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Biomass productivity enhancement and lutein enrichment of an acidic environment microalga

Memoria para optar al grado de doctora
presentada por:

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Carlos Vílchez Lobato

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"Mejora de la productividad de biomasa y enriquecimiento en luteína de una microalga de ambiente ácido"

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Programa de Doctorado Ciencia y Tecnología Química

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"PROFESOR JOSÉ CARLOS VÍLCHEZ MARTÍN"

BIOMASS PRODUCTIVITY ENHANCEMENT AND LUTEIN ENRICHMENT OF AN ACIDIC ENVIRONMENT MICROALGA

"MEJORA DE LA PRODUCTIVIDAD DE BIOMASA Y ENRIQUECIMIENTO EN LUTEINA DE UNA MICROALGA DE AMBIENTE ACIDO"

PROGRAMA DE DOCTORADO

CIENCIA Y TECNOLOGÍA QUÍMICA

MEMORIA PRESENTADA PARA OPTAR AL GRADO DE DOCTOR POR:

Isabel María Vaquero Calañas

Trabajo presentado bajo la dirección de:

Dr. Carlos Vílchez Lobato

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Los hombres ocupan muy poco lugar sobre la tierra…
Las personas mayores no te creerán, seguramente,
pues siempre se imaginan que ocupan mucho sitio.
(“El Principito” - Antoine de Saint-Exupéry)

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(…) Science does not, by itself, advocate courses of human action, but it can certainly illuminate the possible consequences of alternative courses.(…)"

(Carl Sagan)

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"I am not young enough to know everything"

(Oscar Wilde)

ABSTRACT

Cultivation of extremophilic microorganisms has gained interest due to their ability to accumulate and produce high value compounds, namely metabolites, enzymes, carotenoids among others. *Coccomyxa onubensis* is an acid-environment microalga. *Coccomyxa* pigments profile is rich in carotenoids, especially lutein, of which naturally accumulates around 4 to 6 g Kg^{-1} dry weight, that is in accordance with other lutein producing species. Furthermore, *C. onubensis* has the practical advantage of growing well in an extremely selective culture medium at high concentrations of heavy metals, and very low pH which preserves cultures from microbial contamination. This gives the microalga attractive potential as a producer of this photosynthetic pigment.

This thesis was aimed at increasing biomass productivity and carotenoid content of this lutein-rich microalga, *C. onubensis*. **Chapter II** intends to show that acid-environment microalgae can be produced at similar productivities of non-extreme microalgae, with the added advantage of their highly selective culture medium. The existence of carbon concentration mechanisms and conditions that might lead to their increased activity in *C. onubensis* was studied as a tool to increase alga productivity. *C. onubensis* was grown under high and low CO_2 concentrations, showing external and internal carbonic anhydrase activities. Best carbon uptake capacity and growth were showed to occur at acid pH, proving acid-tolerant behavior of *C. onubensis*. Incubation in air followed by shift to high carbon conditions enhanced carbon use efficiency in terms of growth rate and biomass productivity, based on the activity of both carbonic anhydrases. High concnetrations of lutein was accumulated and did not depend on inorganic carbon conditions. Consequently, chapter II showed that repeated cycles of air-incubation and high CO_2-incubation of *C. onubensis* might become a suitable tool to perform production processes of lutein-enriched biomass.

In **Chapter III,** the influence of Cu (II) on productivity and accumulation of value carotenoids of the microalga was studied. Copper was added in range between 0.06 and 0.4 mM, and an increase in algal viability, biomass and carotenoid productivities was obtained. A copper concentration of 0.2 mM was found to be

as the most appropriate one to enhance productivity and lutein accumulation and was used in semicontinuous cultures. *C. onubensis* was found to have great potential as lutein producer when compared to known lutein accumulating microalgae.

At low light intensities, most algae produce more light-harvesting pigments to improve their photosynthetic efficiency while at high light intensities, some algae produce high concentrations of "sunscreen" pigments to protect the cell from excess light. In these terms, the aim of the work described in **Chapter IV** was to study the effect of low to moderate light intensity shifts on lutein accumulation of *Coccomyxa* cultures, doubly aimed at understanding the light-dependent role of main carotenoids in acid-tolerant microalgae and at developing strategies to induce lutein accumulation with applied purposes. *Coccomyxa* reached its maximum growth rates and carotenoid productivities at 400 μmol photons m^{-2} s^{-1} that corresponds to maximal irradiance that reaches the surface of a flat panel reactor placed vertically under summer conditions in southern Spain. Cell density was also shown to influence short-time course evolution of carotenoids, which can be used for increasing lutein content of *C. onubensis* in outdoor production. According to that, the aims of the **Chapter V** were to evaluate the feasibility of outdoor cultivation of the acid-environment alga *C. onubensis* and to assess its potential as alternative source of lutein. *C. onubensis* was cultivated in acid culture medium under springtime outdoor conditions in a pilot tubular photobioreactor in southern Spain. The results showed that *C. onubensis* is able to withstand high temperature levels. Maximum daily biomass productivity was obtained at maximum irradiance of 1000 μmol photons m^{-2} s^{-1}, however higher irradiances were detrimental for the culture. Intracellular lutein content increased at the end of the day, probably related to light absorption functions.

This thesis gives a guideline of some of the parameters that influence both biomass productivity and carotenoid enrichment of the acid-environment microalga *Coccomyxa onubensis*. Therefore, the obtained results should allow to design mass production strategies of a potential large-scale cultivation of acid-environment microalgae and should allow to design lutein-rich biomass production strategies of this microalga.

RESUMEN

El cultivo de microorganismos extremófilos ha ganado interés debido a su capacidad para producir y acumular compuestos de alto valor, como metabolitos, enzimas y carotenoides, entre otros.

Coccomyxa onubensis es una microalga de ambiente ácido rica en carotenoides, especialmente en luteína. *C. onubensis* es capaz de acumular este pigmento fotosintético en alto contenido de manera natural, en torno a 4-6 g Kg^{-1} de su peso seco, valores muy similares a los de especies consideradas productoras de luteína. Además, *C. onubensis* tiene la ventaja de crecer adecuadamente en medios de cultivo extremadamente selectivos, con altas concentraciones de metales pesados y muy bajo pH, previniendo de contaminación microbiana a los cultivos. Esto confiere a *C. onubensis* un atractivo potencial como microalga productora de luteína.

Esta tesis tuvo como objetivo principal incrementar la productividad de biomasa y el contenido en carotenoides de esta microalga. El **Capítulo II** tuvo como propósito mostrar que las microalgas de ambiente ácido pueden generar productividades similares a las de microalgas no extremófilas, con la ventaja añadida de crecer en un medio altamente selectivo. Como herramienta para aumentar la productividad, se estudiaron la existencia de mecanismos concentradores de carbono y las condiciones que pueden provocar un incremento de su actividad. *C. onubensis* creció en altas y bajas concentraciones de CO_2, mostrando actividad de la enzima anhidrasa carbónica, tanto interna como externa. A su vez, la microalga creció bajo distintos valores de pH, mostrando el mejor crecimiento y la mayor capacidad de captura de carbono a pH ácido, lo que proporciona a *C. onubensis* una conducta ácido-tolerante. Cuando los cultivos fueron incubados en condiciones de bajo carbono (solo aire), transfiriéndolos posteriormente a gaseo con alto carbono (aire enriquecido con CO_2), se observó un incremento en la eficiencia de uso del carbono. Este aumento se basó en la actividad de la anhidrasa carbónica y se tradujo en un incremento de la tasa de crecimiento y de la productividad de la microalga. El capítulo II mostró que cultivar *C. onubensis* en ciclos repetitivos consistentes en incubación

en aire seguida de gaseo en aire rico en CO_2 , puede ser una herramienta para la producción de biomasa algal rica en luteína.

En el **Capítulo III** se estudió la influencia del Cu (II) en la productividad y la acumulación de carotenoides. El cobre fue añadido en distintas concentraciones, desde 0.06 a 0.4 mM, provocando en *Coccomyxa* aumentos en productividad y síntesis de carotenoides. La concentración más adecuada resultó ser 0.2 mM, en cultivos semicontinuos. Estos resultados pusieron de manifiesto que *C. onubensis* puede tener un gran potencial como productora de luteína.

A bajas intensidades de luz, la mayoría de las algas producen una mayor cantidad de pigmentos captadores para mejorar su eficiencia fotosintética, mientras que a intensidades altas producen pigmentos antioxidantes para protegerse del exceso de luz. En estos términos, en el **Capítulo IV** se estudió el efecto provocado por cambios de intensidad de luz sobre la acumulación de carotenoides, especialmente luteína, con el doble objetivo de comprender el papel frente a la luz de los principales carotenoides en microalgas ácido-tolerantes y el desarrollo de estrategias para inducir su acumulación. *C. onubensis* alcanzó su máximo crecimiento y productividades de carotenoides a 400 μmol fotones m^{-2} s^{-1}, que corresponde a la máxima irradiancia que alcanza la superficie vertical de un reactor panelar en verano en el sur de España. En este capítulo se mostró que la densidad celular influye en la variación del contenido en carotenoides, lo cual se puede utilizar como estrategia de cultivo exterior.

En esta línea, en el **Capítulo V** se evaluó la viabilidad de cultivos en exterior de *C. onubensis* y su potencial como fuente alternativa de luteína. Para ello *Coccomyxa* creció en un reactor tubular experimental, en condiciones exteriores de primavera en el sur de España. Los resultados mostraron que *C. onubensis* es capaz de soportar altas temperaturas, obteniendo las máximas productividades a 1000 μmol fotones m^{-2} s^{-1}. Por encima de ese valor la luz resultó perjudicial para los cultivos. El contenido en luteína fue máximo al final del día, probablemente debido a su función de absorción de luz.

Esta tesis proporciona una guía de parámetros que influyen en la productividad de biomasa y en el enriquecimiento de carotenoides de la microalga *C. onubensis*. Los resultados obtenidos deben permitir el diseño de estrategias para la producción de cultivos a gran escala de microalgas de ambiente ácido y de producción de biomasa rica en luteína de esta microalga.

CHAPTER I

Introduction, thesis outline and aims

"La ciencia es la progresiva aproximación del hombre al mundo real."

(Max Planck)

1. MICROORGANISMS LIFE OF EXTREME ENVIRONMENTS

Whereas there is no general agreement on how to define an extreme environment, the term is commonly used for any setting that exhibits life conditions detrimental or fatal to humans or higher organisms due to its physicochemical characteristics. These include environments in which physical and chemical conditions differ significantly from those that humans consider 'normal'. That is, temperatures between 20°C and 35°C, neutral pH, pressures around 1 atm, low salinity and adequate concentrations of nutrient (Madigan and Marrs 1997).

Extreme environments can be categorized into several classes. Organisms capable of growth and reproduction in those settings are called extremophiles. Madigan (2003) defined the extreme environments to be typically constant in their physicochemical properties, pointing that constancy as the aspect that in large part forces an extremophilic lifestyle on the local microflora. By this means, most of the extremophile organisms do not just tolerate their extreme living conditions, but actually require them for optimal growth and metabolism (Madigan and Marrs 1997). Most of the extremophiles are unicellular organisms. As a rule, extreme environments show a low diversity of multicellular organisms and only few animals are able to withstand the harsh conditions of particular extreme environments.

Microalgae can be easily found in different extreme habitats, ranging from hyper-saline environments to alkaline or acid environments. Microalgae are unicellular eukaryotic microorganisms (between 1 and 50 μm), which can form colonies or live as individual cells. They are photoautotrophic organisms capable of using light to metabolize carbon dioxide inside energy-rich organic compounds. About thirty five thousand species have been described, but the biodiversity of microalgae has been estimated in two hundred thousand species, including many extremophiles (Norton et al. 1996).

Terms describing extremophiles usually combine its environment-specific prefix with the suffix "-phile" (greek word for "-loving"). Replacing the suffix "-phile" by

"-tolerant" implies that an organism tolerates rather than requires the respective conditions. However, there is no general agreement to define that faculty of tolerance. While some authors describe those organisms as capable to live in both “normal” and specific extreme conditions, other authors describe them as those organisms that are able to survive under its specific environment but having its optimum at more moderate conditions. Different extreme environments and theirs inhabitants are described next.

Extremely hot environments

Environments periodically or consistently above 40°C are called extremely hot environments (Stetter 1998). Thermophiles are named those organisms that display optimum growth between 45-80°C and hyperthermophiles when optimum growth occurs above 80°C. Organisms dependent on moderate temperatures between 10°C and 50°C (optimum 30-40°C) are called mesophiles. (Martinko and Madigan 2006).

Prokaryotes are known to be able to grow at higher temperatures, thus, hyperthermophilic life is mainly represented by deeply branching bacteria (e.g. Aquifex, Thermotoga) and archaea (e.g. Sulfolobus, Methanothermus), (Stetter 1998). The highest temperature for life that has been reported is 122 ºC, for the archae Methanopyrus kandleri (Takai et al. 2008). A thermal degradation of cellular biomacromolecules is the main problem for organisms living in hot environments. That degradation may result, for example, in unfolding of proteins. Some strategies to avoid those problems have been reported, including introduction of proline residues at particular sites into proteins (Imanaka 2008). Many scientists believe that prebiotic molecules and the first living organisms originated in hot environments, thus, hyperthermophiles may provide insight into the early stages of life on Earth.

An example of extremely hot environment is Grand Prismatic, located in the Midway Geyser Basin of Yellowstone National Park (**Figure I.1**), is the largest hot spring in the park and the third largest in the world. Only hyperthermophilic bacteria and archaea are able to live in the nearly boiling water of this hot spring.

Extremely cold environments

These environments are generally defined as those with temperatures below 10°C, such as sea-ice, cold Polar Regions, permafrost soils and glaciers (**Figure I.1**). Cryophiles or psychrophiles are called the organisms that are capable to live under such conditions. According to Morita (1975), these organisms depend on low temperatures (< 0°C to 20°C) and have a growth optimum below 15°C. The all three domains are represented in extremely cold environments. Although bacteria is the most abundant group (especially cyanobacterial genera), also archaea (such as Methanogenium and Methanococcus), protozoans, fungi and microalgae (as *Chlamydomonas nivalis*) have been found (D'Amico et al. 2006, Garrison 1991). A bacteria living in permafrost soil and in sea-ice seems to be the microorganism that is able to support lowest temperature, around −20°C (D'Amico et al. 2006).

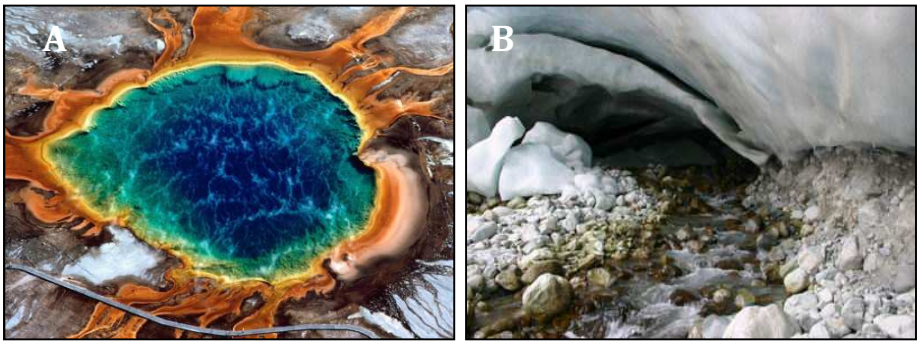

Figure I.1. Extremely hot (A) and cold (B) environments. A. Grand Prismatic (Yellowstone National Park). B. Subglacial stream flowing from the Glacier du Mont Mine, Swiss Alps.

Psychrophiles must face several cold-induced challenges, including decreased membrane fluidity, reduced enzyme activity, decreased rates of transcription, translation and cell division, among others (D'Amico et al. 2006). To overcome these negative effects, psychrophiles strategies involve modifications of the cell membrane composition toward a higher content of unsaturated, branched, or short-chain fatty acids, and large polar head groups (Chintalapati et al. 2004). Psychrophiles also synthesize specific antifreeze proteins, trehalose, and

extracellular polymeric substances (EPS), which play an important role as cryoprotectants to keep the intercellular space liquid and protect the DNA at temperatures below water's freezing point (D'Amico et al. 2006).

High-pressure environments

High-pressure environments are those under extreme hydrostatic or petrostatic pressure. Organisms settings under such conditions are called piezophiles or barophiles, i.e. dependent on pressures greater than atmospheric pressure, 1 atm. (Yayanos 1998).

High pressures affect biological systems, for instance by making the structures more compact (e.g., membranes). Hydrostatic pressure has been shown to exert a considerable influence on many protein-protein interactions, the efficacy of enzymatic catalysis, replication, and translation. Piezophiles have therefore evolved specific adaptations, for example, in terms of membrane lipid composition and cell division. Pressure has also a significant effect on microbial-mediated redox reactions, and metabolic versatility appears to be a specific adaptation to deep environments (Fang and Bazylinski 2008, Lauro and Bartlett 2008).

Hypersaline environments

Environments with salt concentrations greater than that of seawater (35‰) are called hypersaline environments. Organisms that live on such extreme environment are called halophiles. Most halophiles are classified into the archaeal or bacterial domains (Grant et al. 1998, Gunde-Cimerron et al. 2005). However, there are also some eukaryotes, mainly microalgae as *Dunaliella salina*. In the bacterial domain, halophiles represent many different taxonomic groups, whereas those in the archaeal domain fell into a single order and family, namely, Halobacteriales, Halobacteriacae, respectively (DasSarma and DasSarma 2008).

The most significant challenge for halophiles is to prevent the loss of water from the cell into the saline environment, and the accumulation of excess salt concentrations within the cell. To deal with these challenges, halophilic bacteria

and algae accumulate organic compatible solutes, polar, highly soluble molecules uncharged at physiological pH, such as amino acids and their derivatives to counterbalance the osmotic pressure of the surrounding medium (Galinski 1993, Oren 1999).

Extremely dry environments

Environments without free water are considered extremely dry environments and they include hot and cold deserts, and some terrestrial endolithic habitats. An example is the Atacama Desert that is one of the driest places on Earth (**Figure I.2**). Organisms dependent on very dry environments are termed xerophiles.

Figure I.2. The Atacama Desert (Chile).

A strategy to cope with prolonged periods of dryness is to enter the state of anhydrobiosis, which is distinguished by little intracellular water and no metabolic activity. Organisms that can become anhydrobiotic are found among bacteria, yeast, fungi, plants, and even animals such as nematodes and the brine shrimp *Artemia salina* (Rothschild and Mancinelli 2001). The most xerophilic organisms known, thrive in foods preserved by some form of dehydration or enhanced sugar levels, and in hypersaline environments where water availability is limited by a high concentration of salts (Grant 2004). Whereas the former are dominated by xerophilic filamentous fungi and yeasts, high-salt environments are almost exclusively populated by prokaryotes.

High-radiation environments

Environments exposed to high doses of ionizing radiation are high-radiation environments. Ionizing radiation is radiation with sufficient energy to ionize molecules, most commonly ultraviolet (UV) radiation and natural radioactivity. When such radiation passes through living matter, ions and free radicals are produced that react rapidly and modify molecules, being potentially detrimental for life, mainly due to DNA damage resulting from the generation of reactive oxygen species (Cox and Battista 2005). Organisms that are capable of resisting high doses of ionizing radiation are called radio-resistant. There is no clear pattern of evolution among ionizing-radiation-resistant species, and they occur scattered over the three domains of life. The bacterium *Deinococcus radiodurans* (Cox and Battista 2005) and the archaeon *Thermococcus gammatolerans* (Jolivet et al. 2003) are two known redioresistant microorganisms. Increasing the numbers of genome copies or improved enzymatic genome-repair process are some examples of strategies to adapt to such extreme environment.

Alkaline environments

Alkaline environments are called those environments above pH 9 and the organisms dependent on that are termed alkaliphiles (Horikoshi 1998). Alkaline habitats pose particular necessities for the organisms living in, for example, the ability to maintain internal pH at a level not higher than pH 8.5 (Horikoshi 1998).

Some strategies to achieve this include buffering cell cytoplasm using rich polyamines in amino acids with positively charged side groups (lysine, arginine, and histidine), in that way introducing acidic compounds to protect the cell from the alkaline environment (e.g. acidic amino acids and uronic acids) or Na^+-ion channels actively driving the transfer of protons into the cell. The enzymes of alkaliphiles find a variety of industrial applications. For instance, alkaline proteases, protein-hydrolyzing enzymes, are widely used in the detergent industry.

Acidic environments

Especial attention in this thesis is given to acidic environments, generally defined as environments below pH 5, including sulfuric pools, geysers and mining operating areas (Baffico et al. 2004). **Figure I.3** shows two extreme acidic environments. The natural oxidation and dissolution of the sulfidic minerals exposed to oxygen and water results in acid production, and the process can be greatly enhanced by microbial metabolism (Gonzalez-Toril et al. 2003). At the same time, low pH makes metal solubility in water possible, usually cationic forms of many heavy metals, and as a result acidic water tends to have high concentrations of them.

Organisms dependent on acidic conditions are called acidophiles. Bacteria, archaea, fungi and algae are frequently set up under such acidic conditions (Johnson 1998). Some prokaryotes examples are *Ferroplasma acidophilum*, a sulfuric-acid producing archaeon involved in acid mining, and *Thiobacillus ferrooxidans*, the most abundant and active iron/ sulfur-oxidizing bacterium.

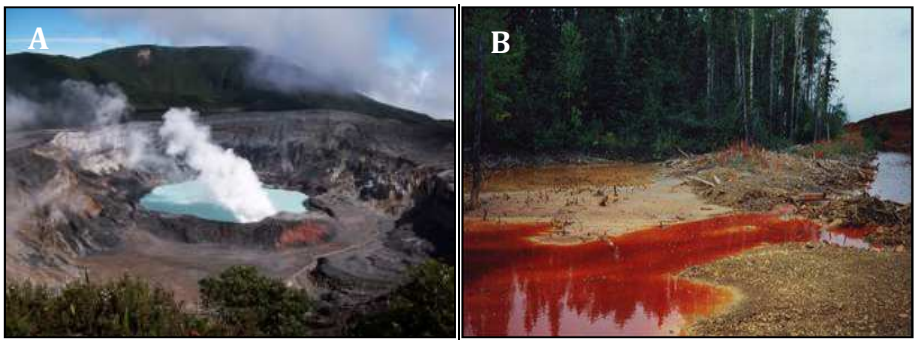

Figure I.3. Two different acidic environments. A. Poas Volcano (Alajuela, Costa Rica), and B. the Tinto River (Huelva, España)

Microalgae are commonly found under acidic environments, including filamentous and unicellular forms, and diatoms (Gyure 1987, Lopez-Archilla 1995). Some examples of acidophilic phototrophic microalgae include *Euglena*

spp., Chlorella spp., Chlamydomonas acidophila, Ulothrix zonata and the microalga studied in this work, *Coccomyxa onubensis*, among others.

The major challenge for these organisms is to maintain their internal cellular pH at a constant, circumneutral level, that is, around pH 7. Individual strategies encompass (i) reinforcement of the cell membrane, (ii) limiting proton diffusion into the cell, (iii) secretion of buffer molecules capable of sequestering protons, including, for example, basic amino acids (lysine, histidine and arginine), and (iv) the ability to actively pump protons out of the cell (Johnson 1998, Rothschild and Mancinelli 2001).

An example of acidic environment is Tinto river, located in Huelva (south of Spain), whose characteristics addressed many scientists to use it as a study model that might explain the possible existence of life on Mars, due to similarities found in between the river and the planet chemical peculiarities (Fernandez-Remolar et al. 2003).

2. ACIDIC MICROALGAE: TINTO RIVER

Tinto River (Huelva, southwester Spain) flows from the Iberian Pyritic Belt down to the Atlantic Ocean (100 km long) and is one of the most extensive examples of extreme acidic environments (Fernandez-Remolar et al. 2003). Tinto River (**Figure I.4**) is an unusual ecosystem due to its size, a rather constant acidic pH (mean value 2.3), high concentrations of aluminum and heavy metals, especially Fe, Cu and Mn. The concentration of the dissolved elements and traces in stream surface waters were analyzed by Ferris et al. (2004). They are shown in **Table I.1.** Tinto River is also characterized by the high level of microbial diversity, mainly eukaryotic (Aguilera 2013).

High concentrations of ferric iron and sulfates are found in its waters as products from pyrite biooxidation, the main mineral component of the system. Ferric iron is maintained in solution due to the acidic pH of the river and is responsible for

the constant pH and its particular water´s red color, probably the main distinctiveness of this river.

Table I.1. Major and trace element concentration for stream water. Values may change with the seasons, mostly due to precipitations. Source: Ferris et al. (2004).

Elements	Concentration (mM)	Detection limit (mM)
Ca	2.48	0.005
Fe	5.78	0.001
K	0.0171	0.010
Mg	9.05	0.005
Na	0.922	0.022
S	27.6	0.006
Si	0.584	0.001
Al	3.18	0.005
As	b.d.	0.001
Ba	0.0002	0.0001
Cd	b.d.	0.0002
Co	0.0200	0.0003
Cr	b.d.	0.0006
Cu	0.0104	0.0002
Li	0.0275	0.001
Mn	0.581	0.0002
Mo	b.d.	0.0002
Ni	0.0044	0.001
Pb	b.d.	0.0005
Sr	0.0091	0.00002
V	b.d.	0.0002
Za	0.0844	0.0002

Tinto River is a very diverse ecosystem which has generated very different environments, which is evidenced by the proliferation of very diverse organisms that build their own ecosystems, although interdependent and interconnected (Lopez-Archilla 2005). Investigations have proved that chemolithotrophic prokaryotes are present in high numbers and also involved in maintenance activities of the system (Lopez-Archilla et al. 2001, Lopez-Archilla 2005). Eighty percent of the prokaryotic diversity in the water column corresponds to three bacterial genus: *Leptospirillum* spp., *Acidithiobacillus ferrooxidans* and *Acidiphilium* spp., all of them conspicuous members of the iron cycle (Gonzalez-

Toril et al. 2003). In addition, fungi, heterotrophic protists and bacteria act as consumers and decomposers (Lopez-Archilla 2005).

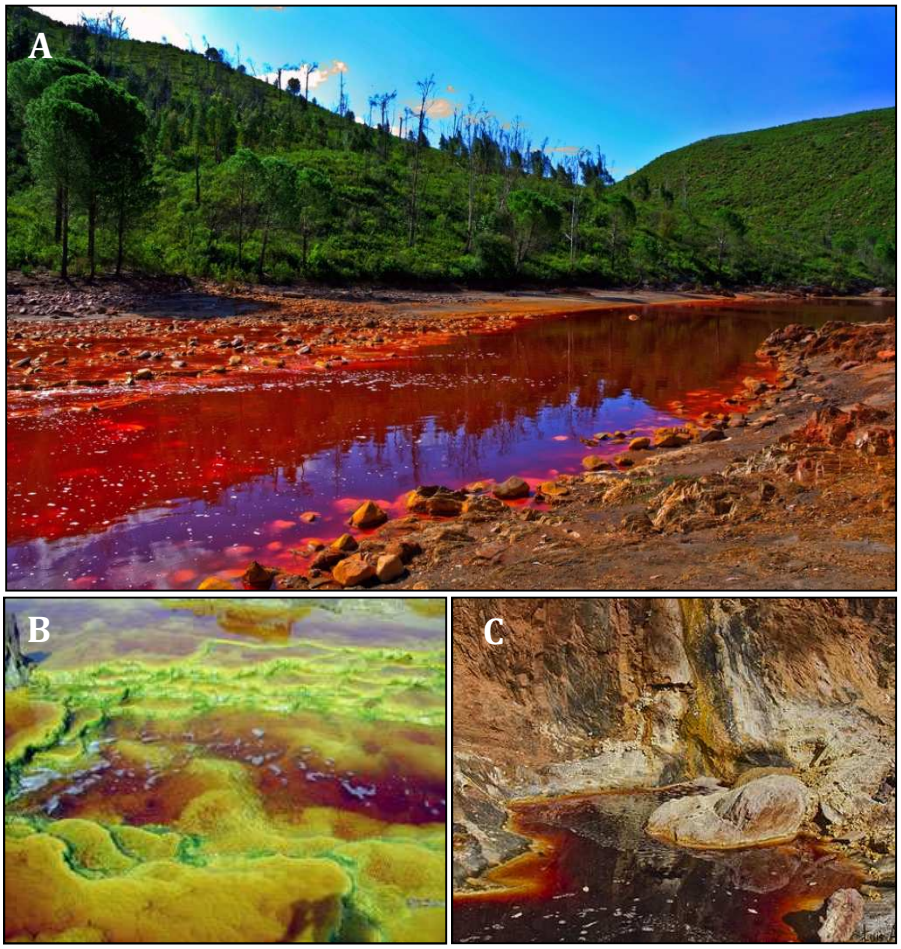

Figure I.4. General view of Tinto River (A). Photosynthetic biofilms formed by acidic microalgae (B). Source of the Tinto River in Peña del Hierro (C).

Acidophilic Microalgal Diversity

Besides its extreme physicochemical water characteristics, what makes Tinto river a unique acidic environment is the unexpected degree of eukaryotic diversity, especially microalgae, found in its waters (Amaral et al. 2002). In fact, eukaryotic organisms are the principal contributors of biomass in the habitat (Garbayo et al. 2012).

Members of the phylum Chlorophyta followed by two filamentous algae (belonging to the genera *Klebsormidium* and *Zygnemopsis*) are the most frequent species (Aguilera 2013). Species related to the genera *Dunaliella* and *Cyanidium* (Rhodophyta), well known for their high metal and acid tolerance, have been also described in the river (Visviki and Santikul 2000). There are not many numbers of diatoms present in the Tinto River. Such low diversity of diatoms in comparison with the diversity found in neighboring freshwaters, supports the idea that there is a threshold between pH 4.5 and 3.5 in which many species of diatoms are eliminated (Aguilera 2013). **Figure I.5** shows two different species of eukaryotic organisms found in the Tinto River, diatoms and the green microalga *Chlamydomonas spp.*

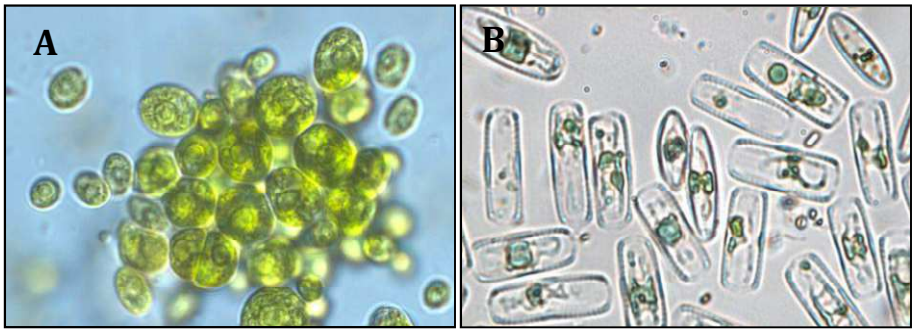

Figure I.5. Microscopy photograph of eukaryotic species isolated from Tinto River: A. Green algae *Clamydomonas spp*. B. Diatoms. Source: Aguilera (2013).

3. PHYSIOLOGICAL ADAPTATIONS OF ACID ENVIRONMENT MICROALGAE

As previously commented, Tinto river is acidic due to a diversity of iron and sulfur-dependent redox reactions which yields sulphuric acid, therefore a low pH river which keeps many metal species in soluble form. The presence of solved metal ions cause stress in different forms to living microorganisms in that acidic habitat. Particularly, metals can be involved in ROS generation (Stohs and Bagchi 1995, Vaquero et al. 2012). Iron ionic species are involved in the so-called Fenton reaction, an intracellular cycle which continuously interferes the antioxidative action of specific enzymes, superoxide dismutase and ascorbate

peroxidase, yielding hydroxyl radicals, the most damaging ROS, which act on DNA, proteins and cell membrane lipids (Dietz et al. 1999) altering their functions for life.

In order to tolerate such adverse conditions, a variety of microorganisms including microalgae, in those acidic habitats developed adaptation mechanisms which specifically allow the cells for resisting high osmotic pressure due to the very high external proton concentration, high concentrations of metal ionic species of iron, copper, zinc and others, and for growing at very low inorganic carbon availability.

Life in so intense stress generating situation seems very difficult, at least in the light of current biochemical knowledge, and instead there is considerable diversity of photosynthetic eukaryotes adapted to such conditions (Amaral et al. 2002). From scientific curiosity, some questions arise whose resolution would help to understand mechanisms of life in extreme conditions, perhaps primitive mechanisms at the base of evolution to adaptation to changing physicochemical conditions. Some of the most relevant questions are: (a) what are the mechanisms by which acidic environments microalgae do control the huge proton gradient across the plasma membrane, without consuming large amounts of energy to maintain cytosolic pH neutral?; (b) what are the mechanisms by which microalgae do capture (or exclude) metal cations at its outer membrane and / or prevent their passage to the cytosol? The metal tolerance by acidic environments microalgae has been shown long ago by several authors (Wood and Wang 1983, Albertano and Pinto 1986), but in practice little or nothing is known about the nature of these mechanisms; and (c) what relationships do exist between eukaryotic microalgae and chemolithotrophs to enable microalgae for having C, N and / or P enough for basal activity?

From a biotechnological perspective, the most intriguing questions are limited to the identification of microalgal metabolic responses to acidic environments oxidative stress conditions (namely, accumulation of metabolites of interest) and the chances of finding suitable conditions for mass production of such microalgae (fast algal growth). Some of these issues are: (a) what sources of

carbon and inorganic nitrogen are preferably used by acid environments microalgae?; (b) what strategies can get acid environment microalgae make the most efficient use of inorganic carbon?; (c) do stress conditions induce a consistent response of antioxidant nature?, (e) and, in such, if so, what bioactive antioxidant molecules can be produced?

Therefore, getting knowledge on the ecophysiology of these acid environment microalgae is particularly attractive since some of the microorganisms specific responses might be of biotechnological utility in the fields of antioxidant molecules production through massive controlled production of a given extreme microalga and of value metal recovery, among others.

Cytosol of acid environment microalgae keeps neutral, spite of the high external proton concentration. Some microalgae are adapted to live at pH values lower than 1. To compensate the that high proton pressure, cell permeability coefficient for protons ranges remains in low values around 100 nms^{-1}, while for non-extreme microorganisms it ranges from 10 to 50,000 nm^{-1} in order to maintain intracellular pH neutral. In acid environment microalgae the cell membrane is apparently very impermeable, so protons are actively pumped out against a passive proton flux on low energy expenses. In some acid environment microalgae, a net positive charge of cell membrane has been described, which reduces energy costs for pumping out protons (Gimmler 2001, Gross 2000).

The high osmotic pressure due to the large proton gradient, the high concentration of sulphate –in Tinto river it can arise 3 g L^{-1}- and the large water content in several metal ions –iron, copper, zinc and others- should drive acid environment microalgae to show certain halotolerance level. The literature references in that respect are scarce, though such that halotolerance response has been probed for some acidophile microalgae (Gimmler y Weis, 1992). Such that fact allows us in this work for holding the thesis that acid environment microalgae might show enhanced biosynthesis of some metabolites usually produced in some microalgal species, namely marine microalgae, in response to high levels of salinity; carotenoids, glycerol and fatty acids are among those

accumulated molecules (Ben-Amotz and Avron 1983, Borowitzka and Borowitzka 1988, Richmond 2004, Ranga Rao et al. 2007).

Because of the low external pH of Tinto River, inorganic carbon (Ci) is mostly as CO_2 at air-equilibrium concentration. Ci concentration is substantially lower at acid pH than in higher-pH waters with bicarbonate as main inorganic carbon source. That situation denotes that carbon availability is a limiting factor for the microorganisms inhabiting acidic waters, limiting its potential for systematic mass production.

The major challenge for the acid-environment microalgae is to maintain their internal cellular pH at a constant, circumneutral level (around pH 7) using appropriate biochemical systems that withstand the proton gradient across the plasma membrane (Gross 2000). Algae have adapted to these challenges through the development of CO_2 concentration mechanisms (CCM). The CCM is a biological adaptation to low carbon dioxide concentrations in the environment. It is a mechanism which augments photosynthetic productivity in algal cells by increasing internal levels of inorganic carbon many times over the environmental concentration of carbon dioxide.

In that respect, knowledge about CO_2 concentrating mechanisms in acidic-environment microalgae is scarce. Verma et al. (2009) proposed the presence of an external carbonic anhydrase (CAext) in a *Coccomyxa* although with a carbon transport facilitating role rather than a concentrator function. These results indicate that there is species-specific variation in the induction mechanism of CCM depending on physiological and ecological conditions (Giordano et al. 2005, Raven 2010).

The extreme conditions of Tinto river are rather recent, so the adaptation of these complex organisms which can be found in neutral aquatic environments nearby, to proton gradients between the inner (pH near neutrality) and outer part of the membranes (pH below 3) of five orders of magnitude and high concentrations of very toxic heavy metals (As, Cu, Zn, Cr, Al), must be relatively fast and efficient (Aguilera and Amils 2005).

Finally, light is another relevant parameter influencing growth of acid-environment microalgae. Tinto River is a low water flow river. The deepest areas along most of the river length are about 1-2 meters, with the exception of the last part of its way out to the ocean. That means that light penetrates reaching to most of the waster column, in depth. Therefore, photosynthetic microorganisms meet most of the requirements needed to proliferate (Lopez-Archilla 2005). In that geographical area of Huelva, irradiance is as high as 2000 μmol photons m^{-2} s^{-1} in summertime. As said. Microalgae have to manage their viability in highly acidic environments with a limited supply of carbon dioxide for photosynthetic function, because of the absence of bicarbonate in the medium. Therefore, some microalgae grow mainly in almost terrestrial locations to increase CO_2 availability in contact with air, or actively move in the water column to areas of greater availability of CO_2. Consequently, these microorganisms are exposed to the high light irradiance of that geographical location and, particularly in summertime, to the associated UV radiations as well. For these reasons, a typical antioxidant response which included an increase accumulation of light-dissipating carotenoids should be expected. From the biotechnological point of view, such that response might be properly controlled in order to use it for production of value carotenoids.

In this item of the Introduction, a brief view has been made of the different adaptation mechanisms of acidic environment microalgae to the light of what has been previously published in literature. In that respect, acidophilic microalgae are gaining interest over last years, but still the number of papers related to physiology of these adapted microorganisms is scarce, hardly some dozens of papers in last three years.

4. MICROALGAL CAROTENOIDS

Commercial interest in microalgae has been increasing in last decades. Nowadays, there are numerous commercial applications of microalgae. Microalgae are a potentially rich source of a vast array of chemical products with applications in nutritional values of animal feed and food, cosmetic,

pharmaceutical and even fuel industries. Moreover, they are cultivated as a source of highly valuable molecules, such as carotenoids, vitamins and antioxidants, fatty acids, and specifically poly-unsaturated fatty acids (Spolaore et al. 2006).

Carotenoids are the most common pigments in nature and are synthesized by all photosynthetic organisms and fungi. Humans cannot synthesize carotenoids although can ingest them in diet and metabolically transform some of them into several derived forms (Jin et al. 2003).

Structure and types

More than 700 carotenoids have been described. Most of them are xanthophylls, which consist of 40 carbon atoms and one or more oxygen moieties (such as lutein and astaxanthin). Hydrocarbons called carotenes, composed solely of carbon (C) and hydrogen (H), constitute less than 10% of all carotenoid species (as β-carotene). They consist of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining non-terminal methyl groups are in a 1,5 – positional relationship. All carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure, having a long central chain of conjugated double bonds, by (I) hydrogenation, (II) dehydrogenation, (III) cyclization, or (IV) oxidation or any combination of these processes.

The colors of these pigments range from yellow to red, and some well-known examples are lycopene (found in tomato fruit), zeaxanthin (maize corn), β-carotene (carrots and the microalga *Dunaliella salina*) and astaxanthin (giving salmon its pink color). But carotenoids are not just a group of natural pigments, having important functional properties. They are considered essential molecules for life due to its functions in photosynthesis, both light-harvesting and photoprotection from excess light by scavenging reactive oxygen species (ROS) like singlet oxygen and other free radicals (Murthy et al. 2005).

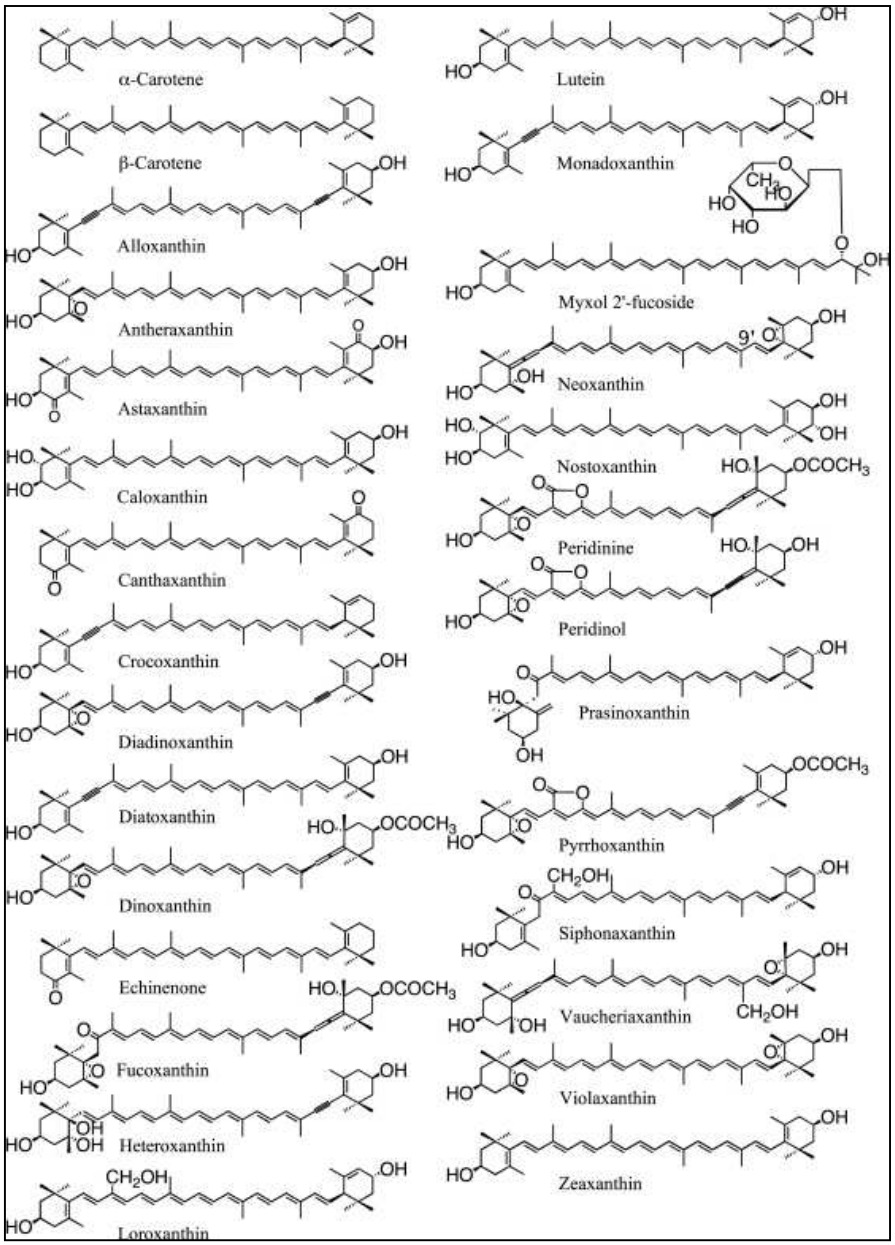

Figure I.6. Structures of main algal carotenoids. Source: Takaichi (2011).

Many kinds of carotenoids are found in algae. Structures of some important carotenoids in algae are illustrated in **Figure I.6**. Among them, about 30 types

may have functions in photosynthesis, and others may be intermediates of carotenogenesis or accumulated carotenoids (Takaichi 2011).

Carotenoids can be used as chemotaxonomic markers in algae since some carotenoids are found only in some algal divisions or classes (Rowan 1989, Mackey et al. 1996). Green algae (Chlorophyta) principally contain β-carotene, violaxanthin, neoxanthin and lutein, as well as chlorophyll *a* and *b* with land plants. Some classes contain additional carotenoids, such as loroxanthin, siphonaxanthin and prasinoxanthin, which are derivatives of lutein, and are class specific.

Carotenoid biosynthesis pathways

Carotenoids are isoprenoids. Despite their diversity of functions and structures, all isoprenoids, including carotenoids, derive from the common five-carbon (C_5) building units' isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP).

Until recently, it was generally assumed that all isoprenoids were synthesized from acetil-CoA via the classical mevalonate pathway to the central precursor isopentenyl diphosphate. A few years ago, a totally different route, in which mevalonate, is not a precursor and where IPP is formed from glyceraldehydes 3-phosphate (GAP) and pyruvate was found in bacteria, green algae and plants (Rohmer et al. 1993, Rohmer et al. 1996, Schwender et al. 1996). This pathway was originally named non-mevalonate pathway or Rohmer pathway, but its name was change after known its first intermediate, deoxyxylulose (DX) 5-phosphate (DXP pathway). Therefore, two biochemical pathways for the synthesis of IPP, and therefore carotenoid biosynthesis, are recognized today.

Figure I.7 shows the general carotenid biosynthetic pathways. The first stage is the synthesis of IPP, common precursor of all terpenoids. In a second phase, the IPP is isomerized to dimethylallyl diphosphate (DMAPP), a compound that undergoes the sequential addition of three molecules of IPP. That reaction produces a compound with 20 carbon atoms that is called geranylgeranyl diphosphate (GGPP) through the enzyme GGPP synthase.

Then, two molecules of GGPP are condensed to form phytoene, a reaction catalyzed by the enzyme phytoene synthase. Phytoene is the precursor compound for all carotenoids and undergoes several desaturations to yield phytofluene, ξ-carotene, neurosporene and finally lycopene. During the third stage, lycopene undergoes a series of metabolic reactions that produce cyclic carotenoids and xanthophylls. For instance, β-carotene is produced due to a cyclization of γ-carotene by the action of lycopene β-cyclase.

Biological function and human health

For photosynthesis, both carotenoids and chlorophylls are necessarily bound to peptides to form pigment-protein complexes in the thylakoid membrane. Some carotenoids are present in the most reaction-center complexes (RC) and the light-harvesting complexes (LHC) of photosystem I (PSI) and photosystem II (PSII). Therefore, carotenoids may act as accessory pigments in light harvesting functions during the light phase of photosynthesis and are also able to photoprotect the photosynthetic machinery from excess light by scavenging reactive oxygen species (ROS) like singlet oxygen and other free radicals (Murthy et al. 2005, Sandmann and Mitchell 2001) .

Under stressful conditions (mainly harmful effect of high light intensities) microalgae viability depends on preventing the overstimulation of photosynthetic RC. Both plants and green algae are able to regulate the capture and flow of excitation energy in the photosynthetic centers through the so-called xanthophyll cycle, also called cycle of VDE or violaxanthin cycle. Through this mechanism, the xanthophylls photosynthetic organisms protect against harmful effects of light (Eskling et al. 1997). Chemically, the xanthophyll cycle is the cyclical interconversion of violaxanthin, antheraxanthin and zeaxanthin. Under high light conditions, violaxanthin is modified to zeaxanthin by the catalytic action of violaxanthin de-epoxidase (Vde) through antheraxanthin. Zeaxanthin is used for dissipating of excess energy from excited chlorophylls (Lohr and Wilhelm 1999). Under non-stress and dark conditions, zeaxanthin epoxidase (Zep) catalyzes the conversion of zeaxanthin to violaxanthin through

antheraxanthin. Violaxanthin is found in the peripheral LHC of PSII (Hager and Holocher (1994).

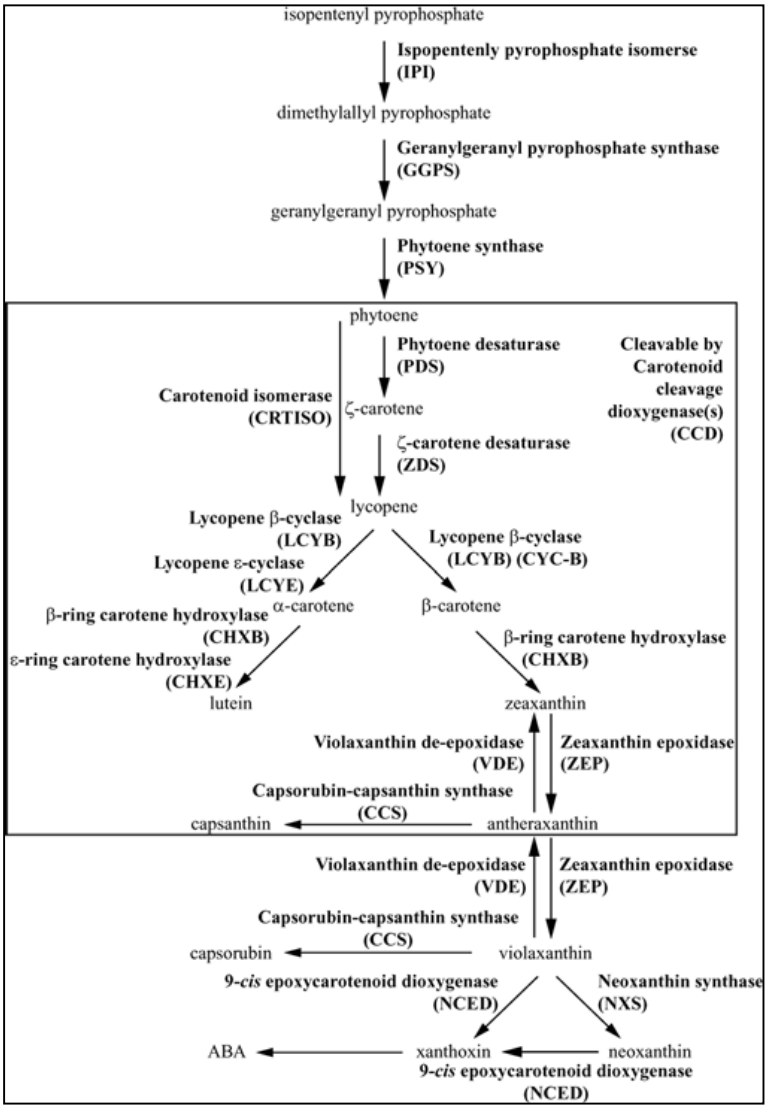

Figure I.7. Diagram of carotenoid biosynthesis pathway. Source: Brian Just, Ph.D. Thesis (2004).

In human health, the most relevant biological functions of carotenoids are linked to their antioxidant properties, which directly emerge from their molecular structure. In recent years, the understanding of ROS-induced oxidative stress mechanisms and the search for suitable strategies to fight oxidative stress has become one the major goals of medical research efforts. A number of studies have been reported which implicate oxidative stress involvement in degenerative pathogenesis, e.g., Alzheimer and Parkinson (Murthy et al. 2005, Guerin et al. 2003). A carotenoid-enriched diet has been found to diminish the risk of suffering from degenerative diseases (Guerin et al. 2003). Additionally, the benefits of carotenoids such as lutein, β-carotene and lycopene, to human health have been shown based on the positive impacts of the antioxidant bioactivity of carotenoids in inmunoresponse modulation, in signaling transduction between cells and in anti-inflammatory response mechanisms (Giovannucci et al. 1995). These positive consequences are the result of either the direct chemical action of carotenoids on biological molecules or through expression of different genes involved in antioxidant responses (Guerin et al. 2003). The main biological functions of carotenoids and benefits to health were widely described by Vilchez et al. (2011) (**Table I.2).**

Lutein is a relevant example of a carotenoid which can be produced with microalgae (Fernandez-Sevilla et al. 2010). Lutein is a xanthophyll that has gathered increasing attention on the grounds of recent studies that show how an adequate intake of this product might help to prevent or ameliorate the effects of degenerative human diseases, such as age-related macular degeneration (AMD) or cataract and also in skin health. It is the only carotenoid that is absorbed in the bloodstream after ingestion and accumulated in the human retina. Lutein is assumed to have a protective effect due to its ability to filter out blue light, therefore inactivating singlet oxygen and quenching active radicals through its antioxidant role. Lutein is largely consumed as food colorant and its yearly sales account for $150,000,000 in the US only. Currently, lutein is obtained from the petals of marigold after an extraction process.

Table I.2. Biological functions, benefits to health and applications of the main carotenoids. (Vilchez et al. 2011).

Carotenoid	Functions and benefits to health
Lycopene	In the prevention of atherosclerosis and acute and chronic coronary syndromes Against prostatic hyperplasia and prostate cancer
β-carotene	Provitamin A function In colorectal cancer Photoprotection of skin against UV light In the prevention of coronary syndromes (accurate and chronic)
Astaxanthin	In benign prostration hyperplasia and prostate and liver tumors Anti-inflammatory properties
Zeaxanthin	Active against liver neoplasm In the prevention of cataracts Helps to maintain a normal vision function To prevent age-related macular degeneration
Lutein	In the prevention of cataracts In prevention of retinitis In the prevention of acute and chronic coronary syndromes and stroke Helps to maintain a normal vision function To prevent age-related macular degeneration To avoid gastric infection by *H.pylori*

Applications and Commercial Value

Carotenoids are traditionally and widely used in food and animal feed as additives due to their color properties (Becker 2004). They are used to reinforce fish color, which increases consumer's perception of quality. An example is the addition of carotenoids to fish feed to impart color to farmed salmon. The

nutraceutical properties of carotenoids also attracted attention of the food industry. However, among the more than 700 carotenoids known, only a few have been used for commercial purposes (mainly β-carotene, lutein, zeaxanthin, astaxanthin and lycopene) (Pulz and Gross 2004).

Large numbers of scientific studies have confirmed the benefits of carotenoids to health and use for this purpose is growing rapidly. Besides, carotenoids have been proposed as added-value compounds that could contribute to make microalgal biofuel production economically feasible (Becker 2004, Pulz and Gross 2004). The main applications of carotenoids are currently as dietary supplements, fortified foods, food color, animal feed and pharmaceuticals and cosmetics.

In economical terms, astaxanthin, β-carotene, lutein, lycopene and cantaxanthin can be considered as to be the most relevant ones among all existing natural carotenoids. β-carotene is probably the most widely known of the carotenoids due to be a vitamin A precursor.

The health industry uses carotenoids in over-the-counter (OTC) dietary supplements and fortified foods. This is one of the fastest growing segments of the industry but is still relatively small compared to the color segment. The pharmaceutical and cosmetics industries also use carotenoids mainly for their coloring properties, though their use by the pharmaceutical and cosmetics companies is growing rapidly due to their nutraceutical properties, as for example the 'beauty pill' containing lycopene. This product belongs to a new market segment known as 'cosmeceuticals', which aims to combine cosmetics and nutraceutical food ingredients to create products to improve skin and hair.

Carotenoids are growing in popularity due to increasing demand for natural products from consumers. Natural carotenoids can be extracted from plant material such as tomatoes, algae and fungi (**Figure I.8**). Individual carotenoids are available in a variety of forms. The most common forms are cold water soluble powder, oil emulsion and beadlets. Concentrations range from 0.2 to 100%. The most common concentration is 10%. Blends or mixed carotenoids are also available containing two or more different carotenoids. Like the individual

carotenoids, blends are available in a variety of forms including, water dispersible powder, oil suspension and beadlet forms. **Table I.3** shows the main applications of microalgae due to their carotenoid contents (Vilchez et al. 2011). The concentration of blends ranges from 1 to 30%, with the most common concentration being 10% (Pulz and Gross 2004, Vilchez et al. 2011).

Figure I.8. Capsules of astaxanthin from *Haematococcus pluvialis.*

In recent years, production of carotenoids has become one of the most successful activities in microalgal biotechnology. The demand for carotenoids obtained from natural sources is increasing. This has promoted major efforts to improve carotenoid production from biological sources instead of chemical synthesis (Del Campo et al. 2007). According to the report published by Business Communications in 2008, the global market for all commercial carotenoids accounted for 766 million dollars, with expectations of rising to 919 million dollars in 2015. In particular, β-carotene market volume in 2007 was 247 million dollars, with expectations of reaching 285 million dollars in 2015. Besides lycopene and β-carotene, xanthophylls lutein, astaxanthin and cantaxanthin appear as the most demanded and valuable carotenoids. Astaxanthin market volume in aquaculture in 2009 was 260 million dollars and about 2500 \$ kg^{-1}. In addition, lutein market volume in 2010 accounted for about 190 million dollars, the carotenoid experiencing the most rapid growth in sales (Fernandez-Sevilla et al. 2010). Therefore, carotenoid-containing microalgae find many applications in a wide range of commercial activities, the reason for which carotenoid-enriched microalgae production is steeply becoming an attractive business.

Table I.3. Main applications of microalgae due to their carotenoid content (Vilchez et al. 2011).

Microalga	Application	Product	Price
Chlorella vulgaris	Aquaculture Cosmetics Nutraceutical Food ingredients	Tablet Dry powder	\$30-100 Kg^{-1}
Isochrisis galbana	Aquaculture cosmetics, nutraceutical	Paste Dry powder	\$100-400 Kg^{-1}
Nannochloropsis gaditana	Aquaculture Cosmetics	Paste Dry powder	\$300 Kg^{-1}
Pavlova lutheri	Aquaculture	Paste Dry powder	< \$300 Kg^{-1}
Phaeodactylum tricornutum	Aquaculture Nutraceuticals	Paste Dry powder	>\$200 Kg^{-1}
Tetraselmis	Aquaculture	Paste Dry powder	\$600-800 Kg^{-1}
Thalassiora weissflogii	Aquaculture	Paste Dry powder	>\$300 Kg^{-1}
Arthrospira	Nutraceuticals cosmetics	Paste Dry powder	>\$200 Kg^{-1}
Haematococcus pluvialis	Aquaculture, nutraceuticals	Dry powder	>\$600 Kg^{-1}
Dunaliella salina	Nutraceuticals, food ingredients	Dry powder Tablets	\$100-400 Kg^{-1}

Factors that influence on carotenoid accumulation

In general, green algae (Chlorophyta) possess a carotenoid composition similar to that of land plants. Main carotenoids in photosynthetic tissue of algae and land plants are lutein (45% of the total), β-carotene (25-30%), violaxanthin (10-15%) and neoxanthin (10-15%) (Britton 1993). However, carotenoid accumulation in algae is dependent on environment growth factors as temperature and light (quality and intensity), salinity, pH, and nutrient limitation, in particular nitrogen or phosphorus.

- Temperature

Temperature is one of the most important factors that influence on growth due to the effect on some enzymes involved in numerous biosynthetic pathways, including carotenoids. Several studies have shown that an increase in temperature might cause high level of oxidative stress and, therefore, an enhancement of carotenoid content in some microalgae (Del Campo et al. 2000, Liu and Lee 2000, Tjahjono et al. 1994).

Temperature also affects the photosynthetic activity (Coles and Jones 2000). In photosynthesis, the photochemical 'light' reactions are mainly affected by irradiance and are not sensitive to temperature. However, the biochemical 'dark' processes are temperature sensitive. At low temperatures the metabolic rate is reduced and consequently less absorbed light energy can be converted into carbohydrates (Coles and Jones 2000, Huner et al. 1998). The slow biochemical reactions then can cause accumulation of light energy and over-excitation of the photosynthetic machinery. In this sense, suboptimal temperatures lead to an imbalance between the light absorbed through photochemistry and the energy utilized through metabolism. Algae use the same defense mechanism, NPQ, to dissipate over-excitation of the photosynthetic machinery at low temperatures as at high irradiances (Huner et al. 1998, Maxwell et al. 1994).

The temperature has been considered as the main factor controlling the growth rate and production of β-carotene from algae *Dunaliella* and *Haematococcus pluvialis* (Bhosale 2004).

- Irradiance

When algae are exposed to excess irradiances respect to that required to saturate photosynthesis, the excess light becomes a stress factor, and can cause photo-damage. To protect the photosynthetic apparatus from oxidative damage, xanthophyll pigments are involved in the quenching of excited chlorophyll and reactive oxygen species (Demming-Adams and Adams 2002).

High light intensities have been showed to induce astaxanthin and lutein biosynthesis enhancement in the *Muriellopsis* and *Haematococcus*, respectively (Del Campo et al. 2000, Park and Lee 2001). Another example of light irradiance influence on carotenoid accumulation in algae is *Dunaliella salina*. Increases in β-carotene and zeaxanthin intracellular content were observed when *Dunaliella salina* was cultured under high light intensities compared to cultures under low light intensities (Jin et al. 2001).

- Nutrient limitation

Certain elements are defined as nutrients because they are essential for life processes in aquatic organisms. Major nutrients include carbon, nitrogen, sulfur and phosphorus. Other potentially important nutrients include calcium, magnesium, sodium and potassium. Micronutrients, those required by plants and animals in very small quantities, might include manganese, copper, zinc, cobalt, and molybdenum (Horne and Goldman 1994).

Nutrient limitation is one of the main regulatory factors for growth. Depletion in nutrients produces a diminution in protein synthesis and variations in carbon fixation processes, resulting in an increment on carotenoid accumulation (Geider et al. 1998). Nitrogen and phosphorus starvation are probably the main nutrient factors influencing carotenoids accumulation. Many studies consider the influence of nutrient limitation in carotenoid content. For example, an increment on carotenoid content was shown under nitrogen starvation and high light intensities in *Haematococcus pluvialis* (Grewe and Griehl 2008).

- Heavy metals and salinity

Heavy metals are essential for life but at higher concentrations can be harmful to algae and other aquatic biota, since produce reactive oxygen species (ROS) formation (Stohs and Bagchi 1995). ROS production is known to be stimulated by various metals belonging to the group of transition elements (Mallick 2004). The variable valence of transition elements allows them to undergo changes in the oxidation state involving one electron, thereby stimulating the formation of free radicals. Reactive forms of oxygen include $O_2^{\cdot}$, $^{\cdot}OH$ and H_2O_2. All these can be highly toxic since are capable to attack several cell constituents, such as amino acids, proteins, carbohydrates, nucleic acids and lipids.

Algae have evolved antioxidant defense mechanisms to combat the presence of ROS with several enzymatic mechanisms, such as superoxide dismutase and catalase among others, and non-enzymatic mechanisms such as tocopherols and carotenoids (Alscher et al. 1997). Indeed, carotenoid biosynthesis is one of the main algal responses to oxidative stress (Bhosale 2004).

However, there are not many studies concerning the response of algal antioxidant systems to metals. Ben-Amotz and Avron (1983) and Schroeder and Johnson (1995) in *Dunaliella bardawil* and Phaffia *rhodozyma*, respectively, studied the enhanced synthesis of carotenoids by free radicals.

Copper is a micronutrient but at higher concentrations can be deleterious to algae. Copper plays an essential role in many processes, as respiration (cytochrome oxidase), the transport of oxygen (hemocyanin) and photosynthesis (plastocyanin). High concentrations of copper have been shown to enhance the β-carotene content and the activity of the enzyme ascorbate peroxidase in *Dunaliella tertiolecta* (Nikookar et al. 2005).

Addition of heavy metals as copper should be expected to induce changes on carotenoid anabolism, especially in algae living in acidic waters of mining areas which normally tolerate high levels of copper in their natural environment (Johnson and Hallberg 2003).

Besides and as previously described in this Introduction section, increased contents of salt in the culture medium have been found to enhance the intracellular carotenoids content in several microalgae species, particularly in those from natural salty water environments like seas and oceans (Richmond 2004).

- pH

Most microalgae have been shown to decrease in carotenoid content when incubated under not optimal pH conditions (Del Campo et al. 2000, Sanchez et al. 2008). However, some microalgae have been shown to improve carotenoid accumulation under extreme pH values, *Chlamydomonas zofingiensis* and *Dunaliella salina* are some examples (Borowitzka 1988, Rise et al. 1994).

5. MICROALGAL CULTIVATION SYSTEMS

Despite the history of microalgal utilization from natural populations is centuries old (*Nostoc* in Asia and *Spirulina* in Africa and Mexico), large scale commercial production of microalgae dates back to the 1950s (Belasco 1997, Grobbelaar 2010). Microalgal biomass production offers a number of advantages over biomass production of other natural materials, including higher productivities, use of otherwise nonproductive land, reuse and recovery of waste nutrients, use of saline or brackish waters, and reuse of CO_2 from power-plants or similar sources. Extensive efforts are already underway to achieve its commercial-scale production.

Microalgae are cultivated in a wide range of different cultivations systems, from open raceway ponds to close photobioreactors. Numerous differences exist between them, but all outdoor microalgae cultivation systems are characterized by their large surfaces to intercept sunlight which provides the energy needed to sustain microalgae growth.

Raceways pond systems

Main characteristic in raceways ponds is that the system is opened and microalgal culture is directly exposed to the atmosphere (**Figure I.9**). Generally, raceway ponds are shallow ponds (between 10 and 50 cm deep) composed of two or four straight channels connected to each others. They incorporate paddlewheels for liquid mixing and circulation. The culture suspension is pumped around in a cycle, being directly illuminated from sunlight via the liquid surface.

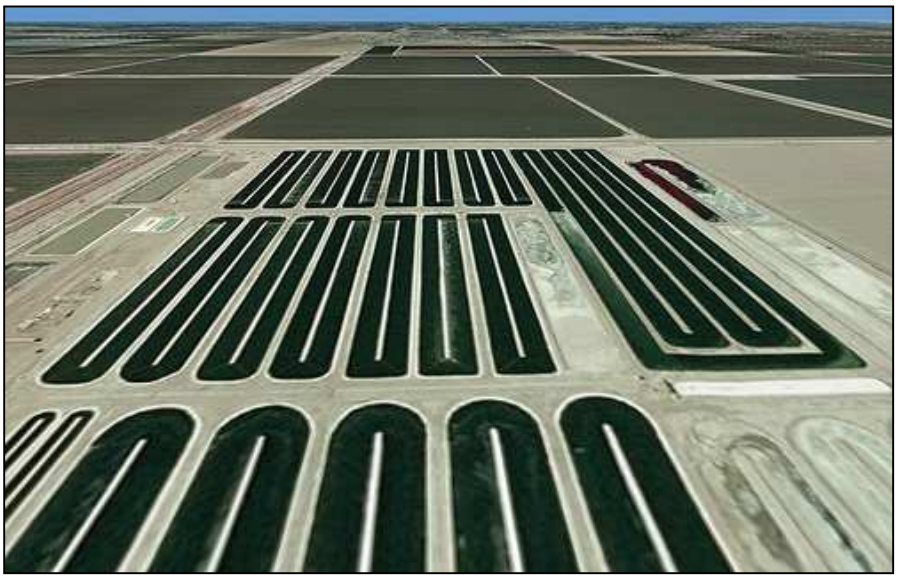

Figure I.9. Open raceway pond. Southern California (USA). Source: http://nanopatentsandinnovations.blogspot.com.es/2012/05/study-algae-could-replace-17-of-us-oil.html

Nutrients are added to the water stream which is used to replace the microalgae culture, continuously harvested. A culture is directly exposed to the atmosphere, the system permits liquid evaporation, helping to regulate the process temperature. Carbon dioxide must be added by gassing the cultures with pure CO_2 or CO_2-rich gasses. This causes significant CO_2 losses to the atmosphere.

Open raceway ponds are easy to build and scale though. On the other hand, the two major factors still limiting large-scale application of the open pond system

are the poor species control and the requirement of lots of open spaces (500-5000 m^2). Other negative aspects are the low biomass concentration in this voluminous system and the difficulty for harvesting small unicellular microalgae cells.

Despite those drawbacks, open ponds are the most popular cultivation systems for the commercial production of microalgae. They are used for the cultivation of some microalgae and cyanobacteria, as *Spirulina,* which is cultivated for the company Cyanotech in Hawai, producing more than 350 ton/year. Other examples are the *Dunaliella salina* and *Chlorella.* These are species which can be grown under specific circumstances preventing their outgrowth (Lee 2001).

Photobioreactors (PBR)

In order to overcome the weaknesses of open raceway ponds, new systems that allow the massive cultivation of microalgae were developed. Those systems are called photobioreactors. In the case of pond systems the sunlight-exposed area is equal to the ground area occupied by the pond. But closed photobioreactors are usually characterized by a larger light exposed surface than the ground area occupied. Therefore, an improved use of light, both natural and artificial, is achieved by using photobioreactors. An increase in the culture productivity is normally accomplished.

Photobioreactors are reasonably independent of climatic variations, allowing for a better control over the factors that influence in algal growth (such as temperature and nutrient concentration). Another benefit of those reactors is that photobioreactors allow for obtaining high cell density cultures. Cell density is up to 16-fold that value achieved in open pond systems (Chini-Zitelli et al. 1999). However, it has been claimed that photobioreactors are unsuited for biomass production because if its high cost production (Waltz 2009).

Many different types of photobioreactors have been developed in last decade (plate, tubular, panelar, plane, bubble column, etc. bioreactors). However, mainly two types, tubular and panelar bioreactors are the most used for algal production.

- Tubular photobioreactors

Tubular photobioreactors are based on transparent tubes in which the algae culture is maintained and exposed to sunlight. A large number of tubes are connected to each other via manifolds and the liquid is continuously pumped through the tubes. That is done to prevent the dark zones in the center of the tubes and to avoid the microalgae cells from settling. Horizontal and vertical tubular photobioreactors can be found (**Figure I.10**).

Figure I.10. Tubular photobioreactor systems. (A) Horizontal tubular photobioreactor, CIECEM, Huelva (Spain). (B) Vertical tubular photobioreactor, California Polythecnic State University, California (USA). http://brae.calpoly.edu/CEAE/images/biofuels3.gif

The length of the tubes and the liquid velocity in the tubes are important design parameters. Typical values are 50 to 100 m for tube length and 0.2 to 0.5 m s^{-1} for the liquid flow velocity. Avoiding oxygen accumulation has become one of the main challenges to overcome in this system. Given the low solubility of oxygen in water, the oxygen partial pressure will quickly rise to levels above those in equilibrium with air. For this reason the microalga culture is continuously pumped through a so-called degassing vessel where the oxygen is allowed to escape. Tubular reactors are used industrially, for example for producing the valuable pigment, astaxanthin, with *Haematococcus*, and also for *Chlorella* and *Nannochloropsis* production.

- Flat panel photobioreactors

Flat panel photobioreactors are vertical bioreactors (**Figure I.11**) composed of several thin rectangular cultivation vessels with a depth in the range of 0.01 to 0.10 m. They are mixed by gassing (CO_2-enriched air) the panels with air which is injected over the full length of the panel bottom. The panels need to be well mixed in order to prevent unequal distribution of nutrients and microalgae. Adding to that, the length also needs to be sufficiently short for the same motive. Therefore, mixing and panel length are important design parameters. The gas leaving the system can be partly recycled in order to increase the residence time of the gas bubbles, allowing for a more efficient use of the carbon dioxide supplied.

For that all, gassing the cultures requires considerable energy. However, the energy requirement for mixing is generally lower in flat panel than in tubular reactors due to the fact that the flat panel reactor does not require such high flow rate. In tubular system, the culture needs to be pumped through the degassing vessel and back, requiring high flow rates.

Flat panel reactors are used in high irradiance areas, in order to achieve light dilution. The light dilution achieved in vertical panel systems will result in a higher photosynthetic efficiency and higher productivity in comparison to horizontal tubular systems or raceway pond (Cuaresma et al. 2011).

Figure I.11. Flat panel bioreactor. Source: http://www.esru.strath.ac.uk/EandE/Web_sites/09-10/ZeroCarbonCommunities/Algaehome.html

Open raceway ponds vs close photobioreactors

It is strongly debated which type of microalgal production technology is the most adequate. Different belief is in respect to the question of which technology is the most promising for future developments and scale up.

The main disadvantage in the use of photobioreactors for biomass production is likely to be the high production costs (Waltz 2009), despite the many benefits. Photobioreactors provide a better control of gas transfer, more uniform temperature, space saving, higher cell density cultures and permit to have single-species culture. Thus, photobioreactors have better productivities. By contrast, open systems suffer from low biomass productivity and high costs of biomass harvesting because of low biomass densities, large land use (500-5000 m^2), losses of carbon dioxide and poor contaminant control possibilities (Posten 2009). However, they are easy to build and scale up.

Norsker et al. (2011) calculate microalgal biomass production costs for the three different microalgal production systems operating at commercial scale: open

ponds (4.95 € per kg), horizontal tubular photobioreactors (4.15 € per kg) and flat panel photobioreactors (5.96 € per kg). However, they state that by means of optimizing irradiation conditions, mixing, medium and carbon dioxide costs, and photosynthetic efficiency of systems, a price of 0.68 € per kg might be achieved.

Probably, the election of using either microalgal production system is related to end product market value. If algal cultivation is performed for production of high value products (as carotenoids), photobioreactors are probable a more practical and efficient system. While, for the industrial-scale production of biomass, open raceway ponds are easier and cheaper.

6. COCCOMYXA ONUBENSIS

An acid-environment microalga, named *Coccomyxa onubensis*, was used in this thesis. *Coccomyxa onubensis* was selected among several microalgae initially isolated from the Tinto river environment. Growth, biochemical properties and potential for high-value products accumulation were considered as selection criteria. *C. onubensis* was elected for being a promising microalga principally because of its rich carotenoid profile, especially lutein, of which naturally accumulates around 4 to 6 g Kg^{-1} dry weight, that is in accordance with other lutein producing species (Fernandez-Sevilla et al. 2010).

Coccomyxa was identified by ribosomal 18S subunit rDNA sequence analysis, registered at GenBank with accession number GU265559 (Garbayo et al. 2012) and finally classified as follows:

Domain: Eukaryotic
Division: Chlorophyta
Class: Chlorophyceae
Order: Chlorococcales
Family: Chlorococcaceae
Genera: *Coccomyxa*

C. onubensis is a unicellular alga belonging to Chlorophyta division. Chlorophyta contains about 7000 different species (Hoek et al. 1995) including very different organism ranging from wall-less unicellular microorganisms to macroscopic filamentous organisms. Chlorophyceae covers the largest number of species of the division. Within this broad class, several important orders including Chlorococcales (to which *Coccomyxa* belongs) are found. Chlorophyceae also encompasses Chaetophorales, Dunaliellales, Microsporales, Oedogoniales, Sphaeropleales, and Volvocales Tetrasporales. (Hoek et al. 1995). Chlorococcales cells normally possess a compact cell wall and one or more chloroplasts, a nucleus, vacuole secretion and reserve substances (starch granules and oil droplets). Some species are characteristic for secondary carotenoids accumulation. Among others, a well-known genus is *Chlorella*, which can be found in freshwater and marine environments. As well *Coccomyxa* genus belongs to the family Chlorococcaceae.

Algae of the genus *Coccomyxa* occurs as free-living forms (found in fresh water and soil), or as phycobionts growing in symbiosis in lichens where it acts as a photosynthetic component (Peveling and Galun 1976). Phylogenetic studies of *Coccomyxa* genus have revealed three main lineages within this genus, corresponding to free-living *Coccomyxa*, those isolated from basidiolichens Omphalina, and *Coccomyxa* isolated from ascolichens belonging to the Peltigerales (Zoller and Lutzoni 2003, Verma et al. 2009). In regard to their life cycles, typically *Coccomyxa* reproduces asexually. The genus *Coccomyxa* was discovered in 1901 by the scientist Schmidle. There are currently 33 recognized species (and subspecies), of which about thirty are currently accepted taxonomically.

Morphologically, *Coccomyxa onubensis* shared with other species acidophilic several common characteristics. Cells are ellipsoidal in shape, size is about 3 mm in length and 2 mm wide, and shows a distinct cell wall. A very large chloroplast occupies about half (visual appreciation) of the total cell volume, observed in **Figure I.12** from SEM micrographs. The nucleus and nucleolus are about 1 mm in length, 1mm wide and 0.25-0.15 mm in diameter, respectively, and are located in the central portion of the microalga.

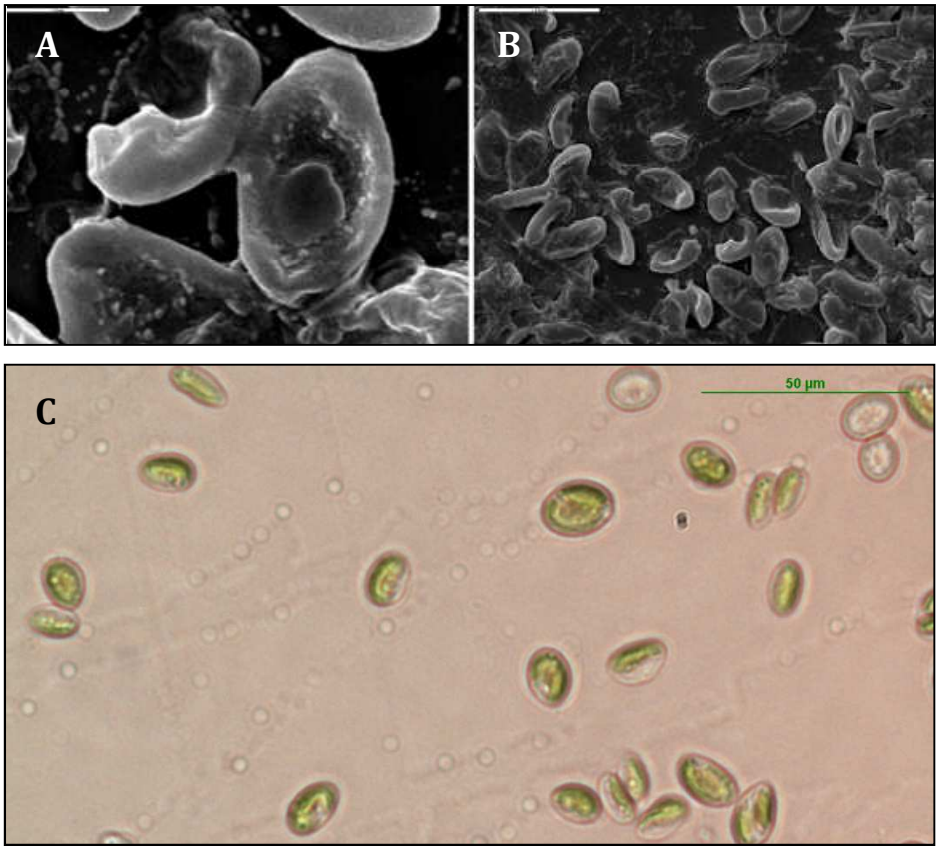

Figure I.12. Micrographs of *Coccomyxa onubensis* cells. A and B. Electron microscope (A: 10000x, B: 2500x). Source: Garbayo et al. (2012). C. Optical microscope (100x).

7. THESIS OUTLINE

This thesis was aimed at increasing biomass productivity and carotenoid content of the novel lutein-rich acid environment microalga, *Coccomyxa onubensis*. **Chapter II** intends to show that acid environment microalgae can be produced at similar productivities of non-extreme microalgae, with the added advantage of their growth in highly selective culture medium. The existence of carbon concentration mechanisms and conditions that might lead to increased activity of *C. onubensis* was studied as a tool to increase microalga productivity. *C. onubensis* was grown under high and low CO_2 levels, showing external and internal carbonic anhydrase activities. Best carbon uptake capacity and growth were found to occur at acid pH, proving acid-tolerant behavior of *C. onubensis*. Incubation in air followed by shift to high carbon concentrations enhanced carbon use efficiency in terms of growth rate and biomass productivity, based on the action of both carbonic anhydrase activities. Lutein was accumulated in the microalga at high concentrations and did not depend on inorganic carbon conditions. Consequently, chapter II showed that repeated cycles of air-incubation and high CO_2-incubation of *C. onubensis* might become a suitable tool to perform production processes of lutein-enriched biomass.

In **Chapter III,** the influence of Cu (II) on productivity and accumulation of value carotenoids of the microalga was studied. Copper was added in range between 0.06 and 0.4 mM, and an increase in algal viability, biomass productivity and synthesis of carotenoids was obtained. A copper concentration of 0.2 mM was found to be as the most appropriate one to enhance productivity and lutein accumulation and was used in semicontinuous cultures. *C. onubensis* was found to have great potential as lutein producer when compared to known lutein accumulating microalgae, due to its facility to live in highly selective environment (low pH and high concentrations of heavy metals).

At low light intensities, most algae produce more light-harvesting pigments to improve their photosynthetic efficiency while at high light intensities, some algae produce high concentrations of “sunscreen” pigments to protect the cell from excess light. In these terms, the aim of the work described in **Chapter IV** was to

study the effect of low to moderate light intensity shifts on lutein accumulation of *Coccomyxa* cultures, doubly aimed at understanding the light-dependent role of main carotenoids in acid-tolerant microalgae and at developing strategies to induce β-carotene and mainly lutein accumulation with applied purposes. *Coccomyxa* cells reached its maximum growth rates and carotenoid productivities at 400 μmol photons m^{-2} s^{-1} that corresponds to maximal irradiance that reaches the surface of a flat panel reactor placed vertically under summer conditions at south Spain. This chapter also shows that cell density influences short-time course evolution of carotenoids, which can be used for increasing lutein content of *C. onubensis* in outdoor production.

According to that, the aims of the **Chapter V** were to evaluate the feasibility of outdoor cultivation of the acid environment microalga *C. onubensis* and to assess its potential as alternative source of lutein. *Coccomyxa onubensis* was cultivated in acid culture medium under springtime outdoor conditions in a pilot tubular photobioreactor in southern Spain. The results showed that *C. onubensis* is able to withstand high temperature levels, since despite of showing some thermodamage, the cultures rapidly recovered. Maximum daily biomass productivity was obtained at maximum irradiance of 1000 μmol photons m^{-2} s^{-1}, however light intensities above that value was detrimental for the algal culture. Intracellular lutein content increased at the end of the day, probable related to light absorption functions.

Chapter V gives the first guidelines of the parameters that influence in a potential large-scale cultivation of acid environment microalgae and should allow to design lutein-rich biomass production strategies of this extremophile microalga.

8. AIMS

Coccomyxa onubensis is green microalga characterized for being able to live in highly selective environment with extreme oxidative conditions, which confers the microalga a competitive advantage over other organisms that cannot survive at such low pH and high concentrations of heavy metals. These features in addition to its potential for high-value products accumulation makes *Coccomyxa onubensis* a very promising lutein accumulating microorganism if incubated under a suitable cultivation strategy.

The main aim of this Thesis is to enhance biomass productivity and carotenoid accumulation, paying special attention to lutein content. To achieve the main objective the following partial objectives were accomplished:

- Improvement of biomass productivity of *Coccomyxa onubensis,* based on efficient inorganic carbon use, and knowledge of strategies of carbon supply for conducting the extremophile algal growth. To do that the following activities were performed:
    - Study of the existence of carbon concentration mechanisms and conditions that might lead to their increased activity in *C. onubensis*, since this acid-environment microalga is expected to have high affinity for carbon dioxide due to the low availability of CO_2 in such acidic media.
    - Comparison of the feasibility of microalgae cultivation at different pH values to assess its ability to grow in terms of proton gradient in different pH ranges.

- Assessing the influence of several copper concentrations on biomass productivity and carotenoid accumulation, paying special attention to the amount of accumulated lutein.

- Understanding of the light-dependent role of main carotenoids in *C. onubensis* and developing strategies to induce β-carotene and, mainly, lutein accumulation. The fundament of this study was based on the fact

that those pigments play an important double role, in light-capturing function (under low light intensities) and in protecting cells against the harmful effects of reactive oxygen species (under high light intensities).

- Assessing the feasibility of outdoor cultivation of *C. onubensis* and evaluating the potential of this microalga as an alternative source of lutein.

CHAPTER II

Efficient inorganic carbon utilization as a tool to enhance acid-environment microalgal growth

"The more original a discovery, the more obvious it seems afterwards"

(Arthur Koestler)

1. ABSTRACT

Besides light intensity, one of the major factors limiting algal productivities is CO_2 availability and uptake. To achieve high productivities of biomass and high value products microalgae cultivation is often performed with overly CO_2 added. *Coccomyxa onubensis* naturally grows under a very low pH range (1-3), so inorganic carbon is mostly present as CO_2 at air-equilibrium concentration. The existence of carbon concentration mechanisms and conditions that might lead to their increased activity in *C. onubensis* was studied as a tool to increase microalga productivity. *C. onubensis* was grown under high and low CO_2 showing external and internal carbonic anhydrase activities. Best growth and carbon uptake capacity occurred at acidic pH, proving acid-tolerant behavior of *C. onubensis*. Incubation in air followed by shift to high carbon conditions enhanced carbon use efficiency in terms of growth rate and biomass productivity, based on the action of both carbonic anhydrase activities. Lutein accumulated in the microalga at high concentrations above 5 mg g^{-1} dry weight and did not depend on inorganic carbon conditions. Therefore, cycles of air-incubation and high CO_2-incubation might become a suitable tool to design mass production strategies of acidophile microalgae, and particularly lutein-rich biomass in the case of *C. onubensis*.

2. INTRODUCTION

The Tinto River, *Coccomyxa onubensis* natural habitat, is an extreme acidic environment characterized by a very low pH (1-3). As a result of such high acidity, inorganic carbon (Ci) is mostly as CO_2 at air-equilibrium concentration. The equilibrium between inorganic carbon forms in aqueous media is pH-dependent. At normal intracellular ionic strength, when the pH level is below 6.4 (first dissociation constant for carbonic acid, pK1), CO_2 predominates; at pH between 6·4 and 10.3 (pK2) HCO_3^- predominates, and above pH 10.3 CO_3^{2-} predominates. Therefore, Ci concentration is substantially lower at acidic pH than in higher-pH waters with bicarbonate as main inorganic carbon source. That situation denotes that carbon availability is a limiting factor for the microorganisms inhabiting acidic waters, limiting its potential for systematic mass production. It is also inferred from the lack of reports on acidophile microalgae biomass production trials. Algae have adapted to these challenges through the development of CO_2 concentration mechanisms (CCM). The CCM is a biological adaptation to low carbon dioxide concentrations in the environment. It is a mechanism which augments photosynthetic productivity in algal cells by increasing internal levels of inorganic carbon many times over the environmental concentration of carbon dioxide.

Previous research on carbon concentration mechanisms (CCM) has revealed that most microalgae and cyanobacteria can import both CO_2 and HCO_3^- through the cell membrane (Maberly et al. 2009; Giordano et al. 2005, Price et al. 2008). Once imported into the cell, CO_2 or HCO_3^- is accumulated mainly as HCO_3^- due to its neutral internal pH. Compared with cyanobacteria, less information is available on Ci transport systems in eukaryotic algae (Yamano and Fukuzawa 2009). However, it has been reported that, in addition to Ci transporters in the cell membrane, eukaryotic algae such as *Chlamydomonas* also have chloroplast Ci transporters, because photosynthesis in eukaryote microalgae occurs in the chloroplast (Yamano and Fukuzawa 2009, Markelova et al. 2009). In addition, CA might contribute to the transport of HCO_3^- into the thylakoid lumen and its conversion into CO_2 (Yamano and Fukuzawa 2009). In algae carbonic anhydrase (CA) has been recognized as one of the essential elements of the CO_2

concentration mechanisms (Jordan and Ogren 1981, Morita et al. 1998, Spalding 1998, Spreitzer 1999). Carbonic anhydrase is a zinc metalloenzyme that catalyses the interconversion of CO_2 and HCO_3^- (Khalifah 1971). The enzyme was first discovered in red blood cells but has also been found in most organisms including animals, plants, archaebacteria, and eubacteria (Hewett-Emmett and Tashian 1996). Carbonic anhydrase (CA) is important in many physiological functions that involve carboxylation or decarboxylation reactions, including both photosynthesis and respiration. In addition, it is clear that CA also participates in the transport of inorganic carbon to active photosynthesizing cells or away from actively respiring cells (Henry 1996). Main function of internal carbonic anhydrase of microalgae is converting bicarbonate to carbon dioxide in the active site of Rubisco, therefore enhancing carbon fixation in microalgae.

Some microalgae living in acidic environments do hardly display CCM under carbon limiting conditions (Colman and Balkos 2005). Accordingly, inorganic carbon (Ci) might take place through the cell membrane by either active or passive diffusion. However, there are other acidophile and acid-tolerant algae those have been showed to have CCM (Raven et al. 1982). In that respect, knowledge about CO_2 concentrating mechanisms in *Coccomyxa* genus is scarce. Verma et al. (2009) proposed the presence of an external carbonic anhydrase (CA_{ext}) in *Coccomyxa* although with a carbon transport facilitating role rather than a concentrator function. These results indicate that there is species-specific variation in the induction mechanism of CCM depending on physiological and ecological conditions (Giordano et al. 2005, Raven 2010).

C. onubensis is expected to have high affinity for carbon dioxide due to the low availability of CO_2 in such acidic media. Therefore, it seems essential to know whether *C. onubensis* possesses CO_2 concentrating mechanisms (CCM) and the potential factors that may lead to increase its activity. One possible line of work is experimenting with high/low CO_2 availability cycles in order to increase CO_2 uptake efficiency.

The low external pH theoretically forces to 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 needed to sustain such systems partially justifies a remarkable activity of PSII, even growing under low carbon conditions.

Coccomyxa onubensis was isolated and identified in previous works of our group (Garbayo et al. 2012). That acid-tolerant microalga was found in screened water samples obtained from acidic drainages in the pyritic belt area around the Tinto River, in the province of Huelva (Spain). The natural habitat of that microalga is highly concentrated in solved metals and poor in nutrient including N and P. Such extreme environment addresses *Coccomyxa* to express some typical antioxidant responses. One of the most attractive physiological responses in terms of commercial applications is large accumulation of lutein, above 5 mg g^{-1} dry biomass and accounting for about 80% of total pigment pool. This makes of *Coccomyxa onubensis* an attractive model acid-tolerant microalga to probe that production of value compounds of extreme microalgae might be feasible. Production of acidophilic or acid-tolerant microalgae meets the advantage of selective growth in acidic culture medium which avoids competition from most of non-adapted microorganisms.

Accordingly, we aimed to study the *Coccomyxa onubensis* growth at different pH values to assess its ability to grow in terms of proton gradient in different pH ranges, and show results that evidence algal productivities similar to those of "common" microalgae if suitable strategies of carbon supply are chosen for conducting the extremophile algal growth.

3. MATERIALS AND METHODS

Microorganism and culture conditions

Coccomyxa onubensis was isolated from acidic waters of the Tinto River (Huelva, Spain). This river has some very special features, such as low pH and a high concentration of heavy metals, especially iron, copper, magnesium and

aluminum (Ferris et al. 2004). An axenic culture of the algae was obtained by streaking it on basal agar medium at pH 2.5, and then was transferred to the liquid medium. *Coccomyxa* has been identified by ribosomal 18S subunit rDNA sequence analysis (Garbayo et al. 2012). Identified 18S subunit rDNA sequence was registered at GenBank with accession number GU265559. According to the chemical composition of the natural environment, cultures were grown at pH 2.5 in a culture medium based on K9 medium (Silverman and Lundgren 1959). A modified K9 medium was prepared according to the following composition: 3.95 g K_2SO_4, 0.1 g KCl, 0.5 g K_2HPO_4, 0.41 g $MgCl_2$, 2.29 g KNO_3, 0.01 g $CaCl_2$, 5 ml Hutner solution (Hutner et al. 1950). The cultures were incubated at 27°C and were illuminated at 150 μE m^{-2} s^{-1} with white fluorescent lamps. The cultures were bubbled without CO_2-added air, under low CO_2 (LC) conditions or bubbled with air containing 5% (v/v) CO_2, high CO_2 (HC) conditions, as control cultures. The bubbled gas flow rate was 190 ml min^{-1} per liter. The described culture conditions were held constant throughout all the experiments.

Microalgal optical density

The optical density of the sample (triplicate) was measured at 750 nm (OD_{750nm}). Demineralized water served as reference. The microalgal samples were diluted using demineralized to achieve an OD_{750nm} value below 1.

Dry Weight measurements

Whatman glass microfiber filters (Ø 47 mm, pore size 0.7 μm) were dried at 95 °C overnight and placed in a desiccator to cool to room temperature. The empty filters were weighed. Approximately 10 mg of sample (triplicate) was filtrated. The filter was rinsed twice with demineralized water to remove adhering inorganic salts. The wet filters containing the samples were dried at 95 °C overnight, allowed to cool to room temperature in a desiccator, and weighed.

Growth rate calculations

Dry weight data were used to calculate growth rates. Specific growth rates of cultures were calculated using the following expression:

$\mu = \mathrm{Ln}(C/C_0)/t$,

where μ is the specific growth rate, C_0 is the initial biomass concentration (dry weight), and C is the biomass concentration at any time t.

In batch culture experiment, specific growth rates were calculated from the linear portion of the biomass time-course evolution, the average growth rates were calculated from the logarithmic growth phase. In semicontinuous cultures, average growth rates were calculated as the average daily growth using dry weight data.

Measurement of CA activity

External carbonic anhydrase (CA_{ext}) activity was determined by the modified potentiometric technique described by Williams and Colman (1993). Cells were harvested by centrifugation at 5,000g for 10 min, three washed with 30 mM Hepes buffer, pH 8.1, and resuspended at a concentration of approximately 25 µg chl mL^{-1})1 in 1.5 mL of the same buffer. To determinate total carbonic anhydrase (CAt) cells were harvested by centrifugation at 5,000g for 10 min, three washed with 30 mM Hepes buffer, pH 8.1, and then, the cells were homogenized using a sonicator. The extract was suspended in 1.5 mL of the same buffer, and an aliquot of the homogenate was taken for CA measurement. Ice-cold, CO_2-saturated distilled water (0.5 mL) was injected into the cell suspension or cell homogenate at 4°C, and the time taken for the pH of the suspension to drop from pH 8.1 to 7.6 was measured.

CA activity was calculated as Wilbur- Anderson (WA) units using the formula (Tc / Ts)-1, where Tc and Ts represent the time taken for the pH change, in the presence and absence of cells, respectively. To determine the effect on

photosynthesis of inhibiting external CA, a sample of cell suspension were treated with acetazolamide (AZA), a CA inhibitor.

Protein Measurement and calibration curve.

The Bradford method (Bradford 1976) was used. This compares the spectrophotometric absorbance of the unknown sample at a wavelength of 595 nm to a calibration curve prepared using standard solutions of a protein. Bovine serum albumin (BSA) was used as a standard.

Quantum Yield (QY)

Fluorescence measurements were made as the maximum quantum yield (QY) of PSII (F_v/F_m). It was measured to evaluate the viability of the cells. It was determined using a pulse amplitude modulation (PAM) (Schreiber et al. 1995). Samples of each culture were previously adapted to darkness for 15 minutes.

Carotenoids determination by HPLC

Carotenoids were extracted using aliquots (1ml) of the cultures. Cells were spinned down for 8 minutes at 13000 rpm. The obtained pellet was placed 60° C water during 5 min. The pellet was resuspended in 1 ml of methanol and the suspension shaken vigorously for 1 min, centrifugated for 8 min at 4400 rpm. Carotenoids and chlorophylls a and b were separated and identified by HPLC (TermoQuest, Thermo Separetion products) with a RP-18 column, using a modified method described by Young in 1997. In the mobile phase, solvent A was ethyl-acetate and solvent B was acetonitrile and water (9:1, v/v). External standards (DHI) and their corresponding calibration curves were used to identify and quantify both lutein and β-carotene.

Statistics

Unless otherwise indicated, tables and figures show means and standard deviations of three independent experiments.

4. RESULTS AND DISCISSION

pH effect on algal growth and lutein content

We aimed to study *Coccomyxa onubensis* growth at different pH values to assess its ability to grow in terms of the proton gradient between the outside and inside the cell membrane, and its ability to use the available inorganic carbon source at different pH values. That information should allow us for defining suitable growth strategies to increase biomass productivity of the acidic-environment microalga, Coccomyxa onubensis. As explained, inorganic carbon form available differs depending on culture medium pH. Based on the need for enhancing inorganic carbon uptake, carbonic anhydrase (CA) acts as carbon concentration mechanism. Several studies describe that CA activity is pH-dependent in some acid-tolerant microalgae (Balkos and Colman 2007, Verma et al. 2009), which external carbonic anhydrase activity is mainly expressed when the microalgae grown at pH up to 5. **Figure II.1** shows growth rates and total CA activities of stabilized *C. onubensis* cultures at the indicated pH values.

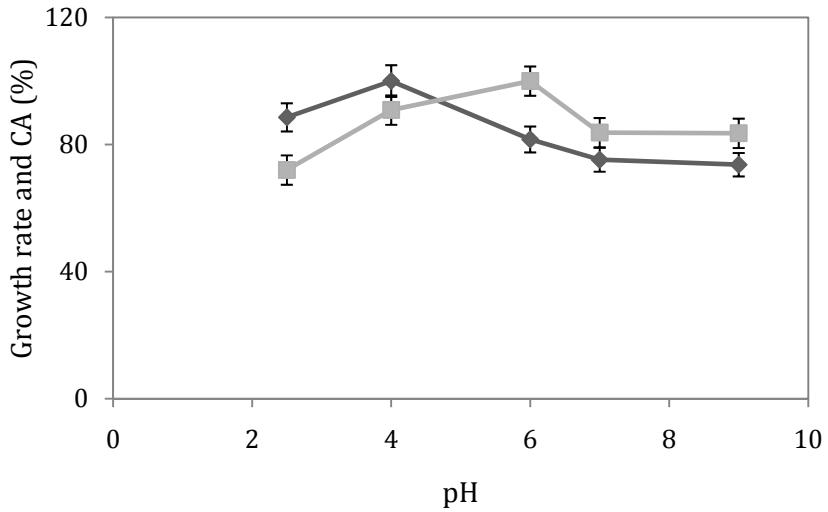

Figure II.1. Specific growth rates (dark symbols) and CA activity (grey symbols) of *C. onubensis* cultures at different pH values. Growth rates were calculated by measuring the stabilized cultures (semicontinuous mode) by optical density at 750 nm. 100% = 0.42 d^{-1} (growth rate) and 0.80 WAU mg^{-1}prot.

The highest cell densities were obtained at pH 4.0 although, actually, *Coccomyxa onubensis* showed growth over the pH range 2.5–9.0. This suggests that *C. onubensis* internal pH should remain neutral enabling the microalga for performing photosynthesis in the whole pH range (2.5-9.0). Particularly, the cultures grown at pH 9.0 lost their cell viability at early growth stages, though still were able to grow at some extend. Therefore, *C. onubensis* can be considered as an acid-tolerant microalga rather than an acidophilic microalga.

Figure II.1 also shows total CA activity, which increased with the pH increase in the culture medium, the maximum being found in cultures at pH 6 and decreasing at higher pH values. Results also show that the lower intensity of the proton gradient, at neutral pH, does not inhibit *C. onubensis* growth. This is opposite to what is observed in stringent acidophilic microalgae as *Dunaliella acidophila* (Gimmler and Weis 1992), but it is in good agreement with Verma et al. (2009) who reported *Coccomyxa sp* growth over the pH range 3.0-9.0, indicating that the microalga should maintain constant internal pH and photosynthesis over that pH range. Interestingly, they observed that external CA of *Coccomyxa sp*. expressed when the alga grew above pH 6.0 only. *Coccomyxa onubensis* apparently shows external CA even growing at pH 2.5, as shown below, perhaps suggesting different adaptation patterns to acidic media within the same microalga genus, depending on the natural habitat of each species.

At neutral pH, HCO_3^- is the predominant inorganic carbon source. If an algal cell can use only CO_2 from the external medium, the rate of photosynthesis will be limited, in the absence of a catalyst, by the rate of spontaneous CO_2 formation from HCO_3^- dehydration. If the photosynthetic rates exceed the calculated uncatalyzed dehydration rate, it would indicate that HCO_3^- is taken up as a source of Ci (Verma et al. 2009). *Coccomyxa onubensis* growth seems to occur more efficiently at pH below 6, where CO_2 is by far the most abundant inorganic carbon source. Therefore, growth should be highly dependent on CO_2 availability. The higher CA activity at intermediate pH values (6-7) seem not to compensate the low CO_2 level available as main carbon source, as inferred from the lower growth rate values obtained at pH above 6. The fact that cell growth occurs at neutral pH suggests that HCO_3^- can also be used as carbon source, but much less

efficiently. Therefore, acidic pH was selected as most suitable to produce *Coccomyxa onubensis*.

When cultivated under suitable conditions, *Coccomyxa onubensis* accumulates lutein at intracellular concentrations above 5 mg g^{-1} dry weight, which are among the highest published for microalgae (Garbayo et al. 2012) and make that microalga attractive for lutein production. **Figure II.2** shows *Coccomyxa onubensis* lutein content, when grown at different pH values. Lutein content (mg g^{-1} DW) significantly differs in cells cultivated at different pH values, the maximum being found in cultures at pH 7.

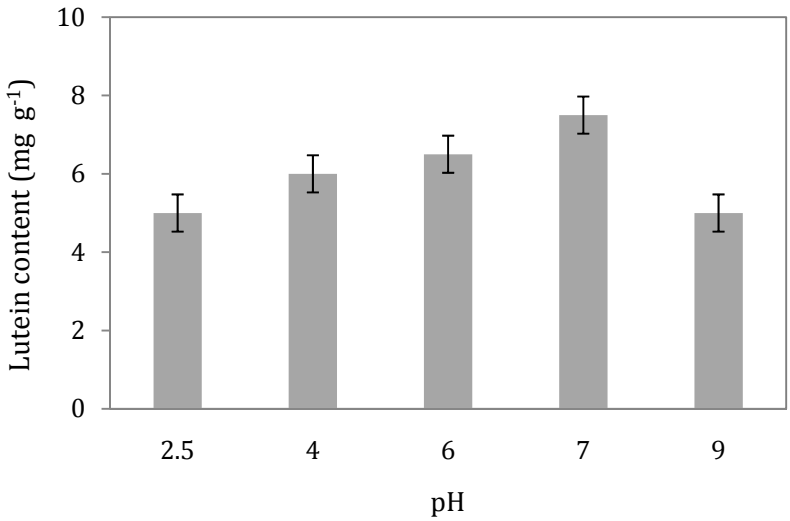

Figure II.2. Lutein content (mg g^{-1} DW) of *C. onubensis* cultures at different pH values.

In previous work we reported that *Coccomyxa onubensis* naturally accumulates a high constitutive pool of lutein (Garbayo et al. 2012), which increases depending on cultivation conditions and accounts for about 60% to 80% of total pigment pool (Vaquero et al. 2012). According to growth rates and lutein accumulation values obtained in *Coccomyxa* cells, pH in the acidic range 2.5-4.0 promote higher lutein productivities, in mg g^{-1} d^{-1}, with a maximum of 2.52. In addition to that, the ability to grow at acidic pH becomes a selective competitive advantage for

Coccomyxa onubensis in continuous production processes, compared to non-acid-tolerant microalgae.

Effect of CO_2 conditions on growth rates, biomass productivities and carotenoids content

According to the results above, pH 2.5 was selected as the most suitable for lutein production with *Coccomyxa onubensis*. As already discussed, CO_2 availability at acidic pH is limited and its uptake by the microalga seems to be enhanced through active carbon concentration mechanisms. CO_2 level should therefore influence such carbon concentration activity and consequently the growth rate of *Coccomyxa onubensis*. Growth rates and carbonic anhydrase activity of *Coccomyxa onubensis* were determined in batch cultures which were bubbled with 5% (v/v) CO_2-enriched air (HC conditions) or only air (LC conditions) under lab culture conditions, continuous light of 150 μmol photons m^{-2} s^{-1} and 27 °C **(Figure II.3).** Optical density at 750 nm was measured as a cell density factor, as mentioned in Materials and Methods section.

Calculated growth rates were approximately 0.23 and 0.17 d^{-1} under HC and LC conditions, respectively, suggesting that *Coccomyxa onubensis* cells grew just slightly faster under HC conditions than under LC conditions. External and total carbonic anhydrase activities (WAU) were measured along the experiment and the average data are represented in **Figure II.3**. Both external and total CA activities per gram of dry weight (WAU g^{-1} DW) were higher under LC conditions than under HC conditions. This result suggests that *Coccomyxa onubensis* might show higher photosynthetic affinity for inorganic carbon under LC conditions than under HC conditions. It seems rather reasonable considering that generally, eukaryotic microalgae and cyanobacteria have developed efficient CO_2-utilization mechanisms and exhibit high affinity for CO_2 when grown under CO_2-limiting conditions (Miyachi et al. 2003). Under saturating CO_2 conditions, microalgae exhibit low affinity for CO_2, as enough CO_2 is available for photosynthesis (Miyachi et al. 2003). In *C. onubensis*, these results reveal a likely existence of an external (in addition to the internal) carbonic anhydrase, which seems to raise its activity when *C. onubensis* grows under low CO_2 levels.

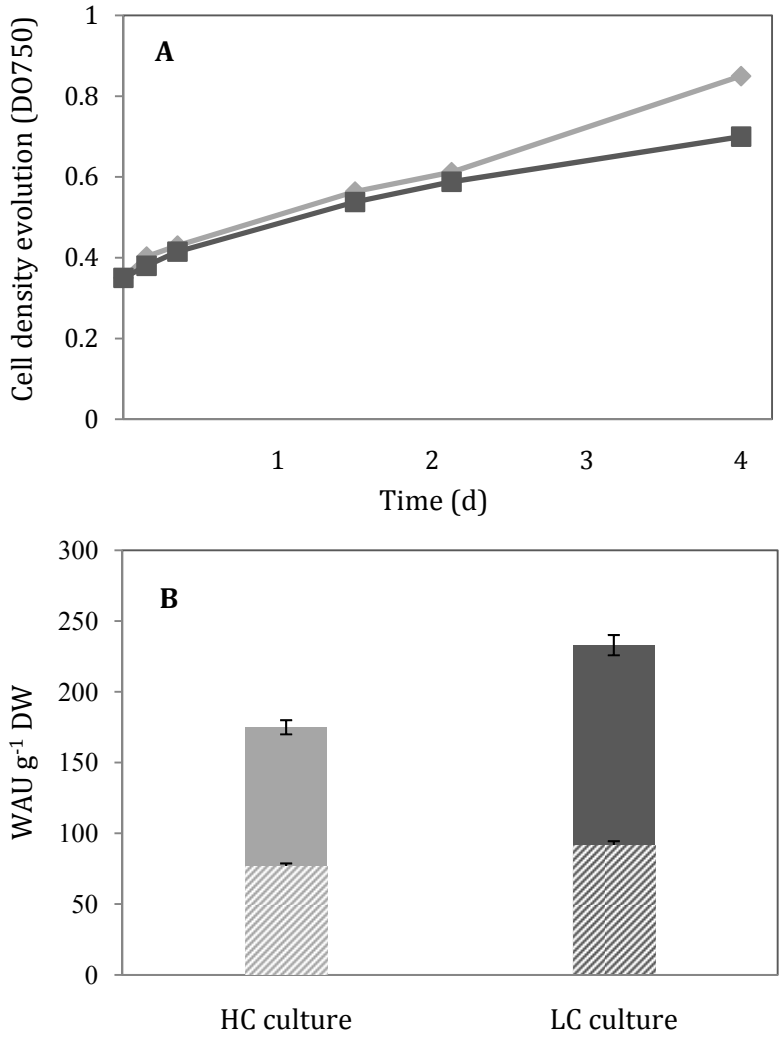

Figure II.3. Cell density evolution (A) and Carbonic anhydrase activity (B) of control culture, HC (light grey) and low CO_2 culture, LC (dark grey) of *Coccomyxa onubensis*. Solid bars show total carbonic anhydrase activity. Cross-hatched bars show external carbonic anhydrase, in HC and LC cultures. Cell density evolution was measured as optical density at 750 nm (OD_{750}). Carbonic anhydrase activities were expressed as WAU g^{-1} DW (dry weight).

According to the obtained results, pre-incubation of *C. onubensis* under LC conditions (air only) might enhance the algal CO_2 uptake capacity, which might further be used to address increased CO_2 uptake by means of transferring LC-

grown cultures to HC conditions. In that respect and aimed at enhancing productivity of *C. onubensis* cultures, experiments addressed to assess CO_2-adquisition capacity of *C. onubensis* were done. By means of a suitable regime of inorganic carbon supply to *Coccomyxa onubensi*s cultures, the microalga might increase its biomass productivity, which would also become essential to achieve high productivities of high value products, namely lutein in *C. onubensis.*

Accordingly, aliquots of *C. onubensis* cultures grew under LC conditions over different specified time periods (6, 12, 24 or 36 hours, respectively). After that period under LC conditions, *C. onubensis* cultures were transferred to HC conditions (5% CO_2-enriched air), accounting for a total experience time of 60 hours, all cultures. A control culture was grown for 60 hours under HC conditions.

Incubation time under LC conditions does influence *Coccomyxa onubensis* growth. During the early growth stages, a growth rate deceleration was observed in those cultures grown under LC conditions, compared to control cultures under HC conditions. However, **Figure II.4** also shows that cultures grown under LC conditions do reactivate their growth when transfered to a carbon-rich environment, reaching higher growth rates and productivities. Suprisingly, after 60 hours of total experience time, those cultures first incubated under LC conditions reached higher cell densities than control cultures (grown for 60 h under high CO_2 conditions), accounting for 40% higher values. This is illustrated in **Figure II.4**, that shows productivities achieved by *Coccomyxa onubensis* batch cultures as a function of air bubbling time in low CO_2 (LC conditions), calculated once *Coccomyxa* cultures had been transferred and incubated under HC conditions.

Figure II.4 shows that biomass productivity of *Coccomyxa* cultures transferred from only air to high CO_2, increases as a function of the residence time of cultures in air. *Coccomyxa onubensis* reaches its maximum productivity (1.8 g L^{-1} d^{-1}) when grown for 36 hours under LC conditions (air bubbling only) followed by 24 hours incubation under high CO_2. That productivity is 4-fold that value achieved by the control culture, which grew 60 hours under 5% CO_2-enriched air.

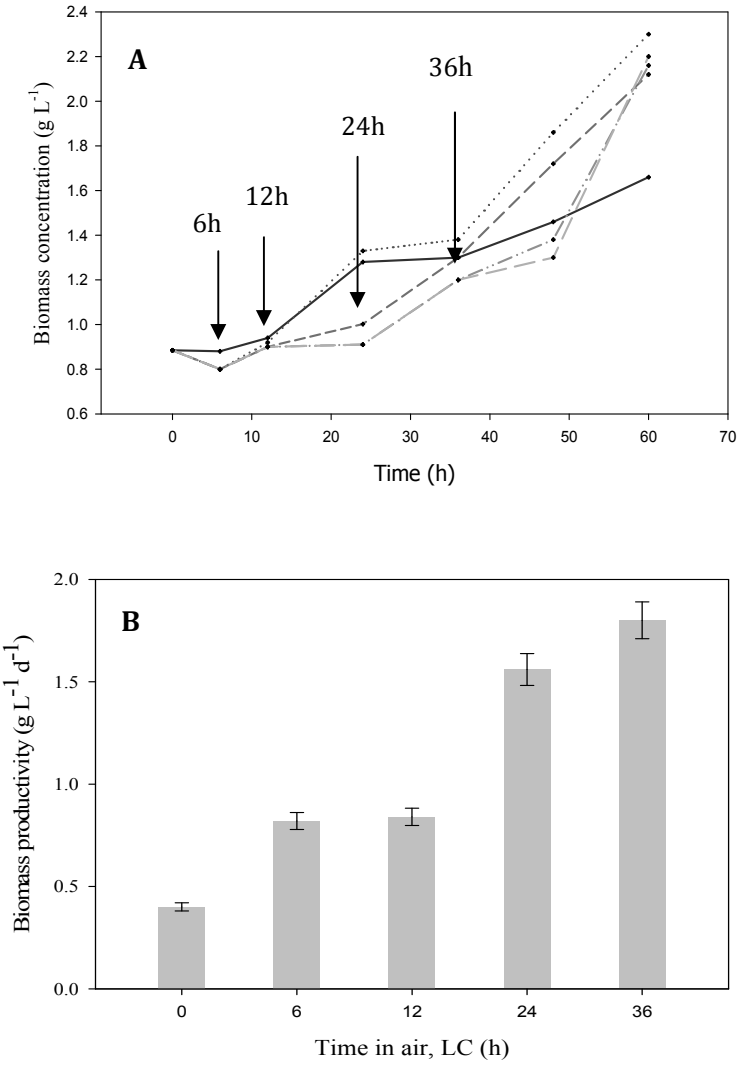

Figure II.4. Growth kinetics from LC to HC conditions (A) and biomass productivity (B) of *Coccomyxa onubensis* cultures 48 hours incubated in only air and further transferred to CO_2-enriched air. Growth kinetics and biomass productivities were calculated from dry weight data and were expressed as g L^{-1} and g L^{-1} d^{-1} respectively. The cultures were grown under different time period of LC. ( ) represents the culture grown 6 hours under LC conditions, ( ) represents the culture grown 12 hours under LC conditions, ( ) represents the culture grown 24 hours under LC conditions, ( ) represents the culture grown 36 hours under LC conditions and, ( ) represent the HC culture (no hours in air). The cultures were cultivated under batch regime and were illuminated with PAR light of 150 µE m^{-2} s^{-1}. At the indicated times, dry weight was determined. Arrows indicate when cultures were transferred from only air to 5% CO_2-enriched air.

Such high productivity data have not been previously described for acidophile microalgae (Beamud et al. 2010, Spijkerman et al. 2011, Visviki and Palladino 2001), meaning that there might be room for improving acidophile microalgae productivities. Moreover such values are comparable to those of microalgae commonly used as biomass producers. These results suggest that the efficient use of CO_2 by *C. onubensis* significantly increases when cells experience low inorganic carbon availability during a certain time period, and that the action of carbon-concentrating mechanisms might be efficiently used to address increased yields of biomass production of acidophile/acidotolerant microalgae, which usually are considered as low growth microalgae (Gross 2000, Pulz and Gross 2004). In once, results would suggest that carbon concentration mechanisms of *C. onubensis* (1) are overactived in air conditions, as expected from carbonic anhydrase (CA) data, and (2) such highly active situation might remain for longer during microalgal re-growth under HC. Excess carbon might be more efficiently transferred into biomass of acidophile microalgae if carbon concentration mechanisms are highly active, which can be achieved by pre-incubation under LC conditions. These results allow to design mass production strategies of acidophile microalgae, where quite elevated algal biomass productivities might be achieved, getting a higher CO_2-use efficiency.

In this manuscript it has been shown that pre-incubation of acid-tolerant microalga cultures in air (low carbon conditions, LC) followed by shift to high carbon conditions (HC) by far enhances carbon use efficiency in terms of growth rate and biomass productivity. In a further experience, it was probed that there is a limit for such carbon use enhanced efficiency and that such limit depends on how long carbon demand is active. The higher productivities of air-preincubated *Coccomyxa onubensis* cultures are consistent with an increased carbon demand. Longer air pre-incubation (LC) times resulted in increased carbonic anhydrase activities (**Figure II.5**), considered as total carbon demanding activity. This is in good agreement with results reported by other authors (Gehl et al. 1990, Lane and Morel 2000, Sültemeyer et al. 1991) and just emphasizes that carbon acquisition mechanisms of microalgae which are expressed for balancing the internal inorganic carbon pool are up-regulated in extreme carbon limiting

situations (Moroney et al. 2011, Moroney and Somachi 1999, Miyachi et al. 2003), as particularly happens in acidic environments.

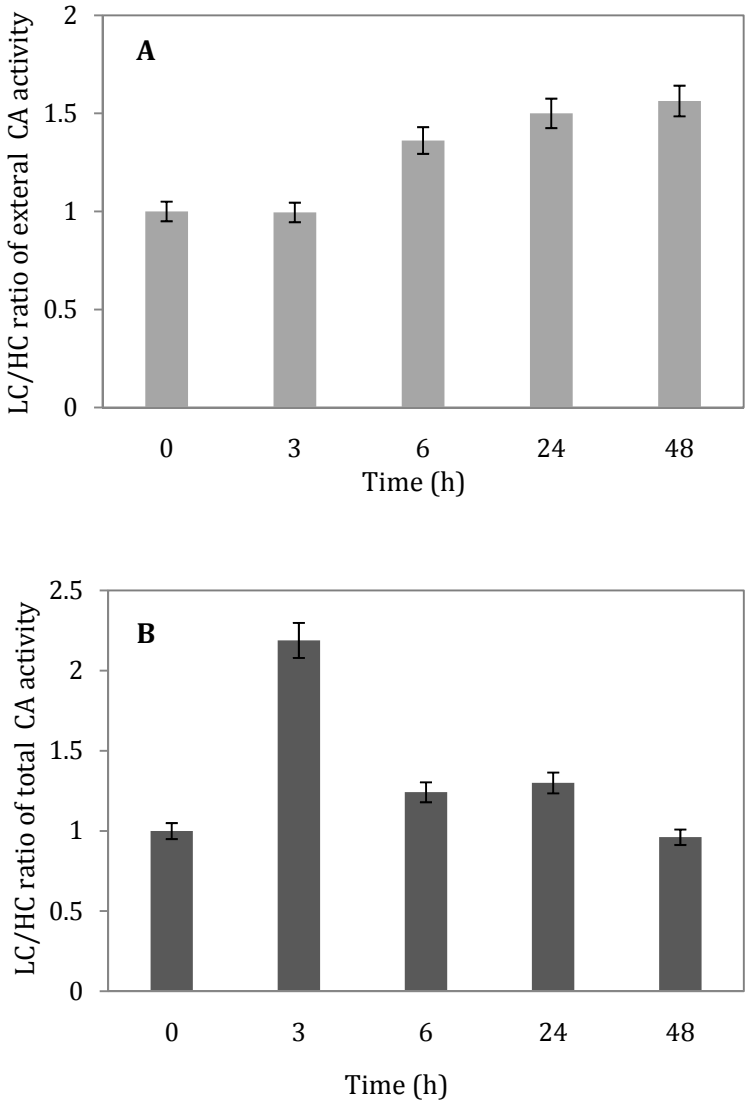

Figure II.5. Ratio of external (A) and total (B) carbonic anhydrase activity of low CO_2 culture (LC) respect to control culture (HC). Both external and total carbonic anhydrase activity were calculated per miligram of protein (WAUext mg^{-1}). Average external CA of 48 HC culture= 0.693±0.055 WAU_{ext} mg^{-1} prot. Average total CA of HC culture during 48 hours= 0.987±0.079 WAU_{ext} mg^{-1} prot.

As shown in **Figure II.5**, both external and total carbonic anhydrase of *C. onubensis* becomes particularly active within the first 24h-48h of incubation in air only (LC) compared to carbonic anhydrase of HC-grown cells (control cultures). Longer incubation periods in air address carbonic anhydrase of LC-grown cells falling down to those values of control cultures under high CO_2. Therefore, the maximal carbon uptake activity of *C. onubensis* cultures can be achieved by air-incubation periods of 24-48h. As previously shown (**Figure II.4**), further shift of air-incubated cultures to HC conditions is followed by massive carbon incorporation into biomass, which seems to be maximal after a period of 24h to 36h under HC conditions.

Longer periods under HC conditions do not improve productivity. In that respect, **Table II.1** shows that in 48h air-incubated *C. onubensis* cultures further transferred to HC conditions for 6 days, carbonic anhydrase activity, growth rate and productivity data at the end of the cultivation period became rather similar to those of control cultures. In addition to that, quantum yield of *C. onubensis* under both cultivation scenarios showed similar values, therefore suggesting no differences in light use efficiency in photosystem II, in good agreement with the similar biomass productivity data obtained.

Table II.1. Growth data and carbonic anhydrase activity of *C. onubensis* subjected to pre-incubation in air (LC conditions) and further growth in high CO_2 (HC conditions) for 6 days. Results are compared with those obtained in cultures grown in HC conditions only.

Inorganic carbon conditions	Growth rate (d^{-1})	Productivity ($g\ L^{-1}\ d^{-1}$)	Total carbonic anhydrase (WAU mg^{-1} prot)	Quantum yield
Control cultures (HC)	0.17	0.19	0.65	0.68
48h air-incubated cultures (LC)	0.21	0.22	0.70	0.77

The existence of external carbonic anhydrase activity in *C. onubensis* is a significant result considering that *C. onubensis* lives naturally in a very low pH environment (below pH 3). Under highly acidic conditions, an extracellular carbonic anhydrase might in principle not be necessary (Gross 2000) as CO_2, and not HCO_3^-, is the available carbon source. As an example of this, according to Geib et al. (1996), *Dunaliella acidophila* showed very low activity of an extracellular carbonic anhydrase activity compared to *Dunaliella* species living at neutral pH. In contrast, acid-tolerant algae, like *Chlorella saccharophila,* seem to have an external CCM (Raven et al. 1982). Other two acid-tolerant microalgae, *Coccomyxa* and *Chlamydomonas spp.* were found to express external carbonic anhydrase when grown in acidic media, but above pH 5 (Verma et al. 2009, Balkos and Colman 2007). Low CO_2 conditions should therefore enhance carbon uptake as appears to happen to other microalgae (Miyachi et al. 2003), although the specific function of external CA at low pH in *Coccomyxa* is still unclear. As described for Verma et al. (2009) the function of external CA at low pH might be aimed at maintaining the equilibrium of CO_2 concentration at the outside part of the cell membrane, therefore speeding CO_2 uptake.

LC conditions seem to undergo some changes in internal CA activity in *C. onubensis*, as well as in external CA. This is in good agreement with results reported in other non-extremophile microalgae as *C. reinhardtii* (Sültemeyer et al. 1991), *Phaeodactylum tricornutum* (Nimer et al. 1997), *Chlorella saccharophila* (Gehl et al. 1990), *Skeeltonema costatum* (Korb et al. 1997) and *Thalassiosira weissflogii* (Nimer et al. 1997, Lane and Morel 2000). Gardner et al. (2012) refer that when algae, especially green algae, are transfered from high CO_2 conditions (1–5%, v/v) to low CO_2 conditions (atmospheric, 0.035%, v/v), a number of carbonic anhydrases and bicarbonate specific transporters are synthesized within a short time period (up to 6 hrs). It is noteworthy that even though the enzyme activity of *Coccomyxa onubensis* appears to reach its maximum value just shortly after (hours) incubation under LC conditions, CA seems to maintain this peak of activity for about 2 days. As explained, the consequence is an increase in biomass productivity when *Coccomyxa onubensis* is transferred to HC conditions, due to higher carbon uptake efficiency and a subsequent more efficient Rubisco activity due to the higher internal CA activity

(Palmqvist 1995). In order to know whether the observed external carbon concentration activity was really due to the external carbonic anhydrase, analyzes were performed with *Coccomyxa* cultures added with an external CA inhibitor, acetazolamide (AZA). Activity of cells plus AZA always resulted in an activity lower than 0.03 WAU g^{-1} dry weight. These results suggest the existence of external carbonic anhydrase activity of *C. onubensis*. However, compared to external CA activities of "common" microalgae, CA values obtained for *C. onubensis* are lower (Badger and Price 1994, Li 2012, Sültemeyer et al., 1995, Sültemeyer et al. 1991). This is predictable considering *C. onubensis* was cultured in low pH and that inorganic carbon is mainly in the form of CO_2 and not as bicarbonate.

C. onubensis accumulates high concentrations of lutein, a well-known carotenoid in the group of xanthophylls (Garbayo et al. 2012, Vaquero et al. 2012). Lutein has recently gained attention as an additive in food industry and especially as a powerful antioxidant, its value being recognized against oxidative diseases such as preventing age-related macular degeneration (Carpentier et al. 2009, Ziegler et al. 1996). Average intracellular lutein content of *Coccomyxa onubensis* is around 6 mg g^{-1} dry weight which is within the range of the most promising lutein producing microalgae species (Fernandez-Sevilla 2010).

For lutein production process it becomes relevant to know whether *Coccomyxa onubensis* shows any variation in its lutein content (mg g^{-1}) if grown under low carbon conditions. **Figure II.6** shows *Coccomyxa onubensis* lutein and β-carotene content evolution when cultured during 48 hours under LC conditions followed by 6 days under HC conditions. A *C. onubensis* control culture grew at HC conditions during the 8 days experiment. Both carotenoids content were identified and quantified by HPLC as mentioned in Materials and Methods section.

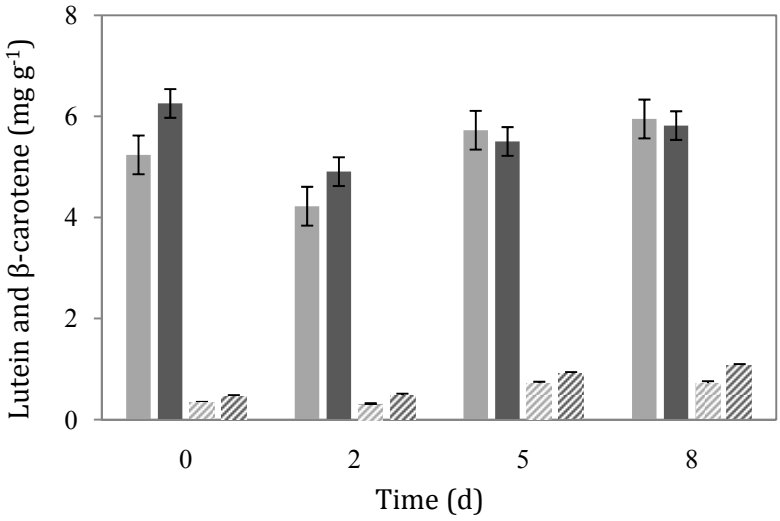

Figure II.6. Lutein and β-carotene content (mg·g^{-1} DW) of *C. onubensis* cultures under different treatments: 48 h incubation in air (LC conditions) and further growth in high CO_2 (dark grey), and constant HC conditions (light grey). Solid bars show lutein content, and cross-hatched show β-carotene content.

Lutein and β-carotene contents (mg g^{-1} DW) seem to remain constant during the whole experiment and CO_2 concentration in the medium (only air or HC conditions) seems not to have any impact in the biosynthesis rate. Average intracellular lutein content is roughly 6 mg g^{-1} dry weight, similar to that of control culture, and β-carotene content accounted for 0.5-0.7 mg g^{-1} dry weight throughout the experience. Accordingly, *C. onubensis* pre-incubation under LC conditions does not result in a decreased intracellular carotenoid content. As growth rate and biomass productivity are enhanced when grown with combined periods of low CO_2 and high CO_2, lutein productivity results therefore enhanced. Consequently, repeated cycles of air-incubation and high CO_2-incubation of *C. onubensis* might become a suitable tool to perform production processes of lutein-enriched biomass.

5. CONCLUSIONS

From the obtained results it might be concluded that incubation of acid-tolerant microalgae cultures (*Coccomyxa onubensis* in this manuscript) in air followed by shift to high carbon conditions by far enhances carbon use efficiency in terms of growth rate and biomass productivity, based on the action of both external and internal carbonic anhydrase activities. Lutein content of *Coccomyxa onubensis* is high and does not seem to depend on carbon level supplied to cultures. Consequently, repeated cycles of air-incubation and high CO_2-incubation of *C. onubensis* might become a suitable tool to perform production processes of lutein-enriched biomass. These results should allow to design mass production strategies of acidophile microalgae, particularly lutein-rich biomass in the case of *C. onubensis*.

CHAPTER III

Cu-mediated biomass productivity enhancement and lutein enrichment of the novel microalga *Coccomyxa onubensis*

"Ciencia es aquello sobre lo cual cabe siempre discusión."

(José Ortega y Gasset)

1. ABSTRACT

The influence of Cu (II) on productivity and accumulation of value carotenoids of a microalga that naturally grows at low pH, *Coccomyxa onubensis*, was investigated. The presence of Cu (II), added in range between 0.06 and 0.4 mM, increases both algal viability and synthesis of carotenoids, mostly lutein and β-carotene. A copper concentration of 0.2 mM was found to be as the most appropriate one to enhance productivity and lutein accumulation and was further used in semicontinuous cultures. Unlike acidophile microalgae, *Coccomyxa onubensis* showed unusual high growth rates (0.50 d^{-1}) when cultured semicontinuously at 0.2 mM Cu (II) and getting an average productivity of 0.42 g $L^{-1}d^{-1}$. Lutein content in 0.2 mM Cu (II) cultures was roughly 50% higher than that obtained for control cultures. *C. onubensis* seems to have great potential as lutein producer when compared to known lutein accumulating microalgae. *Coccomyxa onubensis* is able to live in highly selective environment, which confers the microalga a competitive advantage over other organisms that cannot survive at such low pH and high concentrations of heavy metals. This might make of *Coccomyxa onubensis* a unique alga for large producer in open systems.

2. INTRODUCTION

In recent years, technological developments for large-scale production of microalgae have gained commercial relevance. Currently, the market for microalgae is mostly based on two applications. On the one hand the production of biomass and, secondly, the marketing of high added-value products obtained from them. Cultivation of extremophile microorganisms has gained interest due to their ability to accumulate and produce high value molecules such as enzymes, metabolites and surfactants (Schiraldi and De Rosa 2002). The acid drainage of the Tinto river mining area provide a suitable environment where many extremophile microorganisms grow among high concentrations of heavy metals, low pH and exposed to a high irradiance of UV light. *Coccomyxa onubensis* was isolated from such a sort of oxidative environment.

Previous studies (Casal et al. 2011) showed such *Coccomyxa* microalga has a pretty good growth rate, higher than many acidophile algae (Pulz and Gross 2004, Tittel et al. 2005, Visviki and Santikul 2000) and pretty similar to other common microalgae, having the advantage of growing in a low pH media with an inorganic carbon source which prevents cultures from bacterial contamination. *C. onubensis* accumulates high concentrations of lutein, a well-known carotenoid in the group of xanthophylls. Lutein has recently gained great interest as an additive in food industry and especially as a powerful antioxidant, its value being recognized against oxidative diseases such as preventing age-related macular degeneration (Maci 2010). Average intracellular lutein content of *Coccomyxa onubensis* is up to 6 mg g^{-1} dry weight which is within the range of the most promising lutein producing microalgae species (Salguero et al. 2003).

On the other hand, one of the most abundant metals in the Tinto River, where the microalga of our study was isolated from, is copper. Copper is an essential element for all plants and animals' growth and a regular constituent in the environment (Lewis and Cave 1982). This heavy metal is an essential micronutrient but at higher concentrations can be deleterious to algae and other aquatic biota (Chang and Sibley 1993).

O_2 is able to initiate toxic ROS formation. In higher plants and algae, ROS are always formed by leakage of electrons onto molecular oxygen from the electron transport activities of chloroplasts, mitochondria and the plasma membrane (Foyer et al. 1997). In addition, ROS production is known to be stimulated by various toxic metals belonging to the group of transition elements (Mallick 2004). The variable valence of transition elements allows them to undergo changes in oxidation state involving one electron, thereby stimulating the formation of free radicals. Reactive forms of oxygen include $O_2^{\cdot}$, $^{\cdot}OH$ and H_2O_2. All these can be highly toxic since are capable to attack several cell constituents, such as amino acids, proteins, carbohydrates, nucleic acids and lipids. At the biomembrane level, lipid peroxidation is a very important destructive reaction (Halliwell and Gutteridge 1999).

Interestingly, living cells also have evolved antioxidant defense mechanisms to combat the presence of ROS. These include several enzymatic and non-enzymatic mechanisms such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione reductase (GR), reduced glutathione (GSH), ascorbic acid, tocopherols and carotenoids (Alscher et al. 1997). There are not many studies concerning the response of algal antioxidant systems to metals. Ben-Amotz and Avron (1983) and Schroeder and Johnson (1995) in *Dunaliella bardawil* and *Phaffia rhodozyma*, respectively, studied the enhanced synthesis of carotenoids by free radicals. Indeed, carotenoid biosynthesis is one of the main algal responses to oxidative stress, among which nutrient deficiency, high light intensity, type of light, low temperature, high salinity and metals (Bhosale 2004, Garbayo et al. 2008) are included.

Considering that *Coccomyxa onubensis* is an extremophile alga, which endures high levels of copper in their natural environment (Johnson and Hallberg 2003), addition of this heavy metal in the growth medium should be expected to induce changes on carotenoid anabolism of *Coccomyxa onubensis* which should resulted in the accumulation of carotenoids that could positively affect the production of lutein (Mallick 2004).

3. MATERIALS AND METOHDS

Microorganism and culture conditions

Coccomyxa onubensis was isolated from acidic waters of the Tinto River (Huelva, Spain). This river has some very special features, such as low pH (in between 1.7 and 3.1) and a high concentration of heavy metals, especially iron, copper, magnesium and aluminum. An axenic culture of the algae was obtained by streaking it on basal agar medium at pH 2.5, and then was transferred to the liquid medium. *Coccomyxa* has been recently identified by ribosomal 18S subunit rDNA sequence analysis. Identified 18S subunit rDNA sequence was registered at GenBank with accession number GU265559. According to the chemical composition of the natural environment, cultures were grown at pH 2.5 in a culture medium based on K9 medium (Silverman and Lundgren 1959). A modified K9 medium was prepared according to the following composition: 3.95 g K_2SO_4, 0.1 g KCl, 0.5 g K_2HPO_4, 0.41 g $MgCl_2$, 2.29 g KNO_3, 0.01 g $CaCl_2$, 5 ml Hutner solution (Hutner 1950).

The cultures were bubbled with air containing 5% (v/v) CO_2 as unique carbon source. The bubbled gas flow rate was 190 ml min^{-1} per liter. The cultures were incubated at 28°C and were illuminated at 160 μE m^{-2} s^{-1} with white fluorescent lamps. The described culture conditions were held constant throughout all the experiments.

Experiments in batch reactors

To determine the influence of Cu (II) concentration on the growth rate, productivity and carotenoid accumulation of *C. onubensis*, batch cultures with different concentrations of Cu (II) were performed. Experiments in batch reactors consisted of microalgal growth in culture media supplemented with selected copper concentrations. Culture media was not renewed along the experiment. Growth was followed from the lag phase to the decline phase. Batch experiments were performed in 1l roux flasks, and the algal cultures incubated in a culture room under controlled temperature and continuous white

fluorescent light at a fixed intensity. Firstly, six standard culture media were prepared and supplemented with different concentrations of Cu (II) as $CuSO_4$ $5H_2O$. The Cu (II) final concentrations were 0.06, 0.04, 0.2, 0.4, 2 and 4 mM respectively, as indicated in the Figures in Results and Discussion section. The cultures were incubated at 28°C and were illuminated at 160 μE m^{-2} s^{-1} with white fluorescent lamps.

Semicontinuous cultivation

Some of the experiments in this manuscript were performed in batch reactors run in semicontinuous mode. To do this, algal cultures were always maintained within ranges of optical density (750 nm) which were previously determined as optimal. That was done by diluting the cultures with fresh culture media. The semicontinuous cultures were performed in 1 L roux flasks, and the algal cultures incubated in a culture room under controlled temperature (28ºC) and continuous white fluorescent light at a fixed light intensity of 160 μmol photons m^{-2} s^{-1}.

Dry Weight measurements

To measure dry weight, 10 ml samples of each culture were used. The samples were passed through Whatman glass microfiber filters of 47 mm in diameter and 0.7 μm pore size using a vacuum pump to separate the cells from the medium. Then the filters with the cells were dried in a stove at 100 ºC during 24h.

Growth rate calculations

Dry weight data were used to calculate growth rates. Specific growth rates of cultures were calculated using the following expression:

$$\mu = \mathrm{Ln}(C/C_0)/t,$$

where μ is the specific growth rate, C_0 is the initial biomass concentration (dry weight), and C is the biomass concentration at any time t.

In batch culture experiment, specific growth rates were calculated from the linear portion of the biomass time-course evolution, the average growth rates were calculated from the logarithmic growth phase. In semicontinuous cultures, average growth rates were calculated as the average daily growth using dry weight data.

Photosynthetic activity (oxygen release) kinetics

A Clark-type electrode was used for measuring the photosynthetic activity (Oxygraph DW1/AD, Hansatech Instruments). Oxygen release measurements were made under saturating white light (1000μmol photons m^{-2} s^{-1}) or darkness (respiration) at 25 ° C.

Chlorophyll determination

Chlorophylls were extracted using aliquots (1ml) of the cultures. Cells were spinned down for 8 minutes at 4400 rpm. The obtained pellet was placed in boiling water during 1 min. The pellet was resuspended in 4 ml of methanol and the suspension shaken vigorously for 1 min and centrifugated for 8 min at 4400 rpm. The extraction was based in a modified method described by Lichtenthaler (1987). Chlorophylls were determined spectrophotometrically in the supernatant, using the equation proposed by Wellburn (1994).

Carotenoids determination

Carotenoids were extracted using aliquots (1ml) of the cultures. Cells were spinned down for 8 minutes at 4400 rpm. The obtained pellet was placed in boiling water during 1 min. The pellet was resuspended in 1 ml of methanol and the suspension shaken vigorously for 1 min, centrifugated for 8 min at 4400 rpm. Carotenoids were separated and identified by HPLC (TermoQuest, Thermo Separetion products) with a RP-18 column, using a modified method described by Young (1997). In the mobile phase, solvent A was ethyl-acetate and solvent B was acetonitrile and water (9:1, v/v). External standards (DHI) and their corresponding calibration curves were used to identify and quantify both lutein and β-carotene.

Cell Counting

The number of the cells was determined by microascopy Olympus CH30 and Neubauer chamber.

Statistics

Unless otherwise indicated, tablets and figures show means and standard deviations of the three independent experiments.

4. RESULTS AND DISCUSSION

Growth kinetics of *C. onubensis* in batch reactors

Biomass production is the first essential step in any biotechnological application of microalgae. Therefore, it is important to determine conditions leading to achieve the highest biomass productivity. To be able to define the most appropriate concentration of Cu (II) for *Coccomyxa onubensis* cultivation, its growth under the presence of the different concentrations described above (Materials and Methods section) was studied.

Figure III.1 shows growth rates and biomass productivities of *Coccomyxa onubensis*. Growth rates were quite similar in four cultures, which contained copper concentrations from 0.06 mM (control culture) to 0.4 mM. The results showed that *C. onubensis* experienced a progressive increment in the growth rate due to Cu (II) concentration in the culture medium, achieving the maximum value in the 0.2 mM Cu (II) culture. The 0.4 mM Cu (II) culture also reached a growth rate value higher than that of the control culture. Addition of higher Cu (II) concentrations to the culture medium did not result in increased growth rates, these being significantly lower than that of the control culture.

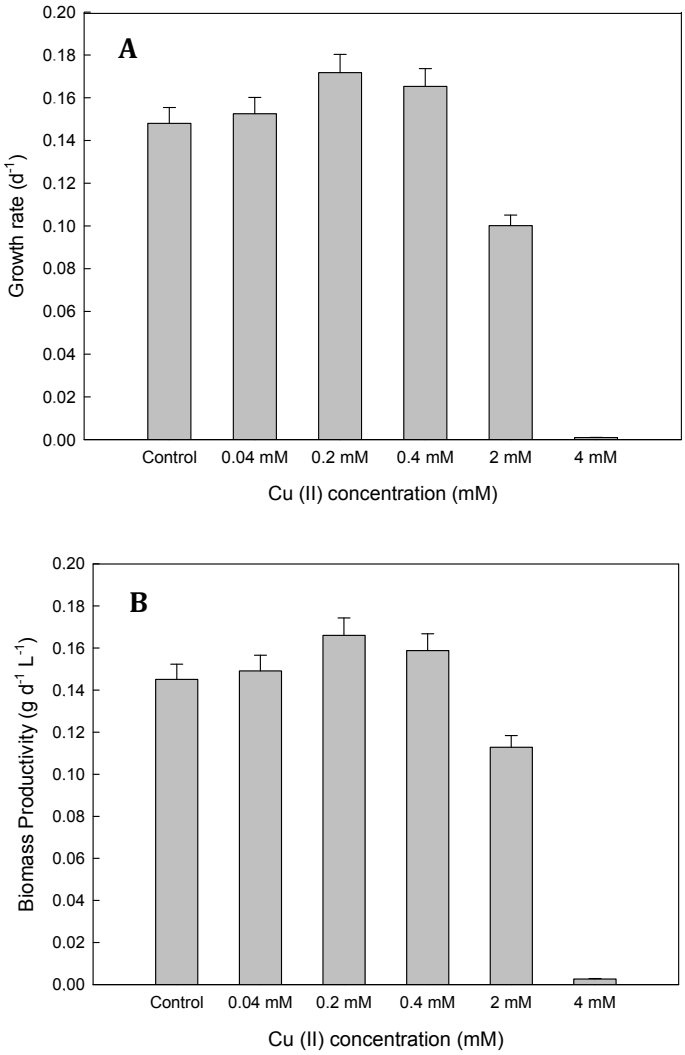

Figure III.1. Growth rates (A) and productivities (B) of *C. onubensis* grown on Cu (II). Standard autotrophic cultures, at the middle of the logarithmic phase of growth, were grown on different Cu (II) concentrations, under the cultivation condition described in Materials and Methods. Growth rates were calculated from the exponential growth phase dry weight data.

Large number of studies shows that addition of low copper concentrations to the medium causes a toxic effect on the microorganism and may even cause cell death. Kalinowska and Pawlik-Skowronska (2010) studied the response to 5μM

copper concentration of two different microalgae, *Stichococcus minor* and *Geminella terricola*, isolated from the Cu-polluted and the unpolluted terrestrial habitats, respectively. About 20% decrease in growth was observed in *S. minor* after 14 days, while decreasing growth in *G. terricola* occurred from the first day of exposure to copper. In both cases the growth of microalgae in copper-added medium was never higher than that of control cultures, revealing the extreme toxicity to algae of very low copper concentrations.

However, *Coccomyxa onubensis* was able to grow in Cu (II)-added culture medium, at less than 0.4 mM Cu (II), reaching higher rates than that of the control culture. This might be due to the natural adaptation of *C. onubensis* to high concentrations of heavy metals such as copper, occurring in its natural habitat. The Tinto river contains a high copper concentration (Johnson and Hallberg 2003), which is even higher than the added one in the experiment, 0.2mM Cu (II). Being that as it, there are no major differences between the growth rates of the different copper-added cultures of *Coccomyxa onubensis*.

Biomass productivity (expressed in g L^{-1} d^{-1}) is another useful parameter to define the appropriated concentration of copper which is necessary to sustain maximal *C. onubensis* growth. **Figure III.1.B** shows the average productivity achieved at each culture. Productivity results showed a similar trend to that obtained in growth rates. The productivity in batch systems, therefore under non optimal conditions, was 0.17 g L^{-1} d^{-1} and was achieved in the culture added with 0.2 mM Cu (II). Cultures between 0.04mM and 0.4 mM also showed better values of productivity than that of the control culture. Extremophile microalgae are known to have not very high productivities (Cassin 1974, Pulz and Gross 2004, Tittel et al. 2005, Visviki and Palladino 2001). However, *Coccomyxa onubensis* seems to have quite reasonable productivity and growth rates considering that it is a microalga from an acidic environment. Copper is an essential micronutrient, out of which photosynthesis would not be a feasible process. However, the concentration of copper in the medium for an efficient algal growth varies depending on particular species of microalgae. *Coccomyxa onubensis*, living in highly stressful conditions of heavy metals (as copper), appears to be able to

withstand higher concentrations of heavy metals than other similar species. So the critical concentration of this metal in the culture medium is around 0.4 $mmolL^{-1}$, i.e. 14.2 mg L^{-1}, which accounts for 100 times greater amount than those critical to other microalgae (Yan and Pan 2002).

Batch systems allow to easily assessing growth parameters of a given microalga. However, culture conditions along the experiment –including cell density, metal concentration, substrates concentration- change significantly. In order to explore the microalgal productivity in long-term processes, closer to actual situations, experiments in semicontinuous mode were performed and further described in this paper. Summing, copper (below 0.4 mM) may be used as a tool to increase the productivity of the microalga cultures without affecting cell viability.

Antioxidant response: carotenoid accumulation

Natural growing conditions of *C. onubensis* become highly oxidative. It usually lives in few inches water of Tinto River, conditions under which cells are exposed to high intensities of PAR and UV light and high concentrations of heavy metal, such as iron, copper, manganese or arsenic. Most of the metals are in soluble chemical forms because of the low pH of the water and cause formation of reactive oxygen species. Thus, cells are expected to react against these chemical species through molecules with antioxidant capacity, mainly carotenoids (Alscher et al. 1997, Mallick 2004, Salguero et al. 2003).

In order to assess the microalgal antioxidant response, lutein and β-carotene content and its cellular biosynthesis rate was determined. The sum of lutein and β-carotene content reaches 90% of total carotenoid cell content. Particularly, from that lutein plus β-carotene intracellular pool, lutein content accounts for about 60% to 80% of it, depending on the copper concentration added into the medium. As shown in **Figure III.2.A**, the highest values of lutein and β-carotene content were obtained for the cultures added with Cu (II) concentrations ranging from 0.04 mM to 0.4 mM. In these cultures the total carotenoid content was over that of the control cultures. That did not happen in the cultures added with the highest concentrations of copper, upper 2 mM Cu (II), where carotenoid content

remained below that in control cultures. The total carotenoid content in the 4 mM Cu (II) culture is negligible, due to the concentration of copper is highly toxic to the cell.

These results should be carefully considered since a high volumetric carotenoid accumulation (total carotenoid content per unit volume of culture) does not necessarily imply an actual intracellular increased pigment biosynthesis rate. To study whether Cu (II) addition into the culture media resulted in a more active carotenoid biosynthesis, the amount of accumulated carotenoids per cell was determined and the results shown in **Figure III.2.B**. The Figure shows that increased Cu (II) concentrations lead to increased carotenoid concentrations per cell in the batch systems. This occurs up to 4 mM Cu (II) which seems to be highly toxic for cell growth. This Figure also has to be analyzed with scientific caution. As such, this graph shows the 2 mM Cu (II) culture to be the most efficient one in cell carotenoid accumulation; however, to the light of cell viability results, this culture is clearly not the most suitable for biomass production as maximum photosynthetic activity was obtained in 0.2 mM Cu (II)-added cultures (210 μmol O_2 mg Chl^{-1} h^{-1}), far from that value obtained in 2 mM Cu (II)-added cultures. As many times stated by other authors, carotenoid accumulation conditions and biomass production conditions might not be coincident, most of times depending on the microalgae species.

Whether carotenoid biosynthesis is actually enhanced by Cu (II) can be assessed by following time course intracellular carotenoid content. From such kinetic data, carotenoid biosynthesis rates at each one of the Cu (II)-added concentrations can be calculated. **Figure III.2.B** shows that biosynthesis occurred between the 8^{th} and 15^{th} days of growth, with a particular intensity in cultures exposed to copper, after there, carotenoid cell content felt drastically, therefore biosynthesis ceased. This contrasts with the control culture where biosynthesis slightly occurred during the whole exponential phase. Interestingly, intracellular carotenoid accumulation rate is culture age-dependent. Carotenoid biosynthesis peaks at mid-late exponential growth phase, declining afterwards. Differences in carotenoid biosynthesis at the different growth phases are

considerably large (**Figure III.2**), the highest cell carotenoid concentration even being 3-fold higher than the lowest one.

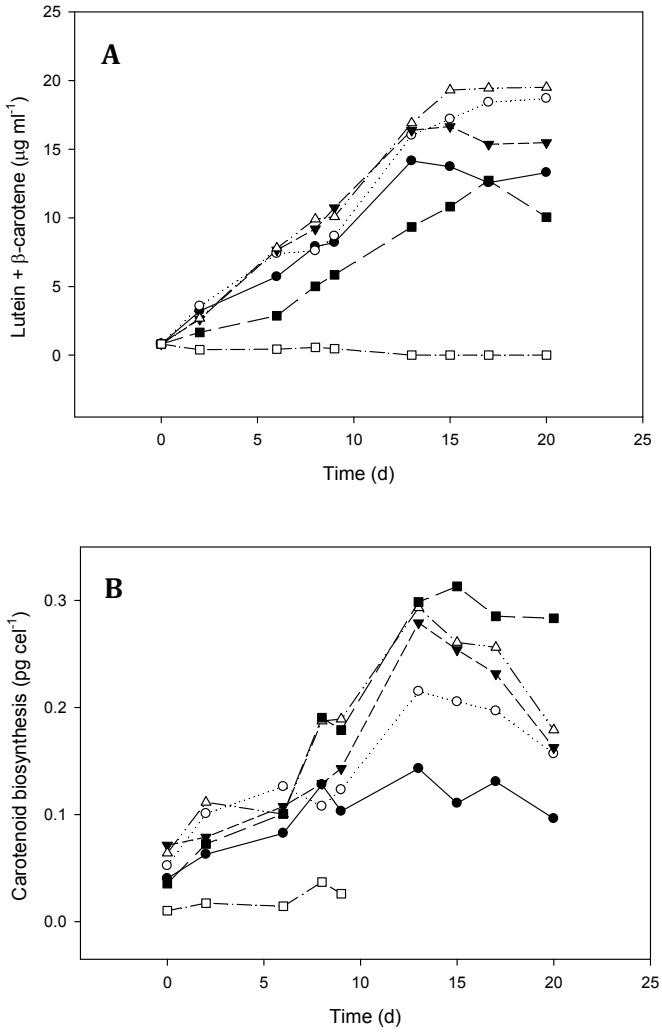

Figure III.2. Effects of various concentrations of copper on lutein and β–carotene accumulation (A) and carotenoid biosynthesis (B) of *C. onubensis*. Standard autotrophic cultures, at the middle of the logarithmic phase of growth, were grown on different Cu (II) concentrations, under the cultivation condition described in Materials and Methods. The different Cu (II) concentration were: (black circle) control culture (0,006mM Cu (II)), (white circle) 0.04 mM Cu (II), (black triangle) 0.2 mM Cu (II), (white triangle) 0.4 mM Cu (II), (black square) 2 mM Cu (II) and 4 mM Cu (II). At the indicated times, both lutein and β–carotene accumulated in the culture (A) and intracellular-content of carotenoids (B) were determined as total lutein and β–carotene content (μg mL^{-1}) by HPLC.

A better picture of carotenoid biosynthesis in Cu-exposed cells is obtained through the calculated carotenoid biosynthesis rates profile, which together with theirs corresponding growth rates are shown in **Figure III.3**. The largest carotenoid biosynthesis rate was obtained from 0.2 mM Cu (II)-added cultures, which also showed a growth rate that accounted for 90% that of control cultures. This means maximum carotenoid production rate and almost maximum biomass production rate.

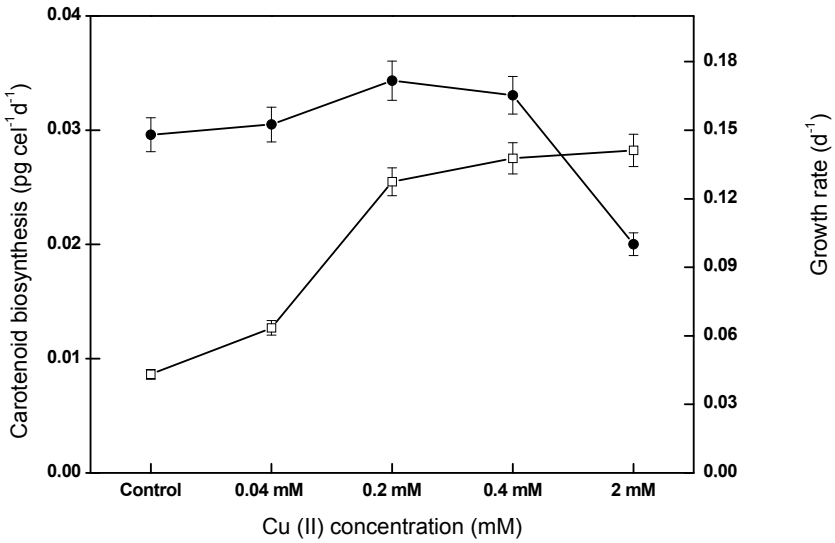

Figure III.3. Carotenoid biosynthesis rate of *C. onubensis* grown on different Cu (II). Carotenoid biosynthesis rate (white square) was calculated from the carotenoid production kinetic in Figure III.2. Growth rates (black circle) are also shown in order to infer correlations between carotenoid accumulation and growth.

Therefore, a suitable strategy for long-term production of lutein should be defined which should consist of continuously maintaining *C. onubensis* cultures at the suitable carotenoid production phase, namely lutein production phase as lutein is the major carotenoid of *C. onubensis*, and at optimal growth conditions. To do that, semicontinuous cultures should be run which consisted of adding a Cu (II) concentration such that rapid growth and high carotenoid biosynthesis rate were simultaneously feasible.

In short, it is possible to say that when copper is added in the culture medium at appropriate concentrations, it stimulates the intracellular accumulation – therefore, biosynthesis- of carotenoids without affecting cell viability, even reaching volumetric accumulation higher than the control culture. The ideal concentration of copper should provide maximum intracellular carotenoid accumulation while sustaining cell growth.

Specific carotenoids were identified and quantified by HPLC. The most abundant pigments were lutein and β-carotene (**Figure III.4)**. We focused the study on these two major pigments, lutein and β-carotene, which along with zeaxanthin, are involved in processes of excess solar energy dissipation.

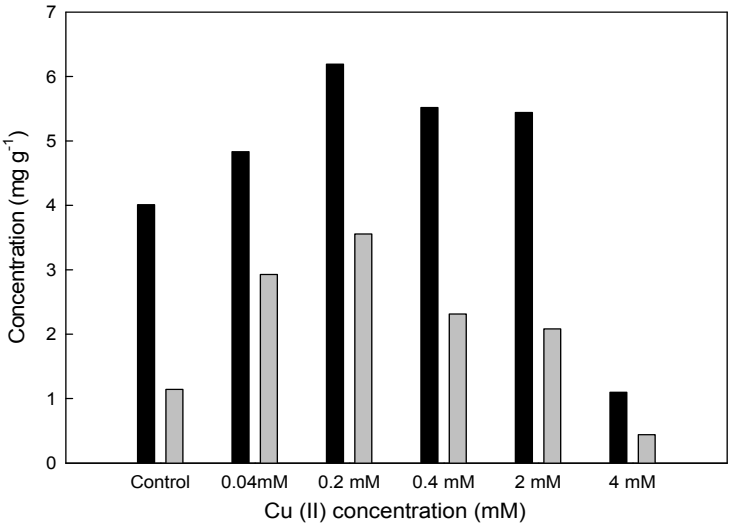

Figure III.4. Lutein and β-carotene concentration of *C. onubensis* grown on Cu (II). Standard autotrophic cultures, at the beginning of the logarithmic phase of growth, were grown on different Cu (II) concentrations under the cultivation condition described in Materials and Methods. Carotenoids were extracted, separated and determinate by following the procedures described in Materials and Methods. Lutein concentration (black bar), β-carotene concentration (grey bar).

As presumed, the highest carotenoid content was reached in the culture added with 0.2 mM Cu (II), accounting for 2.88 milligrams of β-carotene and 6.48 milligrams of lutein per gram of culture (**Figure III.4**).

Furthermore, the concentration of β-carotene and lutein in the control culture is much lower -nearly 3 times lower in β-carotene- than in the cultures supplemented with 0.2mM Cu (II).

Comparing the lutein and β-carotene contents with other microalgae, results show that the lutein content in *C. onubensis*, are similar and even better than those obtained from the most promising lutein producing microalgae species (Ben-Amotz et al. 1992). Regarding the β-carotene content, *Coccomyxa onubensis* does not reach as good results as other β-carotene producing microalgae, such as *Dunaliella* (Ben-Amotz et al. 1992, Mogedas et al. 2009). However, the results obtained in copper added cultures greatly improved the total content of β -carotene in *Coccomyxa onubensis*, around three times higher compared with control culture, as shown in **Figure III.4**.

These findings prompt us to conclude that cultures supplemented with 0.2 mM of copper in the culture medium would be the most suitable one in order to get high lutein and β-carotene productivities. It is also important to note that besides the mentioned optimal copper concentration, the cultures with concentrations in between 0.04 mM and 0.4 mM even accumulate higher concentrations of lutein and β-carotene than those of the control culture, 0.006 mM Cu (II).

Previous studies in other microalgae species have shown that addition of low high concentrations of heavy metals in the culture medium stimulates microalgal growth by increasing cell division (Liu et al. 2008, Nalewajko 1997) as well as pigment content (Mallick 2004, Wang et al. 1995). **Figure III.4** shows that those up to 0.4mM Cu (II) copper-added cultures display quite good growth rates and productivities of *C. onubensis*, while those above 2 mM copper concentrations in the culture medium caused the opposite effect.

Photosynthesis is probably the most sensitive process to oxidative damage by metals, mainly PS II (Heckathorn et al. 2004). There are several effects caused by cell exposure to high concentrations of heavy metals such as copper. Among these effects, the adsorption of ions to the cell wall, the release of extracellular exudates and decrease in the flow of metal through plasma membrane should be

mentioned (Hall 2002). The effect of high concentrations of heavy metals in microalgae induces the activity of antioxidant systems, increasing the activity of enzymes such as superoxide dismutase, catalase and glutathione peroxidase and the synthesis of low molecular weight compounds including glutathione and carotenoids (Pinto et al. 2003, Rick et al. 2006).

Steinbrenner and Linder (2001) have also shown that cultures incubated in heavy metals presence induce the activity of enzymes involved in the carotenoid biosynthesis pathway, which would be consistent with the observed increases in the cellular contents of lutein and β-carotene in ours experiments. Arguably, the oxidative stress caused by copper in high concentrations should lead to physiological changes in the algae aimed at expressing metabolic adaptations to these conditions. Studies on antioxidative enzymes responses of *C. onubensis* to oxidative conditions, including addition of copper, are currently being carried at our laboratories.

Growth kinetic of *C. onubensis* grown on Cu (II) in a semicontinouos process

Once known the influence of copper and the suitable concentration -0.2mM Cu (II)- for both optimal biomass and lutein productivities in batch systems, based on the obtained data *C. onubensis* growth and carotenoid accumulation in semicontinuous mode processes were studied. In the experiment, a culture with 0.2mM Cu (II) added into the culture medium was used, in contrast with a control culture, 0.06mM Cu(II). Both cultures were grown under standard conditions, inoculated from a stock culture at exponential growth phase. Both of them were inoculated with an initial chlorophyll concentration of 10.8 mg ml^{-1} and approximately 1.1 g of dry weight per liter of culture, as shown in **Figure III.5**. In the semicontinuous process, the cultures were maintained within an optical density range between 5 and 7. These values were previously determined as optimal for this microalga growth at the given growth conditions in Materials and Methods. In doing so, the microalgal culture was always kept in about the mid log phase of growth in which carotenoid biosynthesis rate and lutein and β-

carotene content were maximal, according to **Figure III.2** and **Table III.2 and III.3.**

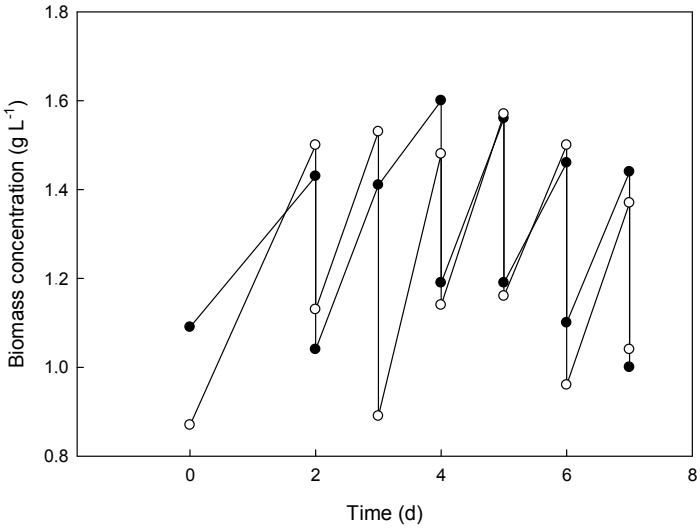

Figure III.5. Semicontinuous cultures of *C. onubensis*. Cells from standard autotrophic cultures, at the beginning of the logarithmic phase of growth, were harvested, washed and resuspended in fresh standard culture medium (black circle, control) and culture medium containing the best Cu (II) concentration previously determined (white circle). The culture was grown in a fluidized bed reactor; initially under batch regime up to an optical density within the range 5-7 was reached. From that moment on, the culture was operated in semicontinuous mode, based on keeping the optical density within the range above mentioned by means of culture dilution. The culture was illuminated with PAR light of 160 μE m^{-2} s^{-1}. At the indicated times, dry weight was determined.

Figure III.5 shows the growth of *C onubensis* in terms of dry weight expressed as g L^{-1}. The culture growth in the 0.2 mM Cu (II) culture was significantly higher than that of the control culture. The first culture, 0.2 mM Cu (II), had to be refreshed every 24 hours in order to maintain the optical density between 5 and 7. An average growth rate of approximately 0.34 d^{-1} was obtained, even peaking at 0.51 d^{-1}. In contrast, in the control culture and under the same culture conditions, the average growth rate was 0.12 d^{-1}, with a peak of 0.17 d^{-1}. Thus, copper added in the culture medium seems to positively affect growth at the studied concentration, 0.2 mM Cu (II).

Table III.1. Productivity and growth rates of 0.2 mM Cu (II) and control cultures. Batch and semicontinuous mode.

	Average productivity $g\ L^{-1}d^{-1}$	Maximum productivity $g\ L^{-1}\ d^{-1}$	Average growth rate d^{-1}	Maximum growth rate d^{-1}
Control culture Semicontinuous mode	0.29	0.37	0.18	0.27
0.2 mM Cu (II) culture semicontinuous mode	0.42	0.59	0.34	0.51
Control culture Batch mode	0.144	0.28	0.15	0.20
0.2 mM Cu (II) culture Batch mode	0.170	0.40	0.174	0.35

The productivity was also quite higher in copper-supplemented cultures. **Table III.1** shows comparative data of productivity and growth rate of that culture grown under copper-induced oxidative stress, and the control one (grown in standard conditions with amended medium K9. **Table III.1** also shows the experimental results obtained in batch system (previous test). Both productivity and growth rates are remarkably different in between the culture with 0.2 mM Cu (II) and the control culture, 0.006 mM Cu (II). It is also very noticeable that the productivity and growth rates are clearly higher in the cultures grown in a semicontinuous system that in those that grew in the batch system. **Table III.1** also shows that *Coccomyxa onubensis* grown in semicontinuous mode has similar growth rate and biomass productivity to those parameters reported for non-extremophile microalgae, which means that mass production with this microalga could be done with at least similar yields to those obtained for non-extremophile ones. In any case *C. onubensis* displays higher yields that those observed for acidophilic microalgae (Tatsuzawa et al., 1996).

Moreover, compared to batch results, growth rate and productivity are higher when the cultures are performed in semicontinuous regime, this highlights the importance of maintaining the most appropriate and productive cultivation conditions, including certain range of DO or dry weight which varies depending on light availability and microalgae species (Prieto 2011).

Table III.2 shows the light-dependent production of oxygen in cultures of *C. onubensis* incubated in the presence of copper. This is a good indicator of the viability of the culture through the photosynthetic activity, measured by an oxygen electrode as indicated in Materials and Methods section. The photosynthetic activity of *C. onubensis* is 20% higher in copper-rich culture. In short, an appropriate copper concentration is essential to provide the energy needed to stimulate the anabolic activity of the microalga, leading to enhanced growth.

Antioxidant response: carotenoid accumulation in semicontinuous regime.

The results obtained on lutein and β-carotene accumulation in semicontinuous process showed that both β-carotene and lutein concentrations increased on copper addition to the culture medium, the same that occurred in the previous experiments conducted in batch.

Tables III.2 and III.3 clearly show an increase in both productivity and cell content in the culture when it might in theory be under the influence of oxidative stress conditions by means of addition copper, both in semicontinuous and in batch cultures. Carotenoid enhancement as an antioxidant response to copper has been previously shown by Mallick (2004) where the impact of Cu on carotenoid and ascorbate pool of *Chlorella vulgaris* is shown. In that work a drastic reduction in ascorbate pool together with an increased carotenoid content was evidenced at higher copper concentrations.

It is also noteworthy that productivities achieved in semicontinuous regime were significantly higher when compared to those in batch cultures, both lutein and β-carotene.

Table III.2. Lutein productivities and content in *C. onubensis*

LUTEIN	Average lutein productivity mg $L^{-1}d^{-1}$	Maximum lutein productivity mg $L^{-1}d^{-1}$	Maximum lutein content mg g^{-1}
Control culture, semicontinuous mode	1.054	2.36	4.07
0.2 mM Cu (II) culture semicontinuous mode	2.124	3.41	6.2
Control culture, batch mode	0.63	0.69	6.003
0.2 mM Cu (II) culture batch mode	0.76	0.80	6.485

Table III.3. β-carotene productivities and content in *C. onubensis*

β-CAROTENE	Average β-carotene productivity mg $L^{-1}d^{-1}$	Maximum β-carotene productivity mg $L^{-1}d^{-1}$	Maximum β-carotene content mg g^{-1}
Control culture semicontinuous mode	0.40	0.50	1.2
0.2 mM Cu (II) culture semicontinuous mode	0.50	1.907	3.55
Control culture batch mode	0.25	0.49	1.04
0.2 mM Cu (II) culture batch mode	0.54	0.68	2.88

The content (mg g^{-1}) of these two pigments is significantly greater in that culture where copper concentration was optimized. Maximum β-carotene and lutein content in 0.2 mM Cu-added semicontinuous culture was about 2-fold and 1.5-fold, respectively, that content in control cultures. Otherwise, lutein content is much higher. This fact implies two evidences. Firstly, *C. onubensis* is a microalga with a great potential as a lutein producer; as inferred from the first set of experiments, *C. onubensis* was able to reach concentrations of lutein never reported previously for other commercial microalgae. Furthermore, lutein can optimally accumulate in viable cells at optimal growth conditions in continuous process, this being a competitive advantage for massive production at large scale. And secondly, these data further proof that copper induces an antioxidant response in the microalga *Coccomyxa onubensis*, producing a relevant increase in carotenoid accumulation.

According with Fernandez-Sevilla et al. review (2010), where the most promising microalgae for lutein production are included, *Muriellopsis sp*, *Chlorella protothecoides* and *Scenedesmus almeriensis* are considered to be the most efficient strains for the lutein production from microalgae. This statement was based on two main factors which make a microalga a good lutein producer: the lutein content and the biomass productivity. In line with its intracellular lutein content **(Table III.2),** if *Coccomyxa onubensis* was cultivated under suitable conditions, could at least be as promising as those promising microalgae, easily obtaining more than 6 mg g^{-1} dry weight in lutein content as light effect is not yet discussed in this manuscript.

Lutein content data obtained in *Coccomyxa onubensis* is within the range of lutein concentrations accumulated by the above mentioned microalgae. Respecting to the biomass productivity, *Coccomyxa onubensis* grown in semicontinuous mode shows similar biomass productivity to those values reported for non-extremophile microalgae (**Table III.1**). In fact, looking at **Table III.2**, where lutein productivity of *C. onubensis* is shown, an average productivity of 2.124 mg $L^{-1}d^{-1}$ was obtained, whereas *Muriellopsis sp,* which is considered as potential

producer of lutein, was reported to have a productivity of 1.4 mg L^{-1} d^{-1} (Fernandez-Sevilla et al. 2010). Both results were given in laboratory conditions.

In conclusion, we can say that the copper enrichment in the culture medium of *C. onubensis* of the order of 0.2 mM Cu (II) seems to be a useful condition to increase algal biomass and to enrich this acidophile microalga with carotenoid antioxidants, in particular the xanthophyll lutein, owing to *C. onubensis* has naturally large quantities of this pigment. These compounds would be accumulated particularly in the logarithmical phase; proof of this can be seen in the high productivities reached in semicontinuous experiments at appropriated stage of growth (5-7 OD). Further studies at larger volume culture system and under outdoor cultivation are needed to prove such that promising potential of *C. onubensis* shown at lab scale.

5. CONCLUSIONS

In this paper, the results obtained allow us to arise the following main conclusions: (1) *Coccomyxa onubensis* cultivation on a copper rich medium (no more than 0.4 mM Cu (II)) enhanced carotenoid accumulation, especially lutein. (2) Copper addiction into the culture medium does not imply a decrease in cell viability, and even enhance the growth rate and biomass productivity if *Coccomyxa onubensis* is cultivated under suitable conditions; and finally, semicontinuous cultivation in Cu-added media clearly improves productivity and growth rate, as well as the intracellular content of lutein, revealing that both optimal biomass and lutein productivities are compatible issues in *C. onubensis* growing in a Cu-enriched culture medium.

CHAPTER IV

Light-mediated lutein enrichment of the acid-environment microalga *Coccomyxa onubensis*

"La felicidad no está en la ciencia sino en la adquisición de la ciencia."

(Edgar Allan Poe)

1. ABSTRACT

Algae fully acclimated to different light intensities express different characteristics. At low light intensities, most algae produce more light-harvesting pigments to improve their photosynthetic efficiency. In contrast, at high light intensities, some algae produce high concentrations of "sunscreen" pigments to protect the cell from exposure to excess ultraviolet and PAR light. *Coccomyxa onubensis* grows selectively at pH 2.5, which is a competitive advantage for massive production. The alga pigment profile is rich in carotenoids, especially lutein. In this work, we studied the effect of low to moderate light intensity shifts on lutein accumulation of *Coccomyxa onubensis* cultures, doubly aimed at understanding the light-dependent role of main carotenoids in acid-tolerant microalgae and at developing strategies to induce β-carotene and mainly lutein accumulation with applied purposes. *Coccomyxa* cells were grown at 50, 140 and 400 μmol photons m^{-2} s^{-1}, reaching its maximum growth rates and carotenoid productivities at 400 μmol photons m^{-2} s^{-1} that corresponds to maximal irradiance that reaches the surface of a flat panel reactor placed vertically under summer conditions at south Spain. Lutein accumulation slightly depended on biomass concentration and maximum productivities of biomass and lutein were achieved in relatively dense cultures of 0.7 and 1 g L^{-1}. Main results indicate that *Coccomyxa onubensis* is a very promising lutein accumulating microorganism if incubated under a suitable cultivation strategy mainly consisting of transferring relatively low cell density cultures either from low to moderate irradiance or from moderate to low irradiance, therefore profiting from either pigment light-capturing or light-dissipation activities.

2. INTRODUCTION

Light is the major factor influencing algal growth, which has been confirmed through studies of the occurring relationships between light intensity and algal density, chlorophyll concentration, and photosynthesis activity (Siegel et al. 2002, Sunda and Huntsman 2004). At low intensities, light can be limiting. Algae photoacclimate by increasing their cellular chlorophyll contents at low intensities, through changes in the numbers of PSU (Raps et al. 1983) and/or in PSU size (Dubinsky et al. 1986, Falkowski and Owens 1980). The opposite, when algae are exposed to excess irradiances respect to that required to saturate photosynthesis, the excess light becomes a stress factor, and can cause photo-damage. Powles (1984) suggest that photo-damage will occur when there is an imbalance between light energy absorption and utilization in PSII. Algae have developed mechanisms to adapt to excess light (Andreasson and Melis 1995, Levy and Gantt 1988, Shapira et al. 1997). Some mechanisms consist in the dissipation of the excess photons absorbed, thorough the light-harvesting antenna of photosystem II(PSII) (Demmig-Adams and Adams 1992). If those relaxation mechanisms are unable to dissipate all of the excess energy, the remaining flux of excess photons leads to the formation of harmful radicals.

Carotenoids play an important role in light capture function and also protecting cells against the harmful effects of reactive oxygen species (ROS) (Choudhury and Behera 2001, Demmig-Adams and Adams 2002, Hu 2004, Krinsky 1989). Some carotenoid pigments may provide effective protection against disadvantageous influence of light (Macintyre et al. 2002, Steiger et al. 1999) by light dissipation through non-photochemical processes. Carotenoids are present in the photosynthetic antenna complexes of plants and algae. These complexes capture the energy in their characteristic wavelengths and transfer it to chlorophylls, expanding the spectrum of light that an organism can use for photosynthesis. LHCII antenna complex described the existence of xanthophylls binding sites. As an example of both roles, light capture and light dissipation, molecules of lutein and neoxanthin take part in light capture and light dissipation; pigments of the so-called xanthophyll cycle do also play a role in light capture –violaxanthin- and in photoprotection through dissipation of excess

energy –zeaxanthin-; and, finally, in detoxification of reactive forms of oxygen that are formed during photosynthesis –several carotenoids and xanthophylls-. In higher plants, Croce et al. (1999) state that two of three binding sites (L1 and L2) posses the highest affinity to lutein, being this pigment the major carotenoid of *Coccomyxa onubensis*, accounting for more than 75% of total carotenoids pool, ranging from 5 to 6 mg g^{-1} dry weight under lab standard conditions.

So far, studies of how light intensity affects *Coccomyxa* growth and role of carotenoids had not been made. Interestingly, some previous works in our laboratory showed *C. onubensis* to have certain sensitivity to high light intensity (summer irradiances), resulting in low growth (Garbayo *et al.* 2012). Acidophile microalgae have been published to grow much slower than the so-called "common" microalgae (Gross 2000, Pulz and Gross 2004). However, we already showed that both suitable culture medium at very low pH and suitable growing conditions (Vaquero et al. 2012), excluding high light intensities, promote growth up to making acidophile microalgae biomass production attractive for trails at outdoor cultivation systems, with the added advantage of the selective low pH culture medium where chances for competitors in open cultures are extremely scarce.

Therefore, meeting at the same time fast growth and suitable conditions to promote lutein accumulation was the current challenge in the research with that acid-tolerant microalga, aimed at proving its value for lutein-rich biomass production processes. Fastest growth might be produced at moderate to high light intensities. A maximal moderate intensity of 400 μmol photons m^{-2} s^{-1} was selected to simulate maximal irradiance that reaches a culture surface in a flat panel photobioreactor placed vertically in summer conditions at Huelva, south Spain (Cuaresma et al. 2011). Regarding lutein accumulation, some chemical parameters (nutrients, meta-ions) have already been shown to enhance its biosynthesis (Casal et al. 2011, Garbayo et al. 2012, Vaquero et al. 2012). In this manuscript, we intend to show that lutein biosynthesis might particularly be activated while *C. onubensis* cultures are in the transient from either low to moderate or moderate to low light intensities.

In this work, we compared steady-state exposures to low light with higher light exposures. Investigations have been focused on various aspects of the differences between acclimated and non-acclimated cell cultures when light shifts from low to moderate intensities and vice versa. Algal growth rate, biomass productivities and variation on chlorophyll and carotenoid contents were studied. Particularly interesting is lutein content, major pigment of *Coccomyxa onubensis*. Several microalgae have been proposed as potentially adequate lutein sources, such as *Muriellopsis sp*, *Chlorella zofingensis*, *Chlorella protothecoides* or *Scenedesmus almeriensis* (Del campo et al. 2001, Del campo et al. 2000, Fernandez-Sevilla et al. 2010, Shi 2000). *Coccomyxa onubensis* accumulates 5-6 mg g^{-1} dry weight, which is within the upper range of lutein concentrations accumulated by the mentioned microalgae. However, compared to continuous cultivation of other lutein producing species, *C. onubensis* has the practical advantage of growing well in an extremely selective culture medium at very low pH which preserves cultures from microbial contamination (Garbayo et al. 2012). This gives the acid-tolerant microalga attractive potential as a producer of this photosynthetic pigment.

3. MATERIALS AND METHODS

Isolation

Coccomyxa onubensis was isolated from acidic waters of the Tinto River (Huelva, Spain). This river has some very special features, such as low pH and a high concentration of heavy metals, especially iron, copper, magnesium and aluminum. An axenic culture of the algae was obtained by streaking it on basal agar medium at pH 2.5, and then was transferred to the liquid medium. *Coccomyxa* has been recently identified by ribosomal 18S subunit rDNA sequence analysis. Identified 18S subunit rDNA sequence was registered at GenBank with accession number GU265559.

Culture conditions

According to the chemical composition of the natural environment, cultures were grown at pH 2.5 in a culture medium based on K9 medium (Silverman and Lundgren 1959). A modified K9 medium was prepared according to the following composition: 3.95 g K_2SO_4, 0.1 g KCl, 0.5 g K_2HPO_4, 0.41 g $MgCl_2$, 2.29 g KNO_3, 0.01 g $CaCl_2$, 5 ml Hutner solution (Hutner *et al.* 1950).

The cultures were cultivated in one-liter roux bottles under constant illumination provided by with fluorescent lamps at three intensities: 50, 140, and 400 (µmols m^{-2} s^{-1}) PAR and with constant bubbling with CO_2: air mixture (5 : 95, v/v) at 27 °C. The bubbled gas flow rate was 190 ml min^{-1} per liter.

Dry Weight measurements and Growth Rate calculations

To measure dry weight, 10 ml samples of each culture were used. The samples were passed through Whatman glass microfiber filters of 47 mm in diameter and 0.7 µm pore size using a vacuum pump to separate the cells from the medium. Then the filters with the cells were dried in a stove at 90 ºC during 24h.

Dry weight data were used to calculate growth rates. Specific growth rates of cultures were calculated using the following expression:

$$\mu = \mathrm{Ln}(C/C_0)/t,$$

where µ is the specific growth rate, C_0 is the initial biomass concentration (dry weight), and C is the biomass concentration at any time t.

In adapted semicontinuous cultures average growth rates were calculated as the average daily growth using dry weight data. When cultures were light shifted, specific growth rates were calculated during the subsequent 24 hours.

QY

Fluorescence measurements were made as the maximum quantum yield (QY) of PSII (F_v/F_m). It was measured to evaluate the viability of the cells. It was

determined using a pulse amplitude modulation (PAM) (Schreiber *et al.* 1995). Samples of each culture were previously adapted to darkness for 15 minutes.

Chlorophyll and carotenoid determination

Carotenoids were extracted using aliquots (1ml) of the cultures. Cells were spinned down for 8 minutes at 13000 rpm. The obtained pellet was placed in 60 ºC water during 5 min. The pellet was resuspended in 1 ml of methanol and the suspension shaken vigorously for 1 min, centrifugated for 8 min at 4400 rpm. Carotenoids and chlorophylls were separated and identified by HPLC (TermoQuest, Thermo Separetion products) with a RP-18 column, using a modified method described by Young (1997). In the mobile phase, solvent A was ethyl-acetate and solvent B was acetonitrile and water (9:1, v/v). External standards (DHI) and their corresponding calibration curves were used to identify and quantify both lutein and β-carotene.

Statistics

Unless otherwise indicated, tables and figures show means and standard deviations of three independent experiments.

4. RESULTS AND DISCUSSION

Light intensity influence on cell viability, growth rate and pigment content

According to scientific literature, almost no attention has been paid to the extreme environment microalgae due to the fact that extreme microalgae productivities are low compared to non-extreme microalgae (Gross 2000). However, under suitable conditions acid environment microalgae show similar growth rates to non-extremophile algae, with the added advantage of growing in very selective acid culture media where very few microorganisms can grow.

In this work, we aim at inducing lutein accumulation and, in addition to that, understanding the role of *Coccomyxa onubensis* main carotenoids, β-carotene

and principally lutein, as light dissipation and/or as light harvesting pigments when the acid-tolerant alga is cultured under different light intensities. *Coccomyxa onubensis* cells grew at 50, 140 and 400 μmol photons m^{-2} s^{-1}, namely low, low-moderate and moderate light intensity, respectively. Biomass concentration of the algal cultures was approximately 0.5 g L^{-1}. Growth rates at those light intensities are shown in **Figure IV.1**. Growth rate enhancement was directly correlated with light irradiance, as expected. The maximal growth rates of *C. onubensis* cultures were obtained at 400 μmol photons m^{-2} s^{-1}, exceeding by 37% those cultures grown at 140 μmol photons m^{-2} s^{-1} and more than 2.5-fold growth rate of those cells grown under continuous light of 50 μmol photons m^{-2} s^{-1}. Moderate light intensity as 400 μmol photons m^{-2} s^{-1} does not seem to cause light stress to *C. onubensis*, at least for cell densities up to 0.5 g L^{-1}. Cells under 50 μmol photons m^{-2} s^{-1} were obviously light-limited, and those under 140 μmol photons m^{-2} s^{-1} also seem to have been light-limited for that biomass concentration. The cell generation times were 1.69 d under 400 μmol photons m^{-2} s^{-1}, 2.3 d under 140 μmol photons m^{-2} s^{-1}, and 4.35 under 50 μmol photons m^{-2} s^{-1}.

Figure IV.1 also shows the ratio between OD_{680}/OD_{750}. This ratio can be used as a relative measurement of the chlorophyll-a content of microalgae cells. OD_{680}/OD_{750} slightly decreased with the increase in light intensity and the maximal ratio was obtained at 50 μmol photons m^{-2} s^{-1}, reaching the minimum value at 400 μmol photons m^{-2} s^{-1}. When photoinhibition occurs, this ratio decreases, since cells are bleached. During this experiment no bleached cells were observed. Considering both, the high specific growth rate and the absence of bleaching, it can be concluded that photoinhibition is not a dominant process under 400 μmol photons m^{-2} s^{-1} when biomass concentration is stabilized at 0.5 g L^{-1} or higher. Consequently, if cultivated in a vertical photobiorreactor in summertime in south Spain or in equivalent latitudes, *Coccomyxa onubensis* should not experience saturating light under midday light irradiance.

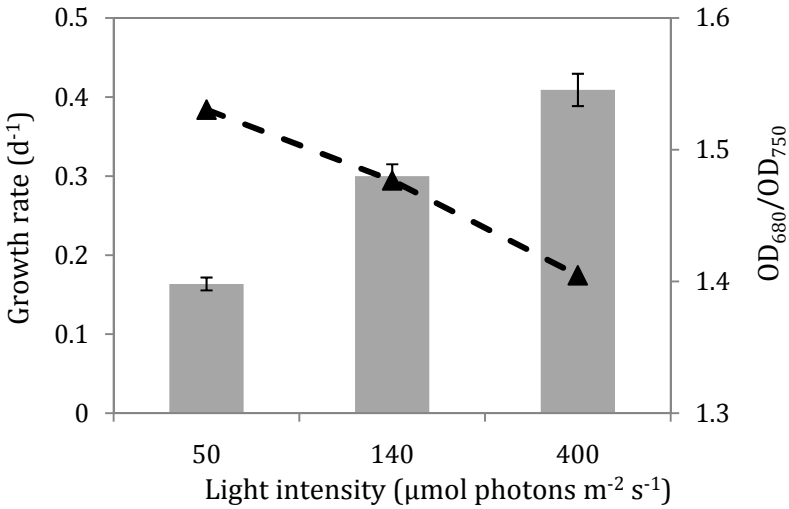

Figure IV.1. Specific growth rates and relative chlorophyll content of *Coccomyxa onubensis* grown at different light intensities (µmol photons m^{-2} s^{-1}). Growth rates (grey bars) expressed by d^{-1} were calculated from dry weight data. Relative chlorophyll-a content (black triangle symbols) is expressed as the ratio between optical density at 680 nm and 750 nm. All data were determined in stabilized cultures at approximately 0.5 g L^{-1}. Cultures were grown as described in Materials and Methods.

Figure IV.2 shows total chlorophyll, lutein and β-carotene contents when the alga was grown at 50, 140 and 400 µmol photons m^{-2} s^{-1} in long-term stabilized 0.5 g L^{-1} cultures. In cells subjected to long term cultivation, photoacclimation takes place (Macintyre et al. 2002). This requires photoregulation of gene expression and several changes at the cellular level. As a cell response, the amount of pigment per cell was inversely correlated with the photon flux density. Lutein and total chlorophyll content of the microalga was higher at low light than at moderate light intensity. Both lutein and total chlorophyll content were approximately 18% greater under low light intensity than under moderate light intensity. Therefore, lutein to chlorophyll ratio was approximately constant during all light conditions tested and that also suggests that lutein and chlorophyll contents are linked in *Coccomyxa* cells incubated at low light intensities, and involved in light capture functions. β-carotene content was quite similar at 50, 140 and 400 µmol photons m^{-2} s^{-1}, roughly 0.6-0.75 mg g^{-1}, being higher at lower light irradiance conditions.

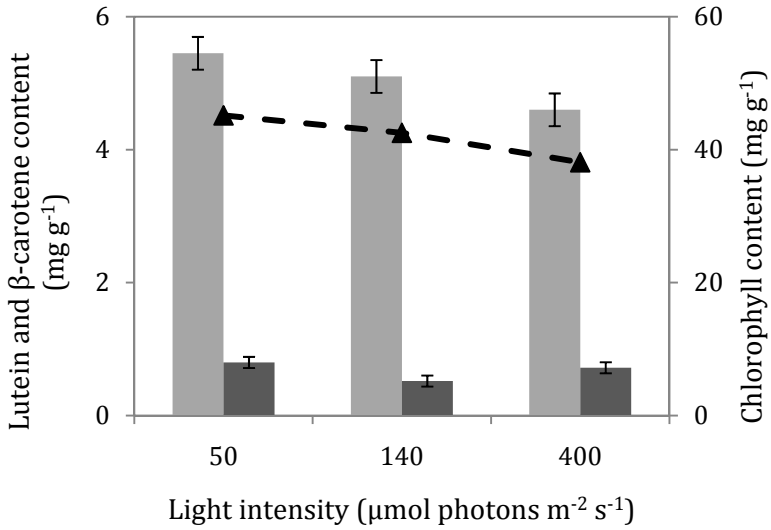

Figure IV.2. Lutein, β-carotene and total chlorophyll contents of *Coccomyxa onubensis* grown at different light intensities (µmol photons m^{-2} s^{-1}). Lutein (light grey bars), β-carotene (dark grey bars) and total chlorophyll (black triangle symbol) were determined in stabilized cultures at approximately 0.5 g L^{-1} by HPLC. Cultures were grown as described in Materials and Methods.

Besides culture medium, light is obviously a driving factor also for growth of acid environment microalgae. At low light intensities, most algae produce more light-harvesting pigments to improve photosynthetic efficiency. Lutein is one of those light-harvesting pigments which take part of the antenna complexes and, consequently, its content seems to be linked to chlorophyll content (Garcia-Camacho et al. 2012, Macintyre et al. 2002, Raps et al. 1983, Richardson et al. 1983). Therefore, at low light intensities, *Coccomyxa onubensis* would be expected to enhance their chlorophyll and lutein contents. In contrast, at high light intensities, some algae produce high concentrations of "sunscreen" pigments to protect the interior of the cell from exposure to excess ultraviolet and PAR light (Demmig-Adams and Adams 2002, Krinsky 1989). From the results in **Figure IV.1** and **Figure IV.2**, it can be concluded that *C. onubensis* rapidly adapts to low light intensities in order to maximize light capture, as inferred from growth rate to light intensity correlation and also from parallel increase in chlorophyll and lutein content, suggesting enhanced biosynthesis of light harvesting complexes. These results are in accordance with the general

algae response when cells are acclimated to low light conditions. Under these limiting conditions for growth, an increment in the light-harvesting pigments is expected to occur in order to capture as much light as possible during light limitation (Garcia-Camacho et al. 2012, Dubinsky and Stambler 2009).

Therefore, moderate light intensity as 400 μmol photons m^{-2} s^{-1} does not seem to cause light stress to *Coccomyxa onubensis*, according to results in **Figure IV.1**. Such that light intensity simulates maximal irradiance that reaches a culture surface in a flat panel photobioreactor placed vertically in summer conditions at Huelva, south Spain (Cuaresma et al. 2011). Consequently, in terms of large-scale, *C. onubensis* cultures should grow well at moderate light intensities, showing moderate to high biomass productivities, maintaining intracellular lutein content above 4.5 mg g^{-1} and lutein productivities of 1.22 mg L^{-1} d^{-1}, whereas at low light intensities, lutein productivities of the alga are much lower. *C. onubensis* reached lutein productivities of 0.84 mg L^{-1} d^{-1} when cells were cultured under 140 μmol photons m^{-2} s^{-1} and 0.34 mg L^{-1} d^{-1} under 50 μmol photons m^{-2} s^{-1}. Compared to data in literature, the obtained results of maximal lutein productivities are interesting for lutein production trials with microalgae (Fernández-Sevilla et al. 2010), having the advantage of sustaining algal growth in very low pH selective culture media (Garbayo et al. 2012).

The light irradiance experienced by *C. onubensis* cultures will be influenced by cell density. Therefore, the effect of increased biomass concentration on biomass productivity and pigments content was studied in adapted cultures at the selected light intensity, 400 μmol photons m^{-2} s^{-1}. **Figure IV.3** shows growth rates (**Figure IV.3A**) and biomass productivities (**Figure IV.3B**) for adapted *Coccomyxa* cells. Adapted cells are defined as those which have been at least 24 hours growing at indicated conditions from their inoculation. *Coccomyxa onubensis* adapted cells increase their growth rates when biomass concentration descends, achieving up to 0.6 d^{-1} when biomass concentration is lower than 0.4 g L^{-1}. From 0.4 to 1 g L^{-1}, *C. onubensis* stabilized cultures reached growth rates around 0.2-0.4 d^{-1}. However, when cells were cultured at higher cell densities, more than 1 g L^{-1}, achieved growth rates were much lower. Cells under those biomass concentrations were obviously light-limited. As expected, biomass

productivities depend on biomass concentrations, reaching its maximum value (0.4 g L^{-1} d^{-1}) when biomass concentration is about 1 g L^{-1}. However, from 1 g L^{-1} onwards, algal biomass productivity decreases. Those results confirm that *C. onubensis* cells under those biomass concentrations were light-limited, in accordance to **Figure IV.3A**.

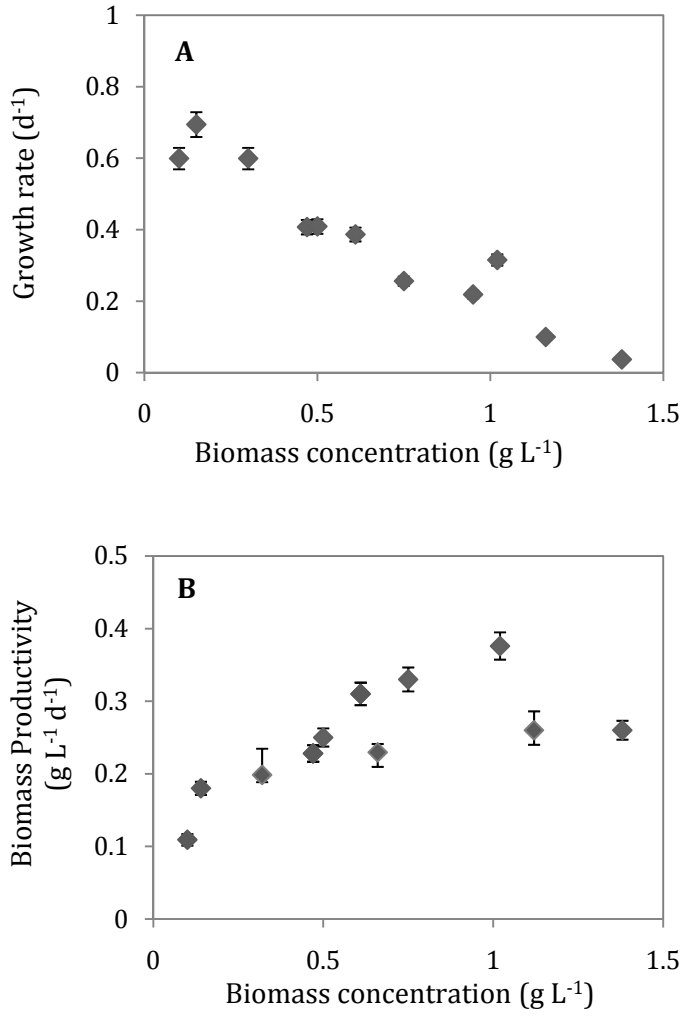

Figure IV.3. Specific growth rates (A) and biomass productivities (B) at different biomass concentrations (g L^{-1}) of *C. onubensis* cultures adapted to 400 µmol photons m^{-2} s^{-1}. Biomass productivities, expressed as g L^{-1} d^{-1}, were calculated from dry weight data in stabilized cultures at 400 µmol photons m^{-2} s^{-1}. Growth rates, expressed as d^{-1}, were calculated by measuring optical density at 750 nm. Cultures were grown as described in Materials and Methods.

Hence, biomass concentration in the reactor medium is linked to light availability and will therefore influence algal productivity. In this manuscript, linear correlation between biomass concentration and biomass productivity –at the studied light irradiances- shows the acid-tolerant microalga can be cultivated yielding productivities that might be suitable for feasible production trials at pilot scale. Moreover, the fact that the irradiance applied to our experiments is equivalent to that at the surface of a vertical panel reactor still leaves the possibility of using intermediate higher irradiances (400-800 μmol photons m^{-2} s^{-1}) open and, also according to the obtained results, higher growth rates might also be obtained.

Having such acceptable biomass productivities, next question is to get knowledge on whether lutein biosynthesis activity does also depend on biomass concentration and, therefore, does result anyhow affected by shadowing effect at high cell densities. Optimal production of lutein-rich biomass of *C. onubensis* at large scale should be consequence of both high growth rates and high intracellular lipid content. **Figure IV.4** shows the content of lutein and total chlorophyll at different biomass concentrations in *C. onubensis* cultures illuminated with moderate light intensity, 400 μmol photons m^{-2} s^{-1}.

Lutein content and total chlorophyll were calculated in adapted cultures. Cultures are considered adapted when pigment content becomes constant, i.e. in photoacclimated cultures. Garcia-Camacho et al. (2012) affirm that during photoacclimation, microalgae respond to the light available by varying their cell pigment content. In *C. onubensis,* lutein and total chlorophyll content were maximal (about 8.5 and 45 mg g^{-1} dry weight, respectively) almost at the highest biomass concentration tested, 1 g L^{-1}. These results are in accordance with data in **Figure IV.2**, in view of the fact that carotenoid accumulation of *C. onubensis* is similar under low light intensities and when cultured at high biomass concentrations. Under both conditions, cell cultures become darker because of the self-shadowing effect. Thus, an increment in the light-harvesting pigments (such as lutein and chlorophyll) occurs.

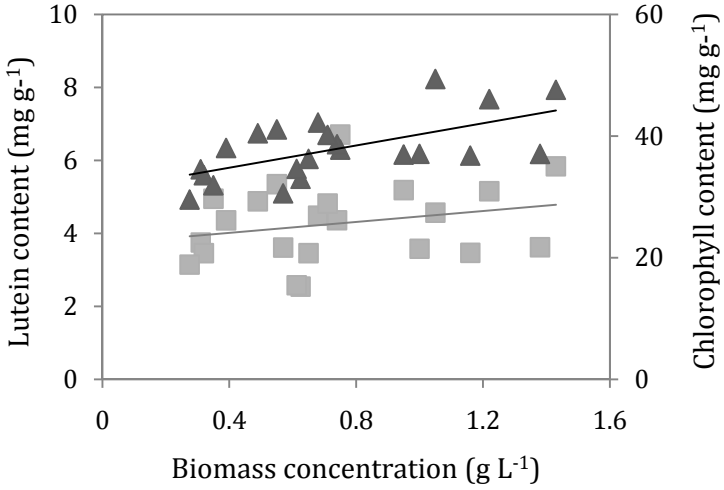

Figure IV.4. Lutein and total chlorophyll contents (mg g^{-1}) of *Coccomyxa onubensis* grown at different biomass concentration at 400 µmol photons m^{-2} s^{-1}. Lutein (light grey square symbols) and total chlorophyll (dark grey triangle symbols) were determined by HPLC, in cell extracts of stabilized adapted cultures. Biomass concentrations were calculated from dry weight data at 400 µmol photons m^{-2} s^{-1}. Cultures were grown as described in Materials and Methods.

Interestingly, unlike chlorophyll content, maximal lutein content (greater than 6 mg g^{-1}) might be achieved for biomass concentrations above 0.5-0.6 g L^{-1}, reaching its maximum at 1 g L^{-1} or more. Consequently, a suitable biomass concentration in the reactor really improves lutein productivity without losing biomass productivity of the acid-tolerant microalga.

Considering the maximal growth rate at 1 g L^{-1} and the corresponding lutein content, a maximal lutein productivity of about 3.5 mg L^{-1} d^{-1} can be calculated for *C. onubensis* at the tested light intensity. These data suggest that *C. onubensis* lutein production capacity at moderate light intensity is in the range of that published for other non-extremophile microalgae, like *Chlorella zofingiensis* (3.4 mg L^{-1} d^{-1} , Del Campo et al. 2000) and *Scenedesmus almeriensis* (4.77 mg L^{-1} d^{-1} , Sanchez et al. 2008), which are among the microalgae considered as promising for lutein production. More studies to assess lutein production capacity at higher light intensities and in outdoor systems remain to be done with *Coccomyxa onubensis*.

To the view of the obtained results, lutein accumulation in *Coccomyxa* cells is particularly enhanced as a result of productive growth at stable low photons flux density, and such lutein accumulation seems to be linked to increased chlorophyll content. However, if lutein biosynthesis was enhanced as a consequence of sudden increase in light irradiance, it might provide another strategy to help increasing lutein yield. Besides, it would also provide knowledge on the cell response to excess light energy and therefore on the antioxidative role of lutein.

Therefore, a deeplier study on how shift in light intensity affects chlorophylls and lutein content in *Coccomyxa onubensis* was done. To induce light stress, low light acclimated cells were transferred to higher irradiance and the kinetics of biomass and pigments were analyzed. In addition, moderate light acclimated cells were transferred to lower irradiance for similar study. Lutein and β-carotene content, and chlorophyll content of the microalga were measured every two hours after shift.

Light intensity shifts influence on growth rate, biomass productivity and pigment content

Coccomyxa onubensis cultures were grown in semicontinuous mode and stabilized at 0.5 g L^{-1} and 1.5 g L^{-1}. Once stabilized, cultures shifted from moderate to low light intensity, and vice versa. Namely, shifts from 400 to 50 μmol photons m^{-2} s^{-1} and 140 to 400 μmol photons m^{-2} s^{-1} were performed. *C. onubensis* cultures that remained under initial light conditions at low-moderate light intensity (140 μmol photons m^{-2} s^{-1}) were grown as control cultures. **Figure IV.5** shows growth rate (A) and biomass productivity (B) of *C. onubensis* cultures at 0.5 and (light grey bars) 1.5 g L^{-1} (dark grey bars), once light shifts occurred.

Greatest growth rates and biomass productivities were obtained when light intensity shifted from low to moderate values (400 μmol photons m^{-2} s^{-1}). That trend is unlikely cell density, so that similar trends are observed in both 0.5 and 1.5 g L^{-1} stabilized cultures. Those cultures stabilized at 0.5 g L^{-1} showed larger increases in growth rates when shifted from low to moderate light intensity,

exceeding by 70% growth rates of those cultures shifted from moderate to low light intensity and more than 3-fold those cultures grown in continuous light of 140 µmol photons m^{-2} s^{-1} (**Figure IV.5A**).

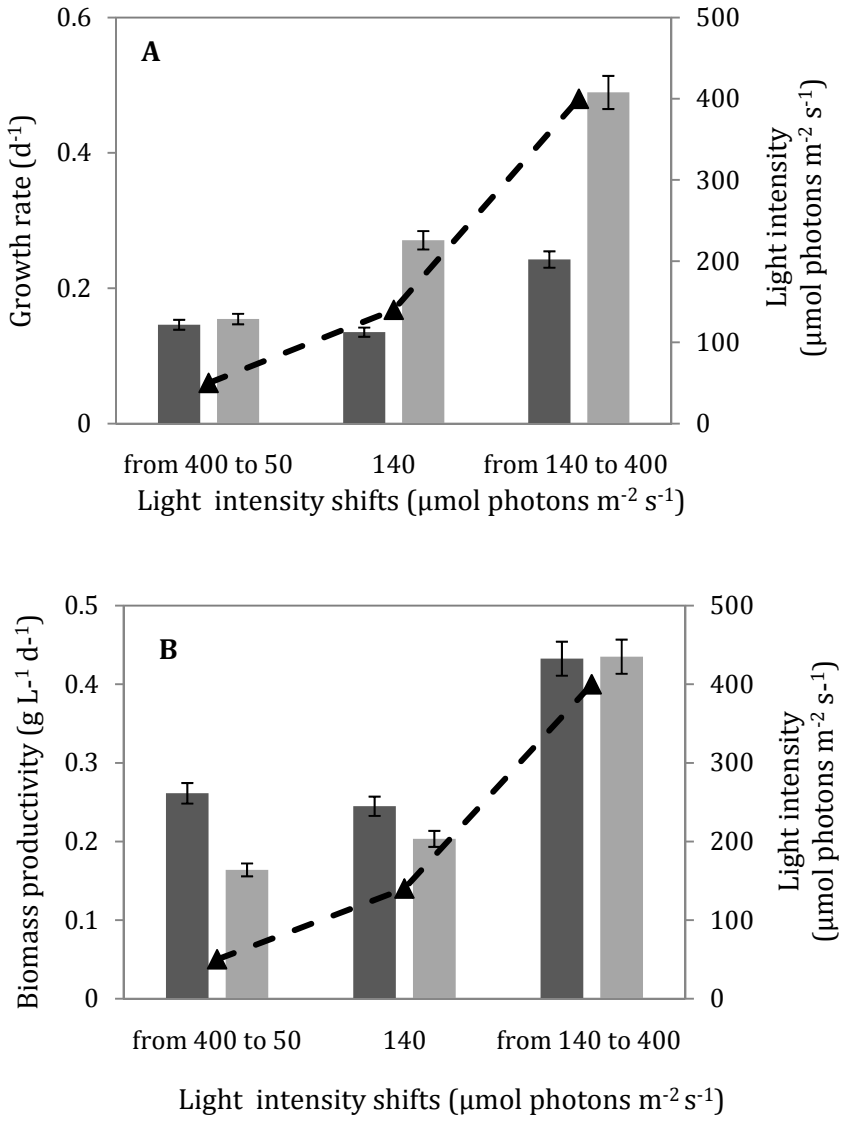

Figure IV.5. Growth rates and biomass productivities of *C. onubensis* subjected to different light shifts. Growth rates (A), expressed as d^{-1}, were calculated by measuring optical density at 750 nm. Biomass productivities (B), expressed as g L^{-1} d^{-1}, were calculated from dry weight data. Dark grey bars represent *Coccomyxa onubensis* cultures stabilized at 1.5 g L^{-1}. Light grey bars represent *Coccomyxa onubensis* cultures stabilized at 0.5 g L^{-1}. Black triangle symbols represent light intensities (µmol photons m^{-2} s^{-1}). Cultures were grown as described in Materials and Methods.

Therefore, if photon flux density decreases, cells become obviously light-limited, improving its growth rates when transferred to a higher light intensity. Such increase in growth is particularly noticeable in low cell density cultures (0.5 g L^{-1}). More dense cultures, 1.5 g L^{-1}, do not experience such a high growth rate rising when shifted to 400 μmol photons m^{-2} s^{-1}. Accordingly, it might be assumed that such moderate light intensity is even limiting for dense cultures of *C. onubensis*.

Figure IV.5B shows biomass productivities of *C. onubensis* cultures shifted from moderate to low light intensity and vice versa, in cultures at 0.5 and 1.5 g L^{-1} of biomass. As said before, greatest biomass productivities were obtained when light intensity shifted from low to moderate values. Although best growth rates are obtained for less dense cultures (0.5 g L^{-1}), best biomass productivities are obtained in very dense cultures due to their larger cell concentrations, as already discussed for results in **Figure IV.3**. Therefore, biomass productivity of dense cultures equals or even surpasses that of cultures with 0.5 g L^{-1}. Best productivity was 0.43 g L^{-1} d^{-1}, relevant biomass productivity considering that *C. onubensis* is an extremophile alga, which faces limited inorganic carbon availability at very low pH (Diaz and Marbely 2009). This value, 0.43 g L^{-1} d^{-1}, is still a bit low when compared to non-extremophile microalgae productivities, but it is two-fold that rate determined for other acid-environment strains (Tittel et al. 2005, Visviki and Palladino 2001).

Shift from moderate to low light intensity: lutein content

Coccomyxa onubensis major pigment contents (lutein, total chlorophyll and β-carotene) obtained from light shifts experiences are shown in **Figure IV.6**. Cultures were shifted from moderate to low light intensity, 400-50 μmol photons m^{-2} s^{-1} (A) and from low light to moderate intensity, 50-400 μmol photons m^{-2} s^{-1} (B). Cell density influences time course of cell pigment content of *Coccomyxa onubensis.*

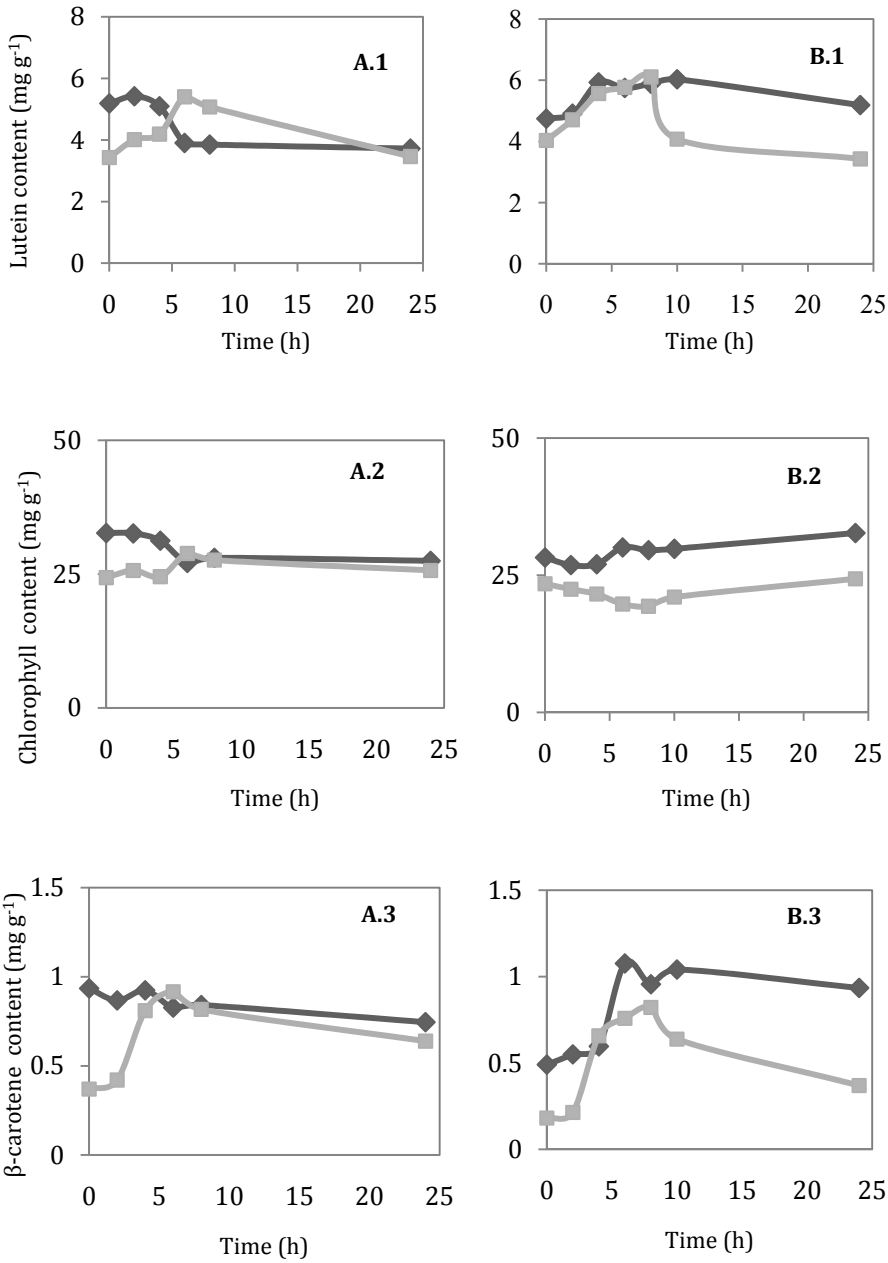

Figure IV.6. Lutein (1), total chlorophyll (2) and β-carotene (3) contents of *Coccomyxa onubensis* cultures shifted from moderate to low light intensity, 400-50 μmol photons m^{-2} s^{-1} (A) and from low light to moderate intensity, 50-400 μmol photons m^{-2} s^{-1} (B). Dark grey symbols represent *Coccomyxa* cultures grown at 1.5 g L^{-1} and light gray symbols represent *Coccomyxa* cultures grown at 0.5 g L^{-1}. Zero time indicates light intensity shifts. Both lutein total chlorophyll and β-carotene were determined by HPLC. Cultures were grown as described in Materials and Methods.

When *C. onubensis* is transferred to low irradiance, the time-course main carotenoids trend seems to depend on biomass concentration. In low cell density cultures, lutein and β-carotene increased. These results are according to those in **Figure IV.2**. However, *C. onubensis* dense cultures (1.5 g L^{-1}), shortly after having been transferred to low irradiance, followed the opposite trend in terms of variation of the content of three pigments.

This could be due to the fact that moderate light (400 μmol photons m^{-2} s^{-1}) is insufficient for sustaining basic anabolism in high cell density cultures (1.5 g L^{-1}). This was supported by **Figure IV.3**, which showed decreased growth rates for dense cultures incubated under 400 μmol photons m^{-2} s^{-1}. Therefore, dense cultures of *C. onubensis* tend to decrease its content in chlorophyll and lutein when dramatically decreases the amount of photons.

Particularly, in cultures shifted from moderate to low irradiance, the increased lutein content in low density cultures is in good agreement with the light harvesting role for lutein and β-carotene (Garcia-Camacho et al. 2012, Grossman et al. 1995). Kirk (1994) affirmed that increasing the number of those light-harvesting pigments is thought to afford a competitive advantage to the organism in an environment where sunlight is limiting. By decreasing the amount of light reaching the system, the microalga is expected to increase the content of lutein and β-carotene due to its light harvesting role, which is linked to their presence in light harvesting complexes and therefore, to the presence of chlorophyll. Several authors clearly advocate the increase of carotenoids and chlorophylls in darkness or low light (Garcia-Camacho et al. 2012, Kirk 1994, Smith et al. 1990).

For dense cultures, the observed opposite trend (decreased lutein content) might be related to very low energy availability that should be required for sustaining basic anabolic activities of dense cells cultures. In that respect, to effectively address cell growth, maintenance energy requirements should be properly satisfied (Zijffers et al. 2010). The need of light energy might be not satisfied for much higher cell density cultures if light intensity does not supply enough number of photons (Cuaresma et al. 2011). In a much more dense

cultures subjected to low light intensity, content of light harvesting pigments is by far enough as to cope with the incoming photon flux density, which might anyhow explain the different trend in lutein content.

Shift from low to moderate light intensity: lutein content

Figure IV.6B shows lutein, β-carotene and total chlorophyll contents of *C. onubensis* cultures when shifted from low to moderate irradiance. Some significant differences were observed in the intracellular content evolution of the three photosynthetic pigments. Cultures were previously adapted to low photons flux densities and, therefore, to cellular metabolic activity limited by light. Cells were then exposed to a sudden increment in irradiance and a larger number of photons.

In lower density cultures, when light increases from low to moderate light intensity lut/chl ratio increases during the first hours after irradiance shift. Interestingly, 48 hours afterwards intracellular lutein content decreases to levels prior to that light shift occurred. That decrease is probably caused by an algal acclimation to moderate light intensity. Even though irradiance increase is far from becoming photoinhibitory, according to the increase in lutein and β-carotene *Coccomyxa* cells seem to experience a temporary excess light effect. Subsequently, lutein might be overproduced as an antioxidant algal response. However, in high density cultures under the same light conditions, lut/chl ratio remains constant. Though lutein content increases during the first hours from light shift (**Figure IV.6**), that increase on lutein content is not as noticeable as for low density cultures and an increment in chlorophyll content was also produced, revealing predominance of light capture functions in the cell response of dense cultures.

According to the obtained results, the increased lutein content might be related to antioxidant microalgal response mechanisms. When algae are exposed to irradiances in excess of that required to saturate photosynthesis, the excess light could become a stress factor. Light energy would be used by photosynthesis to producing higher concentration of NADH (Dat et al. 2000, Demmig-Adams and

Adams 1992, Mathis 1983, Moller 2001). Most NADH would be used by photosynthesis to assimilate nutrients and to manufacture organic molecules. Among these processes, NADH can also be consumed in the synthesis of neutral lipids that requires a large amount of NADH (Hu et al. 2008) and thus relaxes the overreduced electron transport chain under high light. Furthermore, the TAG and carotenoid synthesis pathway are usually correlated in algae (Rabbani et al. 1998, Zhekisheva et al. 2002) preventing or reducing excess light. Even though 400 µmol photons m^{-2} s^{-1} should not be excess light in terms of photosynthetic capacity of microalgae (Osmond et al. 1997), microalgae can eventually experience a shift to higher light intensity as excess light while adapting to new light conditions (Melis 1999).

Moreover, when light become a stress factor several protective mechanisms are activated (Asada et al. 1998, Asada 1999) including mechanisms related with light-harvesting antenna of photosystem II. Excess photons absorbed by the antenna can be dissipated through down-regulation of PSII activity associated with the proton gradient generated across the thylakoid membrane (Demmig-Adams and Adams 1992). However, if these relaxation mechanisms are unable to dissipate all of the excess energy, the remaining flux of excess photons leads to over-production of reactive oxygen species, due to a more intense respiratory activity (Moller 2001, Nohl 2003). Carotenoids, including β-carotene and lutein, play an important role in protecting cells against the harmful effects of reactive oxygen species (ROS). The defense against harmful ROS could be one of the reasons for the increase of carotenoid content during the first hours after shift to a higher light intensity. Such response is in good agreement with the fact that, unlike the stable chlorophyll content time course evolution, lutein and β-carotene contents show net rapid increase. That statement is confirmed in **Figure IV.7**, which shows the relative variation of lutein and β-carotene contents respect to total chlorophyll content through the lutein/chlorophyll and β-carotene/chlorophyll ratios. Those ratios were calculated for low and high cell density cultures of *C. onubensis*, 0.5 and 1.5 g L^{-1} respectively. In that respect, Andreasson and Melis (1995) have shown a declining amount of LHCII when low

light grown cells of the green alga *Dunaliella salina* were transferred to high light.

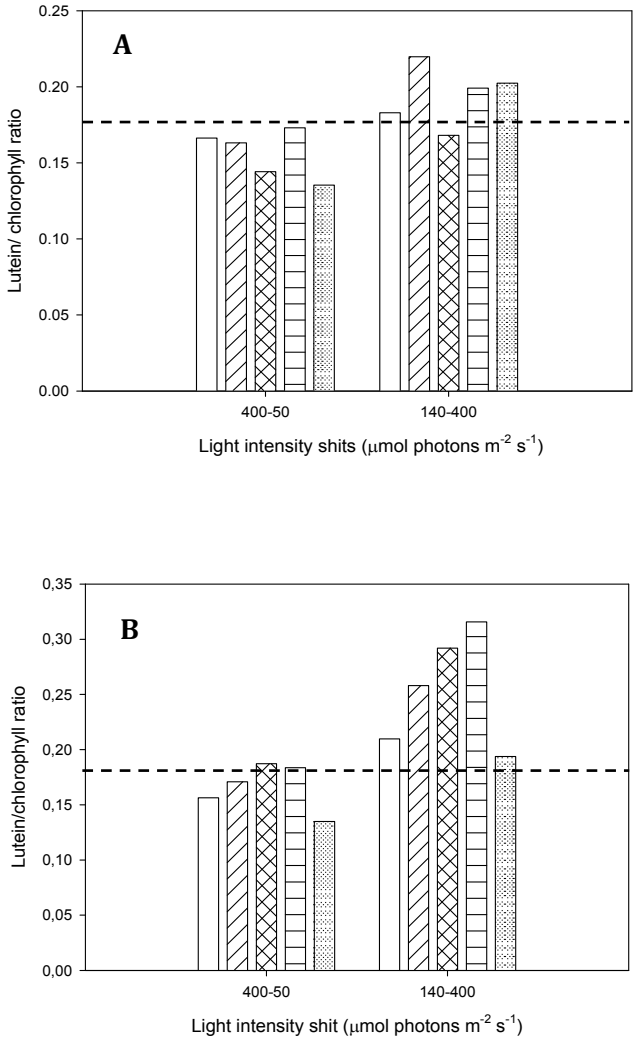

Figure IV.7. Lutein/ chlorophyll ratio of *Coccomyxa onubensis* cultures shifted from 400 to 50 and from 140 to 400 μmol photons m^{-2} s^{-1}. (A) *Coccomyxa* cultured at 1.5 g L^{-1} and (B) *Coccomyxa* cultured 0.5 g L^{-1}. (▭) represent the lutein/chlorophyll immediately after light shift; (▨) represent the lutein/chlorophyll ratio 2 hours after light shift, (▨) represent the lutein/chlorophyll ratio 4 hours after light shift, (▭) represent the lutein/chlorophyll ratio 6 hours after light shift, and (▭) represent the lutein/chlorophyll ratio 24 hours after light shift. Dashed grey lines represent the average lutein/chlorophyll ratio at 140 μmol photons m^{-2} s^{-1} at 1.5 in figure A and 0.5 g L^{-1} in figure B.

Consequently, when *C. onubensis* grows at 1.5 g L^{-1}, the shift to moderate irradiance seems not to apparently be experienced as excess light, thus light does not become a stress factor. This may be due to the high cell density of the culture itself, which is 3-fold the lowest cell density studied. Antioxidant response based on increased pigment biosynthesis would not be necessary to mitigate harmful effects of reactive oxygen species. Therefore, the observed increase in the intracellular content of carotenoids should be related to light absorption functions, as suggested from the parallel increase in chlorophyll content, and might be used for conducted lutein production processes.

Main results of this manuscript indicate that *Coccomyxa onubensis* is a very promising lutein accumulating microorganism if incubated under a suitable cultivation strategy mainly consisting of transferring relatively low cell density cultures (about 0.7-1 g L^{-1}) either from low to moderate irradiance or from moderate to low irradiance. In both cases, first over a period of hours, lutein content increases up to more than 6 mg g^{-1} (even up to 8 mg g^{-1}) industrially relevant concentration of xanthophyll. According to the obtained results discussed in this manuscript, physiological responses to each one of those irradiance shifts might be of different nature, either light capture (moderate to low irradiance shift) or partly excess light defense mechanism (low to moderate irradiance shift), though the final result is the same. In good agreement with the obtained results, it might be proposed that a continuous outdoor production trial of lutein rich *Coccomyxa onubensis* biomass should include harvesting at either beginning or end of the day, where eventually cell lutein concentration should increase.

Looking at end process application, operating under 400 μmol photons m^{-2} s^{-1}, so-called moderate light intensity in this manuscript, e.g. maximal summer irradiance in south Spain on a vertical panel reactor (Cuaresma et al. 2011), dense cultures of *Coccomyxa onubensis* will be suitable to accumulate lutein at high productivity.

5. CONCLUSIONS

In this paper, the results obtained allow us to arise the following main conclusions: (1) Typical average wintertime light intensity in south Spain, 400 μmol photons m^{-2} s^{-1} (moderate light), does not cause light stress in *Coccomyxa* cells, and furthermore, at such irradiance *C. onubensis* reached its maximum biomass and lutein productivities; (2) Cell density influences growth rates and productivities of biomass and lutein, which optimal values were achieved when the alga grows at a biomass concentration from 0.7 to 1 g L^{-1} DW; (3) Cell density influences short-time course evolution of carotenoids, which can be used for increasing lutein content of *C. onubensis*; and, finally (4) *Coccomyxa onubensis* is a very promising lutein accumulating microorganism if incubated under a suitable cultivation strategy, mainly consisting of transferring relatively low cell density cultures either from low to moderate irradiance or from moderate to low irradiance, or selecting the suitable day time for the harvesting step in outdoor production processes.

CHAPTER V

Coccomyxa onubensis growth and lutein accumulation in a pilot tubular photobioreactor under spring outdoor conditions

"There are no such things as applied sciences, only applications of science."

(Louis Pasteur)

1. ABSTRACT

Coccomyxa onubensis is an acid-environment microalga. *Coccomyxa* pigment profile is rich in carotenoids, especially lutein, of which naturally accumulates around 4 to 6 mg g^{-1} dry weight, that is in accordance with other lutein producing species. Furthermore, *C. onubensis* has the practical advantage of growing well in an extremely selective culture medium at very low pH which preserves cultures from microbial contamination. This gives the acid-tolerant microalga attractive potential as a producer of this photosynthetic pigment.

The aims of the present study were to evaluate the feasibility of outdoor cultivation of *C. onubensis* and to assess the potential of this microalga as an alternative source of lutein. *Coccomyxa onubensis* was cultivated in acid culture medium under springtime outdoor conditions in south of Spain. A 6 liters pilot tubular photobioreactor (PBR) was used to study the influence of environmental light and temperature conditions on outdoor algal cultivation. The results showed that *C. onubensis* is able to withstand high temperature levels. Although some thermodamage was found, the cultures rapidly recovered. However, a light intensity above 1000 μmol photons m^{-2} s^{-1} was detrimental. Maximum daily productivity was obtained for *C. onubensis* cultured in a cloudy day (maximum irradiance of 1070 μmol photons m^{-2} s^{-1}), achieving 0.32 g L^{-1} d^{-1}. Average intracellular lutein content was around 4.5-5 mg g^{-1} when *C. onubensis* was cultured under suitable conditions. An increase at the end of the day was observed, probable related to light absorption functions.

This work gives the first guidelines of the parameters that influence in a potential large-scale cultivation of acid environment microalgae and should allow to design lutein-rich biomass production strategies of these extremophile microalga.

2. INTRODUCTION

Commercial interest in microalgae has been increasing in last decades. Microalgae are a potentially rich source of a vast array of chemical products with applications in the feed, food, nutritional, cosmetic, pharmaceutical and even fuel industries.

The history of microalgal utilization from natural populations is centuries old (Nostoc in Asia and Spirulina in Africa and Mexico). However, large scale commercial production processes only date back to the 1950s, when *Chlorella* and *Arthrospira* started to be produced as an alternative nutritious source (Belasco 1997, Grobbelaar, 2010). Together with the availability of nutrients, light and temperature are the main parameters that influence in the productivity of the microalga cultures (Richmond 1990). Under outdoor conditions, the daily solar cycles determine both light and temperature cycles. Solar radiation comprises a broad range of wavelengths although only the photosynthetically active radiation (PAR, 400–700 nm) are useful for photosynthesis. Light should be managed as efficiently as possible. In an algal culture, light availability is a function of the irradiance on the culture surface, the length of the light path from the surface to any point inside the culture, and the attenuation produced by the cells within the reactor (Acien-Fernandez et al. 1998). The light path length inside a photobioreactor is a function of the angle of incidence of the radiation and the system geometry (Alfano et al. 1986). The attenuation produced by the cells (mutual-shading effect) depends on the biomass concentration and the extinction coefficient of the biomass.

Concerning irradiance on the culture surface, several circumstances might occur. When the level of irradiance is low, photolimitation occurs. The photosynthetic machinery receives less photon than it can process, and algal growth is light-limited. However, the efficiency of photosynthesis is usually high under such conditions, since the larger part of the light energy absorbed by the algae can be allocated to biomass growth (Macintyre et al. 2002). Under high light intensities, irradiance might be saturating for photosynthesis process and photoinhibition could take place. Photoinhibition occurs because excessive light damages the

photosystem II of the photosynthetic apparatus (Jensen and Knutsen 1993). Consequently, growth becomes limited by the dark reactions of photosynthesis and photosynthetic efficiency decreases (Macintyre et al. 2002). The excess of light energy absorbed will usually be dissipated via a number of processes which are usually referred to non-photochemical quenching (NPQ) (Huner et al. 1998, Maxwell and Jonhnson 2000). Thanks to these processes microalgae could be able to acclimate to high sunlight levels preventing photoinhibition. Moreover, algae have mechanisms to prevent photoinhibition and photolimitation processes. Some of those mechanisms are related to carotenoids, since those pigments play an important double role, in light capture function (under low light intensities) and also protecting cells against the harmful effects of reactive oxygen species (under high light intensities) (Choudhury and Behera 2001, Demmig-Adams and Adams 2002, Hu 2004, Krinsky 1989).

Also temperature affects the photosynthetic activity and the growth of microalgae (Coles and Jones 2000). In photosynthesis, the photochemical 'light' reactions are mainly affected by irradiance and not sensitive to temperature. However, the biochemical 'dark' processes are temperature sensitive. At low temperatures the metabolic rate is reduced and consequently less absorbed light energy can be converted into carbohydrates (Coles and Jones 2000, Huner et al. 1998). The slow biochemical reactions then can cause accumulation of light energy and over-excitation of the photosynthetic machinery. In this sense, suboptimal temperatures lead to an imbalance between the light absorbed through photochemistry and the energy utilized through metabolism. Algae use the same defense mechanism, NPQ, to dissipate over-excitation of the photosynthetic machinery at low temperatures as at high irradiances (Huner et al. 1998, Maxwell et al. 1994). However, various authors stated that when temperature is controlled within a narrow band, light availability becomes the only factor determining the growth rate (Lee and Low 1992, Torzillo et al. 1991).

Coccomyxa onubensis is an extremophile microalga that naturally lives in Rio Tinto (southern Spain), an acidic river that exhibits very low pH (1.5-3) with high concentrations of sulphuric acid and heavy metals, including Fe, Cu, Mn, Ni, Al, among others (Lopez-Archilla 2005). Moreover, *C. onubensis* pigment profile

is rich in carotenoids, especially lutein, accumulating from 4 to 6 mg g^{-1} dry weight of lutein. All these give the acid-tolerant microalga attractive potential as a producer of this photosynthetic pigment.

In acid environment microalgae, some studies on the influence of environmental variables on biomass productivity have been carried out (Casal et al. 2011, Cuaresma et al. 2006, Visviski and Palladino 2001). However, very few have been performed under outdoor conditions. One of the main reasons to explain this could be the fact that acidophile/acid-tolerant microalgae cannot compete with the so-called "common" microalgae due to slow growth. (Gross 2000, Pulz and Gross 2004). However, relatively fast growth of *Coccomyxa onubensis* could be possible under suitable conditions. Moreover, the extreme acid culture medium that prevents outdoor cultivation from non-desired microorganism's growth should become one of the main advantages for large production of *C. onubensis.* Actually, previous studies (Chapter IV) have revealed that this acid-tolerant microalga can be cultivated in the lab yielding productivities that might be suitable for feasible production trials at pilot scale.

Main aims of this work are to assess the ability of *Coccomyxa onubensis* to grow under natural high irradiance and temperature and to determine the influence of irradiance and temperature on lutein accumulation, to obtain overall strategies that will allow to achieve the maximum biomass and lutein productivity of the alga.

Dissolved oxygen (DO) was also measured to assess the adverse effects of dissolved oxygen on photosynthesis (Carvalho et al. 2006). The removal of photosynthetically generated oxygen is necessary. The adverse effects of dissolved oxygen are widely reported as its accumulation is one of major problems in the design and operation of closed photobioreactors (Camacho-Rubio et al. 1999). It is therefore necessary to assess the effect of dissolved oxygen, and to consider this when designing tubular reactors.

The present work used an experimental tubular reactor at pilot scale with a total length of 13 meters and a bubbled vertical column that acts as a degasser. The

results reported here should allow the design and development of improved systems for the efficient production of lutein-rich cells of this acid-tolerant microalga at large scale.

3. MATERIALS AND METHODS

Microalgae and growth medium

Coccomyxa onubensis was isolated from acidic waters of the Tinto River (Huelva, Spain). This river has some very special features, such as low pH and a high concentration of heavy metals, including iron, copper, magnesium and aluminum. An axenic culture of the algae was obtained by streaking it on basal agar medium at pH 2.5, and then was transferred to the liquid medium. *Coccomyxa* has been recently identified by ribosomal 18S subunit rDNA sequence analysis. Identified 18S subunit rDNA sequence was registered at GenBank with accession number GU265559. According to the chemical composition of the natural environment, cultures were grown at pH 2.5 in a culture medium based on K9 medium (Silverman and Lundgren, 1959). A modified K9 medium was prepared according to the following composition: 3.95 g K_2SO_4, 0.1 g KCl, 0.5 g K_2HPO_4, 0.41 g $MgCl_2$, 2.29 g KNO_3, 0.01 g $CaCl_2$, 5 ml Hutner solution (Hutner *et al.* 1950).

The microalga was grown photoautotrophically in a pilot tubular photobioreactor aerated to avoid dissolved oxygen accumulation, under controlled pH at 2.5. The cultures were bubbled with CO_2 air mixture, containing 5% (v/v) CO_2.

Photobioreactor (PBR)

The microalgal cell cultures were cultivated in an experimental tubular reactor at pilot scale with a total length of 13 meter. The tubular reactor is composed of transparent acrylic pipes of 21 cm inside diameter and 25 cm outside diameter, PVC pressure pipes, bubbled vertical column that acts as a degasser (PEHD) and spray cooling system. Total volume capacity of the system is 6 liters, of which

approximately 4.5 L are within the pipes and 1.5 L within the degasser. The flow rate throughout the tubes was aprox. 6-8 L min^{-1}. A schematic diagram showing the concept and the components of the photobioreactor used in this study is shown in **Figure V.1**.

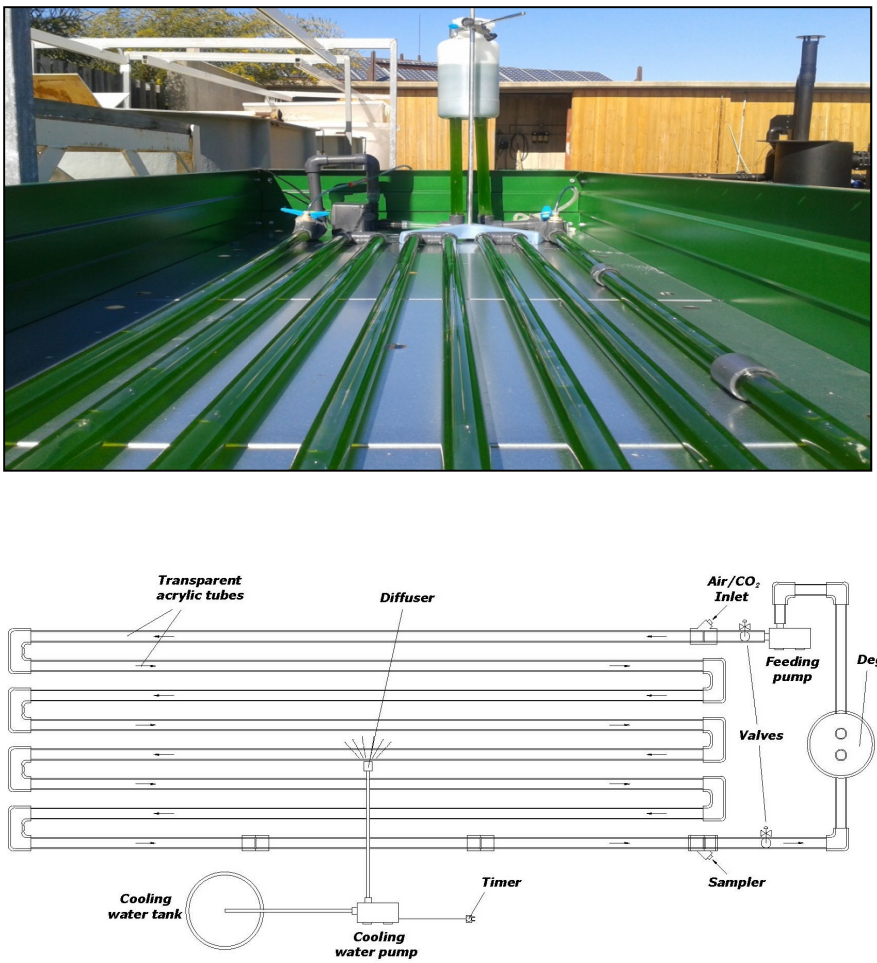

Figure V.1. Picture and schematic diagram (top view) of the pilot tubular photobioreactor used for growth of *Coccomyxa onubensis*

Experimental conditions

Since light and temperature are the main parameters that influence in the productivity of the microalga cultures, those parameters were specially considered to determine the conditions that allow/forbid efficient outdoor algal cultivation.

During the experiment four different situations were studied.

1. Irradiance and temperature naturally varied as typical in spring time at the study site, in which the cell culture endured high levels of light intensity (1800 μmol photons m^{-2} s^{-1}) and maximum temperature (40ºC in the culture) at early afternoon. Henceforth it will be called "VHLT" (Very High light and temperature).
2. Irradiance varied as typical spring radiation at the study site, and temperature was semi-controlled through a spray cooling system. Cell culture reached a maximum light irradiance of 1800 μmol photons m^{-2} s^{-1} and temperature of 32ºC in the culture. Henceforth, it will be called "VHL" (Very high light).
3. Irradiance at the surface of the culture was mitigated by a mesh. Cell culture reached a maximum light irradiance of 650 μmol photons m^{-2} s^{-1} and temperature of 36ºC in the culture. Henceforth, it will be called "ML" (Moderate light).
4. Irradiance and temperature were naturally restrained (cloudy days), still irradiance achieved fairly high levels. Cell culture reached a maximum light irradiance of 1070 μmol photons m^{-2} s^{-1} and temperature of 31ºC in the culture. Henceforth, it will be called "HL" (High light).

Dry weight measurements and growth rate calculations

To measure dry weight, 10 mL samples of each culture were used. The samples were passed through Whatman glass microfiber filters of 47 mm in diameter and 0.7 μm pore size using a vacuum pump to separate the cells from the medium. Then the filters with the cells were dried in a stove at 90 ºC during 24h. Dry

weight data were used to calculate growth rates. Specific growth rates of cultures were calculated using the following expression:

$$\mu = Ln(C/C_0)/t,$$

where μ is the specific growth rate, C_0 is the initial biomass concentration (dry weight), and C is the biomass concentration at any time t. Biomass accumulation was calculated using dry weight data.

Quantum yield (QY)

Fluorescence measurements were made as the maximum QY of PSII (F_v/F_m). It was measured to evaluate the viability of the cells. It was determined using a pulse amplitude modulation (PAM) (Schreiber et al. 1995). Samples of each culture were previously adapted to darkness for 15 minutes.

Chlorophyll and carotenoid determination by HPLC

Carotenoids were extracted using aliquots (1mL) of the cultures. Cells were spinned down for 8 minutes at 13000 rpm. The obtained pellet was placed in 60 ºC water during 5 min. The pellet was resuspended in 1 mL of methanol and the suspension shaken vigorously for 1 min, centrifugated for 8 min at 4400 rpm. Carotenoids and chlorophylls were separated and identified by HPLC (TermoQuest, Thermo products) with a RP-18 column, using a modified method described by Young in 1997. In the mobile phase, solvent A was ethyl-acetate and solvent B was acetonitrile and water (9:1, v/v). External standards (DHI) and their corresponding calibration curves were used to identify and quantify both lutein and β-carotene.

Measures of dissolved oxygen (DO)

DO concentrations reflect an equilibrium between oxygen-producing processes (*e.g.* photosynthesis) and oxygen-consuming processes (*e.g*. aerobic respiration, nitrification, chemical oxidation), and the rates at which DO is added to and removed from the system by atmospheric exchange (aeration and degassing).

Dissolved oxygen concentrations were measured at the surface of the degasser. It was assumed that it was well mixed and that readings taken at this point were representative of the sump contents. DO was determined using a portable oximeter (Crison, OXI 45+)

Statistics

Unless otherwise indicated, tables and figures show means and standard deviations of three independent experiments.

4. RESULTS AND DISCUSSION

The aim of this work is to evaluate environmental conditions that influence algal growth, biomass productivity and lutein accumulation of outdoor cultures of *Coccomyxa onubensis*. Since, light and temperature are the main parameters that influence in productivity of the microalga cultures, those parameters were specially considered to determine those conditions that allow/forbid a proper outdoor algal cultivation. A 6 liters PBR was used under springtime outdoor conditions in south of Spain as in Materials and Methods section is explained. The pH was 2.5 during all the study.

Diurnal evolution of growth parameters

Temperature and light were studied in order to determine the main parameters values that avoid outdoor algal growth. Four different conditions were studied (see Materials and Methods section).

Figures V.2, V.3 and **V.4** show time course evolution in the daylight hours of incident light intensity (**Figure V.2**), relative variation of biomass (**Figure V.3**) and QY variation (**Figure V.4**) of *C. onubensis* cultures under the different conditions mentioned above.

Effect of light irradiance on the culture (natural temperature variation) was studied according to the following premises:

1) ML conditions. Semi-controlled temperature and light irradiance by covering the PBR with a mesh. **Figures 2A**, **3A** and **4A** (light grey squares). Maximum light intensity was roughly 650 μmol photons m^{-2} s^{-1}.
2) HL conditions. Naturally produced in cloudy days during spring time at the study site. **Figures 2A, 3A** and **4A** (dark grey squares). Maximum light intensity was roughly 1000 μmol photons m^{-2} s^{-1} and it was reached among 13:00-15:00h.

Effect of the temperature on the culture (natural variation of light irradiance) was studied according to the following premises:

3) VHLT conditions. Light and temperature varied as natural outdoor conditions. **Figures V.2B, V.3B** and **V.4B** (light grey triangles). This situation corresponds to a typical spring radiation at the study site. The temperature of the *C. onubensis* culture fluctuated between 16°C in the early morning and 40°C during the early afternoon (13:00-15:30 h).
4) VHL conditions. Light irradiance varied as environmental outdoor conditions, and temperature was semi-controlled by a spray cooling system. **Figures V.2B, V.3B** and **V.4B** (dark grey triangles). The temperature of the *C. onubensis* culture fluctuated between 16°C in the early morning and 32°C during the early afternoon, as the cooling system's capacity matched against extreme temperatures. Maximum PAR light of 1800 μmol photon m^{-2} s^{-1} was reached during 3 hours at early afternoon.

Figure V.2 shows incident light intensities along the daylight hours. **Figure V.2A** shows that the cultures where light intensity did not exceed 1000 μmol photon m^{-2} s^{-1}, specifically, ML culture reached a maximum light intensity of 650 μmol photon m^{-2} s^{-1} and HL cultures reached a maximum light intensity of 1070 μmol photon m^{-2} s^{-1}. **Figure V.2B** shows the cultures where light intensity greatly exceeded 1000 μmol photon m^{-2} s^{-1} (both VHL and VHLT cultures achieved more than 1800 μmol photon m^{-2} s^{-1}). VHLT is named as the control condition since it represents a typical diurnal variation of light intensity at the study site (southern

Spain) during a 10 hours period corresponding to the daylight hours. Maximum temperature of 40º and maximum PAR of 1800 µmol photon m^{-2} s^{-1} were reached between 13:30-15:30 h local time. Light irradiance was higher than 1000 µmol photons m^{-2} s^{-1} for more than 6 hours per day.

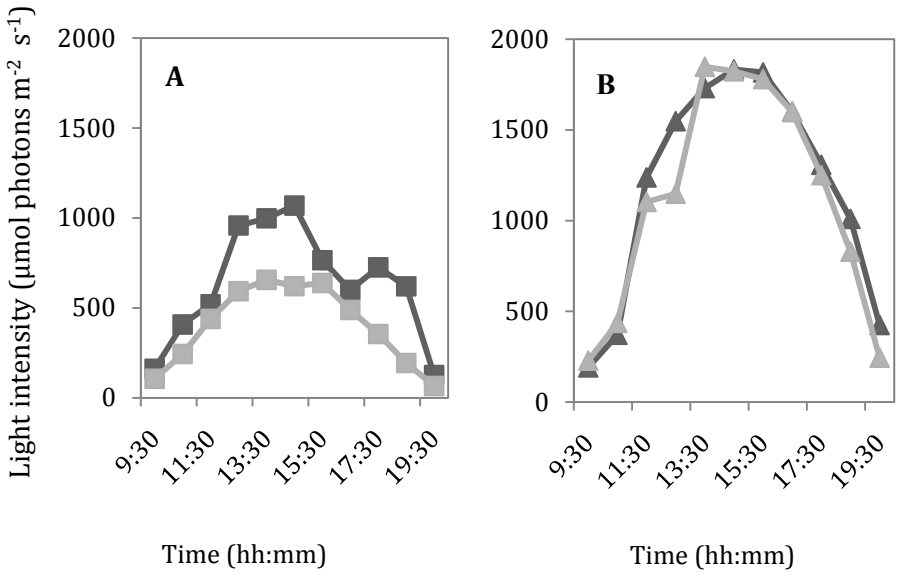

Figure V.2. Diurnal variation of light intensity in *C. onubensis* cultures under outdoor conditions. Figure V.2A exemplifies two cultures with maximum light intensity lower than 1000 µmol photons m^{-2} s^{-1}. Light grey squares show a ML day. Dark grey squares show a HL day. Figure V.2B. shows two typical sunny days where cultures reached light intensities higher than 1000 µmol photons m-2 s-1. Light grey triangles show a VHLT day. Dark grey squares show a VHL day. All conditions occurred during spring time.

Microalgae cultivated under outdoor conditions are subjected to daily variations in growth rate over the solar period. During daylight, biomass generation through photosynthesis is the main observable fact that occurs in the culture, in addition to photorespiration and dark respiration (Goldman 1979), while during the night, biomass is lost because of dark respiration (Molina-Grima et al. 1999, Torzillo et al. 1991, Tredici et al. 1991). All those phenomena are a function of the culture conditions. For that reason, culture conditions should be suitably

addressed to achieve maximum biomass generation and minimum biomass night loss, hence maximum productivity.

Diurnal variation of biomass growth of *C. onubensis* obtained under ML, HL, VHL and VHLT conditions are shown in **Figure V.3. Figure V.3A** shows biomass evolution in those cultures where light intensity did not largely exceed 1000 μmol photon m^{-2} s^{-1}, specifically, ML culture reached a maximum light intensity of 650 μmol photon m^{-2} s^{-1} and HL cultures reached a maximum light intensity of 1070 μmol photon m^{-2} s^{-1}. **Figure V.3B** shows biomass evolution in those cultures where light intensity greatly exceed 1000 μmol photon m^{-2} s^{-1} (both VHL and VHLT cultures achieved more than 1800 μmol photon m^{-2} s^{-1}, as described previously in this manuscript).

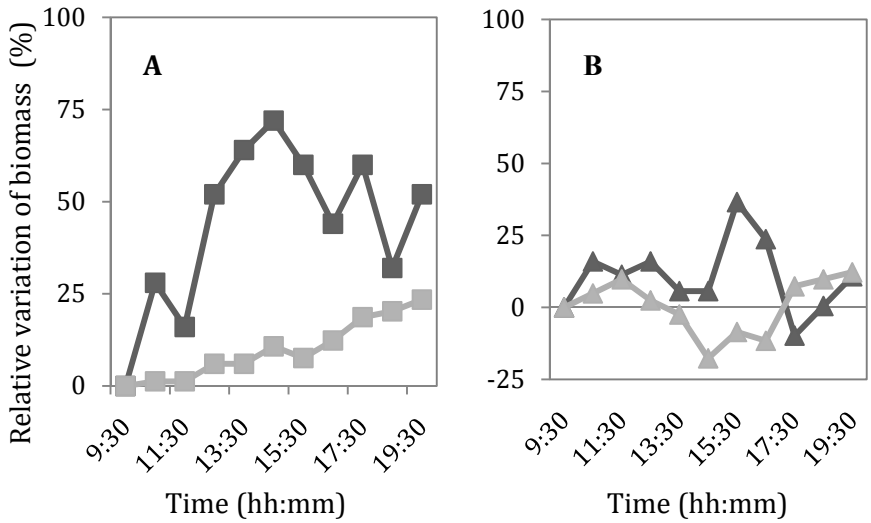

Figure V.3. Relative variation of biomass of *C. onubensis* cultures under outdoor conditions along the daylight time. Figure V.A exemplifies two cultures with maximum light intensity lower than 1000 μmol photons m^{-2} s^{-1}. Light grey squares show a ML day. Dark grey squares show a HL day. Figure V.B shows two typical sunny days where cultures reached light intensities higher than 1000 μmol photons m^{-2} s^{-1}. Light grey triangles show a VHLT day. Dark grey squares show a VHL day. All conditions occurred during spring time. Initial biomass concentration of HL= 0.7 g L^{-1}. Initial biomass concentration of ML= 1.5 g L^{-1}. Initial biomass concentration of VHL= 0.8 g L^{-1}. Initial biomass concentration of VHLT= 0.85 g L^{-1}.

Figure V.3 shows that biomass growth profiles differ from each other significantly under those different conditions, even when the initial biomass concentration was similar in all the cases (roughly 0.7-0.85 g L^{-1}) except in the ML culture which initial biomass concentration was 1.5 g L^{-1}. When light irradiance was higher than 1200 μmol photons m^{-2} s^{-1} (**Figure V.3B**) no biomass increase was observed. That absence of biomass increment occurred independently on temperature control. Therefore, the final dry cell increment after 10 hours was lower than 1 g L^{-1}, under both VHL culture (maximal temperature of 32 ºC) and VHLT culture (maximal temperature of 40 ºC) conditions. That value corresponds to an approximately 12% increase in biomass during daylight hours.

During the night, the accumulated biomass was consumed by respiration, so that the biomass concentration at the beginning of the next day equaled that of the previous day and the overall daily specific growth rate was zero. The very high light intensity that *C. onubensis* cells received during the illuminated period, affects the cellular metabolism and biomass loss from respiration, determining the net biomass productivity of the system (Richmond 1990, Torzillo et al. 1991). Sanchez-Miron et al. (2002) affirm that during the night, as well known, some of the accumulated carbohydrate pool is oxidized to support metabolism for cell maintenance. Thus, the carbohydrate content of the biomass at the start of each day is normally less than at the end of the previous daylight period, resulting in losses of biomass.

Focusing on **Figure V.3A**, an increase in algal biomass was produced during the daylight hours. However, relative biomass concentration profiles show some differences depending on cultivation conditions. After 10 hours, the HL cultures reached about 2-fold higher cell densities than those cultures where light intensity was artificially reduced through a mesh (ML cultures).

Under cloudy day conditions (HL culture), the more irradiance, the highest biomass accumulation occurs. Therefore, maximal biomass accumulation is observed during the early evening, in coincidence with higher irradiance on the culture surface (**Figure V.3A**). These results indicates that *Coccomyxa* is able to

withstand relatively high light levels (1200 μmols photons m^{-2} s^{-1}), still not saturating for the alga, and consequently, photo-damage is not produced under these conditions.

Obviously, no photo-damage was produced in ML cultures, since these cultures received moderate light intensity, bounded to 50-650 μmol photons m^{-2} s^{-1} during the daylight hours (due to the use of the mesh). Moreover, irradiance greater than 400 μmols photons m^{-2} s^{-1} was only held for 6 hours per day. Previous studies determined that moderate light intensity as 400 μmol photons m^{-2} s^{-1} does not seem to cause light stress to *Coccomyxa onubensis*, even when cultured under low cell densities (see chapter IV). Therefore, these moderate to low light irradiances might be suitable for growth of *C. onubensis* cultures, especially in dense cultures.

It is known that the concentration of biomass in a reactor is one of the most important factors acting in the light availability. Acien-Fernandez et al. (1998) mentioned that in an algal culture, light availability is a function of the irradiance on the culture surface, the length of the light path from the surface to any point inside the culture, and the attenuation produced by the cells within the reactor. The attenuation produced by the cells (mutual-shading effect) depends on the biomass concentration and the extinction coefficient of the biomass. Therefore, lower irradiance on the culture surface added to the higher biomass concentration could explain the differences in biomass accumulation and therefore diurnal growth rate in the two stuations shown in **Figure V.3A**. Nevertheless, in ML as in HL, biomass accumulation during daylight hours overcomes the possible biomass lost during the night period. Even in ML culture (the less biomass productive) biomass accumulation exceeds 23% of the initial biomass, offsetting the nighttime biomass losses.

Conversely, a completely different story is shown in **Figure V.3B**. During the first hours of daylight, alga grows successfully, when light intensity does not exceed 1200 μmols photons m^{-2} s^{-1} (**Figure V.3B**), still harmless to cell growth of *C. onubensis*. After that, biomass accumulation decreases and cell growth falls yielding a negative balance, losing up to 17% of their biomass when irradiance

and temperature are maxima, 1800 μmols photons m^{-2} s^{-1} and 40 ºC respectively. In the last hours of daylight, cell growth occurs and cell biomass again accumulates, reaching levels of around 10-12% over the initial biomass concentrations. These results show that *Coccomyxa* is under stressful light conditions in both cases, since similar trends are observed under both VHL and VHLT cultures (semi-controlled and uncontrolled temperature) conditions.

As a result of that, ML and HL cultures (irradiance on the culture surface lower than 1200 μmol photons m^{-2} s^{-1}) were able to accumulate biomass during the solar period so that the daily growth rates were possitive, while VHL and VHLT cultures were not able to do it even when all cultures, ML, HL, VHL and VHLT cultures, were in the same location, with the same PBR and operated during the same time of the year. The difference on the biomass accumulation between them can be explained by intense photoinhibition produced under the highest light irradiances.

That demonstrates that light intensity is probably the most critical factor influencing the productivity of that microalga cultures. Thereby, VHL and VHLT conditions for *Coccomyxa* growth were defined for light intensities higher than 1200 μmol photons m^{-2} s^{-1}, since it drives to substantial reduction in growth rate and photosynthetic rate, as shown in **Figure V.3** and **V.4**. ML and HL are identified as comparatively suitable conditions for *C. onubensis* growth.

Difference on temperature did not cause significant influence on algal growth. These results are in accordance to experiences with other microalgae. Sanchez et al. (2008) observed as *Scenedesmus almeriensis* was able to grow under high temperature levels. The steady state of the microalga biomass concentration increased with temperature attaining maximum values between 30 and 40°C and the thermal death of the culture took place at 48°C.

In terms of cell viability, **Figure V.4** shows maximal quantum yield (QY) of PSII (F_v/F_m) under the same HL and ML (A) and VHL and VHLT (B) conditions**. Figure V.4A** shows that the cultures where light intensity did not exceed 1000 μmol photon m^{-2} s^{-1}, specifically, ML culture reached a maximum light intensity of 650

μmol photon m^{-2} s^{-1} and HL cultures reached a maximum light intensity of 1070 μmol photon m^{-2} s^{-1}. **Figure V.4B** shows the cultures where light intensity greatly exceed 1000 μmol photon m^{-2} s^{-1} (both VHL and VHLT cultures achieved more than 1800 μmol photon m^{-2} s^{-1}). VHLT is defined as the control condition as explained previously in this manuscript.

HL and ML are shown in **Figure V.4A**. This Figure shows that maximal quantum efficiency of PS II was similar in both culture conditions, around 0.7 which is a typical value for non-stressed microalgae cells. That confirmed that *C. onubensis* cultured under light intensities below 1000 μmol photons m^{-2} s^{-1}, is apparently not subjected to light stress, since maximum quantum yield (QY) remained stable under those conditions. However, **Figure V.4B** shows that when light intensity is more than 1000 μmol photons m^{-2} s^{-1}, QY appears unsteady during the daylight hours, getting the lowest values between the midday and the early afternoon, around 0.5.

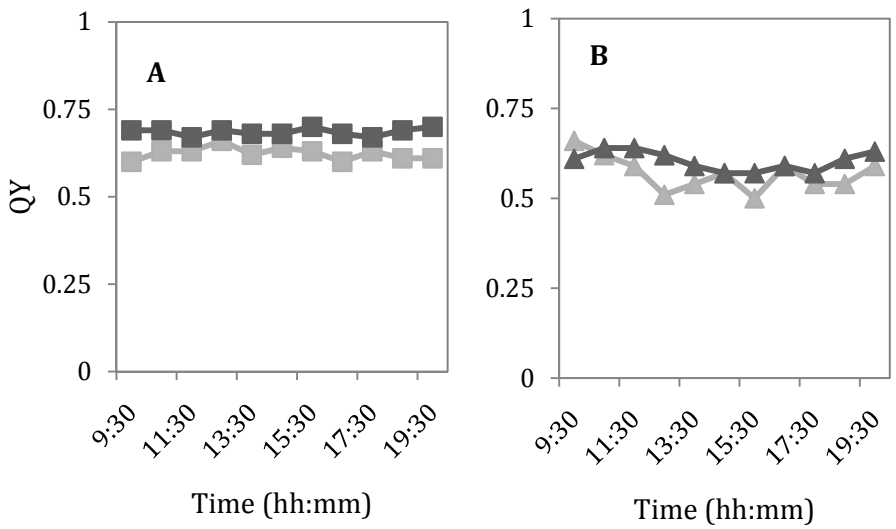

Figure V.4. Maximal quantum yield (QY) of PSII (F_v/F_m) of *Coccomyxa* cells cultured under outdoor conditions, along the daylight time. Figure V.A exemplifies two cultures with maximum light intensity lower than 1000 μmol photons m^{-2} s^{-1}. Light grey squares show a ML day. Dark grey squares show a HL day. Figure V.B shows two typical sunny days where cultures reached light intensities higher than 1000 μmol photons m^{-2} s^{-1}. Light grey triangles show a VHLT day. Dark grey squares show a VHL day. All conditions occurred during spring time.

Especially negative values of QY were obtained in the VHLT culture, where temperature and irradiance varied normally according to outdoor conditions. Minimum values of QY were observed, dropping from 0.64 in the early morning to 0.51 in only four hours, remaining in this low value during some hours. Many organisms show a typical midday depression in the photosynthetic yield between noon and the early afternoon hours. This diurnal pattern is believed to be mainly due to dynamic photoinhibition (Hanelt et al. 1994).

However, *Coccomyxa* VHLT culture recovers its moderate highest QY values at the end of the evening, achieving photosynthetic activity efficiency similar to VHL culture, whose maximum temperature was 32ºC. As a result of this, although some photodamage was observed, cultures rapidly recovered at the end of the day.

This reduction in the efficiency of the cells to utilize the available light at midday is probably due to some photoinhibition. Excessive light energy absorbed by the light-harvesting complexes (LHC) and not used for photochemistry or dissipated as heat, may cause photoinhibition, that is, a permanent or slowly reversible damages to the photosynthetic apparatus (Falkowski et al. 1994). Photoinhibition is a common phenomenon in photosynthetic organisms, including algae, in response to high levels of solar radiation. Various degrees of photoinhibition or even irreversible photodamage have been described (Häder et al. 1998, Hanelt et al. 1992, Krause and Weis 1991). The existence of photoinhibition in outdoor conditions has been reported extensively (Acien Fernandez et al. 1998, Molina-Grima et al. 1996, Rebolloso-Fuentes et al. 1999, Vonshak and Guy 1992).

However, attending to Figure V.4A, *C. onubensis* does not present photoinhibition pattern when is cultured under light intensity lower than roughly 1000 μmols photons m^{-2} s^{-1} , since QY remains stable and around 0.7, typical value for non-stressed microalgae cells. On the contrary, reversible photoinhibition is present when light intensity is higher than that irradiance, as QY recovered at the end of the daylight period. Quantitatively speaking, photoinhibition level is similar or even lower to that observed in other green algae studied (Molina-Grima et al.

1996, Rebolloso-Fuentes et al. 1999), though for *Coccomyxa* it occurs, on average, at lower irradiances.

It will be interesting in the future to determine if this behavior is endogenously regulated depending on irradiance and light quality. In that respect, a remarkable observation is the fact that solar UV-B had a significant share in photoinhibition under natural conditions even though the incident UV-B energy represents only a small fraction in solar radiation (Häder et al. 2003).

In conclusion, the present studies indicate that *C. onubensis* adapts its photosynthetic capacity to the changing light conditions in its natural habitat. These organisms should have developed effective mechanisms to counteract excessive irradiation and rapidly optimize their photosynthetic apparatus to the environmental conditions. In addition to that, *Coccomyxa onubensi*s resulted not being particularly tolerant to high irradiances.

Diurnal evolution of lutein and β-carotene

As said in Introduction section, one of the main aims in this work is to assess outdoor cultivation of this acid-tolerant microalga for production of lutein. Lutein has gathered increasing attention due to help to prevent or diminish age-related macular degeneration (AMD). Currently lutein is obtained from marigold, where the lutein content is as low as 0.03% w/w (Piccaglia et al. 1998). However, continuous reports of lutein-producing microalgae pose the question if those microorganisms can become an alternative source. Several microalgae have higher lutein contents than most marigold crops. *Muriellopsis sp*. and *Scenedesmus almeriensis*, described by Del Campo et al. (2000) and Sanchez et al. (2008) to be potential lutein producers, have been tested in outdoor growth conditions. With that reported information, it can be inferred that production of lutein with microalgae might compete with the use of marigold. Fernandez-Sevilla et al. (2010) explained that the most significant factors known to affect lutein content are irradiance, pH and temperature, although not forgetting nitrogen availability and source, salinity or ionic strength, the presence of oxidizing substances and, definitely, growth rate.

The effect of the light and temperature conditions were studied on biosynthesis of main *C. onubensis* carotenoids. **Figure V.5** shows lutein content and lutein/chlorophyll ratio when the alga was grown under HL and ML and unfavorable conditions (VHL and VHLT).

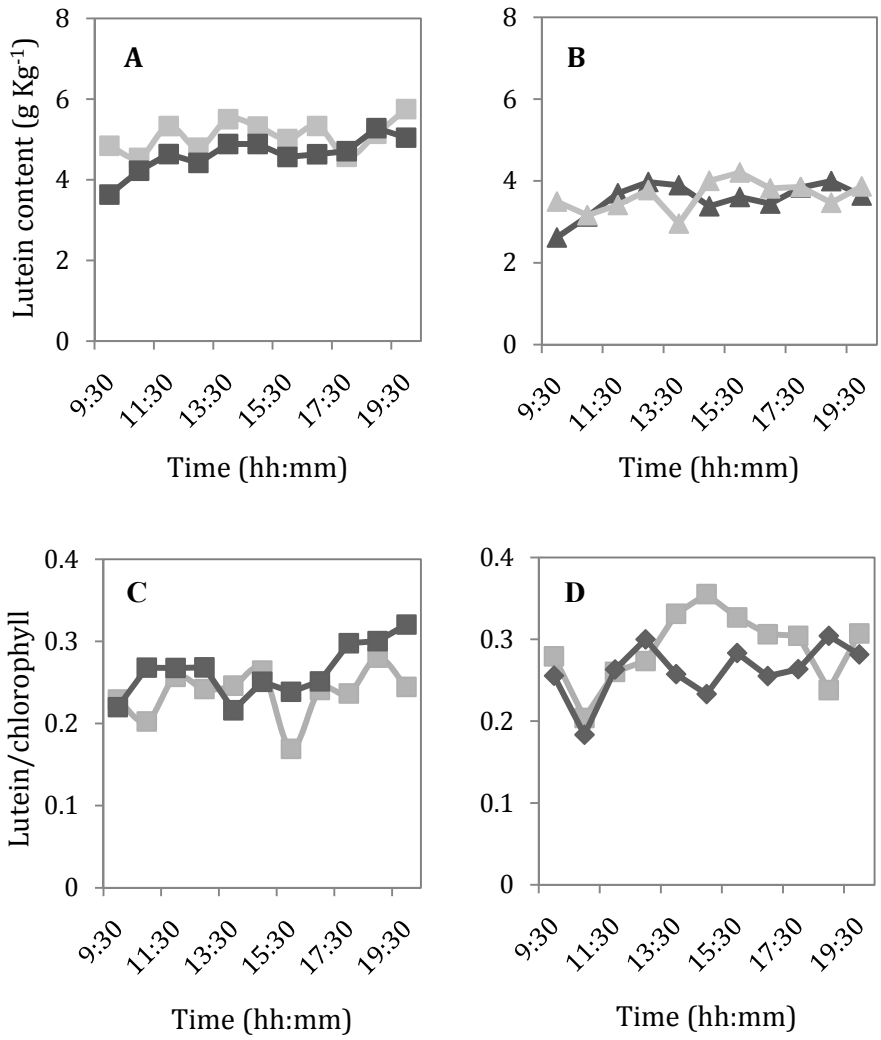

Figure V.5. Lutein content (mg g^{-1}) and lutein/chlorophyll ratio of *C. onubensis* cultivated under outdoor conditions along the daylight time. (A) Lutein content under HL and ML conditions (light intensity lower 1000 µmol photons m^{-2} s^{-1}). (B) Lutein content under VHL and VHLT conditions (light intensity higher 1000 µmol photons m^{-2} s^{-1}). (C) lutein/ chlorophyll ratio under HL and ML conditions. (D) lutein/chlorophyll ratio under VHL and VHLT. Light grey squares show a ML day. Dark grey squares show a HL day. Light grey triangles show a VHLT day. Dark grey squares show a VHL day. All conditions occurred during spring time (April).

Figure V.5 shows than *C. onubensis* lutein content under ML and HL conditions is quite higher than under VHL and VHLT conditions. Under ML and HL conditions, average lutein content is around 4.5 mg g^{-1} (when light is reduced by a mesh) and 5 mg g^{-1} (in a cloudy day), getting its maximum cell content (nearly 6 mg g^{-1}) at the end of the daylight (from 17:30), when light irradiance is decreasing (**Figure V.5A**). *C. onubensis* seems to rapidly adapt to lower light intensities in order to maximize light capture. These results are in accordance to other reports regarding microalgal cells acclimation to low light conditions (Dubinsky and Stambler 2009, Garcia-Camacho 2012). At low light intensities, most algae produce more light-harvesting pigments to improve photosynthetic efficiency, lutein being one of those light-harvesting pigments which take part of the antenna complexes and, consequently, its content seems to be linked to chlorophyll content (Garcia-Camacho 2012, Macintyre et al. 2002, Richardson et al. 1983). This relationship between lutein and chlorophyll can be seen in **Figure V.5C**, which shows the ratio between lutein and chlorophyll in *C. onubensis* during daylight hours in the HL and ML conditions described above. **Figure V.5C** shows lutein/chlorophyll ratio fairly stable throughout the day, showing the connection between lutein and chlorophyll contents in non-photodamaged cells.

Therefore, the observed increase in the intracellular content of carotenoids should be related to light absorption functions, as suggested from the parallel increase in chlorophyll content, and might be used for conducting lutein production processes.

Coccomyxa onubensis cultured under VHL and VHLT conditions accumulated a lower lutein content compared to that of cultures under HL and ML conditions. **Figure V.5B** shows similar lutein content trends when *C. onubensis* is cultured under both controlled and not controlled temperature, hardly exceeding 4 mg g^{-1} of dry weight. However, in terms of lutein/chlorophyll ratio, those previously discussed parallel variations were not observed (**Figure V.5D**). Lutein/chlorophyll profiles in cultures which temperature was controlled and not controlled differ from each other significantly, even when the initial lutein/chlorophyll ratio values (0.25-0.27 g L^{-1}) were similar. Thus, the culture that varied by external environmental conditions (under not controlled

temperature) had a higher lutein/chlorophyll ratio during the maximum irradiance and temperature (early afternoon, 40ºC). This lifted ratio occurred despite lutein content did not increase excessively, leading to assume that it is due to a drastic decrease in chlorophyll content.

Figure V.5 shows that, unlike for chlorophyll, high temperature of the culture (reaching up to 40 ° C) does not significantly affect intracellular content of lutein, obtaining the same profile for cultures at 32 °C and at 40 °C. This is in accordance to other lutein producer microalgae. Del Campo et al. (2007) reviewed the influence of temperature on carotenoid content. Sanchez et al. (2008) reported a direct influence of temperature on lutein content. Their results showed experimental data that probed increased lutein content of the biomass with temperature, at every irradiance and only at the highest temperature of 45°C, with the culture intensely stresses due to thermal stress. The lutein content of the biomass decreased compared to the value measured at 35°C.

Figure V.6 shows β-carotene content (mg g^{-1}) and β-carotene/chlorophyll ratio when the alga was grown under all tested conditions. **Figure V.6A** shows β-carotene content (mg g^{-1}) when *C. onubensis* was grown under HL and ML conditions (when light is lower than 1000 μmol photons m^{-2} s^{-1}). Some differences can be observed in the two runs. When light is reduced by a mesh, average β-carotene content is roughly 1 mg g^{-1} DW, while in a cloudy day, HL conditions, average β-carotene content is rather lower (0.7 mg g^{-1} DW). Moreover, the β-carotene/chlorophyll ratio is also higher in ML than in HL *C. onubensis* cultures (**Figure V.6C**).

Since in cloudy days irradiance was higher than in days where light was attenuated by mesh, the highest content of β-carotene appears to occur when the light is relatively low. These results suggest that β-carotene has in *C. onubensis* a predominant role as light-harvesting pigment, similarly to lutein, under non-photodamaging irradiances. However, lutein seems better withstand high irradiances, as no decrease in lutein content was appreciated when light was in a maximum of 1000 μmol photons m^{-2} s^{-1} as **Figure V.5A** shows for HL culture.

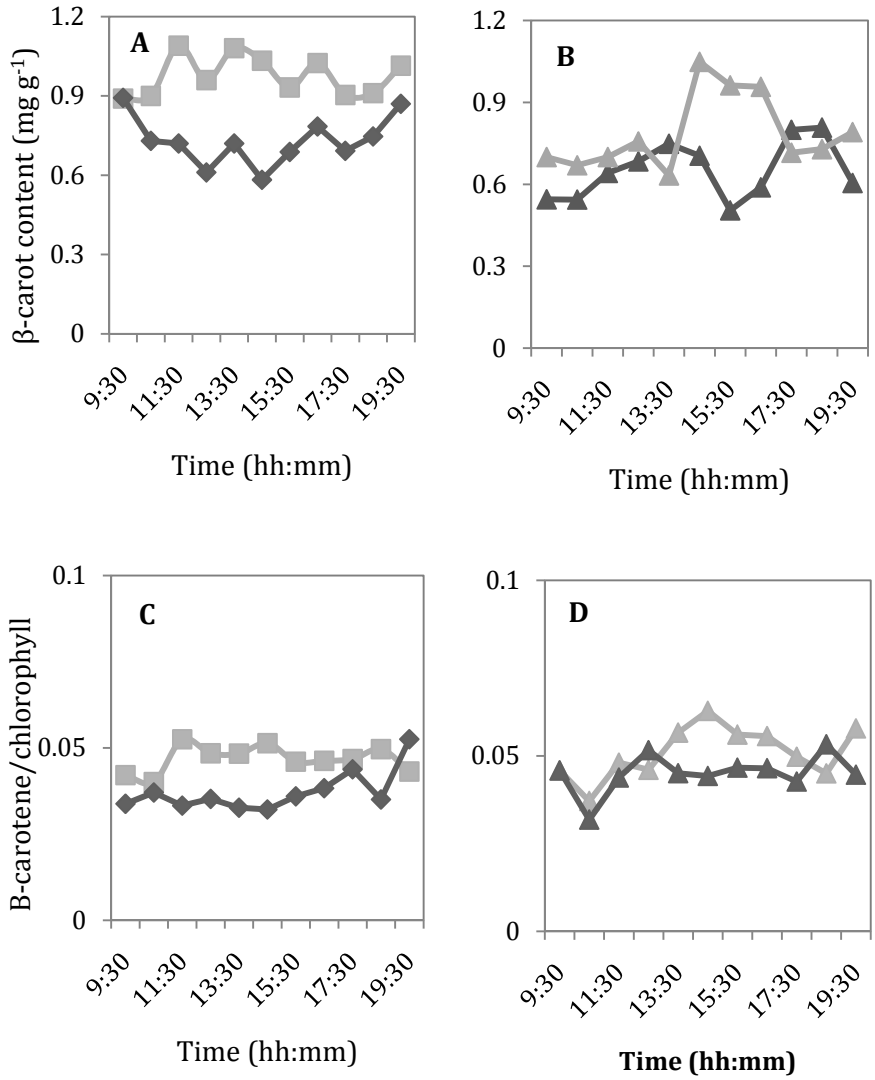

Figure V.6. β-carotene content (mg g^{-1}) and β-carotene/chlorophyll ratio under HL and ML, VHL and VHLT conditions. (A) β-carotene content under HL and ML conditions (light intensity lower 1000 µmol photons m^{-2} s^{-1}). (B) β-carotene content under unVHL and VHLT conditions (light intensity higher 1000 µmol photons m^{-2} s^{-1}). (C) β-carotene/chlorophyll under HL and ML conditions (light intensity lower 1000 µmol photons m^{-2} s^{-1}). (D) β-carotene/chlorophyll under unVHL and VHLT conditions (light intensity higher 1000 µmol photons m^{-2} s^{-1}). Dark grey squares show a cloudy day. Light grey squares show artificially light limited day. Dark grey triangles show a temperature controlled *C. onubensis* culture. Light grey triangles show a not controlled temperature *C. onubensis* culture. All conditions occurred during spring time (April).

That light-harvesting behavior is in accordance to what other authors described for other microalgae (Kirk 1994, Smith et al., 1990). By decreasing the amount of light reaching the system, the microalga is expected to increase the content of lutein and β-carotene due to its light harvesting role, which is linked to their presence in light harvesting complexes and therefore, to the presence of chlorophyll. Moreover, an increase in both lutein and β-carotene contents at the end of the daylight hours due to the irradiance attenuation was observed (**Figure V.5A** and **Figure V.6A**). That increase in the intracellular content of carotenoids should be related to light absorption functions, as suggested from the parallel increase in chlorophyll content, and might be used for conducting lutein production processes.

Powles (1984) and Ridley (1982) affirmed that excess irradiation lead, in addition to photoinhibition, to photodestruction of chlorophyll and carotenoids. Ben-Amotz et al. (1989) showed that the two β-carotene isomers accumulated in *Dunaliella bardawil* are photodestroyed at different rates. In parallel, carotenoid pigments may provide effective protection against disadvantageous influence of high light (Demmig-Adams 2002, Steiger 1999) by light dissipation through non-photochemical processes. Therefore, carotenoids take part in light capture function and also protecting cells against the harmful effects of reactive oxygen species (ROS). In the case of *C. onubensis*, β-carotene and especially lutein could also play that double function, in light capture function under low light intensities, and also protecting cells against the harmful effects of reactive oxygen species under high light intensities (Chapter IV).

Alternatively, an increase on the β-carotene/chlorophyll ratio was also observed at high temperature cultures (**Figure V.6D**). Furthermore an increment in the β-carotene content was also observed under those culture conditions. **Figure V.6B** shows that the culture directly subjected to external environmental conditions (VHLT culture) had a higher β-carotene content during the early afternoon at 40 °C, reaching more than 1 mg g^{-1} DW at 14:30. However, when *C. onubensis* grew under the same light irradiance but different temperature conditions, 32 ºC (VHL culture), intracellular β-carotene content decreased during the same time slot, hardly exceeding 0.5 mg g^{-1} DW. Consequently, intracellular β-carotene content

seems to be influenced by temperature. As discussed, this is in accordance to other authors who assume that temperature directly influences on carotenoid content, especially β-carotene content (Del Campo et al. 2007). In addition, in *Dunaliella salina,* the extent of carotenoid accumulation in oil globules in the inter thylakoid spaces of their chloroplast depends on temperature, as well as high salinity, high light intensity, and nitrogen limitation. Under these conditions, up to 12% of the algal dry weight is β-carotene (Ben-Amotz 2004).

According to the obtained results discussed in this manuscript, it might be proposed that continuous outdoor production of lutein rich *Coccomyxa onubensis* biomass should include harvesting at either beginning or end of the day, where eventually cell lutein concentration should increase.

Time-course evolution of biomass accumulation and productivity

Light intensity equal or lower than 1000 μmol photons m^{-2} s^{-1} was found as feasible conditions for the outdoor cultivation since *C. onubensis* was able to grow during the daylight hours, overcoming the biomass lost during the night and, therefore, accumulating biomass during the day. Moreover, in terms of cell viability, **Figure V.4** showed that the maximum quantum yield of PS II was a typical value for non-stressed microalgae cells.

C. onubensis cells grown in cloudy day (maximum irradiance of about 1000 μmol photons m^{-2} s^{-1}) and cells cultured in a sunny spring day and protected from excess light by a mesh were tested. **Figure V.3** showed that, under both conditions, *C. onubensis* was able to grow. Results of *C. onubensis* biomass accumulation when cultivated for several days under batch conditions, in the outdoor PBR and under most productive culture conditions previously determined are shown in **Figure V.7**.

Figure V.7 shows relative biomass accumulation along the days of *C. onubensis* cultures in a cloudy spring day (dark grey symbols) and, in a sunny spring day where light irradiance were reduced by a mesh (light grey symbols).

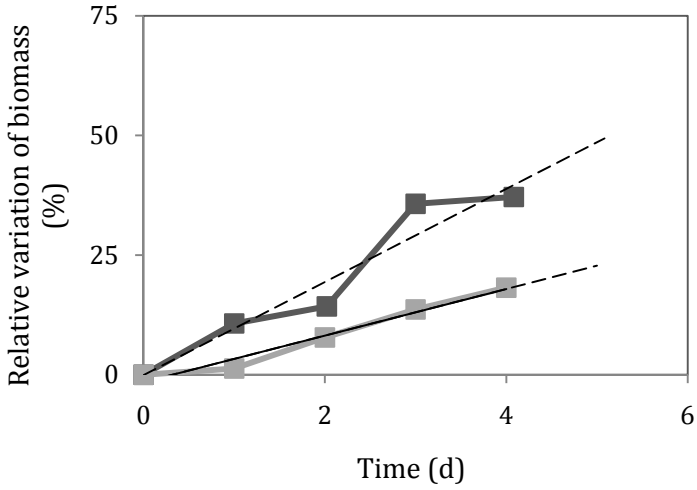

Figure V.7. Relative biomass accumulation of *Coccomyxa onubensis* under HL and ML outdoor conditions. Dark grey squares show a cloudy day. Light grey squares show a day artificially light limited by a mesh. Starting biomass concentration was 0.7 g L^{-1} and 1.5 g L^{-1} respectively. Both conditions occurred during spring time (April).

Coccomyxa onubensis was able to grow and accumulate biomass under both outdoor studied conditions. Although the most stable biomass accumulation was achieved for the culture grown under natural light reduced in intensity by a mesh, the highest accumulation of biomass was obtained for *C. onubensis* grown under a natural cloudy day. After four run days, *C. onubensis* increased its biomass up to almost 40%. On the other hand, when *C. onubensis* was cultured under reduced irradiance, biomass accumulation after four days was 18% of starting biomass. Average growth rate under that condition was 0.042 d^{-1}, while average growth rate when *C. onubensis* was cultured in a cloudy day was on average 0.08 d^{-1}, being 2-fold higher than those cultures grown under artificially attenuated light (**Table V.1**).

Those significant differences can be discussed on two facts. First, light intensity in the culture grown under reduced irradiance was much lower, having a maximum irradiance of 650 µmol photons m^{-2} s^{-1} compared to 1070 µmol photons m^{-2} s^{-1}, and second, cell density in the culture grown under attenuated irradiance was rather higher than that of the culture grown in a natural cloudy day, 1.5 g L^{-1} and 0.7 g L^{-1} of starting biomass, respectively.

Table V.1. Biomass productivities and growth rates under HL and ML conditions.

Culture conditions			Growth rate (μ, d^{-1})			Productivity ($g\ L^{-1}\ d^{-1}$)		
Cultures	Max. Irradiance ($\mu E\ m^{-2}\ s^{-1}$)	Max. T (ºC)	Global value	Max. daily	Max. solar period	Global value	Max. daily	Max. solar period
HL	1070	31	0.08	0.38	0.87	0.063	0.32	0.66
ML	650	36	0.042	0.062	0.490	0.070	0.100	0.864

That situation provides an obvious decrease in the photon flux density inside the more dense culture. According to Raven (1994) if photon flux density decreased, cells might become light-limited, decreasing their growth rates. Such a decrease in growth is particularly noticeable in high cell density cultures (1.5 $g\ L^{-1}$). It might be assumed that such moderate light intensity is even limiting for dense cultures of *C. onubensis*, which means that there is still room for improving growth rates and productivity of that acid extreme environment microalga.

Maximum apparent growth rates of *C. onubensis*, calculated on a given day of experience, are in the range of those determined for other acid-environment strains (Tittel et al. 2005, Visviki and Palladino 2001). Consequently, finding ways to maintain such growth rates over a longer time period would be fairly appropriate for sustaining outdoor production of *C. onubensis.*

Table V.1 also shows biomass productivities of *C. onubensis* grown in the two mentioned conditions. Maximum daily productivity was obtained for *C. onubensis* cultured in a cloudy day, achieving 0.32 $g\ L^{-1}\ d^{-1}$ on a given day. However, total productivities after the four days studied were not so high.

Paradoxically, despite *C. onubensis* had substantially better growth rates under natural conditions than under artificially controlled conditions, both situations result in similar productivities. After 5 days, *C. onubensis* achieved 0.063 $g\ L^{-1}\ d^{-1}$

when cultured in a cloudy spring day and 0.070 g L^{-1} d^{-1} when cultured in a sunny spring day where light intensity was reduced by a mesh. That is probably due to the higher cell density of that last culture. These results prove that biomass productivities largely depend on biomass concentrations for *Coccomyxa* cells, in spite of *Coccomyxa* cells might be light-limited under those biomass concentrations, in accordance to **Figure V.7**.

It is also noticeable that maximum productivity during the daylight hours was about 0.8 g L^{-1} d^{-1}. These results do not take into account the loss of night biomass produced by the lack of light and hence, lack of photosynthesis. It might be possible to improve the results if *C. onubensis* was incubated under a suitable cultivation strategy that might include use of reduced carbon substrates in dark or even the use of urea, easily used by *C. onubensis* (Casal et al. 2011).

Dissolved Oxygen variation under outdoor conditions

Time course of dissolved oxygen evolution is usually a reflect of photosynthetic activity. Therefore, measures of dissolved oxygen (DO) were necessary for assessing possible inhibition of photosynthetic activity. **Figure V.8** shows time-course evolution of DO (%) respect to the variation of biomass and maximum quantum yield of *Coccomyxa onubensis* during daylight hours under ML outdoor conditions. In this case, the DO rose from about 7:30 h to 10:00 h, as the irradiance and growth rate increased. During midday, the rate of photosynthesis smoothly declined. This behavior could be attributed to a soft photoinhibition effect caused by the higher external irradiance, being the culture sensitive to that increase in irradiance. In order to verify the existence of photoinhibition in outdoor conditions, the variation of photosynthetic activity of the cells during the day was assessed by QY. The results in Figure V.8 show that the photosynthetic activity kept fairly stable during all the daylight period, as no loss in the light use efficiency was recorded. Similar behavior was observed in terms of biomass concentration, that is, a decrease in biomass accumulation was not observed. Consequently, photoinhibition is not produced. In fact, the cultures rapidly recovered and from midday forward the DO increased up to the end of the daily solar time.

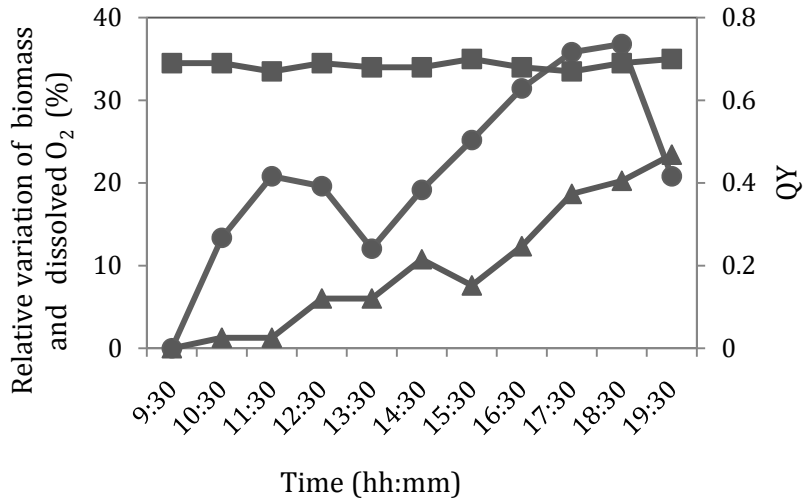

Figure V.8. Relative variation of biomass, variation of dissolved oxygen (%) and maximum quantum yield of *Coccomyxa onubensis* under HL and ML outdoor conditions (day artificially light limited by a mesh). Triangle symbols show the relative variation of biomass, expressed in %. Dot symbols show relative abundance of oxygen, expressed in %. Square symbols show the maximum quantum yield. Starting biomass concentration was 1.5 g L^{-1}. Starting dissolved oxygen was 97%.

If oxygen concentration exceeding saturation occurs in algae cultures, photo-oxidative damage occurs to the chlorophyll reaction centers, inhibiting photosynthesis and reducting productivities (Molina-Grima et al. 2003, Pulz 2001). The adverse effects of dissolved oxygen are widely reported as its accumulation is one of major problems in the design and operation of closed photobioreactors (Camacho-Rubio et al. 1999). It is therefore necessary to assess dissolved oxygen, and to consider this when designing tubular reactors. In this case the experimental tubular PBR used for growing *C. onubensis* does not allowed to accumulate elevated quantities of DO, since little variation of DO values along the daylight were observed, reaching a maximum value of 130 % (exceeding only a 37% over starter value).

These results indicate that the photosynthetically generated oxygen does not inhibit basic algal metabolism. Indeed, this problem is avoided in PBR by means of a bubbled open vertical column in which surface oxygen can easily leaves the culture medium, and the bubbles can be controlled by varying the flow rate. The

small capacity of 6 L and total length of 13 meters of the PBR also helps to avoid major oxygen accumulation.

Carvahlo et al. (2006) explained that efficient gas transfer is a critical aspect of PBR design and operation, both to provide sufficient CO_2 as a source of inorganic carbon for cell growth (especially for freshwater species) and to remove photosynthetically generated O_2 that can inhibit photosynthetic efficiency or be directly toxic to microalgae at high concentrations.

The findings of this work open possibilities for production of lutein-rich biomass of the acid-environment microalga *C. onubensis* in PBR systems, with the advantage of low pH culture medium which greatly limits contamination by other microorganisms, particularly microalgae.

5. CONCLUSIONS

From the obtained results it might be concluded that incubation of acid-tolerant microalgae cultures (*Coccomyxa onubensis* in this manuscript) is able to withstand high temperature levels (up to 40ºC). In terms of growth rate and biomass productivity, irradiance higher to 1000 μmol photons m^{-2} s^{-1} was detrimental to the culture, named VHL and VHLT conditions in this work. Under HL and ML conditions, *Coccomyxa onubensis* is able to suitably accumulate biomass, mantaining fairly high its lutein content. Intracellular lutein content increased at the end of the day, probable related to light absorption functions. Consequently, *C. onubensis* might become a suitable tool to perform production processes of lutein-enriched biomass. According to the obtained results discussed in this manuscript, it might be proposed that a continuous outdoor production of lutein rich *Coccomyxa onubensis* biomass should include harvesting at either beginning or end of the day, where eventually cell lutein concentration should increase. In summary, these results should allow to design mass production strategies of acido-tolerant microalgae, particularly lutein-rich biomass in the case of *C. onubensis*.

CONCLUSIONS

CONCLUSIONES

When you make the finding yourself – even if you're the last person on Earth to see the light – you'll never forget it.

(Carl Sagan)

CONCLUSIONS

From the obtained results in **chapter II,** it can be concluded that:

- Incubation of *Coccomyxa onubensis* cultures in air and followed by shift to high carbon conditions, by far enhances carbon use efficiency in terms of growth rate and biomass productivity, based on the action of both external and internal carbonic anhydrase activities. This concept should in principle be applied more efficiently to acid-tolerant microalgae.
- Lutein content of *Coccomyxa onubensis* is high and does not seem to depend on carbon level supplied to cultures. Consequently, repeated cycles of air-incubation and high CO_2-incubation of *C. onubensis* might become a suitable tool to perform production processes of lutein-enriched biomass. These results should allow designing mass production strategies of acidophile microalgae, particularly lutein-rich biomass in the case of *C. onubensis*.

From the obtained results in **chapter III,** it can be concluded that:

- *Coccomyxa onubensis* cultivation on a copper rich medium (no more than 0.4 mM Cu (II)) enhanced carotenoid accumulation, especially lutein, and even enhanced growth rate and biomass productivity.
- Semi-continuous cultivation in Cu-added media clearly improves growth rate, productivity and as well as the intracellular content of lutein, revealing that both optimal biomass and lutein productivities are compatible issues in *C. onubensis* growing in a Cu-enriched culture medium.

From the obtained results in **chapter IV,** it can be concluded that:

- Under moderate light intensity of 400 μmol photons m^{-2} s^{-1} (typical average wintertime light intensity in southern Spain), *Coccomyxa* cells experienced no light stress and reached its maximum biomass and lutein productivities.

- Cell density influences growth rates and productivities of biomass and lutein. Particularly, cell density influences short-time course evolution of carotenoids, which can be used for addressing increased lutein contents in cultures of *C. onubensis*.
- These results allow to design cultivation strategies to produce lutein-rich *Coccomyxa onubensis* biomass, consisting of transferring relatively low cell density cultures either from low to moderate irradiance or from moderate to low irradiance, or selecting the suitable day time for the harvesting step in outdoor production processes.

From the obtained results in **chapter V,** it can be concluded that:

- *Coccomyxa* is able to withstand outdoor incubation at high temperature levels (up to 40°C), therefore showing certain thermo-tolerance.
- If illuminated at a maximal irradiance of 1000 μmol photons m^{-2} s^{-1}, *Coccomyxa onubensis* outdoor cultures were able to suitably accumulate biomass, mantaining lutein content fairly high. Higher irradiances were detrimental to the culture.
- Intracellular lutein content increased at the end of the natural day light-times, probably due to the cell need of enhancing light-capturing functions in which lutein is involved.
- According to the obtained results, it might be proposed that continuous outdoor production of lutein-enriched *Coccomyxa* biomass should include harvesting at either beginning or end of the day.

This thesis gives a guideline of some of the parameters that influence on both biomass productivity and carotenoid enrichment of the acid-environment microalga *Coccomyxa onubensis*. The obtained results should be useful to define biomass production strategies of large-scale cultivation of acid environment microalgae and, particularly, should allow to design lutein-rich biomass production processes of the extreme environment microalga *Coccomyxa onubensis*.

CONCLUSIONES

De los resultados obtenidos en el **capítulo II,** se puede concluir que:

- La incubación de *Coccomyxa onubensis* en aire y su posterior transferencia a condiciones de alto CO_2, aumenta en gran medida la eficiencia de uso del carbono en términos de tasa de crecimiento y productividad, basada en la actividad interna y externa de la enzima anhidrasa carbónica.
- El contenido en luteína de *C. onubensis* es elevado y no parece depender de los niveles de carbono añadido a los cultivos. Consecuentemente, cultivar *C. onubensis* en ciclos repetitivos consistentes en la incubación en aire seguida de transferencia a alto CO_2 , puede ser una herramienta para la producción de biomasa algal rica en luteína. Estos resultados permitirían diseñar estrategias de producción de microalgas acidófilas/acidotolerantes.

De los resultados obtenidos en el **capítulo III,** se puede concluir que:

- Cultivar *Coccomyxa onubensis* en un medio rico en cobre (hasta 0.4 mM Cu (II)) aumenta la acumulación de carotenoides, especialmente en luteína, aumentando también la tasa de crecimiento y la productividad de biomasa de la microalga.
- Cultivos semi-continuos con medio rico en cobre claramente producen una mejora en la productividad y la velocidad de crecimiento, así como un aumento en el contenido intracelular de luteína. Esto revela que los óptimos de ambas productividades -biomasa y luteína- son compatibles cuando *C. onubensis* crece en un medio rico en cobre.

De los resultados obtenidos en el **capítulo IV,** se puede concluir que:

- Bajo la intensidad de luz moderada de 400 μmol fotones m^{-2} s^{-1} (intensidad de luz media en invierno en el sur de España), las células de *C. onubensis* no experimentan estrés lumínico, alcanzando sus máximas productividades de biomasa y luteína.

- La densidad celular influye en la velocidad de crecimiento y en la productividad de biomasa y luteína. En particular, la densidad celular influye en la evolución a corto plazo de carotenoides, pudiéndose usar para aumentar el contenido en luteína en cultivos de *C. onubensis*.
- Estos resultados permiten diseñar estrategias de cultivo para la producción de biomasa de *Coccomyxa* rica en luteína. Las estrategias consistirían en transferir cultivos de densidad celular relativamente baja, desde intensidad de luz baja a moderada o desde intensidad moderada a baja, así como en seleccionar el momento del día más apropiado para el cosechado en procesos de producción exterior.

De los resultados obtenidos en el **capítulo V,** se puede concluir que:

- *C. onubensis* es capaz de superar altas temperaturas exteriores (hasta 40° C), mostrando cierta termo-tolerancia.
- En condiciones exteriores con una irradiancia de hasta 1000 μmol fotones m^{-2} s^{-1}, *C. onubensis* es capaz de acumular biomasa, manteniendo un contenido en luteína moderadamente alto. Irradiancias por encima de esos niveles fueron perjudiciales para los cultivos.
- El contenido intracelular en luteína aumentó en las últimas horas de luz del día, debido probablemente a la necesidad celular de aumentar sus funciones de captura de luz, siendo la luteína uno de los principales pigmentos con esta función.
- De acuerdo con los resultados obtenidos, se podría proponer que una posible producción continua en exterior de biomasa de *Coccomyxa* rica en luteína, debe incluir el cosechado al principio o al final del día.

Esta **tesis** proporciona una guía sobre algunos de los parámetros que influyen tanto en la productividad de biomasa como en el enriquecimiento de carotenoides de la microalga de ambiente ácido *Coccomyxa onubensis*. Los resultados obtenidos deben permitir el diseño de estrategias para la producción de cultivos a gran escala de microalgas de ambiente ácido y estrategias de producción de biomasa rica en luteína de esta microalga.

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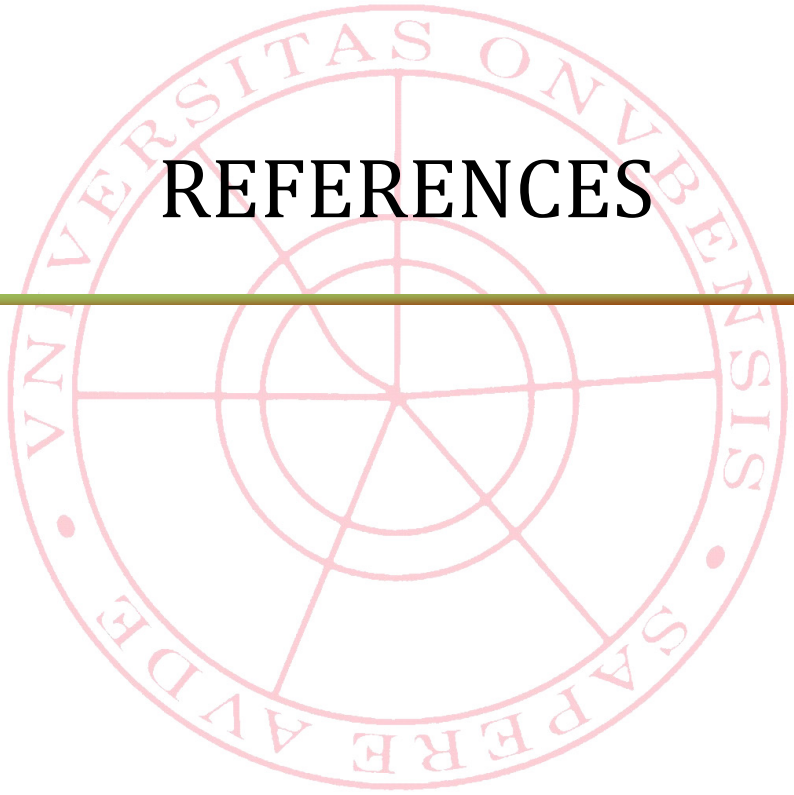

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"Lo más importante nunca se ve…"
("El Principito" - Antoine de Saint-Exupéry)

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NOMENCLATURE

"Caminante no hay camino, se hace camino al andar (…)"

(Antonio Machado)

A	absorbance
AMD	age-related macular degeneration
ATP	adenosine-5'-triphosphate
AZA	acetazolamide, carbonic anhydrase inhibitor
CA	carbonic anhydrase
CAext	external carbonic anhydrase
CAint	internal carbonic anhydrase
Car	Carotenoid content, mg g^{-1}
CCM	carbon concentration mechanisms
cel	cell
Chl	Chlorophyll content, mg g^{-1}
Ci	inorganic Carbon
DO	dissolved oxygen, %
DW	dry weight, mg g^{-1}, g Kg^{-1}
Fv/Fm	maximum PSII quantum yield
HC	high CO_2
HL	high light intentsity
HPLC	high pressure liquid chromatography
LC	low CO_2
LHC	light harvesting complex
ML	moderate light intentsity

NADPH	nicotinamide adenine dinucleotide phosphate
NPQ	non photochemical quenching
OD	optical density
OD750nm	optical density meassured at 750 nm
PAM	pulse amplitude modulation
PAR	photosynthetically active radiation (400 – 700 nm)
PBR	photobioreactor
pK1	first dissociation constant of carbonic acid
pK2	Second dissociation constant
PSI	photosystem I
PSII	photosystem II
ROS	Reactive oxygen species
T	Temperature, °C
QY	maximum PSII quantum yield
VHL	very high light intensity
VHLT	very high light and temperature
v/v	volume/volume
WAU	Units of carbonic anhydrase activity using Wilbur-Anderson formula
μ	specific growth rate, d^{-1}
μmax	maximal specific growth rate, d^{-1}

ANNEXES

"Nuestra recompensa se encuentra en el esfuerzo y no en el resultado.

Un esfuerzo total es una victoria completa".

(Mahatma Gandhi)

ANNEX 1. PUBLICATIONS

Vaquero I, Ruiz-Dominguez MC, Marquez M, Vilchez C (2012). Cu-mediated biomass productivity enhancement and lutein enrichment of the novel microalga *Coccomyxa onubensis*. Process Biochem 47:694-700.

Garbayo I, Torronteras R, Forjan E, Cuaresma M, Casal C, Mogedas B, Ruiz-Dominguez MC, Marquez M, **Vaquero I**, Fuentes JL, Fuentes R, Gonzalez M, Vilchez C (2012) Identification and physiological aspects of a novel carotenoid-enriched metal resistant microalga isolated from an acidic river in Huelva (Spain). J Phycol 47:607-614.

Vázquez M, **Vaquero I**, Ruiz MC, Márquez M, Mogedas B, Vílchez C (2011) Hacia nuevos alimentos funcionales: producción de microalgas enriquecidas en caroteno. Alimentaria 427: 83-86

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Vaquero I, Mogedas B, Ruiz-Domínguez MC, Vega JM, Vílchez C (2013). Light-mediated lutein enrichment of an acid environment microalga. Process Biochem Submitted.

Forján E, **Vaquero I**, Ruiz-Domínguez MC, Gojkovic Ž, Vázquez M, Márquez M, Mogedas B, Bermejo E, Girlich S, Dominguez MJ, Navarro F, Cuaresma M, Vega JM, Vílchez C, Garbayo I (2013). Microalgae: fast-growth sustainable green factories. Algal Research. Submitted.

Ruiz-Domínguez MC, **Vaquero I,** Obregón V, de la Morena B, Vega JM, Vílchez C (2013) Growth and lipid accumulation of an acidophile microalga under nutrient deprivation. The Scientific World Journal. Submitted.

ANNEX 2. TABLE LEGENDS

ANNEX 3. FIGURE LEGENDS

The research described in this thesis was financially supported by grant AGR-4337 (Proyecto de Excelencia, Junta de Andalucía).