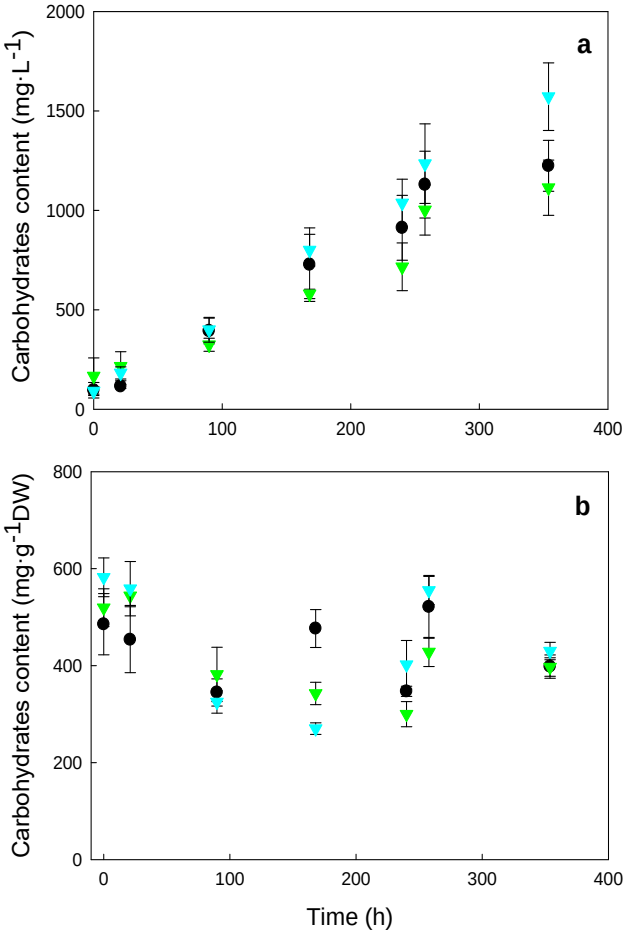


Figure 3.15. Total carbohydrates content of *B. braunii* race A, expressed as (a) milligrams per liter of culture broth and (b) milligrams per gram of dry weight, during the cultivation in the different media in Roux flasks. (●) Reference medium; (▼) M21m; (▼) M44m. Error bars show standard deviation of replicates.

In order to evaluate total carbohydrates productivity, it was calculated for each sampling point. Accumulated productivity values, calculated from the beginning of the experiment, are shown in Figure 3.16. As it can be seen, maximal carbohydrates productivity value was reached after 240 hours of cultivation, which concurs with the highest cellular carbohydrates content.

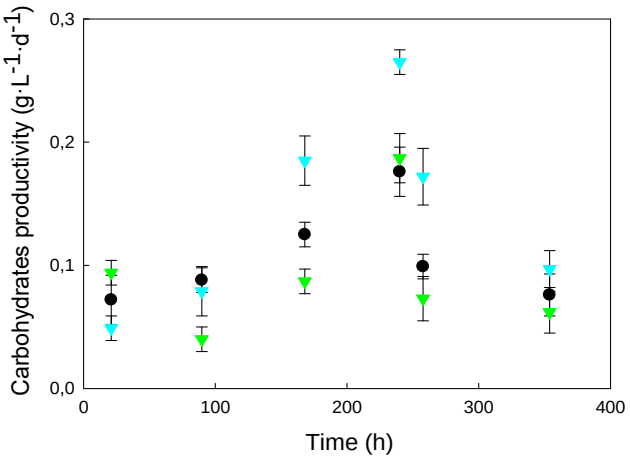


Figure 3.16. Accumulated carbohydrates productivity of *B. braunii* race A, calculated since the beginning of the experiment and expressed as grams of total carbohydrates per liter of culture broth per day, during the cultivation in the different modified media in Roux flasks. (●) Reference medium; (▼) M21m; (▼) M44m. Error bars show standard deviation of replicates.

It has been proposed that a solution to facilitate the production of high-value products of microalgae is to reuse the cell mass for continuous production (Hejazi and Wijffels, 2004), which is known as “milking” process. Considering the above, extracellular carbohydrates (EPS) are interesting from the point of view of milking and, therefore, EPS content in culture broth was also analyzed. For that purpose, biomass was removed from culture broth by centrifugation and supernatant was freeze dryed (Figure 3.17.). A normal carbohydrates analysis was performed in that fraction.

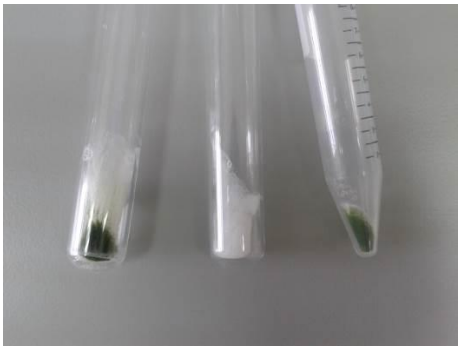


Figure 3.17. Picture of different fractions of *B. braunii* race A after freeze drying during 24 hours. From left to the right: Total culture broth, supernatant (after centrifugation) and biomass pellet (after centrifugation).

As it can be seen in Figure 3.18., M44m showed the highest EPS productivity and Reference medium and M21m resulted in similar values at the end of the cultivation. Considering the point of interest (240 hours), the productivity was also higher in M44m. It confirmed the potential of that medium for production of biomass and carbohydrates (total and EPS) in *B. braunii* race A.

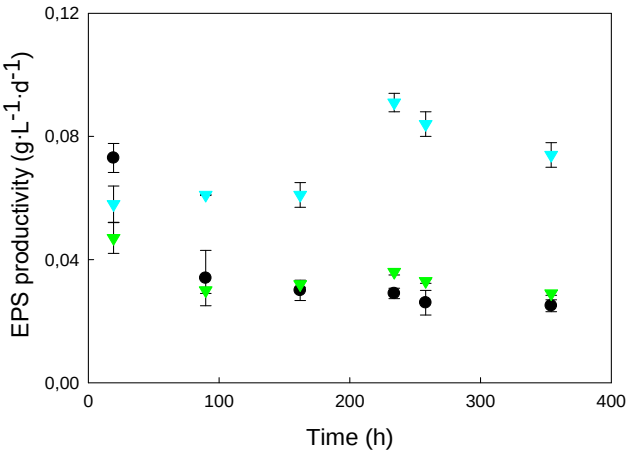


Figure 3.18. Accumulated (exo)polysaccharides productivity of *B. braunii* race A, calculated since the beginning of the experiment and expressed as grams per liter of culture broth per day, during the cultivation in the different modified media in Roux flasks. (●) Reference medium; (▼) M21m; (▼) M44m. Error bars show standard deviation of replicates.

Since medium-large scale cultivation requires to optimize productivity, selection of final media was done according to the values found at that moment (240 h) (Table 3.6.). M44m resulted in higher productivity of carbohydrates (total and EPS), as well as of biomass, compared to the Reference medium. Besides, it can be also observed that the cultivation in Roux flasks, where the CO_2 availability is higher, improved the productivities (biomass and carbohydrates) obtained with such medium respect to the experiment in the well-plates. All of this justified the selection of M44m for *B. braunii* race A.

Table 3.6. Biomass, total carbohydrates and (exo) polysaccharides volumetric productivities (P_V), of *B. braunii* race A, expressed as grams per liter of culture broth per day, after 240 hours of cultivation.

Culture media	Biomass P_V ($g \cdot L^{-1} \cdot d^{-1}$)	Carbohydrates P_V ($g \cdot L^{-1} \cdot d^{-1}$)	EPS P_V ($g \cdot L^{-1} \cdot d^{-1}$)
Reference	**0.233±0.014**	**0.176±0.022**	**0.029±0.004**
M21m	0.245±0.008	0.187±0.019	0.036±0.002
M44m	0.264±0.018	0.265±0.048	0.091±0.003

3.3.2. Validation of pre-selected media in Roux flasks for *B. braunii* race B.

As in the case of *B. braunii* race A, a pre-adaption phase was also carried out for *B. braunii* race B, after which biomass was harvested and inoculated in nutrient replete media. Culture showed a short lag phase of 50 hours followed by a linear growth phase which lasted until 330 hours of cultivation (Figure 3.19.). Similar growth pattern was found for all the media assayed. However, M22 showed a slightly higher biomass concentration at the end of the cultivation (2.73 $g \cdot L^{-1}$), possibly ought to the combination of both, its higher amount of iron and the presence of selenium.

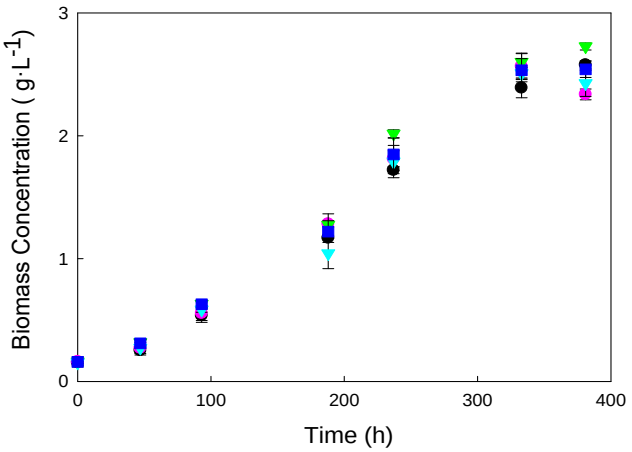


Figure 5.19. Biomass concentration evolution of *B. braunii* race B, expressed as biomass dry weight in grams per liter, during the cultivation in the different media in Roux flasks. (●) Reference medium; (●) M11; (▼) M22; (▼) M28 and (■) M35. Error bars show standard deviation of replicates.

Although differences in terms of biomass concentration were not important, visual observation of cultures revealed differences (Figure 3.20.). At the end of the experiment Reference medium showed a pale green colour while the rest of the media showed a darker green colour.

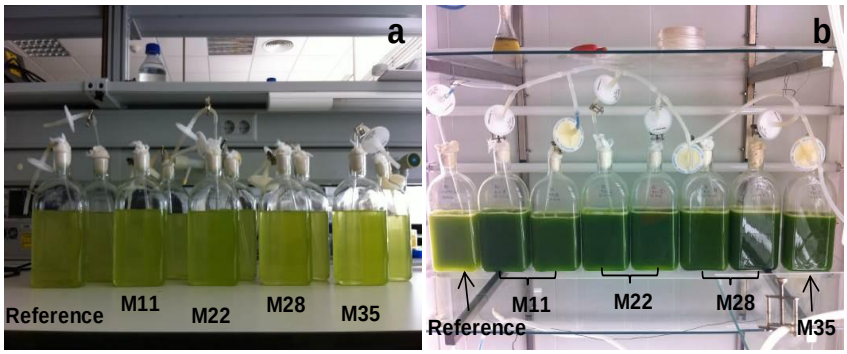


Figure 3.20. Picture from different cultures of *B. braunii* race B in Roux flasks at the beginning (a) and at the end of the experiment (b). From the left to the right: Reference medium, M11, M22, M28 and M35 (cultures are shown in duplicate).

As it was already observed in the other *Botryococcus* race, higher chlorophyll content was expected in the greener cultures, which was confirmed by the optical density ratio 680nm/750nm that was lower for the Reference medium (Figure 3.21a). In terms of photosynthetic efficiency, all the cultures showed similar values (Figure 3.21b).

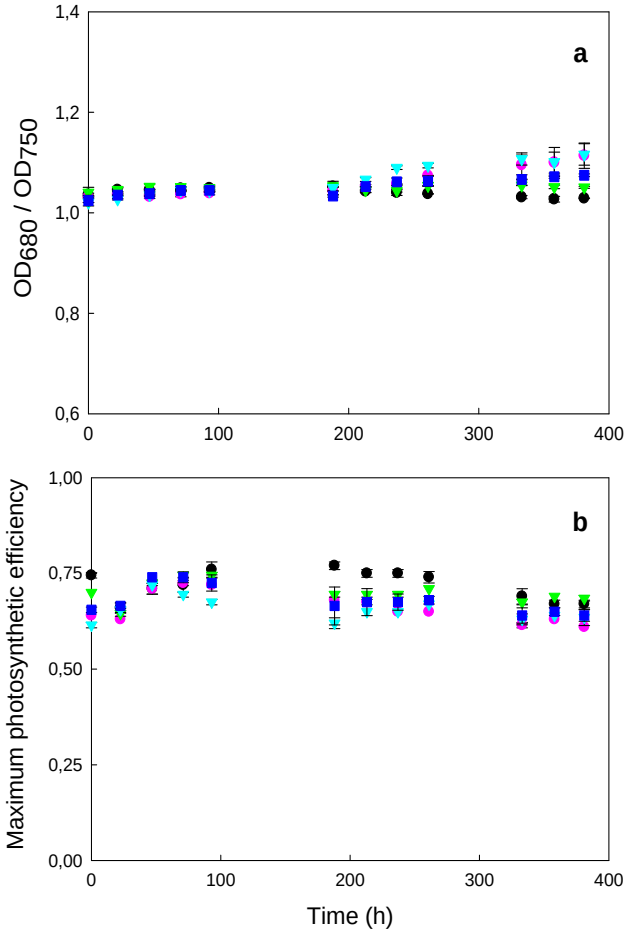


Figure 3.21. Relative celular chlorophyll content (a) expressed as the ratio between optical densities at 680 nm and 750 nm and maximum photosynthetic efficiency of PSII (F_v/F_m) (b), during the cultivation in the different media in Roux flasks. (●) Reference medium; (●) M11; (▼) M22; (▼) M28; (■) M35. Error bars show standard deviation of replicates.

3.3.2.1. Hydrocarbons accumulation in *B. braunii* race B cultivated in different media in Roux flasks.

Hydrocarbons content was punctually analysed during the entire cultivation in total broth samples (Figure 3.22.). The high initial intracellular hydrocarbons content (time zero) observed in Figure 3.22b was related to the previous adaptation experiment carried out. At that moment biomass was exposed for the first time to the different media and hydrocarbons accumulation took place. After that period, real experiment started and biomass was harvested and inoculated in fresh media again, where nutrients and light were fully available. It implied the hydrocarbons content to decrease as the cultivation conditions were considered as optimal again.

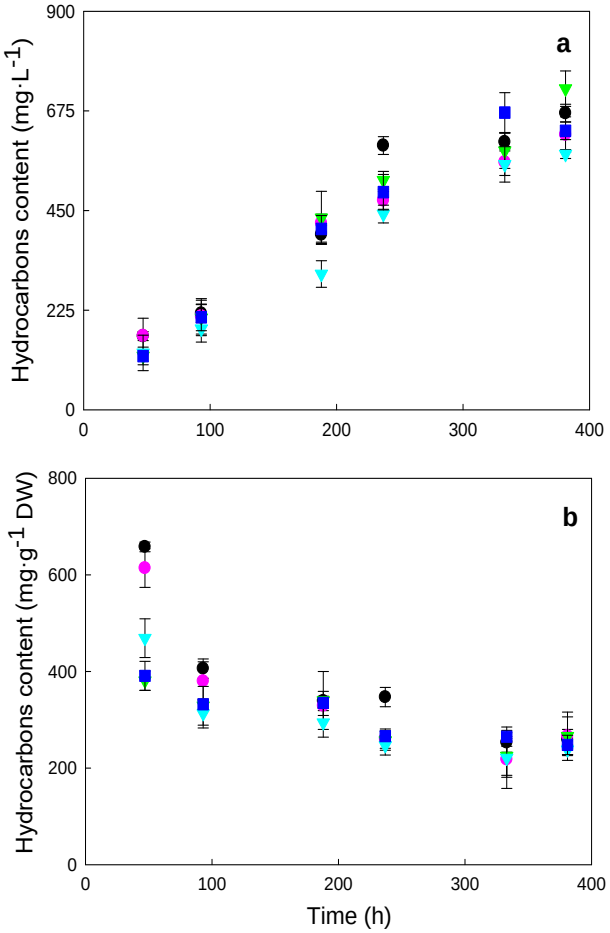


Figure 3.22. Hydrocarbons content of *B. braunii* race B, expressed as (a) milligrams per liter of culture broth and (b) milligrams per gram of dry weight, during the cultivation in the different media in Roux flasks. (●) Reference medium; (●) M11; (▼) M22; (▼) M28; (■) M35. Error bars show standard deviation of replicates.

No important differences were observed between the cultures, although a slightly higher hydrocarbons content was found in M35 after 330 hours of cultivation, while the rest of the media showed similar values (around 30% of dry weight).

On the other hand, calculation of biomass and hydrocarbons productivities was done based on dry weight data and hydrocarbons content (Table 3.6.).

Table 3.6. Biomass and hydrocarbons volumetric productivities (P_V) of *B. braunii* race B, expressed as grams per liter of culture broth per day.

Culture media	Biomass P_V ($g \cdot L^{-1} \cdot d^{-1}$)	Hydrocarbons P_V ($g \cdot L^{-1} \cdot d^{-1}$)
Reference	**0.158±0.002**	**0.055±0.005**
M11	0.167±0.003	0.042±0.003
M22	0.189±0.003	0.048±0.001
M28	0.165±0.010	0.041±0.002
M35	0.162±0.010	0.043±0.003

M22 resulted in the highest biomass productivity but it contains selenium (25 µM) (Table 3.1.). It could be an indicator that Se plays a role in biomass accumulation in this race. On the other hand, regarding the hydrocarbons, it was observed that none of the pre-selected media for this microalga resulted in better productivities compared to the Reference medium. Therefore, considering that M22 contained selenium it was decided to select M11 as the final media for the hydrocarbons race.

3.4. Benefits of culture media optimization in microalgae cultivation.

The advantages of optimizing microalgal culture media are based on the fact that selecting a suitable culture medium can enhance the quality and quantity of biomass produced, and therefore it has to be considered as one of the most important tasks in industrial production (Monserrat et al. 1993; Gong and Chen 1997).

Summarising the main results obtained in this Chapter of the Thesis, it could be concluded that:

1. Literature review allowed comparison of *B. braunii* Reference medium (Chu 13, Gouveia et al. 2017) with the calculated average nutrients concentration for the identified media. Together with the biological importance of each element, it was allowed the identification of macro- and micronutrients to be optimized. Such elements were nitrogen, phosphorus, iron, molybdenum and, considering its toxicity, selenium.

2. Most of the new media resulted in better biomass productivities than the obtained in the Reference medium when they were evaluated in microwell-plates, which seems to indicate that there was a certain nutrient limitation in the Reference medium. However, carbohydrates and hydrocarbons productivity were only improved for some of the new culture media. The improvement in biomass production and photosynthetic productivity would be desirable in a cultivation strategy of 2-phases, in which biomass is first obtained and then stressed in order to improve the production of metabolites of interest. Thus, two different media could be used to carry out each phase: first a nutrient replete medium which enhances growth and photosynthetic efficiency, and then a medium in which certain degree of nutrients limitation is present to enhance product accumulation in these two races.

3. According to the biomass and product productivities, as well as considering avoiding the presence of selenium, the best media for each race were pre-selected to be further validated. M15, M21 and M44 for *B. braunii* race A, and M11, M22, M28 and M35 for race B.

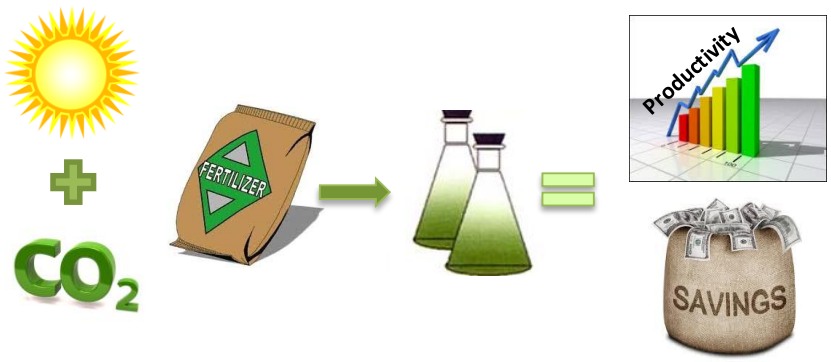
4. Validation in Roux flasks revealed that for *B. braunii* race A the best productivities were reached with M44. Considering that it contained selenium, it was decided to carry out another experiment where it was removed from the medium (M44m). That second experiment demonstrated that the lack of selenium does not affect the performance of *B. braunii* race A since biomass and carbohydrates productivities were even a slightly higher than previously. Therefore M44m was selected as the final medium for *B. braunii* race A.

5. Validation in Roux flasks revealed that for *B. braunii* race B similar biomass and hydrocarbons productivities were achieved. However, considering the need to remove selenium M11 was selected as the final medium for *B. braunii* race B.

Once the optimized media, in terms of biomass and product productivities, were selected for both *B. braunii* races (A and B), the next targets to be achieved were to ease the preparation of the culture media and to reduce production costs as much as possible. For that, it was decided to grow these microalgae in fertilizer-based media, which is the issue of the next Chapter of the Thesis.

Chapter 4

COST REDUCTION AND PRODUCTIVITY ENHANCEMET IN THE CULTIVATION OF *Botryococcus braunii* RACES A AND B USING COMMERCIAL FERTILIZERS



The work presented in this Chapter will be submitted as:
Bermejo, E., Montero, Z., González, C., Cuaresma, M. Cost reduction and productivity enhancement in the cultivation of *Botryococcus braunii* strains using commercial fertilizers.

4. COST REDUCTION AND PRODUCTIVITY ENHANCEMENT IN THE CULTIVATION OF *Botryococcus braunii* RACES A AND B USING COMMERCIAL FERTILIZERS.

In Chapter 4 of this Doctoral Thesis, several experiments were carried out with the aim of further improving the productivities, with respect to the selected media in Chapter 3, and, at the same time, of reducing costs in the microalgae cultivation process.

As it has been mentioned previously, the culture medium composition in microalgae cultivation significantly affects the production of biomass and the total productivity of biochemical compounds. However, the economic viability in the production of compounds of interest from microalgae highly depends on reducing costs during the cultivation, being the source of nutrients one of the main limitations in the cultivation at large scale (Nayak et al. 2016).

During the last decades, as mentioned in the Introduction Chapter, several studies were undertaken for cultivation of different microalgal species by replacing the chemicals compounds by agricultural fertilizers as nutrients, which is cheaper and simpler to prepare (Fábregas et al. 1987; Valenzuela-Espinoza et al.1999; Kanlis et al. 2004; Silva-Benavides 2016).

In that sense, with the aim of reducing costs during the cultivation process, or even increasing biomass and product productivities, it was proposed to cultivate both *B. braunii* races (A and B) in culture media based on agricultural fertilizers (NPKs). For that, as a first approach, these microalgae were cultivated with different nitrogen sources in order to identify the preferred nitrogen source and, therefore, to know which fertilizers could be more suitable to cultivate them. Subsequently, the suitability of several fertilizers containing the preferred nitrogen source was assessed. Finally, the fertilizers resulting in the highest productivities for both *B. braunii* races, were decided to be further optimized and nitrogen concentrations above and below the Reference one were tested to check if productivities could be enhanced.

4.1. Evaluation of different nitrogen sources for the cultivation of *B. braunii*.

Evaluation of different nitrogen sources was carried out in order to identify the microalgae preferred source from those present in most of the fertilizers (nitrate, ammonium or urea) with the aim of selecting the most suitable NPKs. Considering all *B. braunii* strains might present a similar behavior in terms of nutrients uptake and growth (Gouveia et al. 2017), the experiment was carried out with *B. braunii* race A. For that purpose, the optimal growth media of *B. braunii* race A was used as Reference (M44m, see Chapter 3) and it was modified with the different nitrogen sources while preserving the final N concentration. Biomass growth in the modified media was accordingly evaluated and compared to the growth obtained in the original Reference media (Figure 4.1.).

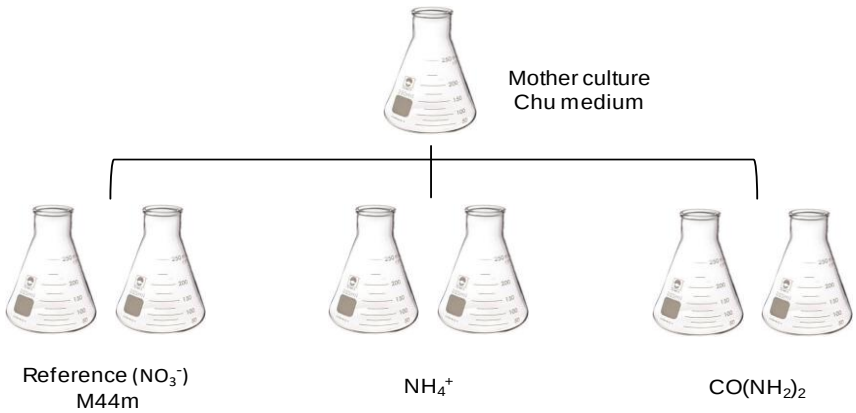


Figure 4.1. Experimental conditions evaluated during the assessment of the preferred nitrogen source for the cultivation of *B. braunii* in liquid cultures. A mother culture growing in Chu medium (Gouveia et al. 2017) was used to inoculate the different experimental conditions. All the conditions were assessed in duplicate.

As previously commented, in order to resemble the nitrogen molar composition of the Reference medium (M44m), the amount of nitrogen used was approximated to the content of this compound in the Reference medium (22 mM).

Figure 4.2. shows the evolution of biomass concentration of the different cultures, measured as biomass dry weight. As it can be seen, the growth was similar between the cultures with nitrate and those with urea, although it was

slightly higher with nitrate at the end of the cultivation; both cultures were still in the linear growth phase after 264 hours of cultivation. On the other hand, cultures with ammonium showed hardly any growth.

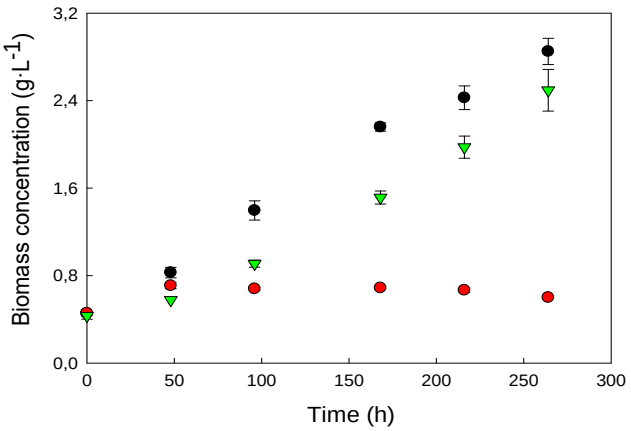


Figure 4.2. Evolution of biomass growth in *B. braunii* cultures with different nitrogen sources, expressed as biomass dry weight. (●) Reference medium (NO_3^-); (▼) $CO(NH_2)_2$; (●) NH_4^+. Error bars show standard deviation of replicates.

Same pattern was found for the maximum photosynthetic efficiency of Photosystem II (F_v/F_m) (Figure 4.3.). Cultures which contained nitrate and urea followed the same tendency during the whole experiment, with the exception of the first 96 hours. During that period a lag phase was found in the culture with urea due to the adaptation to the new nitrogen source. That lag phase was not observed in the Reference culture because the nitrogen source was the same as the mother culture media. However, after that adaptation, both cultures showed efficiency values above 0.6, which is considered an optimum value for microalgae (Schreiber et al. 1995). However, in the case of the cultures with ammonium the photosynthetic efficiency was continuously decreasing, resulting in a final value below 0.4.

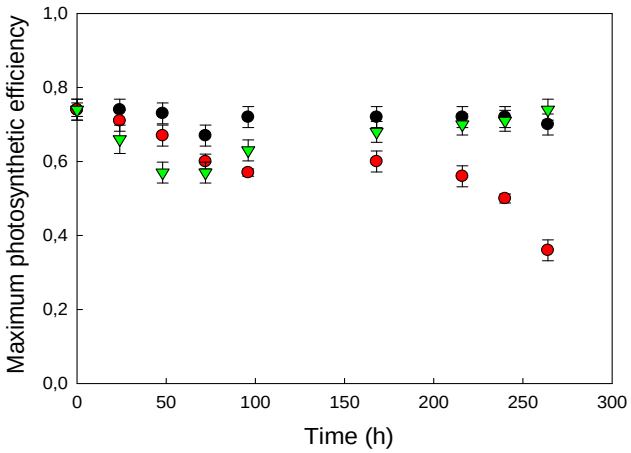


Figure 4.3. Evolution of maximum photosynthetic efficiency of PSII (F_v/F_m) in *B. braunii* cultures with different nitrogen sources. (●) Reference medium (NO_3^-); (▼) $CO(NH_2)_2$; (●) NH_4^+. Error bars show standard deviation of replicates.

Regarding the effect in the biochemical composition of *B. braunii* cultivated in the different nitrogen sources, the total carbohydrates content was evaluated at the beginning and at the end of the experiment. Total culture broth was analysed in order to also analyse the exopolysaccharides, and as it can be seen in Table 4.1. the highest values were found in the cultures with nitrate as nitrogen source. Similar content, expressed as amount per biomass dry weight, was found in the cultures with urea and ammonium after 264 hours of cultivation.

Table 4.1. Total carbohydrates content per volume of culture ($mg \cdot L^{-1}$) and per gram of biomass dry weight ($mg \cdot g^{-1}$) at the beginning (0 h) and at the end (264 h) of *B. braunii* cultivation with different nitrogen sources.

Nitrogen source	**Total carbohydrates content t (0h)**		**Total carbohydrates content t (264h)**	
	$mg \cdot g^{-1}$	$mg \cdot L^{-1}$	$mg \cdot g^{-1}$	$mg \cdot L^{-1}$
Reference (NO_3^-)	291.966±0.032	132.636±0.015	302.630±0.004	691.510±0.010
$CO(NH_2)_2$			123.238±0.006	204.979±0.011
NH_4^+			121.523±0.035	72.914±0.021

The lower growth and carbohydrate content at the end of the experiment can be related to a decrease of culture pH when using urea and, more specially, ammonium. Differences in culture broth pH with growth could be expected. The absorption of nitrate results in a pH increase that is normally buffered by the addition of carbon dioxide to the media via aeration, so that it rarely affects growth to an appreciable extent. On the contrary, when nitrogen is supplied as ammonium salts, the absorption of the ammonium ion may cause the medium to become too acid to support growth (Lupi et al. 1994). During this first experiment, pH was not corrected and, considering the pH value of the ammonium cultures showed a decreasing trend since the beginning (data not shown), the low pH values at the end of the experiment might have resulted in the cease of growth.

This experiment showed that nitrate is the preferred nitrogen source for *B. braunii*. However, the cultures were not under pH control neither they were previously adapted to the new nitrogen sources. Therefore, it was decided to assess the potential benefit of pH control and pre-adaptation in the final *B. braunii* performance with the different nitrogen sources.

4.1.1. Cultivation of *B. braunii* in cultures with nitrate and ammonium under pH control.

In order to confirm the hypothesis that cultures with ammonium did not grow (section 4.1.) due to problems to maintain the pH, it was decided to perform a test in which such parameter was daily measured and corrected up to its original value (7.2) each time it dropped below 6. For that, *B. braunii* was cultivated with ammonium and a Reference culture with nitrate was also monitored. Biomass dry weight and maximum photosynthetic efficiency of Photosystem II (F_v/F_m) were evaluated (Figure 4.5.).

Evolution of biomass concentration (Figure 4.5a.) showed that *B. braunii* cultivated with ammonium was able to grow under controlled pH conditions, although the biomass density values reached by the culture with nitrate were much higher. Maximum photosynthetic efficiency of Photosystem II (F_v/F_m) (Figure 4.5b.) followed the same trend during the whole cultivation period, with the exception of the first 48 hours. As it happened before in the culture with urea, the initial drop in the photosynthetic efficiency in the culture with ammonium might be

related to the adaptation to the new nitrogen source. Nevertheless, efficiency values for both cultures were always above 0.6.

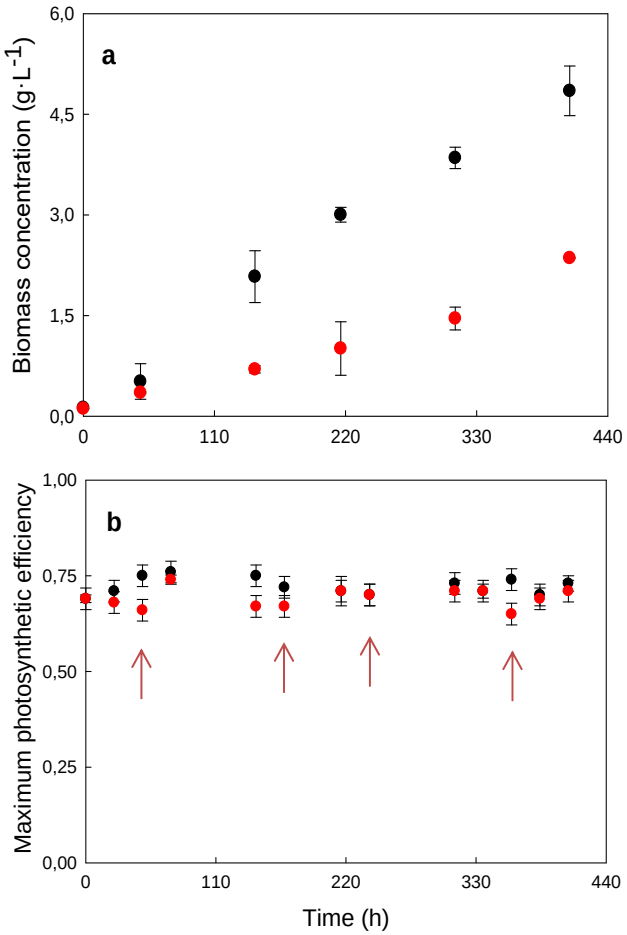


Figure 4.5. Evolution of biomass content expressed as biomass dry weight (a) and maximum photosynthetic efficiency of PSII (F_v/F_m) (b) in *B. braunii* cultures with different nitrogen sources under controlled pH. (●) Reference medium (NO_3^-); (●) NH_4^+. Light red arrows represent the moment in which pH was corrected. Error bars show standard deviation of replicates.

As previously hypothesized, pH control allowed *B. braunii* to grow in the culture medium with ammonium. However, the biomass produced was only half of the biomass produced in the culture with nitrate, which led to disregard that nitrogen source for further experiments.

It is known that nitrogen sources must be first reduced by the microalgae to the ammonium form, through a variety of pathways, in order to be assimilated into amino acids (Cai et al. 2013). Thus, considering it requires less energy for assimilating into amino acids, ammonium is the preferred nitrogen form for many microalgae (Hellebust and Ahmad 1989). However, in the case of *B. braunii*, as well as for other microalgae such as *Dunaliella tertiolecta* and some *Chlorella spp.* the same it is not applicable (Chen et al. 2011; Li et al. 2013; Muthuraj et al. 2014).

Since the favorable nitrogen source for growth is different from species to species, and considering that urea showed a good growth performance (section 4.1), it was decided to adapt the biomass, via repeated-batch cultivation, to that nitrogen source to further evaluate its potentiality.

4.1.2. Repeated-batch cultivation of *B. braunii* in cultures with nitrate and urea as nitrogen source.

In order to assess the *B. braunii* growth and carbohydrate production in urea adapted cultures, the cultivation was performed in repeated-batch mode. For that, cultures were maintained in an optical density range between 0.5 and 1.0 approximately, measured at 750 nm, via punctual dilutions. Several cycles were carried out in order to ensure the steady state was reached.

Normalized optical density at 750 nm is shown in Figure 4.6. As it can be seen, the differences in the growth pattern for both nitrogen sources were minimal at the end of the experiment.

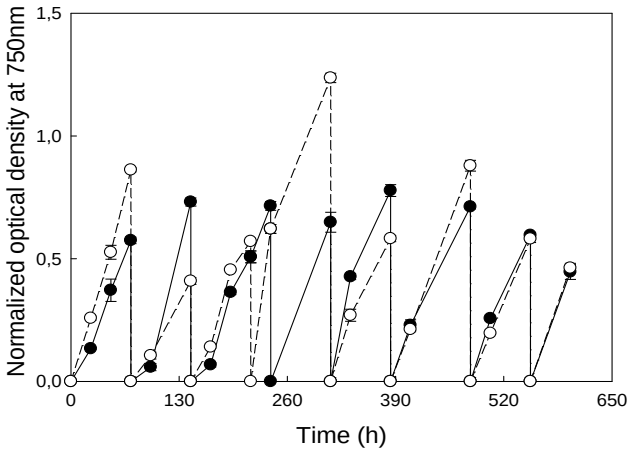


Figure 4.6. Evolution of normalized optical density measured at 750 nm (Ln (N/N_0)), during repeated-batch cultivation of *B. braunii* with NO_3^- (-●-) and $CO(NH_2)_2$; (-○-). Error bars show standard deviation of replicates.

Steady state was considered to be reached after 400 hours of cultivation, when it was observed similar growth rate during the last 3 growth cycles for both nitrogen sources (Table 4.2.). Nitrate and urea cultures showed a specific growth rate of 0.20 d^{-1} and 0.21 d^{-1}, respectively, calculated as the average value of the last 3 cycles.

Table 4.2. Specific growth rate (μ) and biomass volumetric productivity (P_V) in each growing cycle of *B. braunii* cultivated with nitrate and urea.

Growth Cycles	**Nitrate** (NO_3^-)		**Urea** ($CO(NH_2)_2$)	
	μ (d^{-1})	**Biomass P_v** ($g \cdot L^{-1} \cdot d^{-1}$)	**μ** (d^{-1})	**Biomass P_v** ($g \cdot L^{-1} \cdot d^{-1}$)
1	0.191±0.005	0.322±0.005	0.287±0.001	0.417±0.007
2	0.244±0.006	0.330±0.005	0.136±0.001	0.278±0.007
3	0.179±0.006	0.240±0.006	0.190±0.016	0.235±0.008
4	0.216±0.022	0.308±0.045	0.205±0.007	0.312±0.028
5	0.260±0.006	0.347±0.013	0.194±0.005	0.316±0.008
6	0.178±0.002	0.227±0.018	0.220±0.003	0.340±0.007
7	0.199±0.003	0.300±0.009	0.194±0.005	0.403±0.041
8	0.223±0.006	0.360±0.018	0.232±0.019	0.464±0.018

Apart from biomass cell density, measured as optical density, dry weight was followed during each cycle and it allowed to calculate the volumetric biomass productivities of the different cultures. It was observed that productivity in the culture with urea improved during the repeated-batch cultivation, which resulted in productivity values higher than in the cultures with nitrate after six growth cycles (400 hours of experiment), as it is showed in Table 4.2.

According to the results, cultures with urea were able to adapt to the new conditions and showed similar growth compared to the cultures with nitrate. Furthermore, productivity seemed to be higher with urea after the adaptation, which was an interesting finding considering, that most of the commercial fertilizers are urea-based.

4.2. Cultivation of *B. braunii* races A and B in different commercial fertilizers.

As it was demonstrated in the previous section (6.1.2), urea is a suitable nitrogen source to grow the microalgae *B. braunii* once adaptation takes place, and the search of adequate commercial fertilizers for its cultivation was carried out accordingly. The selected fertilizers (NPKs) for both *B. braunii* races, already available at the research group, presented urea as main nitrogen source although they also contained a small fraction of ammonium. As negative control, one fertilizer based in ammonium was also selected (fertilizers composition shown in Table 5, section 3.1.2.2. of Materials and Methods). In addition to the NPKs, sufficient supplement of other nutrients for microalgae to grow is the first key step to produce a large amount of microalgae biomass (Lam and Lee 2012). In this sense, magnesium and calcium, as well as a micronutrients solution (AG Complex, Agralia S.L.) containing other essential trace elements were added to media based on the NPK fertilizers (See Materials and Methods section 3.1.2.2.).

As it was already made in section 4.1. for the evaluation of the nitrogen sources, the amount of fertilizers to be used in the preparation of the different culture media was calculated based on the nitrogen content of the Reference media (M44m -Chapter 3- for race A, and Chu -Gouveia et al. 2017- for race B). However, these amounts resulted in precipitation problems during the preparation

of the final media. This, together with the necessity of a further reduction in the media cost, and considering that a certain degree of nutrients limitation was proven to enhance product accumulation (Chapter 3), led to the employment of approximately one-third and one-half of the corresponding nitrogen content in the Reference media M44m and Chu, respectively (Table 4.3.). On the other hand, the amount of micronutrients solution (AG Complex) added to each medium was adjusted according to the iron concentration in each Reference medium, as indicated in section 3.1.2.2. of Materials and Methods (micronutrients composition shown in Table 6).

Table 4.3. Composition of the Reference culture media and the fertilizer-based media (NPKs) for *B. braunii* race A and race B. Reference medium for *B. braunii* race A was the media coded as M44m, and for *B. braunii* race B was Chu medium. The volume of the commercial NPKs and micronutrients solution (AG complex) used in the preparation of the media is shown in $mL \cdot L^{-1}$. The ratio Nitrogen/Phosphorus (N/P) is shown, as well as the final media composition in millimol per liter (mM).

	***B. braunii* race A**					***B. braunii* race B**				
	$mL \cdot L^{-1}$	N [mM]	P [mM]	N/P	Fe [mM]	$mL \cdot L^{-1}$	N [mM]	P [mM]	N/P	Fe [mM]
Reference	---	22.25	0.60	37	0.04	---	3.96	0.60	6.70	0.05
12-6-4(3)	2	11.09	1.01	11	---	0.50	2.77	0.25	11	---
4-10-10	5	13.30	4.23	3.10	---	1	2.66	0.84	3.10	---
18-6-6	1	8.01	0.52	15.30	---	0.25	2	0.13	15.30	---
2.4.-4.8.-6	1	10.84	3.82	2.80	---	2	2.17	0.76	2.80	---
8-6-6	2	7.27	0.99	7.30	---	0.50	2.18	0.30	7.30	---
AG Complex	0.44	---	---	---	0.04	0.06	---	---	---	0.05

4.2.1. Growth and carbohydrates accumulation in *B. braunii* race A cultivated in different commercial fertilizers.

In order to study the effect of each fertilizer on *B. braunii* growth, and based on the previous experience for the assessment of the preferred nitrogen source, an adaptation period was needed before the experiment, which has been reported to be essential to get biomass growth comparable to that of the controls (Voltolina et al. 1998). For that purpose, repeated-batch cultivation was performed until adaptation was observed (Figure 4.7.) At that moment, the last growing cycle was carefully analysed in terms of biomass and product formation.

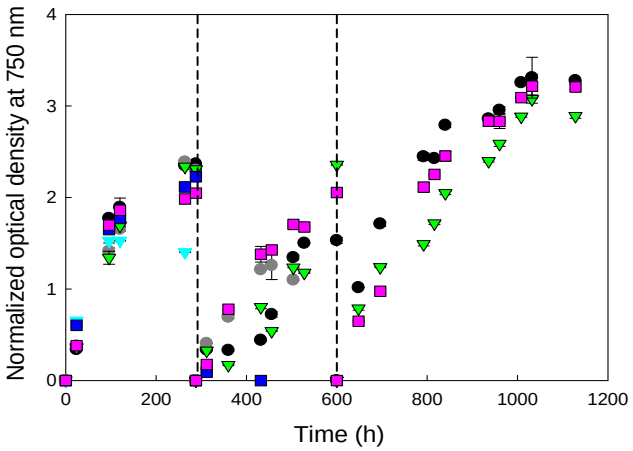


Figure 4.7. Evolution of *B. braunii* race A biomass density in the different media, expressed as normalized optical density at 750 nm (Ln (N/N_0)), during the repeated-batch cultivation; (●) Reference medium (M44m); (●) NPK 18-6-6; (▼) NPK 2.4.-4.8.-6; (▼) NPK 4-10-10; (■) NPK 12-6-4(3); (■) NPK 8-6-6. Dash lines represent the punctual dilutions carried out and error bars show standard deviation of replicates.

During this repeated batch cultivation it was observed that *B. braunii* race A was able to grow with only two of the five selected fertilizers, which were those with the code 2.4-4.8-6 and 8-6-6. 18-6-6 cultures were contaminated with other microalgae, 4-10-10 with fungi and 12-6-4(3) culture collapsed. Probably, the loss of viability when *B. braunii* was subjected to the NPK-based media for the first time might allow other microorganisms to overcome its growth. In this way, the final growing cycle for this microalga was analysed only for those two fertilizers and compared to the growth in the Reference medium, which was the optimized one in Chapter 3 (M44m). Measurements of optical density, photosynthetic efficiency as well as pH, due to the presence of ammonium in the media, were daily taken. Each time the pH dropped below 6, it was corrected up to its original value (7.2). On the other hand, punctual samples were taken (every approximately 48 hours) for the determination of dry weight and carbohydrates content.

Figure 4.8. shows the evolution of biomass concentration of each culture after the adaptation phase (last growing cycle) measured as biomass dry weight. As it can be observed, it was slightly higher in the Reference medium during almost the entire experiment and it only showed a lower value than the 8-6-6

fertilizer-based medium at the end of the final growth curve. Dry weight values of *B. braunii* race A grown in the 2.4-4.8-6 fertilizer-based medium were minor along the entire cultivation.

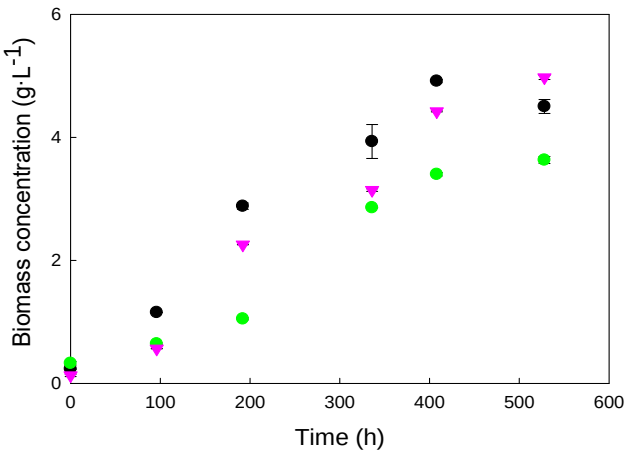


Figure 4.8. Evolution of biomass concentration, expressed as dry weight in grams per liter, in *B. braunii* race A cultures with different media during the final growth cycle. (●) Reference medium (M44m); (●) NPK 2.4-4.8-6; (▼) NPK 8-6-6. Error bars show standard deviation of replicates.

Same pattern was found for the maximum photosynthetic efficiency of Photosystem II (F_v/F_m) (Figure 4.9.). Minor differences were observed between the Reference medium and the 8-6-6 fertilizer-based medium, moreover both cultures showed an efficiency above 0.6, which is considered an optimal value for microalgae (Schreiber et al. 1995). Although the 2.4-4.8-6 fertilizer based medium followed the same tendency, it punctually showed lower efficiency values which coincided with pH decreases. Photosynthetic efficiency recovered as soon as the pH was corrected to its original value.

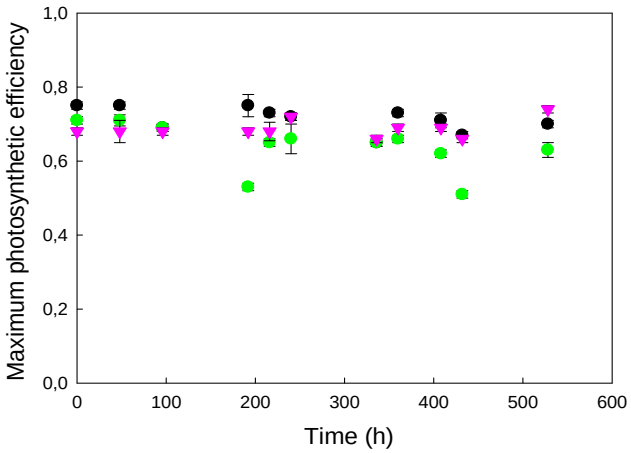


Figure 4.9. Evolution of maximum photosynthetic activity of PSII (F_v/F_m) in *B braunii* race A cultures in the different media during the final growth cycle. (●) Reference medium (M44m); (●) NPK 2.4-4.8-6; (▼) NPK 8-6-6. Error bars show standard deviation of replicates.

Regarding the possible effect in the biochemical composition of *B. braunii* race A when cultivated with the fertilizer-based media, the total carbohydrates as well as the (exo)polysaccharides (EPS) content was punctually evaluated during the final growth curve (Figure 4.10.). As it can be seen in Figure 4.10a, maximal total carbohydrate content (38% dw) may be found for the Reference medium, followed by the 8-6-6 fertilizer-based medium (31% dw) at the end of the cultivation, when cultures approximated the end of the linear growth phase. 2.4-4.8-6 fertilizer-based medium presented a similar trend during the whole period but with a carbohydrates content much lower. On the other hand, Figure 4.10b shows that the medium based in the 8-6-6 fertilizer was the one which produced a higher amount of EPS during the last 200 hours of cultivation, reaching its highest value at the end of the curve (22% of its dry weight).

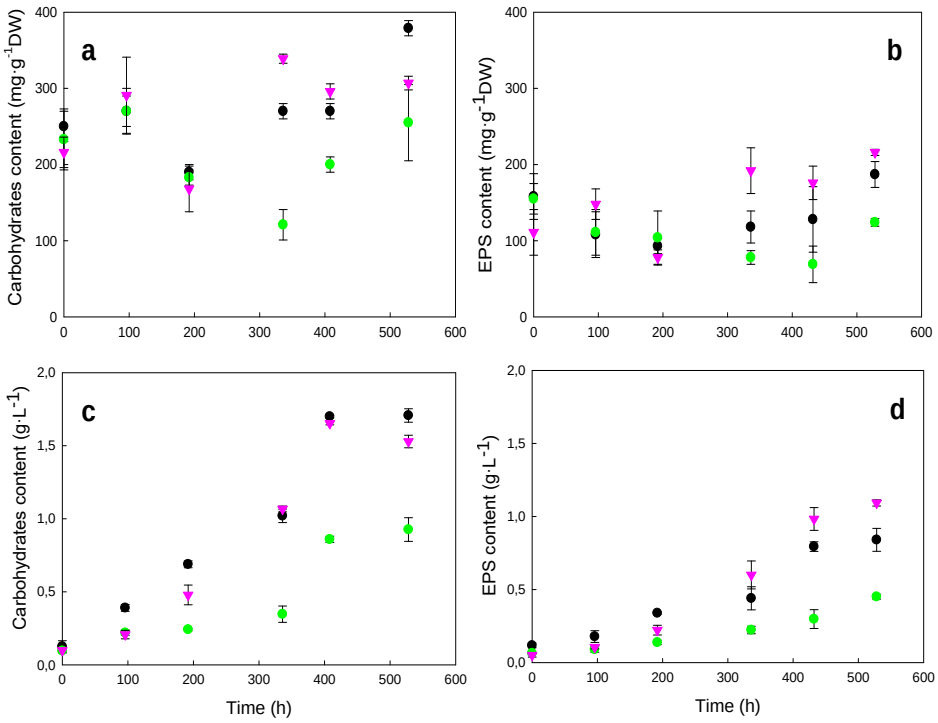


Figure 4.10. Evolution of total carbohydrates and EPS content expressed as milligrams per gram of biomass dry weight (a, b), and as milligrams per liter of culture (c, d), in *B braunii* race A cultures with different media during the final growth cycle. (●) Reference medium (M44m); (●) NPK 2.4-4.8-6; (▼) NPK 8-6-6. Error bars show standard deviation of replicates.

Maximum carbohydrate productivity value was reached at the end of the cultivation, which concurs with the highest cellular carbohydrate content. Since medium-large scale cultivation requires to optimize productivity, selection of final media was done according to the values found at that moment (Table 4.4.). 8-6-6 fertilizer-based medium resulted in the highest EPS and biomass productivities, showing only slightly lower total carbohydrates content respect to the Reference medium.

Table 4.4. Biomass, total carbohydrates and (exo)polysaccharides volumetric productivities (P_V), of *B. braunii* race A, expressed as grams per liter of culture broth per day, at the end of the cultivation in different fertilizer-based media.

Culture media	Biomass P_V ($g \cdot L^{-1} \cdot d^{-1}$)	Carbohydrates P_V ($g \cdot L^{-1} \cdot d^{-1}$)	EPS P_V ($g \cdot L^{-1} \cdot d^{-1}$)
Reference (M44m)	0.183±0.023	0.069±0.002	0.033±0.005
2.4-4.8-6	0.150±0.008	0.038±0.004	0.018±0.002
8-6-6	0.212±0.027	0.065±0.003	0.047±0.003

4.2.2. Growth and hydrocarbons accumulation in *B. braunii* race B cultivated in different commercial fertilizers.

As already done with *B. braunii* race A, *B. braunii* race B was adapted to the different media via repeated-batch cultivation, where only optical density measurements at 750 nm were taken (Figure 4.11.), and after which the last growing cycle was carefully analysed. However, unlike the previous case, the microalga was able to grow in all the fertilizer-based media assessed.

Despite the optimized media for this race was M11 (Chapter 3), the similar productivity values and the lower nitrogen content of the initial Chu medium (Gouveia et al. 2017) would result in more optimal conditions for the cultivation feasibility and, therefore, it was selected as Reference medium in this Chapter.

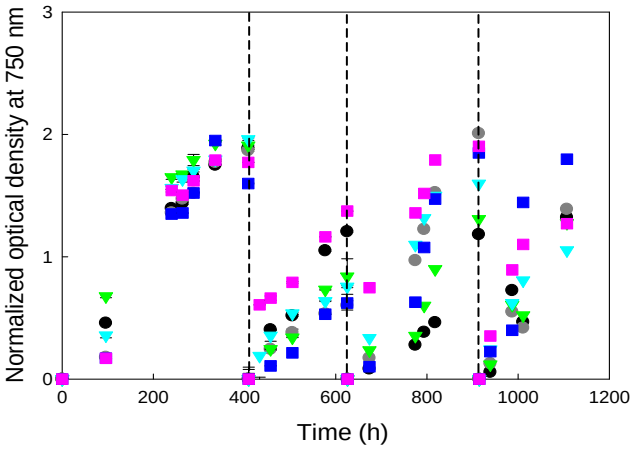


Figure 4.11. Evolution of *B. braunii* race B biomass density in the different media, expressed as normalized optical density at 750 nm (Ln (N/N_0)), during the adaptation period in repeated-batch cultivation. (●) Reference medium (Chu); (●) NPK 18-6-6; (▼) NPK 2.4.-4.8.-6; (▼) NPK 4-10-10; (■) NPK 12-6-4(3); (■) NPK 8-6-6 . Dash lines represent the punctual dilutions carried out and error bars show standard deviation of replicates.

Once again, optical density, photosynthetic efficiency as well as pH measurements, due to the presence of ammonium in the media, were daily taken. Each time the pH dropped below 6, it was corrected up to its original value (7.2). To determine dry weight and hydrocarbons content, during the last cycle, samples were punctually taken (every approximately 48 hours).

As for the other race, the evolution of biomass concentration of each culture was represented as biomass dry weight (Figure 4.12.). As it can be observed, the same pattern was followed for all the cultures, and although most of the fertilizer-based media showed similar biomass concentrations when compared to the Reference medium, differences were major for the culture with the fertilizer 12-6-4(3), which resulted in the lowest values. On the other hand, 4-10-10 fertilizer-based medium showed slightly higher biomass concentration during the entire cycle.

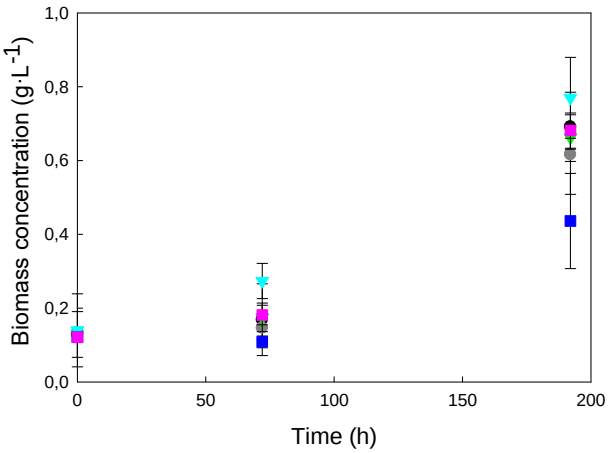


Figure 4.12. Evolution of biomass growth, expressed as dry weight in grams per liter, in *B braunii* race B cultures with different media during the final growth cycle. (●) Reference medium (Chu); (●) NPK 18-6-6; (▼) NPK 2.4-4.8-6; (▼) NPK 4-10-10; (■) NPK 12-6-4(3); (■) NPK 8-6-6. Error bars show standard deviation of replicates.

Regarding the maximum photosynthetic efficiency of Photosystem II (Figure 4.13.), no big differences were found between the different cultures, with the exception of 18-6-6 and 8-6-6, which punctually showed lower efficiency values, which coincided with pH increases. However, photosynthetic efficiency recovered as soon as the pH was corrected to its original value. In general, all fertilizer-based media resulted in slightly lower photosynthetic efficiency values compared to the Reference medium, although viability of the cultures was not compromised.

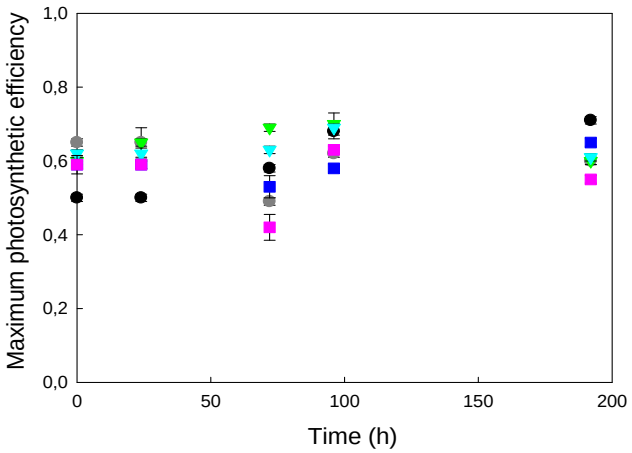


Figure 4.13. Evolution of maximum photosynthetic efficiency of PSII (F_v/F_m) in *B. braunii* race B cultures with different media during the final growth cycle. (●) Reference medium (Chu); (●) NPK 18-6-6; (▼) NPK 2.4-4.8-6; (▼) NPK 4-10-10; (■) NPK 12-6-4(3); (■) NPK 8-6-6. Error bars show standard deviation of replicates.

Regarding the cellular content of the most interesting metabolite from *B. braunii* race B, Figure 4.14. shows the results from the total hydrocarbons analysis. The high hydrocarbons content observed for all cultures at the beginning of the final growth curve might be related to the longer incubation time carried out during the pre-adaptation period, after which biomass was harvested and inoculated in fresh nutrients-replete media, where more light was available. Thus, as the cultures grew, the hydrocarbons concentration tends to decrease.

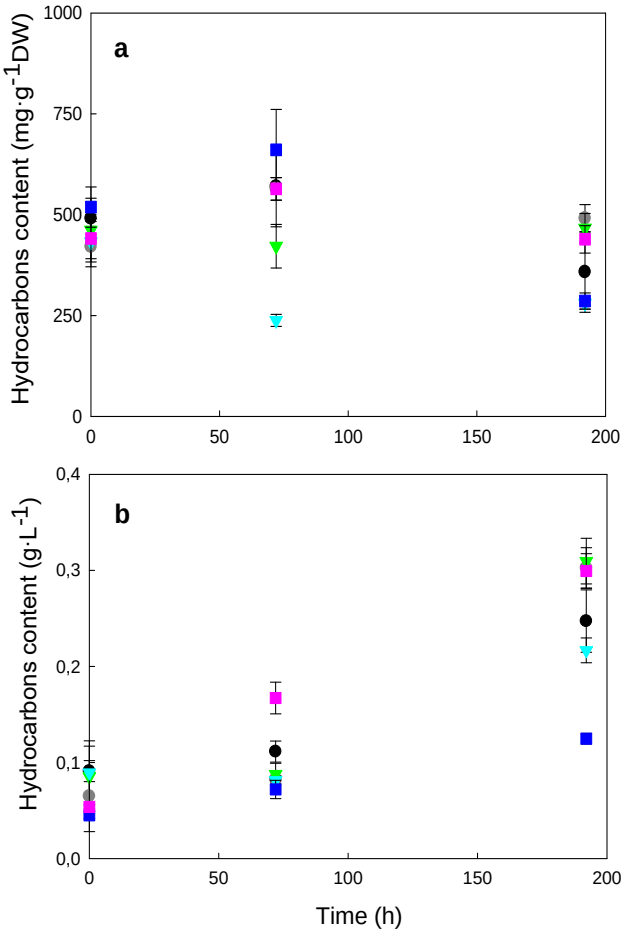


Figure 4.14. Evolution of hydrocarbons content, expressed as milligrams per gram of biomass dry weight (a) and as milligrams per liter of culture, in *B. braunii* race B cultures with different media during the final growth cycle. (●) Reference medium (Chu); (●) NPK 18-6-6; (▼) NPK 2.4-4.8-6; (▼) NPK 4-10-10; (■) NPK 12-6-4(3); (■) NPK 8-6-6. Error bars show standard deviation of replicates.

Regarding the hydrocarbons content, expressed as percentage of biomass dry weight, most of the fertilizer-based media (18-6-6, 2.4-4.8-6 and 8-6-6) presented higher content (49.1, 46.7 and 43.9%) compared to the Reference medium at the end of the experiment (35.8%). These values were slightly higher than the values found in literature for *B. braunii* race B (between 30-40%$_{dw}$) (Metzger and Largeau 2005). On the other hand, two of the fertilizer-based media

(4-10-10 and 12-6-4(3)) showed slightly lower values (28.1% and 28.6% respectively).

With the obtained data, volumetric biomass and hydrocarbons productivities were calculated (Table 4.5.), highlighting that the 8-6-6 fertilizer-based medium resulted in 1.6-fold higher hydrocarbons productivity than the Reference one. Besides, only two fertilizers media resulted in lower hydrocarbon productivity values (4-10-10 and 12-6-4(3)). Regarding the biomass productivity, no important differences were observed between all the media assayed, with the exception of 12-4-6(3).

Table 4.5. Biomass and hydrocarbons volumetric productivities (P_V) of *B. braunii* race B, expressed as grams per liter of culture broth per day, at the end of the cultivation in different fertilizer-based media (NPKs).

Culture media	**Biomass P_V** ($g \cdot L^{-1} \cdot d^{-1}$)	**Hydrocarbons P_V** ($g \cdot L^{-1} \cdot d^{-1}$)
Reference (Chu)	0.063±0.002	0.019±0.002
18-6-6	0.058±0.001	0.030±0.001
2.4-4.8-6	0.060±0.002	0.028±0.004
4-10-10	0.063±0.003	0.016±0.002
12-6-4(3)	0.045±0.002	0.010±0.001
8-6-6	0.061±0.005	0.031±0.003

4.2.3. Media cost calculation and selection of the best fertilizer-based medium for both *B. braunii* races.

Considering the results from the previous sections (4.2.1. and 4.2.2.), it is evident that *B. braunii* growth conditions, including media composition, substantially affect the productivity of the cultures. But another point to bear in mind in order to select the best media is the final media cost. Thus, the price of all fertilizer-based media was calculated and compared to the price of the Reference media for each microalga. As it can be seen in Table 4.6., fertilizer-based media presented much lower prices than their respective Reference culture media. The price was calculated according to the real price of the commercial fertilizers and

the price of the Reference media was calculated based on the price of the different chemicals from the Sigma-Aldrich website.

Table 4.6. Comparison between fertilizer-based media (NPKs) and Reference media costs for *B. braunii* race A and race B.

			***B. braunii* race A**			***B. braunii* race B**		
	NPK (€/ton)	AG Complex (€·L^{-1})	NPK (mL·L^{-1})	AG Complex (mL·L^{-1})	Final Price (€·L^{-1})	NPK (mL·L^{-1})	AG Complex (mL·L^{-1})	Final Price (€·L^{-1})
Reference	---	---	---	---	0.258	---	---	0.073
18-6-6	274	4.8	1	0.044	0.0004852	0.250	0.058	0.0003469
2.4-4.8-6	240	4.8	10	0.044	0.0026112	2	0.058	0.0007584
4-10-10	226	4.8	5	0.044	0.0013412	1	0.058	0.0005044
12-6-4(3)	230	4.8	2	0.044	0.0006712	0.500	0.058	0.0003934
8-6-6	210	4.8	2	0.044	0.0006312	0.500	0.058	0.0003834

In general terms, results showed that biomass productivity was not strongly influenced by the usage of fertilizers whereas carbohydrates and hydrocarbons productivities were substantially improved by some of them. Therefore, considering the easiness of operation when preparing medium based in fertilizers, their lower price, and that both *B. braunii* strains resulted in better productivities of their corresponding products, it was proposed to cultivate both strains in 8-6-6 fertilizer-based media. Comparing its composition to both Reference media (M44m and Chu), the fact that the medium based on fertilizer 8-6-6 presented a much lower nitrogen concentration could have strengthened the increase in the production of the metabolites of interest. A study carried out by Singh and Kumar (Singh and Kumar 1992) demonstrated that N limitation, as well as other stress conditions, may promote the biosynthesis of hydrocarbons and carbohydrates in *B. braunii*. However, N starvation can present many negative effects in microalgae cultivation such as, damaging the synthesis of proteins participating in photosystems PSI and PSII, and decreasing the synthesis of photosynthetic pigments (Markou et al. 2012b). Nevertheless, no effect was found in the biomass productivity when using the fertilizer 8-6-6, which can be considered a positive outcome compared to other N-limiting media. In that sense, to find the optimized

limited media should be considered an affordable approach for the production of microalgae rich in products of interest while preserving biomass growth.

4.3. Assessment of *B. braunii* races A and B cultivation in different 8-6-6 fertilizer-based medium concentrations.

Previous experiments carried out in section 4.2. revealed the potentiality and suitability of the commercial fertilizer Agroliq NPK 8-6-6 (Agralia Fertilizantes, S.L.) to cultivate *B. braunii* races A and B. In this regard, it was decided to further optimized the amount of fertilizer to be used in order to achieve even better productivities and/or reduce the media cost. Table 4.7. shows the volumes and consequent nitrogen concentrations used for each microalgal race. Fertilizer concentration was adjusted to one-half (8-6-6(1/2)) and 2-fold (8-6-6(2x)) the concentration used previously (sections 4.2.1. and 4.2.2.) with each *Botryococcus* race.

Table 4.7. Different concentrations of 8-6-6 fertilizer used for *B. braunii* race A and race B. Composition of each Reference culture medium is also shown. The volume of the commercial NPKs and micronutrients solution used in the preparation of the media is shown in $mL \cdot L^{-1}$. The ratio Nitrogen/Phosphorus (N/P) is shown, as well as the final media composition in millimol per liter (mM).

	B. braunii race A					***B. braunii race B***				
	$mL \cdot L^{-1}$	N [mM]	P [mM]	N/P	Fe [mM]	$mL \cdot L^{-1}$	N [mM]	P [mM]	N/P	Fe [mM]
Reference	---	22.253	0.602	22	0.041	---	3.956	0.602	6.600	0.054
8-6-6 (1/2)	1	3.635	0.495	7.300	---	0.300	1.090	0.148	7.300	---
8-6-6	2	7.270	0.989	7.300	---	0.600	2.181	0.297	7.300	---
8-6-6 (2x)	4	14.539	1.978	7.300	---	1.200	4.362	0.593	7.300	---
AG Complex	0.044	---	---	---	0.040	0.058	---	---	---	0.053

4.3.1. Growth and carbohydrates accumulation in *B. braunii* race A cultivated in different concentrations of 8-6-6 fertilizer-based medium.

As in the previous sections, an adaptation period was carried out. During the repeated-batch cultivation the daily determination of biomass density allowed to maintain the different cultures in an optimal range (optical density at 750 nm was approximately maintained between 0.4 and 2.5) by punctual dilutions. During

1000 hours of cultivation, pH was maintained at 7.2 ± 0.5 and maximum photosynthetic activity of Photosystem II (PSII) was daily monitored. Biomass dry weight and carbohydrates content (total carbohydrates and EPS) were determined during the first and the last growing cycle as representative from non-adapted and adapted cultures respectively.

Figure 4.15. shows the evolution of biomass concentration of the different cultures, measured as normalized optical density at 750 nm. As it can be seen, the different batch cycles shortened with the periodic dilution as a mean of growth adaptation. Differences in biomass growth were not major between Reference medium and fertilizer-based media 8-6-6 control and 8-6-6(1/2), whereas in the culture with the fertilizer 8-6-6(2x) almost no growth was observed until its death at 380 hours of cultivation.

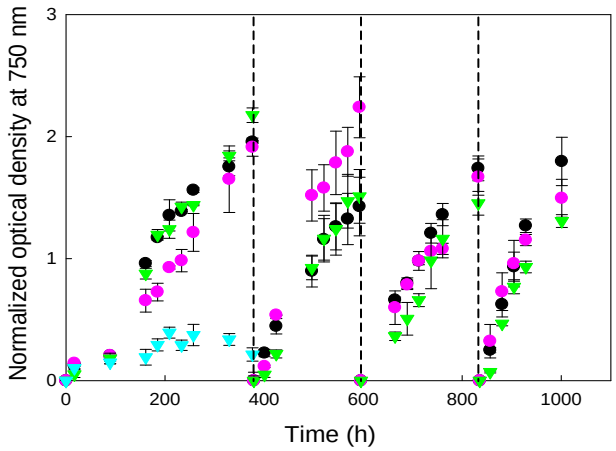


Figure 4.15. Evolution of *B. braunii* race A biomass density in the different media, expressed as normalized optical density at 750 nm (Ln (N/N_0)), during the repeated-batch cultivation; (●) Reference medium (M44m); (▼) NPK 8-6-6(1/2); (●) NPK 8-6-6 control; (▼) NPK 8-6-6(2x). Dash lines represent the punctual dilutions carried out and error bars show standard deviation of replicates.

Same pattern was found for the maximum photosynthetic efficiency of Photosystem II (F_v/F_m) (Figure 4.16.). Minor differences were observed between the Reference media and fertilizer-based media 8-6-6 control and 8-6-6(1/2), which showed a photosynthetic efficiency above 0.6. However, photosynthetic efficiency of the culture with the fertilizer 8-6-6(2x) was continuously decreasing since the beginning of the experiment until the culture death.

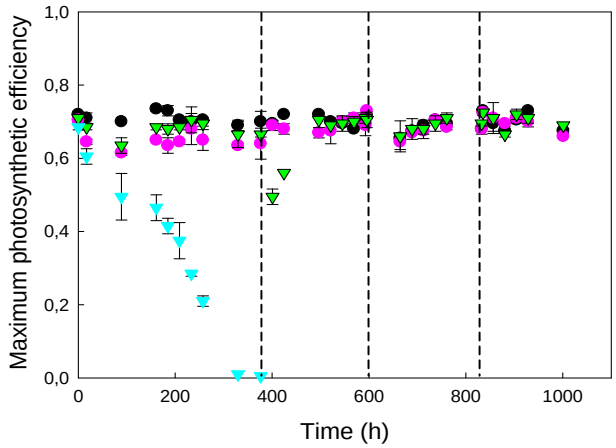


Figure 4.16. Evolution of *B. braunii* race A maximum photosynthetic efficiency of PSII (F_v/F_m) during the repeated-batch cultivation. (●) Reference medium (M44m); (▼) NPK 8-6-6(1/2); (●) NPK 8-6-6 control; (▼) NPK 8-6-6(2x). Dash lines represent the punctual dilutions carried out and error bars show standard deviation of replicates.

The biomass collapse observed in the culture with 8-6-6(2x) might be attributed to the higher concentration of ammonium in the medium. In aqueous solution, ammonium ion (NH_4^+) exists in equilibrium with the un-ionised or free ammonia (NH_3), which has been reported to be toxic to several photosynthetic organisms (Abeliovich and Azov 1976), according to the dissociation equation:

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$$

Because of the equilibrium, increasing one of them automatically increases the other. Furthermore, this equilibrium mainly depends on pH. Thus, as the algal density increased the pH in the medium also increased due to carbon dioxide assimilation. This condition would have forced the reaction to move to the left increasing the concentration of ammonia which has a toxic effect on the microalga and, consequently, affecting the microalgal growth and photosynthetic efficiency.

A close look at the biomass dry weight during the non-adapted and the adapted growth phases confirmed the above mentioned results (Figure 4.17.). During both phases, biomass cell concentration followed a similar trend under all conditions, with the exception of 8-6-6(2x). However, the control culture with the

initial fertilizer concentration (8-6-6) resulted in a slightly higher biomass concentration at the end of the adapted phase compared to the Reference media and 8-6-6(1/2) (14% and 31.5% respectively). The fertilizer 8-6-6(2x), due to the cell collapse, could not be kept until the adapted phase was reached and, therefore, no data were available.

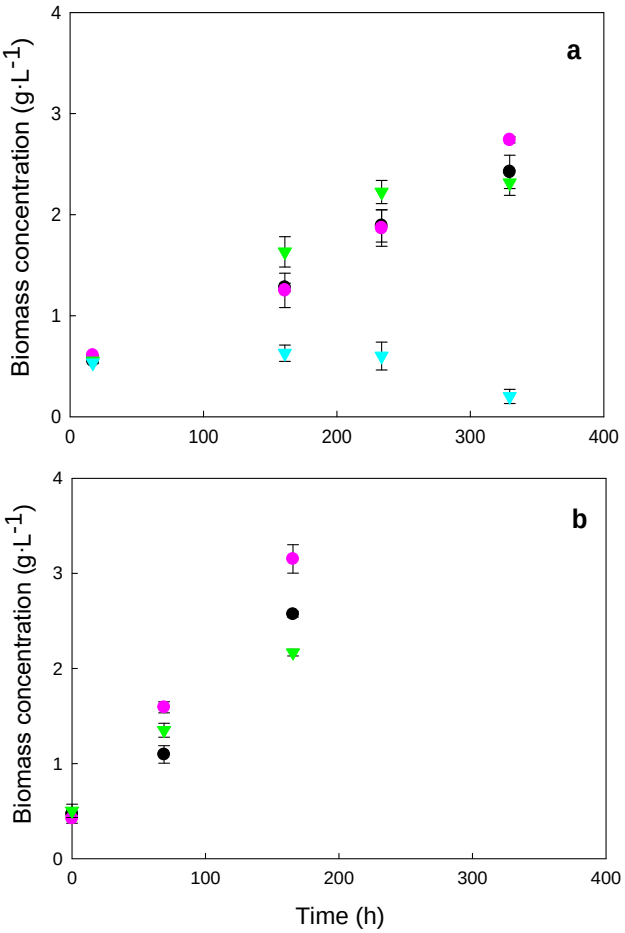


Figure 4.17. Evolution of *B. braunii* race A biomass dry weight, expressed as dry weight in grams per liter, during the non-adapted phase (a) and the adapted phase (b). (●) Reference medium (M44m)**;** (▼) NPK 8-6-6 (1/2); (●) NPK 8-6-6 control; (▼) NPK 8-6-6(2x). Error bars show standard deviation of replicates.

Volumetric biomass productivities were greatly improved during the repeated-batch cultivation, which resulted in productivity values up to 2.4-fold

higher at the end of the adaptation phase when compared to the first growing cycle (non-adapted) (Table 4.8.). The 8-6-6 control culture (2ml·L^{-1} NPK) resulted in a 29.6% higher volumetric productivity when compared to the Reference media (M44m), whereas it dropped a 20.7% for the medium with fertilizer 8-6-6(1/2). Noteworthy, it is important to address that the reduction in biomass volumetric productivity is accompanied of a considerably easiness of operation, which is very important at large scale operation, and a major reduction in media price when using the commercial fertilizer at half of its concentration. Therefore, such concentration cannot be discharged until the analysis of the carbohydrate content is done.

Table 4.8. Biomass volumetric productivity (P_V) of *B. braunii* race A, expressed as grams of biomass produced per liter of culture broth and per day, calculated during the non-adapted (first growing cycle, 233 hours length) and the adapted (last growing cycle, 166 hours length) phases.

Culture Media	**Biomass P_V ($g \cdot L^{-1} \cdot d^{-1}$)**	
	Non-adapted phase	Adapted phase
Reference (M44m)	0.148±0.016	0.304±0.028
8-6-6 (1/2)	0.186±0.012	0.241±0.003
8-6-6 Control	0.140±0.032	0.394±0.015
8-6-6 (2x)	---	---

Regarding the effect of different fertilizer concentrations in the biochemical composition of *B. braunii* race A, the carbohydrates content was evaluated during the repeated-batch cultivation. As it can be seen in Figure 4.18., during the final growth cycle, cultures were able to produce higher amount of carbohydrates in a shorter period of time. Nonetheless, EPS content dropped a lot at the end of the adapted phase, after 1000 hours of batch cultivation. That lower measured EPS content must be due to the fact that the adapted period was ceased when cultures were still in linear growth phase, and it has been studied that EPS concentration starts to increase once the stationary phase is reached (Moreno et al. 1998; Banerjee et al. 2002).

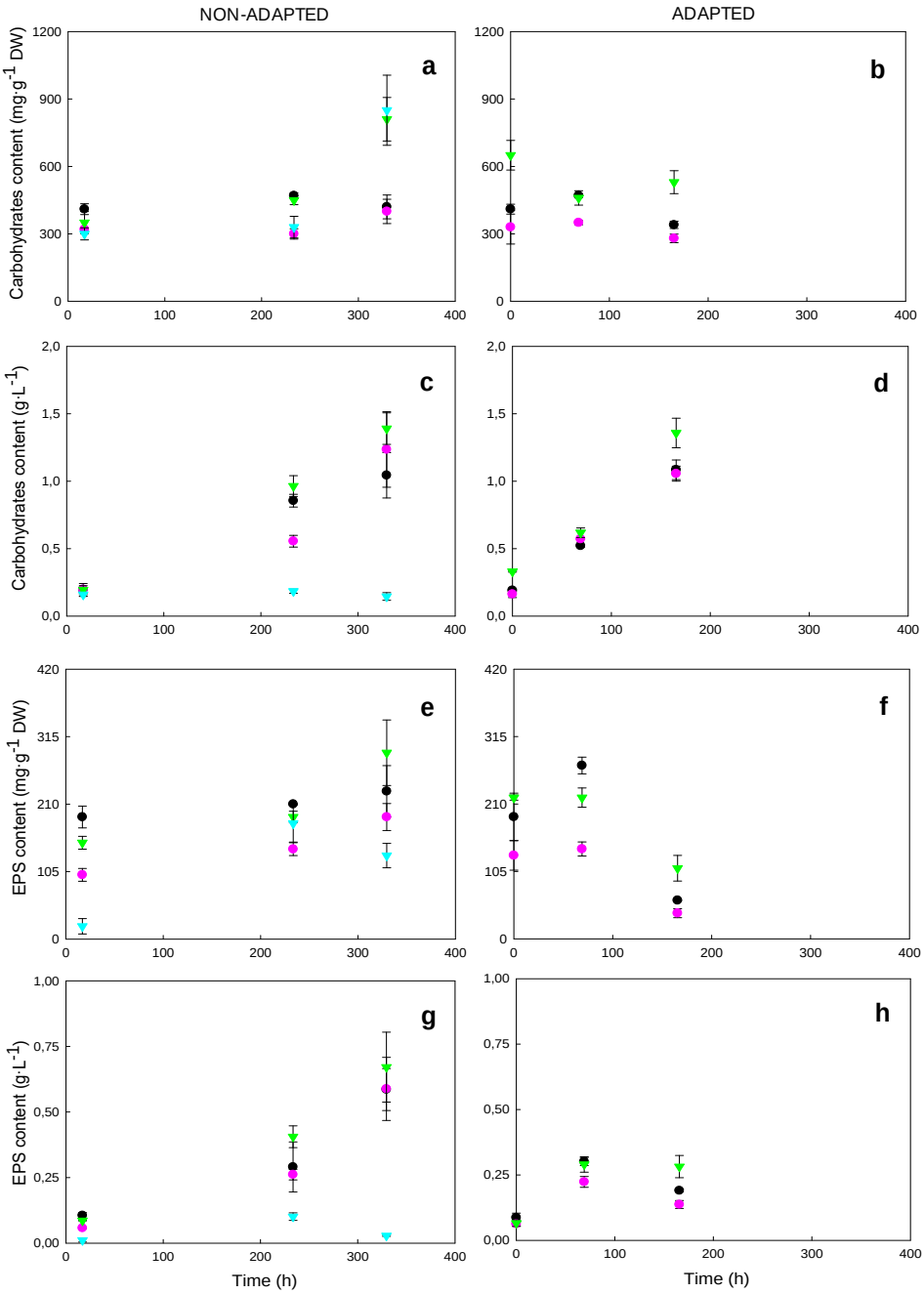


Figure 4.18. Evolution of total carbohydrates (a, b) and EPS content (e, f), expressed as milligrams per gram of biomass dry weight, during the non-adapted phase (a,e) and the adapted phase (b, f), and evolution of total carbohydrates (c, d) and EPS content (g, h), expressed as grams per liter of culture broth, during the non-adapted phase (c, g) and the adapted phase (d, h). (●) Reference medium **(**M44m**);** (▼) NPK 8-6-6(1/2); (●) NPK 8-6-6 control; (▼) NPK 8-6-6(2x). Error bars show standard deviation of replicates.

Table 4.9. shows that final total carbohydrates volumetric productivity was similar between the 8-6-6 control fertilizer-based medium and the Reference one, whereas for the fertilizer 8-6-6(1/2) it was a slightly higher. Different pattern was found for the EPS volumetric productivity between the Reference medium and the 8-6-6 control fertilizer-based medium whose value was twice the value of the Reference one, and also for the fertilizer (1/2), which presented a productivity 26.7% lower. However, taking into account what was above mentioned about the higher EPS content during the stationary phase, even a better productivity value for the fertilizer control would be expected if the growth cycle had been extended. All of this must be considered in order to select the most appropriate harvesting time, depending on the desired product (total carbohydrates or EPS).

Table 4.9. Carbohydrates and EPS volumetric productivities (P_V) of *B. braunii* race A, expressed as grams of biomass produced per liter of culture broth and per day, calculated during the non-adapted (first growing cycle, 233 hours length) and the adapted (last growing cycle, 166 hours length) phases.

Culture Media	**Carbohydrates P_V ($g \cdot L^{-1} \cdot d^{-1}$)**		**EPS P_V ($g \cdot L^{-1} \cdot d^{-1}$)**	
	Non-adapted phase	Adapted phase	Non-adapted phase	Adapted phase
Reference (M44m)	0.066±0.002	0.129±0.003	0.021±0.003	0.015±0.001
8-6-6(1/2)	0.085±0.005	0.149±0.010	0.031±0.008	0.011±0.006
8-6-6 Control	0.040±0.002	0.129±0.009	0.017±0.001	0.031±0.003
8-6-6(2x)	---	---	---	---

4.3.2. Growth and hydrocarbons accumulation in *B. braunii* race B cultivated in different concentrations of 8-6-6 fertilizer-based medium.

During the entire batch cultivation of *B. braunii* race B, the daily determination of biomass density allowed, as in the case of *B. braunii* race A, to maintain the different cultures in an optimal range (optical density at 750 nm was approximately maintained between 0.4 and 1.5) by punctual dilutions. During almost 800 hours of cultivation pH was maintained at 7.2 ± 0.5 and maximum photosynthetic activity of Photosystem II (PSII) was daily monitored. Biomass dry

weight and total hydrocarbons content were determined during the first and the last growing cycle as representative from non-adapted and adapted cultures respectively.

Figure 4.19. shows the evolution of biomass concentration of the different cultures, measured as normalized optical density at 750 nm. As it can be seen, from the second dilution the cycles became shorter, and not major differences were found between the growth trends of each medium.

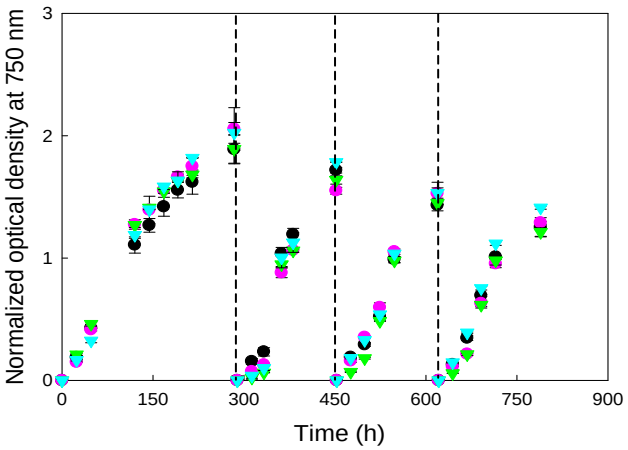


Figure 4.19. Evolution of *B. braunii* race B biomass density in the different media, expressed as normalized optical density at 750 nm (Ln (N/N_0)), during the repeated-batch cultivation; (●) Reference medium (Chu); (▼) 8-6-6(1/2); (●) NPK 8-6-6 control; (▼) NPK 8-6-6(2x). Dash lines represent the punctual dilutions carried out and error bars show standard deviation of replicates.

Regarding the maximum photosynthetic efficiency of Photosystem II (F_v/F_m) (Figure 4.20.), it can be noticed that both the Reference medium and the fertilizer 8-6-6(2x) followed the same pattern during the entire experiment, with efficiency values above 0.6. Nonetheless, maximum photosynthetic efficiency values of 8-6-6 control and 8-6-6(1/2) showed a steep decrease at the middle of each growing cycle, being more prominent for the second one, although it could be recovered after each dilution step. That might be due to the fact that the microalga needs a period of time to adapt its metabolism to the nutrients limitation at which the biomass could be exposed at the end of each cycle, especially in the case of the fertilizer (1/2).

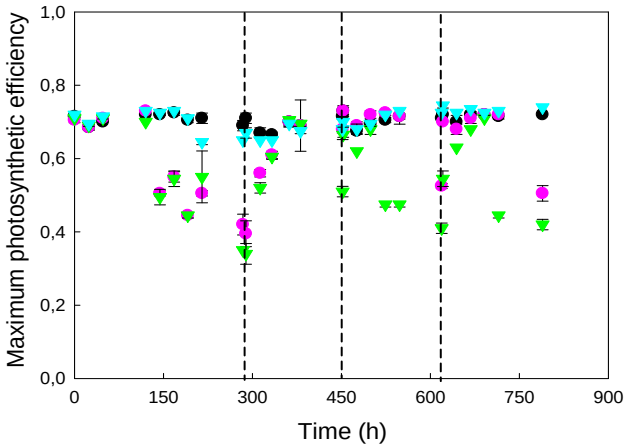


Figure 4.20. Evolution of *B. braunii* race B maximum photosynthetic efficiency of PSII (F_v/F_m) during the repeated-batch cultivation. (●) Reference medium (Chu); (▼) 8-6-6(1/2); (●) NPK 8-6-6 control; (▼) NPK 8-6-6(2x). Dash lines represent the punctual dilutions carried out and error bars show standard deviation of replicates.

Biomass cell concentration, measured as biomass dry weight, was determined during the non-adapted and the adapted phases. As it can be seen in Figure 4.21., biomass production was only slightly higher in the culture with 8-6-6(2x) during the adapted phase, while the culture with 8-6-6(1/2) showed lower biomass content, probably related to the lower amount of available nutrients in that media. Unlike what happened with the race A, race B was able to grow in fertilizer 8-6-6(2x), which could be due to the fact that the Reference medium of this race (Chu) had a much lower nitrogen concentration (5.6-fold lower) than that of the race A. Therefore, the 2x fertilizer concentration in this case did not present an ammonium content as high as the race A, being possible its growth.

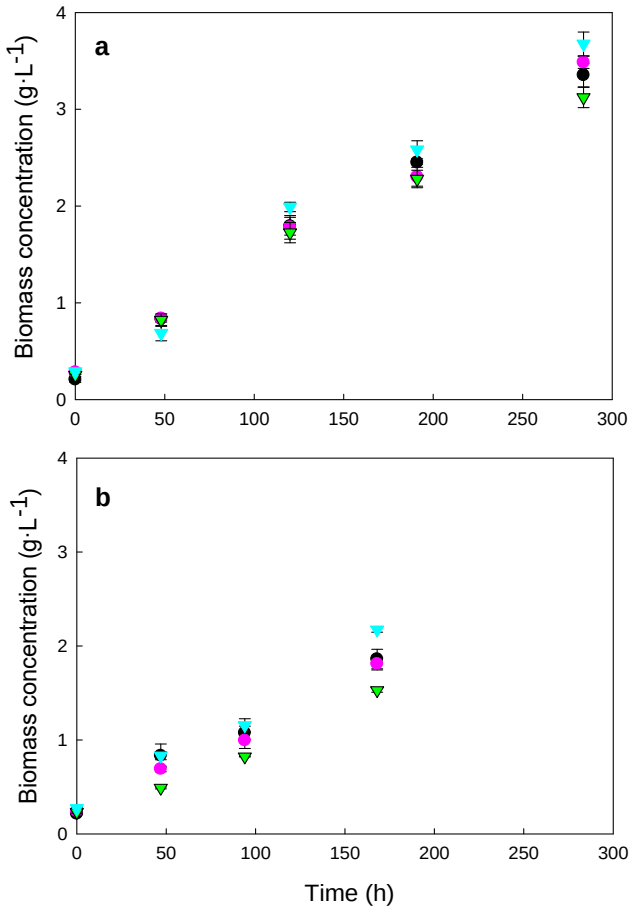


Figure 4.21. Evolution of *B. braunii* race B biomass dry weight, expressed as dry weight in grams per liter, during the non-adapted phase (a) and the adapted phase (b). (●) Reference medium (Chu); (▼) 8-6-6(1/2); (●) NPK 8-6-6 control; (▼) NPK 8-6-6(2x). Error bars show standard deviation of replicates.

As well as for the biomass content results, Table 4.10. shows that biomass productivities were lower for all the media in the adapted phase, although the fertilizer 8-6-6(2x) resulted in the highest biomass productivity. Those lower biomass productivity values, when culturing for a long period of time on nitrogen-limited media, may be indicative, as it has been already reported (Zhila et al. 2005), of an increase in the production of hydrocarbons, and/or other metabolites by the microalga.

Table 4.10. Biomass volumetric productivity (P_V) of *B. braunii* race B, expressed as grams of biomass produced per liter of culture broth and per day, calculated during the non-adapted (first growing cycle, 191 hours length) and the adapted (last growing cycle, 168 hours length) phases.

Culture Media	Biomass P_V ($g \cdot L^{-1} \cdot d^{-1}$)	
	Non-adapted phase	Adapted phase
Reference (Chu)	0.282±0.005	0.235±0.010
8-6-6(1/2)	0.255±0.024	0.185±0.005
8-6-6 Control	0.254±0.020	0.224±0.015
8-6-6(2x)	0.288±0.010	0.271±0.025

Regarding the hydrocarbons production, in Figure 4.22. it can be visualized how cells growing in cultures with fertilizer-based media, at the end of the experiment, tended to float up more than Reference medium cells, which could be related to a major hydrocarbons content. Also colour changes could be observed. Cultures with 8-6-6(1/2) showed a more orange-yellowish color along the time the experiment lasted, which might be due to carotenoids production since it has already been stated that race B is also a good producer of carotenoids (Grung et al. 1989). In addition, it has also been reported for *B. braunii* that a lower nitrogen concentration results in an increase in total carotenoids content (Venkatesan et al. 2013). In that sense, it could be thought that the nitrogen limitation in *B. braunii* race B produces higher carotenoids content at the expense of a decrease in biomass and/or hydrocarbons content.

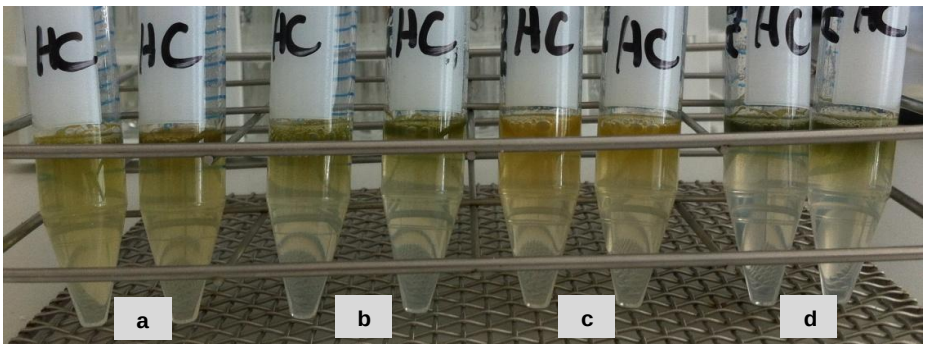


Figure 4.22. Final time samples of *B. braunii* race B, growing in different cultures media. (a) Reference medium (Chu); (b) 8-6-6 control; (c) 8-6-6(1/2); (d) 8-6-6(2x).

The analysis of the total hydrocarbons content (Figure 4.23.) confirmed the visual observations, showing that the fertilizer-based media presented higher hydrocarbons content than the Reference media at the end of the adapted phase. However, the hydrocarbons cellular content was similar for all the media assayed. Although the cellular content during the adapted phase was higher than the non-adapted phase, to further enhance products content, in addition to adaptation via repeated-batch cultivation, it could be necessary to carry out a stress strategy, as it has been performed in other studies (Cheirsilp and Torpee 2012; Mulders et al. 2014).

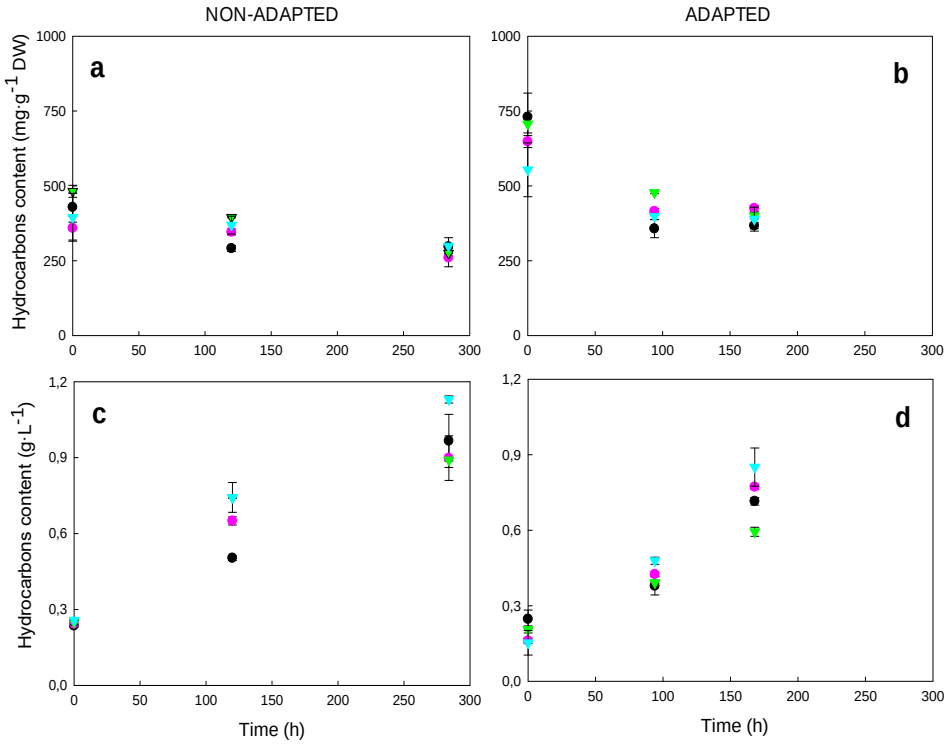


Figure 4.23. Evolution of hydrocarbons content, expressed as milligrams per gram of biomass dry weight (a,b) and grams per liter of culture broth (c, d) during the non-adapted phase (a, c) and the adapted phase (b, d). (●) Reference media (Chu); (▼) 8-6-6(1/2); (●) NPK 8-6-6 control; (▼) NPK 8-6-6(2x). Error bars show standard deviation of replicates.

In terms of volumetric hydrocarbons productivity, Table 4.11. shows that both fertilizer-based media, 8-6-6 control and 8-6-6(2x) resulted in major values at

the end of the experiment respect to the non-adapted phase, being a 29.8 and 49.2% higher, respectively, than the Reference medium.

Table 4.11. Hydrocarbons volumetric productivity (P_V) of *B. braunii* race B, expressed as grams of biomass produced per liter of culture broth and per day, calculated at the end of the non-adapted and the adapted phases.

Culture Media	Hydrocarbons P_V ($g \cdot L^{-1} \cdot d^{-1}$)	
	Non-adapted phase	Adapted phase
Reference (Chu)	0.065±0.005	0.067±0.007
8-6-6 (1/2)	0.054±0.004	0.055±0.003
8-6-6 Control	0.055±0.002	0.087±0.002
8-6-6 (2x)	0.074±0.005	0.100±0.005

In general terms, *B. braunii* race B results showed that biomass productivity maintained similar values using the mentioned concentrations for the fertilizer 8-6-6 in repeated-batch cultivation. Nevertheless, regarding the biochemical composition of hydrocarbons, the productivity improved with two of the fertilizer concentrations assayed (8-6-6 control and 2x). In that sense, bearing in mind that biomass productivities did not dramatically decrease, those two concentrations must be considered feasible for the hydrocarbons production, especially the concentration 2x, since it was the one that presented the most promising results.

4.4. Commercial fertilizers advantages in microalgae cultivation.

The advantages of commercial fertilizers usage in microalgae cultivation are mainly related to the fact that they might reduce part of the microalgal production costs, which could enhance the biotechnological use of these microorganisms on a large scale.

Summarizing the main results obtained in this Chapter of the Thesis, it could be concluded that:

1. Urea could be used as a suitable nitrogen source to cultivate *B. braunii,* since it was possible to improve its biomass and product

productivities when it was adapted to this compound via repeated-batch cultivation.

2. The presence of ammonium in the media entailed that cultures suffered a continuous decrease in pH (especially in race A), which compromises *Botryococcus* growth. However, pH control allowed to cultivate *B. braunii* although lower biomass density was reached.

3. *B. braunii* race A and B were able to grow in fertilizer-based media.

4. Fertilizers-based media were easier to prepare and their prices were considerably lower compared to both *B. braunii* races (A and B) Reference media (M44m and Chu, respectively).

5. Final productivity results suggested that certain nutrient limitation (fertilizer-based medium 8-6-6 control) is acceptable for the cultivation of *B. braunii* races A and B since they presented similar biomass productivity values to their respective Reference media at the end of the repeated-batch cultivation.

6. Products productivity of both *B. braunii* races, A and B, were improved with the usage of certain fertilizers-based media concentrations, probably due to its lower nitrogen content. However, whereas for *B. braunii* race A was needed to reduce the concentration of nutrients even more (fertilizer-based medium 8-6-6(1/2)) to obtain a slightly higher carbohydrates productivity, in the case of the race B, as the nitrogen concentration in the Reference medium was already low, the highest hydrocarbons productivity was reached with 2-fold the concentration of the fertilizer control (fertilizer-based medium 8-6-6(2x).

Therefore, considering the major benefits related to media preparation and media cost, and the productivity values, it can be concluded that the use of fertilizers should be considered as a promising tool for the sustainable production of *Botryococcus*. Furthermore, from the results obtained it is proposed to choose the fertilizer 8-6-6 to cultivate both *B. braunii* races, although using different concentrations: 0.3 $ml \cdot L^{-1}$ for race A and 1.2 $ml \cdot L^{-1}$ for race B.

Chapter 5

General Discussion

5. GENERAL DISCUSSION.

5.1. Background.

The fact that microalgae are identified with high potential for the production of high-value products as well as biochemicals can easily be justified. Microalgae are very attractive for the purpose of producing energy-rich molecules as they are photosynthetic organisms that can live in various aqueous environments, such as saline or seawater. This gives them a low water footprint and moreover, they do not have to compete with cultivated farmland. Although they are not superior to higher plants concerning photosynthetic efficiency, microalgae do have high growth rates and they provide much higher oil yields than higher plants such as palm, soybean or rapeseed oil, and do not produce lignocelluloses. Microalgae do not only use sunlight as energy source, but they are also very efficient in using fertilizers and waste streams as nutrient source. Despite all of this, a key challenge in microalgae cultivation is to find strains that not only produce important biomass or commercial biomolecules amounts, but also grow well under industrially relevant outdoor conditions. In view of this, extremophilic microalgae should be taken into account as they are able to survive in a variety of extreme environments where competitors are poorly developed and, at the same time, to produce interesting compounds.

The aim of this Doctoral Thesis was to assess the effects of certain cultivation conditions over the growth and metabolites productivity of the acidophile microalga *Coccomyxa onubensis* and two strains belonging to the genus *Botryococcus braunii* (race A and race B). *Coccomyxa onubensis* is a novel eukaryotic green microalga isolated from an acidic environment, which is already known to accumulate carotenoids when it is grown under different stress conditions (Casal et al. 2011; Vaquero et al. 2012). On the other hand, *Botryococcus braunii* is a green colony-forming microalga which has the almost unique capacity to synthesise, accumulate and excrete large amounts of long chain hydrocarbons and/or interesting groups of polysaccharides which can be further converted into bio-chemicals, such as polyesters and polyethylene, which are two of the most common plastics. In this sense, and as part of the tasks developed by our Research Group (Biotecnology of Algae Group) within the European Project "SPLASH" (Sustainable Polymers from Algae Sugars and

Hydrocarbons), funded by the Seventh Framework Programme, Part II of this Thesis was focused on the production of biopolymers using the microalga *B. braunii* as renewable raw material.

The results obtained from all the experiments carried out for the completion of this Thesis have shown that biomass and metabolites productivity can be improved for the three mentioned microalgal strains when they are cultivated under different conditions.

5.2. Stress conditions as a tool to enhance carotenoids production in *Coccomyxa onubensis*.

Having been reported that the production of metabolites of commercial interest and algal biomass can be enhanced under environmental stress conditions (Mata et al. 2010; Mulders et al. 2014; González et al. 2015), it was decided to assess the effect of temperature and UV-radiation on *C. onubensis* growth and metabolites (carotenoids, lipids), since such microalga is able to survive under extreme conditions in its natural environment and, therefore, it must have developed special strategies to cope with these factors.

Temperature is a very sensitive factor for microalgal growth in general and, more specifically, for metabolic activities in microalgal cells. Added to this, the temperature also stimulates the reactions involved in the carotenogenic route of microalgae as part of the response to oxidative stress (Bhosale 2004). On the other hand, intensity and quality of the light, are also fundamental in the development of the photosynthetic organisms. Light effects on the biochemical composition of photosynthetic algae are greatly influenced by photoadaptation and photoinhibition processes. In these processes, algal cells show important changes in their cellular composition, often with alterations in their ultrastructure and physiological behaviour, in order to increase the efficiency of photosynthesis and growth (Benavente-Valdés et al. 2016).

According to the results obtained in this Thesis, it can be said that *C. onubensis* is able to improve its growth and carotenoids productivities until a temperature of 40 ºC from which its cell viability is affected. However, although at 35 ºC carotenoids productivity was higher than in control conditions (25 ºC), it was

not greatly improved. This limited improvement might be due to the cellular acclimation of the microalga to the high temperature conditions in its natural habitat. Therefore, in order to further increase the production of carotenoids, it should be considered to cultivate *C. onubensis* at such temperature in combination with other stress factors, which has already been demonstrated to work for other microalgal species (Pisal and Lele 2005).

Regarding the UV-radiation, the results showed that when UVA radiation is added to PAR *C. onubensis* biomass and carotenoids productivities were greatly improved. The accumulation of such pigments must be a molecular adaptation mechanism as response to the oxidative stress induced by UVA rays. However, cultures subjected to PAR+UVB radiation were not able to deal with the cellular damaged produced by such radiation and, thus, their biomass and carotenoids productivities were negatively affected.

As it is already known that UV-radiation induces metabolic changes in microalgae that alters the biomass composition and increases the lipids fraction (Guihéneuf et al. 2010; Khozin-Goldberg et al. 2011), *C. onubensis* lipids content was also analyzed. Similar results to that for carotenoids were obtained since UVA radiation also produced a highly positive effect in *C. onubensis* lipids productivity, whereas UVB radiation only presented a slight improvement respect to the control. Besides, UVA radiation stimulated PUFAs accumulation whereas in the case of UVB radiation, MUFAs content was higher. This behaviour makes sense as it is known that many algae react to the oxidative stress modifying their fatty acids biosynthetic pathways towards the formation and accumulation of storage lipids (SFAs and MUFAs) which has a more active role in the stress response, in addition to functioning as carbon and energy storage under such conditions (Hu et al. 2008). Considering all these results, it must be said that the usage of UVA radiation as a tool to increase the productivity of compounds such as carotenoids and lipids is a good strategy in *C. onubensis* cultures.

Another hypothesis in this Thesis was that the harmful effects of UV-radiation on the cellular metabolism of living organisms also stimulates the accumulation of reactive oxygen species (ROS). To avoid the damage generated by the presence of ROS molecules, microorganisms have developed enzymatic and non-enzymatic defense mechanisms (Mallick and Mohn 2000; Schützendübel

and Polle 2002). The so-called antioxidant enzymes are proteins that catalyze the detoxification of ROS. In this study, the activity of the enzymes ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (GPX) were assessed. It could be seen that the activity of every enzyme increased when the microalga was subjected to UV-radiation (either UVA or UVB), demonstrating that the incubation in the presence of ultraviolet radiation triggers situations that produce oxidative stress, with the consequent increase in the activity of enzymes which participate in antioxidant defense mechanisms, and experimentally ratifies the existing relationship between ultraviolet radiation A or B and the generation of oxidative stress. On the other hand, according to the results, APX activity was, in general, much more activated by UV-radiation than the other two studied enzymes, and as it is known that this enzyme is presented in high concentrations in chloroplasts (Noctor and Foyer 1998), it might be considered that UV-radiation acts directly on such type of organelles.

Overall, according to the obtained results from the experiments with *C. onubensis,* its capacity to accumulate carotenoids when it is cultivated under stress conditions was demonstrated. Therefore, and considering that the accumulation of lipids was only studied under UV-radiation, which showed promising results, it would be interesting to assess its potential in the production of other metabolites, such as lipids, proteins or carbohydrates, using, in addition to the conditions assayed in this Thesis, other stress strategies (nutrient limitation/starvation, high/low light intensities). Besides, the effect of growing the microalga under more than one unfavorable condition at the same time must be something to bear in mind for future studies, in order to reach better productivities. Added to the above, and considering the results obtained from the experiments carried out with *B. braunii*, another future interesting approach would be to assess the usage of commercial fertilizers for *C. onubensis* cultivation.

5.3. Media optimization as a tool to optimize *Botryococcus braunii* productivity.

Culture media optimization is an important factor to take into account in microalgae cultivation due to the current high costs associated with the biomass and metabolites production. Previous research studies showed that in the

development of microalgal products, one of the major tasks is to select a suitable culture medium (Monserrat et al. 1993; Gong and Chen 1997), so that the quality and quantity of biomass can be enhanced.

Another microalga of interest for the production of metabolites is *Botryococcus braunii*. *B. braunii* is a microalga which has the almost unique capacity of accumulation and excretion of large amounts of long-chain hydrocarbons and interesting groups of polysaccharides, which can be convertible into bio-polymers. And these bio-polymers can be used, for instance, in the production of either polymer fiber applications or packaging. Nevertheless working with this microorganism presents a number of challenges considering the extreme variability between the different races, their slow growth and their low productivities (Banerjee et al. 2002). As hypothesis, it could be said that the composition of the commonly used culture media and the morphology of the colonies might limit the availability and diffusion of nutrients, and, therefore, the growth of *B. braunii* (Li and Qin 2005). Therefore, in this Thesis it was decided to improve growth and products productivity of two races of *B. braunii* (A and B) by means of the culture media optimization. At the same time, the selected media to cultivate *B. braunii* contained selenium (Se), which is toxic in the chemical form in which it is present. Then, a second target in the media optimization was to reduce or avoid Se usage at medium-large scale cultivation.

The results indicated that for *B. braunii* race A (carbohydrates producer) a nitrogen amount 7-fold higher than the Reference medium, while maintaining the same phosphorus concentration, significantly improved biomass and carbohydrates productivities when cultures approximated the stationary phase, which it has been reported to be the moment in which carbohydrates concentration starts to increase (Moreno et al. 1998; Banerjee et al. 2002). On the other hand, it has been proposed that a solution to optimize the production of high-value products from microalgae is to reuse the cell mass for continuous production, which is known as “milking” process (Hejazi and Wijffels, 2004). Considering the above, extracellular carbohydrates (EPS) are interesting from the point of view of milking and therefore the EPS content in culture broth was also analyzed, and the results showed that the best medium for its production was the same as in the case of the total carbohydrates. Finally, selenium removal from the culture medium of *B. braunii* race A did not compromise microalgal biomass and

carbohydrates content, which is an important achievement in terms of safety and environmental protection.

In the case of *B. braunii* race B (hydrocarbons producer) no important differences were observed between the evaluated culture media and the Reference one in terms of biomass and hydrocarbons productivity when cultivated in Roux flasks, so the selection was based mainly on the lack of selenium. This may indicate that this race is not so sensitive to the nutrients composition of the medium as the race A when hydrocarbons are targeted as the main product.

5.3.1. Commercial fertilizers for the production of *Botryococcus braunii*.

To obtain biochemical compounds with high added value at a commercial level, it is necessary to produce biomass on a large scale (Liu and Hu 2013), which implies a high demand of optimum analytical grade reagents and a considerable consumption of time due to the preparation of traditional media. All this also has the disadvantage of raising the production costs of microalgae cultures (Molina et al. 2003; Borowizka 2005). Thus, with the purpose of reducing costs and even reaching better productivity results, some studies have been carried out with agricultural fertilizers as culture media for different species of microalgae (Fábregas et al. 1987; Kanlis et al. 2004; Nayak et al. 2016; Silva-Benavides 2016). In this sense, it has been recognized that the replacement of chemical compounds by such fertilizers must be a way to make microalgae cultivation even easier and cheaper (Scardoelli-Truzzi and Sipaúba-Tavares 2017).

According to the above, in this Thesis it was decided to use commercial fertilizers (NPKs) to further improve the productivities, with respect to the selected media previously optimized and, at the same time, to reduce costs in the microalgae cultivation process. For that, it was firstly analyzed the preferred nitrogen source (nitrate, ammonium, urea) for *B. braunii*. The results of this study revealed urea as a good compound for *B. braunii* which was an interesting finding since most of the commercial fertilizers are urea-based.

From the point of view of preparation and costs the fertilizers-based media were easier to prepare and their prices were considerably lower compared to both

B. braunii races (A and B) media (the ones optimized previously). According to biomass productivity, results indicated that certain nutrient limitation (fertilizer-based medium 8-6-6 control) is acceptable for the cultivation of *B. braunii* races A and B since they presented similar biomass productivity values to their respective Reference media at the end of the repeated-batch cultivation. On the other hand, products productivity (carbohydrates and hydrocarbons) of both *B. braunii* races, A and B, were improved with the usage of certain fertilizer-based media, probably due to their lower nitrogen content.

Overall, taking in mind the major benefits related to media preparation and media cost, and the productivity values, it can be said that the use of fertilizers should be considered as a promising tool for the sustainable production of *B. braunii*, which can be applied to other microalgae.

To summarize, it must be said that the culture media optimization for microalgal cultivation can be used as a suitable strategy to enhance biomass and metabolites productivity. Nevertheless, with the aim of reducing costs as much as possible to produce biomass on a large scale, an interesting approach is the use of agricultural fertilizers, as they are cheaper and easier to prepare. Besides, it was demonstrated that its usage does not compromise the optimized microalgal productivity values.

5.4. Recommendations for future research.

Considering that microalgae are important future bio-resources for various industrial applications and according to the results obtained in this Doctoral Thesis, future research could focus on:

1. Assessing the effect of combined stress factors for *C. onubensis* in order to evaluate the influence in microalgal biomass and metabolites production and to find the adequate combination for the optimal carotenoids production.
2. Assessing *C. onubensis* potential in the production of other metabolites different from carotenoids, such as lipids or carbohydrates, using different stress strategies, like for example, nutrients limitation/starvation or high/low light intensities.

3. Assessing the antioxidant enzymatic activity potential of *C. onubensis* under different conditions in order to evaluate its potential use in the food/feed, cosmetic and/or clinical sector.

4. Optimizing *C. onubensis* culture medium. As good results were obtained for *B. braunii,* it would be interesting to find the most suitable nutritional conditions in which the acidophile microalga is able to enhance its carotenoids production and, at the same time, reducing production costs.

5. Testing the optimized selected media for *B. braunii* races A and B under outdoors conditions in photobioreactors (PBRs) in order to analyze if the results on a large scale would still be as promising as they were on lab scale.

6. Assessing the influence of a longer *B. braunii* cultivation time in order to identify the optimal moment for maximal metabolites accumulation. Moreover, the comparison between metabolite accumulation after the first exposure to a new culture media and once the cultures are adapted might be interesting in order to evaluate if a production approach in 2-phases is more preferable than continuous adapted cultures.

7. Assessing the microalgal biomass production with the obtained optimal culture media and, subsequently, subjecting the cultures to stress conditions in order to test if it would be possible to have a higher accumulation of metabolites (2-phases cultivation).

8. Assessing the carotenoid-producing capacity of *B. braunii* race B. It has been stated that race B is also a producer of carotenoids (Grung et al. 1989). Considering the promising results of carotenoids accumulation obtained for *C. onubensis* when it was subjected to stress strategies, an interesting proposal would be to test the response of *B. braunii*, regarding the production of such compounds when it is cultivated under the assayed concentrations of fertilizers as well as under conditions such as UV-radiation or high temperature.

IV. CONCLUSIONS

CONCLUSIONES

Microalgas aisladas del Río Tinto

1. Se aislaron y caracterizaron diferentes microalgas del ambiente ácido del Río Tinto. Especialmente, una de ellas mostró un gran contenido de luteína, por lo que fue seleccionada y finalmente identificada como *Coccomyxa onubensis*. El alto contenido de luteína debe ser la consecuencia de vivir en ambientes extremos donde las microalgas necesitan desarrollar mecanismos especiales, como la expresión de una respuesta antioxidante típica, para hacer frente a tales condiciones.

C. onubensis bajo condiciones de estrés

2. Los cultivos de *C. onubensis* pueden ser cultivados en un rango de temperatura de 25 ºC a 35 ºC, dando como resultado una mejor productividad de biomasa y carotenoides. Sin embargo, existe un valor óptimo a partir del cual el crecimiento de las microalgas comienza a disminuir. Para *C. onubensis* dicho valor fue de 35 ºC, ya que la microalga no pudo mantener su viabilidad celular a 40 ºC. Además, la productividad de carotenoides a 35 ºC fue superior a la obtenida para el control, aunque no en gran medida. Por lo tanto, para aumentar aún más la producción de carotenoides, se debería considerar el cultivo de *C. onubensis* a dicha temperatura en combinación con otros factores de estrés.

3. El cultivo de *C. onubensis* bajo radiación UVA mejoró enormemente las productividades de biomasa y carotenoides. Sin embargo, los cultivos sometidos a la radiación UVB no fueron capaces de compensar el daño celular producido por dicha radiación y, por lo tanto, sus productividades de biomasa y carotenoides se vieron negativamente afectadas. Con respecto a la productividad de lípidos de *C. onubensis*, se puede concluir que la radiación UVA también produjo un efecto altamente positivo, mientras que la radiación UVB solo presentó una ligera mejora con respecto al control.

4. La radiación UV mejoró la capacidad enzimática antioxidante de las enzimas catalasa (CAT), ascorbato peroxidasa (APX) y guaiacol peroxidasa (GPX) en cultivos de *C. onubensis*. La radiación UVA tuvo más influencia en la actividad CAT, mientras que las actividades APX y GPX fueron influenciadas principalmente por la radiación UVB.

Optimización de los medios de cultivo para dos razas de *Botryococcus braunii* (A y B)

5. La realización de una revisión bibliográfica permitió la comparación del medio de Referencia de *B. braunii* (Chu 13, Gouveia et al. 2017) con la concentración promedio calculada de nutrientes de otros medios identificados para crecer microalgas. Esta comparación, junto con la importancia biológica de cada elemento, permitió la identificación de macro- y micronutrientes que debían ser optimizados, dando lugar a 46 nuevos medios de cultivo. Dichos elementos fueron nitrógeno, fósforo, hierro, molibdeno y, considerando su toxicidad, selenio.

6. Mediante el experimento en placas de micropocillos y su posterior validación en matraces Roux se lograron conseguir mejores productividades en base a los nuevos medios definidos.

7. El medio de cultivo finalmente seleccionado, que presentaba una cantidad de nitrógeno siete veces mayor manteniendo la misma concentración de fósforo que el medio de Referencia, mejoró significativamente las productividades de biomasa y carbohidratos para la raza A de *B. braunii* (productora de carbohidratos) en los ensayos en matraces Roux. Con respecto al contenido de exopolisacáridos (EPS), el cual es importante desde el punto de vista de reutilizar la biomasa para una mayor producción, los resultados siguieron el mismo patrón que para los carbohidratos totales.

8. En el caso de la raza B de *B. braunii* (productora de hidrocarburos), los resultados obtenidos de los ensayos en matraces Roux no mostraron diferencias importantes entre los medios de cultivo evaluados y el de

Referencia, en términos de productividad de biomasa e hidrocarburos, por lo que la selección se basó principalmente en la ausencia de selenio.

9. De los resultados obtenidos se concluyó que la falta de selenio en los medios no afectó a la productividad de biomasa y metabolitos (carbohidratos e hidrocarburos) de las razas (A y B) de *B. braunii,* lo cual es un logro importante en términos de seguridad y protección del medio ambiente.

Uso de fertilizantes comerciales en el cultivo de dos razas (A y B) de *Botryococcus braunii*

10. La urea podría utilizarse como una fuente de nitrógeno adecuada para cultivar *B. braunii*, ya que fue posible mejorar sus productividades de biomasa y de producto cuando se adaptó a este compuesto mediante el cultivo por lotes repetidos.

11. La presencia de amonio en los medios implicó que los cultivos sufrieran una disminución continua del pH (especialmente en la raza A), lo que comprometía el crecimiento de *Botryococcus*. Sin embargo, el control de dicho parámetro permitió cultivar *B. braunii* aunque alcanzando una menor densidad de biomasa.

12. Las razas A y B de *B. braunii* fueron capaces de crecer en medios basados en fertilizante, los cuales fueron más fáciles de preparar y sus precios fueron considerablemente más bajos en comparación con los medios de Referencia para ambas razas -A y B- (M44m y Chu, respectivamente).

13. Los resultados finales de productividad sugirieron que cierta limitación de nutrientes, como la que presentaba el medio de cultivo basado en el fertilizante 8-6-6 control, es aceptable para el cultivo de las razas A y B de *B. braunii*, ya que ambas alcanzaron valores de productividad de biomasa similares a sus respectivos medios de Referencia al final del cultivo por lotes repetidos. Por otro lado, la productividad de los productos (carbohidratos e

hidrocarburos) de ambas razas de *B. braunii*, A y B, se mejoró con el uso de ciertas concentraciones de los medios a base de fertilizante, probablemente debido a su menor contenido de nitrógeno. Sin embargo, mientras que para la raza A de *B. braunii*, fue necesaria una concentración de nutrientes aún menor (medio a base de fertilizante 8-6-6(1/2)) para obtener una productividad de carbohidratos ligeramente más alta, en el caso de la raza B, como la concentración de nitrógeno en el medio de Referencia ya era baja, la mejor productividad de hidrocarburos se alcanzó con una concentración 2 veces mayor que la del fertilizante-control (medio basado en fertilizante 8-6-6(2x)).

14. Teniendo en cuenta los principales beneficios relacionados con la preparación de los medios y el coste de los mismos, así como los valores de productividad, se puede concluir que el uso de fertilizantes debe considerarse una herramienta prometedora para la producción sostenible de microalgas.

CONCLUSIONS

Microalgae isolated from Río Tinto

1. Different microalgae were isolated and further characterized, from the acidic environment of Río Tinto. Especially, one of them showed a large content of lutein, which was selected and finally identified as *Coccomyxa onubensis*. The high lutein content must be the consequence of living in extreme environments where microalgae need to develop special mechanisms, as the expression of some typical antioxidant response, to cope with such conditions.

C. onubensis under stress conditions

2. *C. onubensis* cultures can be cultivated in a temperature range from 25 ºC to 35 ºC, resulting in even better biomass and carotenoids productivities. However, there is an optimum value from which microalgae growth starts to decrease. For *C. onubensis* such value was 35 ºC since the microalga could not maintain its cell viability at 40 ºC. Besides, carotenoids productivity at 35 ºC was higher than the obtained for the control, but it was not greatly improved. Therefore, in order to further increase the production of carotenoids, it should be considered the cultivation of *C. onubensis* at such temperature in combination with other stress factors.

3. *C. onubensis* cultivation under UVA radiation greatly improved its biomass and carotenoids productivities. However, cultures subjected to UVB radiation were not able to deal with the cellular damaged produced by such radiation and thus, their biomass and carotenoids productivities were negatively affected. Regarding *C. onubensis* lipids productivity, it can be concluded that UVA radiation also produced a highly positive effect whereas UVB radiation only presented a slight improvement respect to the control.

4. UV-radiation enhanced the antioxidant enzymatic capacity of catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) enzymes in

C. onubensis cultures. UVA radiation had more influence in CAT activity, whereas APX and GPX activities were mainly influenced by UVB radiation.

Culture media optimization for two *Botryococcus braunii* races (A and B)

5. Literature review allowed comparison of *B. braunii* Reference medium (Chu 13, Gouveia et al. 2017) with the calculated average nutrients concentration for the identified media. Together with the biological importance of each element, it was allowed the identification of macro- and micronutrients to be optimized, being eventually defined 46 new culture media. Such elements were nitrogen, phosphorus, iron, molybdenum and, considering its toxicity, selenium.

6. The micro-well plates experiment and its subsequent validation in Roux flasks allowed to accomplish better productivity values based on the new culture media.

7. The finally selected culture medium, which presented a nitrogen amount 7-fold higher but maintaining the same phosphorus concentration as the Reference medium, significantly improved biomass and carbohydrates productivities for *B. braunii* race A (carbohydrates producer) cultivated in Roux flasks. Regarding EPS content, which is important from the point of view of reusing the biomass for further production, results followed the same pattern as those for total carbohydrates.

8. In the case of *B. braunii* race B (hydrocarbons producer), the results obtained from its cultivation in Roux flasks did not show important differences between the evaluated cultures media and the Reference one in terms of biomass and hydrocarbons productivity, so the selection was based mainly on the lack of selenium.

9. It was concluded from the results obtained, that the lack of selenium in the media did not affect any microalgae races (A and B) biomass and

metabolites (carbohydrates and hydrocarbons) productivity, which is an important achievement in terms of safety and environmental protection.

Commercial fertilizers usage in the cultivation of two *Botryococcus braunii* races (A and B)

10. Urea could be used as a suitable nitrogen source to cultivate *B. braunii,* since it was possible to improve its biomass and product productivities when it was adapted to this compound via repeated-batch cultivation.

11. The presence of ammonium in the media entailed that cultures suffered a continuous decrease in pH (especially in race A), which compromises *Botryococcus* growth. However, pH control allowed to cultivate *B. braunii* although lower biomass density was reached.

12. *B. braunii* races A and B were able to grow in fertilizer-based media which were easier to prepare and their prices were considerably lower compared to both *B. braunii* races (A and B) Reference media (M44m and Chu, respectively).

13. Final productivity results suggested that certain nutrient limitation, as the one presented by the fertilizer-based medium 8-6-6 control, is acceptable for the cultivation of *B. braunii* races A and B since they reached similar biomass productivity values to their respective Reference media at the end of the repeated-batch cultivation. On the other hand, products productivity (carbohydrates and hydrocarbons) of both *B. braunii* races, A and B, were improved with the usage of certain fertilizers-based media concentrations, probably due to its lower nitrogen content. However, whereas for *B. braunii* race A was needed to reduce the concentration of nutrients even more (fertilizer-based medium 8-6-6(1/2)) to obtain a slightly higher carbohydrates productivity, in the case of the race B, as the nitrogen concentration in the Reference medium was already low, the highest hydrocarbons productivity was reached with 2-fold the concentration of the fertilizer control (fertilizer-based medium 8-6-6(2x).

14. Considering the major benefits related to media preparation and media cost, and the productivity values, it can be concluded that the use of fertilizers should be considered as a promising tool for the sustainable production of microalgae.

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ANNEXES

List of publications

Published

- **Bermejo, E.**, Ruíz-Domínguez, M.C., Cuaresma, M., Vaquero, I.M., Vega, J.M., Vílchez, C., Garbayo, I. (2018). Production of lutein and polyunsaturated fatty acids by the acidophilic eukaryotic microalga *Coccomyxa onubensis* under abiotic stress by salt or ultraviolet light. Journal of Bioscience and Bioengineering, 125(6): 669-675. doi.org/10.1016/j.jbiosc.2017.12.025.

- Navarro, F., Forján, E., Vázquez, M., Montero, Z., **Bermejo, E.**, Castaño, M.A., Toimil, A., Chagüaceda, E., García-Sevillano, M.A., Sánchez, M., Domínguez, M.J., Pásaro, R., Garbayo, I., Vílchez C., Vega, J.M. (2016). Microalgae as a safe food source for animals: nutritional characteristics of the acidophilic microalga *Coccomyxa onubensis.* Food & Nutrition Research, 60: 30472.

- Forján, E., Navarro, F., Cuaresma M., Vaquero I., Ruíz-Domínguez, M.C., Gojkovic, Ž., Vázquez M., Márquez M.C., Mogedas B., **Bermejo, E.,** Girlich, S., Domínguez, M.J., Vílchez, C., Vega J.M., Garbayo, I. (2015). Microalgae: Fast-Growth Sustainable Green Factories. Critical Reviews in Environmental Science and Technology, 45:1705-1755.

Submitted for publication

- Cubero, R., Wang, W., Martín, J., **Bermejo, E.**, Sijtsma, L., Togtema, A., Barbosa, M., Kleinegris, D.M.M. Milking exopolysaccharide from *Botryococcus braunii* CCALA778 by membrane filtration. Submitted to Algal Research.

To be submitted

- **Bermejo, E.**, Muñoz, A., Ramos-Merchante, A., Vílchez, C., Garbayo, I., Cuaresma, M. Optimized media for growth and carbohydrates and hydrocarbons production in two *Botryococcus braunii* strains.

- **Bermejo, E.**, Montero, Z., González, C., Cuaresma, M. Cost reduction and productivity enhancement in the cultivation of *Botryococcus braunii* strains using commercial fertilizers.

Participation in congresses

- IX Jornadas de Acuicultura en el Litoral Suratlántico, Cartaya (Huelva), Spain (2018).
- 6th Congress of the International Society for Applied Phycology, Nantes, France (2017).
- EU Algaemap Roadmap Conference, Olhão, Portugal (2016).
- XIII National Meeting of Nitrogen Metabolism, Villanueva de la Serena (Badajoz), Spain (2016).
- IV SOLABIAA Congress, Florianópolis, Brazil (2015).
- XV National Congress of Aquaculture (NCA) and I Iberian Congress of Aquaculture (ICA), Huelva, España (2015).
- Alg'n'Chem, Montpellier and Narbonne, France (2014)
- Young Algaeneers Symposium (YAS), Montpellier and Narbonne, France (2014).
- 5th Congress of European Microbiologists (FEMS 2013), Leipzig, Germany (2013).

About the author

Elisabeth Bermejo Padilla was born in Sevilla, Spain, on April the 9th 1985. In 2004 she started her studies on “Marine Sciences” in the Faculty of Marine and Environmental Sciences, University of Cádiz, Spain. During the last year of her studies, she moved to Genoa for an “Erasmus” internship at University of Genoa, Italy. In 2010 she graduated and started a two years MSc on Enviromental, Industrial and Food Biotechnology at Pablo Olavide University, Sevilla. In 2011 she carried out her practices for the Master’s degree in the Environmental Protection department of the energy company CEPSA, Huelva, Spain.

In 2012, after finishing her MSc’s degree she started to collaborate inside the Biotechnology of Algae (Bital) Group within the Chemical and Material Sciences department, University of Huelva. In December 2013 she obtained a contract in the European Project “SPLASH” (Sustainable PoLymers from Algae Sugars and Hydrocarbons) from the Seventh Framework Programme, in which she was working up to January 2017.

In 2014 she started her PhD studies in which she included most of the results obtained from the work carried out as part of the Bital Group as well as from the work carried out in SPLASH, focusing on the optimization of the culture media and conditions to obtain added-value products from microalgae. During her four years of PhD she attended different congresses and conferences, as well as European Project meetings, and she had the opportunity to move to Wageningen for a research internship in AlgaePARC, Wageningen University and Research, The Netherlands. From July to October 2017 she was also working in the European Project “MIRACLES” (Multi-product Integrated bioRefinery of Algae: from Carbon dioxide and Light Energy to high-value Specialties), in which she studied the effects of some fertilizers-based media and the addition of the surfactant Pluronic F68, in the growth and products accumulation of the microalga *Chlorella sorokiniana.*

Elisabeth is currently looking for a job in which she can utilize and further develop the knowledge and skills that she acquired during her PhD studies.

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