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Outdoor production of *Isochrysis galbana* (T-iso) in industrial scale photobioreactors and modelling of its photosynthesis and respiration rate

Tesi di laurea in Dinamica del fitoplancton

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1. INTRODUCTION

1.1. Microalgae

Algae are primarily oxygen-releasing photosynthetic organisms with simple body plans having no roots, stems, or leaves. They are mostly aquatic organisms and they can be found attached to a substrate like plants, some are motile like animals, some are simply suspended in water, some grow loosely on soil, trees, and animals, and some form symbiotic relationships with other organisms. They do not belong to a single monophyletic group and therefore cannot be easily defined. The internal cell structure and morphology of algae is vey various. Eukaryotic algal cells are characterized by a nucleus and usually one or more chloroplasts, mitochondria, Golgi bodies, endoplasmic reticulum, and other typical eukaryotic organelles (Richmond and Hu, 2013). Numerous books, scientific journals, and internet websites are dedicated exclusively to compiling our knowledge of algae. Nevertheless, it is still difficult to give a precise and clear definition for "algae" (Lee, 2008; Graham et al., 2009). Microalgae are unicellular species, and they can be found individually, or in colonies (in chains or groups). Some species are very small, and their sizes can range from a few micrometers (μm) to some hundreds of micrometers.

Microalgae have a long evolutionary history that has determined the presence of organisms with very diversified characteristics. For this they have different shapes and colours. This morphological variation exists not only among species but also among different life stages of the same species (Richmond and Hu, 2013). They appear in different forms as amoeboid, palmelloid, coccoid,

filamentous, flagellate, and sarcinoid. Large biomolecules are classified into broad major groups: carbohydrates, lipids, nucleic acids, and proteins. These groups are similar to those found in other living organisms, but the algae provide an exceptional diversity of biomolecules (Richmond and Hu, 2013). In recent years commercial companies have shown interest in this diversity, and today algae are grown for the express purpose of harvesting these compounds. There are extensive publications on algal biochemistry, and the precise biochemical product can be manipulated to some degree by altering the growth conditions (Hu, 2004; Beer et al., 2009). The diversity of algae is amazing at several levels. Species diversity is measured by the number of described species and there is general agreement that many species have not yet been described (Andersen, 1992; Norton et al., 1996). Algal diversity can be measured according to different purposes of study: biochemical pathways, ecological roles, endosymbiotic genomes, morphology, and reproductive strategies. The recent discovery of cryptic endosymbiotic genomes is significant, for example, predominately green algal genes in diatoms (Moustafa et al., 2009). Endosymbiotic events, and even horizontal gene transfers, have been major genetic mixing pots that have shuffled genomes, created gene duplications, and allowed for gene replacements. These have contributed significantly to algal diversity at all levels. (Richmond and Hu, 2013)

Despite their diversity, microalgae are specially adapted to an aquatic environment. They are mainly photosynthetic organisms, and therefore necessary for life on earth. Recent studies show they produce approximately half of the atmospheric oxygen and use

simultaneously the greenhouse gas carbon dioxide to grow photoautotrophically (Falkowski e Raven, 2007). Microalgae perform the primary production and they are also at the base of the food web and provide energy for all the trophic levels above.

1.2. Mass cultivation of microalgae

Over recent decades, interest in microalgae has grown enormously. In particular, due to their biodiversity these organisms are used in many sectors: the production of chemicals, dietary supplements, and in the aquaculture sector (Muller-Feuga, 2013). Microalgae can be also used in wastewater treatment and they have been proposed as biofuels source to reduce global warming problem (Chisti 2007; Benemann and Oswald 1996). Whatever the final application of microalgae its production is based on the same principles as light availability, sufficient mass and heat transfer and adequate control of culture parameters (Acièn et al 2013). The present production of microalgal biomass is low (9,000 t dry matter per year) but the price of biomass is high (from 30 to 300 € kg-1), nevertheless the size of these markets is growing considerably (Brennan and Owende 2010). The biomass productivity of any microalgae culture system is a function of environmental conditions and usually geographical and climatic conditions in the selected location determines overall year-round conditions such as solar radiation and temperature (Incropera and Thomas 1978). To optimize the biomass productivity, the microalgae are grown inside structures called photobioreactors (PBR). Their design and orientation has to be selected to maximize the amount of solar energy intercepted,

whereas the geometry of the reactor determines the distribution of the intercepted radiation on the culture surface (Acien et al. 2001; Sierra et al. 2008). Solar radiation on the reactor surface along with biomass concentration determine the average irradiance inside the culture, and consequently the growth rate and achievable biomass productivity (Acièn et al. 2013). Light is the main factor that influences photosynthesis and, therefore, the maximum productivity can be achieved only with an adequate design of the reactor and of its operation mode according to the light availability.

Currently, there are different types of photobioreactors which are classified according either to their design or the best way to accomplish microalgae growth requirements. The simplest and cheapest are the open reactors. They have the advantage of high solar radiation availability, low power consumption and sufficiently low mass transfer cost, but have disadvantages related to an easy contamination risk and a limited control of culture conditions (Acièn et al. 2013). The most common are the raceways, and they are typically used on a commercial scale for the cultivation of microalgae and cyanobacteria, such as *Arthrospira platensis* and *Dunaliella salina*, although low-scale production of other strains has also been reported (Richmond and Cheng-Wu 2001; Jiménez et al. 2003; Moreno et al. 2003; Moheimani and Borowitzka 2007; Radmann et al. 2007).

Fig. 1 – Pre-industrial open raceways in the campus pilot plant. (University of Almeria)

In recent decades the closed photobioreactors have been developed. Their advantage, as compared to open ponds or tanks, is that the light path length is noticeably reduced leading to higher cell densities, which diminishes the chance of contamination and facilitates harvesting. Furthermore, cultures in closed PBRs do not suffer from severe evaporative losses and temperature, pH changes and growth conditions can be more easily controlled, therefore, it is possible to improve the production and quality of the resulting biomass and to reduce the production costs (Norsker et al. 2011; Acién et al. 2013).

The most widely used closed system is the tubular photobioreactor. It consists of a solar collector tubing through which the culture flows, recirculated by aeration or mechanical pumps. To remove

oxygen, the culture is passed through a bubble column or tank, where air is supplied to avoid the achievement of damaging (dissolved) oxygen levels. In this bubble column, air can be supplied but also CO2 can be injected for carbonation, and a heat exchanger can be included for temperature control (Acièn et al 2013). The helical tubular system is laid vertically as an arrangement of transparent, coiled polyethylene tubes arranged around an open circular framework (Watanabe et al. 1995; Zhu and Jiang 2008). In these structures the presence of the tubes causes reciprocal shading. This variable, in addition to the tube diameter, determines the solar radiation availability for this type of photobioreactor.

The main disadvantage of these reactors is the power consumption necessary to recirculate the liquid in the tubular loop. Nevertheless they allow the production of a wide variety of microalgae strains compared to the other systems.

Fig. 2 – Pre-industrial tubular photobioreactors in the campus pilot plant. (University of Almeria)

Another type of closed photobioreactor is the flat panel. It consists of joined transparent plates to store the culture, where the culture is illuminated from one or both sides and stirred by aeration. Flat panels are promising although currently show different problems: fouling, maintenance work, and unavailability of suitable machinery for process automation.

Fig. 3 – Flat panel in the campus pilot plant. (University of Almeria)

In addition, different reactors can be used at the same time in the called hybrid systems to improve the entire system: for example the use of closed photobioreactors to maintain and produce inoculum for large open reactors. Whatever the photobioreactor design their productivity is severely limited by the amount of energy received from the sun (Acién et al. 2012).

1.3. Microalgae for Aquaculture

With an expanding aquaculture industry, the demand for live microalgae is also increasing.

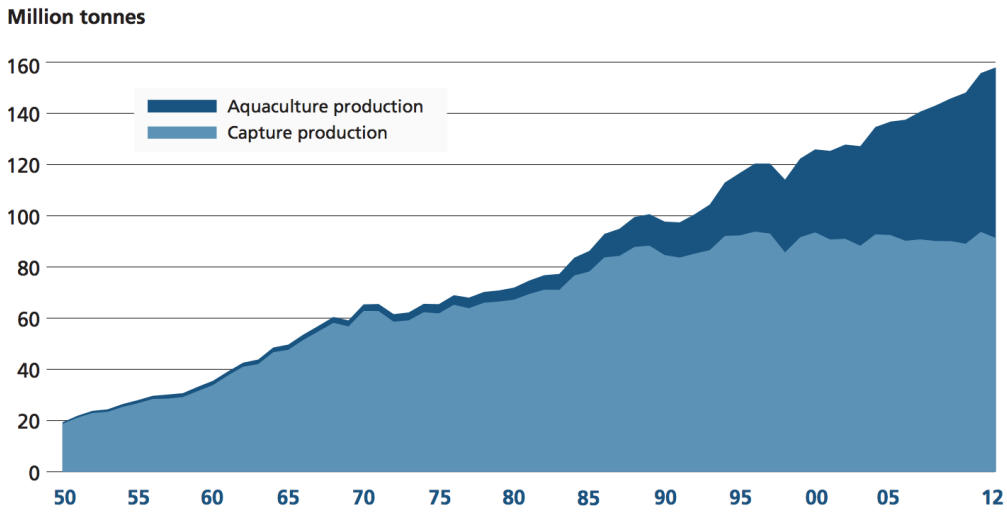

Fig. 4 – The state of world fisheries and aqualculture. (FAO 2014)

For this reason the demand of oil and proteins to prepare feeds is increasing and, at the same time, that of live microalgae required for early larvae stages development. Therefore, their use as food in aquaculture remains the major reason for their production (Brown et al., 1997; Pulz and Gross, 2004). Microalgae are an essential food source in the rearing of many species, especially the larvae and spat of bivalve molluscs, penaeid prawn larvae and live food organisms such as rotifers which, in turn, are used to rear the larvae of marine finfish and crustaceans. The importance of algae in aquaculture is not surprising as algae are the natural food source of these animals (Borowitzka and Siva, 2007). There are two main sources of algal species used in aquaculture. These are: natural

populations of phytoplankton, either as they are found in nature or from cultures enriched by adding nutrients; and unialgal cultures. Unialgal cultures are fundamental when a high quality feed source with known nutritional properties is required. Bacteria-free algal cultures also reduce the risk of introducing unwanted pathogens into the animal cultures which can result in mortality of some of the animals cultured. In recent years extensive studies have been undertaken to determine the nutritional requirements of the target species and the biochemical composition of algae which possibly can be used as a food source (de Roeck-Holtzhauer et al., 1993; Brown and Farmer, 1994). Filtering mollusks consuming natural phytoplankton represented 18% of total aquaculture production (Richmond and Hu, 2013). Using microalgae, these animal productions are completely sustainable. Traditional microalgae production in aquaculture represents one of the main problems in the development of a number of processes due to the typically low productivity and poor quality of the resulting biomass, along with contamination and high production costs (Muller-Feuga, 2013; Boeing, 2000). The microalgal production systems used by fish farmers are normally simple and traditional with few, if any, control systems, but this is expensive and leads to variable microalgal biochemical composition. Optimization of microalgal culture conditions is therefore necessary to reduce production costs and meet the demand of hatchery managers for an easy, cheap and reliable method of producing microalgae of high nutritional value (Coutteau and Sorgeloos, 1992). Continuous and semicontinuous culture is an attractive alternative to batch production.

1.4. *Isochrysis galbana* (T-iso) production and models

Several strains belonging to the genus *Isochrysis* (Prymnesiophyceae) are among the most widely used microalgae in aquaculture. *Isochrysis aff. galbana Green 'Tahiti'* (T-iso) is one of most commonly used strains because of its biochemical profile (fatty acids, ascorbic acid, etc.) (Muller-Feuga, 2013). T-iso is supposed to be an excellent candidate for mass culture because of its ability to adapt to a wide range of temperatures and photon flux densities (Saoudi-Helis et al., 1994; Marchetti et al., 2012).

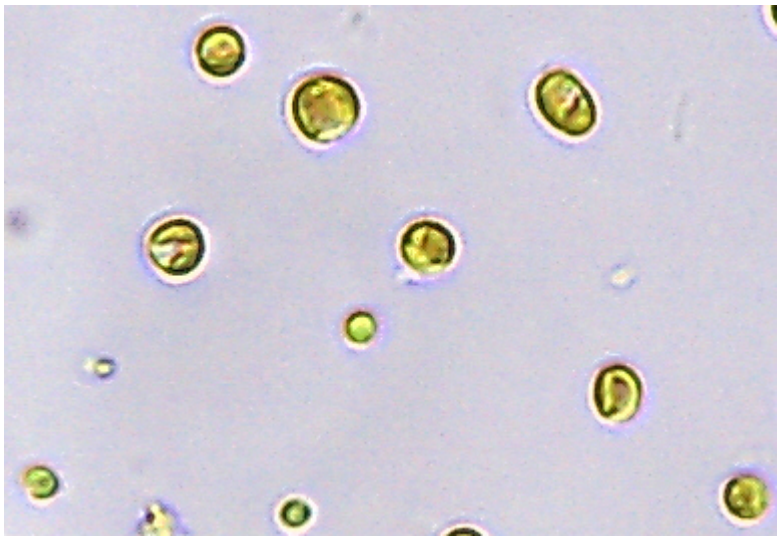

Fig. 5 - Sample of *Isochrysis galbana* in the pre-industrial tubular. photobioreactors (Estación Experimental Las Palmerillas, Almeria)

However very few references are reported about the outdoor production of this strain, the commercial production of this strain being much lower than others as *Nannochloropsis* or *Tetraselmis* also used as feed for early larvae stages.

Whatever the reactor and the microalgae used, outdoor photobioreactors are subject to daily variations in culture

parameters that influence their overall yield (Camacho et al., 1999; van Bergeijk et al., 2010; Mendoza et al., 2013; Fernández et al., 2014). In order to adequately design outdoor photobioreactors, it is necessary to know the influence of culture conditions on the photosynthesis rate as a primary step in determining the biomass productivity of any photosynthetic system. The utilization of average variable values, whether daily or weekly, only provides partial information. Greater accuracy can be obtained by using instantaneous variable values, and models which take into account the rapid response of microalgae cells to culture condition variations (Costache et al., 2013). There are already publications on models that describe the photosynthetic response to environmental changes under nutrient-unlimited culture conditions such as irradiation, temperature, pH and dissolved oxygen. The photosynthesis rate has been reported as increasing hyperbolically with irradiance up to values above which it decreases due to photoinhibition (Molina et al. 1996). Researchers have developed several models on irradiation (Platt et al., 1980; Eilers and Peeters, 1988; Papadakis et al., 2005; Bernard and Rémond, 2012). Another important factor influencing photosynthesis is temperature, and the models produced up to now have been able to describe the most optimal condition of growth and productivity (Bernard and Rémond, 2012; Ras et al., 2013; Guedes et al., 2011; Perez et al., 2008). Less information has been reported on the influence of pH or dissolved oxygen on the microalgae photosynthesis rate. Large variations in pH can reduce microorganism productivity (Camacho et al., 1999; Ying et al., 2014; not et al., 2015); but these values remain almost stable (7.5 - 8.0) in closed tubular photobioreactors

because of the on-demand injection of CO_2. Conversely, in this type of photobioreactor, the high concentration of dissolved oxygen can increase considerably (Camacho et al., 1999; Acién et al., 2013). Several studies reported dissolved oxygen concentrations as high as 500 %Sat. causing photosynthesis inhibition and eventually leading to culture death (Weissman et al. 1988; Marquez et al. 1995; Singh et al. 1995; Mendoza et al., 2013). In addition, it can be useful to observe and to model the respiration trend to understand how, and how much, this activity influences photosynthesis with accurate values of net photosynthesis being obtained by adding the values for oxygen consumed during the dark period to the oxygen production during the light period.

2. OBJECTIVES

The objectives of this work were to study the influence of major environmental parameters (irradiance, temperature, pH and dissolved oxygen) on determining the net photosynthesis and the respiration rates of *Isochrysis galbana*. The results were used to develop an overall model that allows the simulation of the net photosynthesis and respiration rates under different culture conditions. The model was validated by experimental data assembly obtained under different culture conditions to those tested. Furthermore, the model was used to simulate the daily behaviour of outdoor cultures performed in tubular photobioreactors, thus verifying their validity. An other objective was to demonstrate the feasibility of the outdoor production of this strain at industrial scale, and to determine the optimal conditions to perform it. Experiments were performed in continuous mode, modifying the dilution rate, also monitoring the environmental conditions (solar irradiance, temperature), and the culture parameters (dissolved oxygen, temperature, pH). The biomass productivity and quality of biomass produced was analysed as dependent variables, in addition to the quantum yield and overall behaviour of the cultures. This research represents a necessary step in the scale-up of whatever microalgae production process, to prevent fails due to inadequate performance of the strain at real scale/conditions.

3. MATERIALS AND METHODS

3.1. Microorganisms and culture conditions

The strain used, *Isochrysis aff. galbana* (T-Iso), was obtained from the Universidad de Santiago de Compostela. Stock cultures were maintained photoautotrophically in spherical flasks (1.0 l capacity) using Algal medium (Bionova, Santiago de Compostela, Spain). The inoculum was maintained with aeration and an on-demand CO_2 supply under continuous illumination at 100 µE m^{-2} s^{-1}, at 25 °C.

Fig. 6 – Inoculum of *Isochrysis galbana* (Estación Experimental Las Palmerillas, Almeria)

For the experiments, the inoculum was transferred to laboratory-scale tubular photobioreactors and to industrial-scale outdoor tubular photobioreactors (T-PBRs). Details of the reactors used are next provided.

3.2. Laboratory photobioreactors

To simulate the environmental conditions prevailing into the cultures performed in outdoor T-PBRs, an indoor system simulating outdoor conditions was developed. Thus, laboratory culture was performed in a stirred tank reactor made with a section of the same polymethylmethacrylate (PMMA) tube than that used in outdoor T-PBRs (0.09 m diameter, 0.5 m high, 0.8 l capacity), obtained only by covering one of the extremes. The reactor thus build-up was inoculated with 20% of the volume of inoculum and completing with fresh Algal medium. Then, it was operated in batch mode to achieve a biomass concentration higher than 1.0 g/L, next being operated in semicontinuous mode, by replacing 20% of the culture volume daily with fresh medium, till the steady state is reached, after 10 days. The reactor was magnetically stirred to simulate the mixing existing in outdoor T-PBRs. Concerning illumination, the reactor was illuminated artificially by one side simulating the solar cycle, using four Philips PL-32 W/840/4p white-light lamps, with a maximal irradiance of 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (measured in the centre of the reactor when empty). The light lamps used mainly provided disperse radiation, but because lamps were only located in one side of the reactor the light distribution approaches that found inside the

greenhouse where the outdoor T-PBRs are located. The pH was controlled by on-demand injection of pure CO_2 at pH 8.0. Whereas the temperature was controlled by regulating the air temperature in the chamber on which the reactor was located.

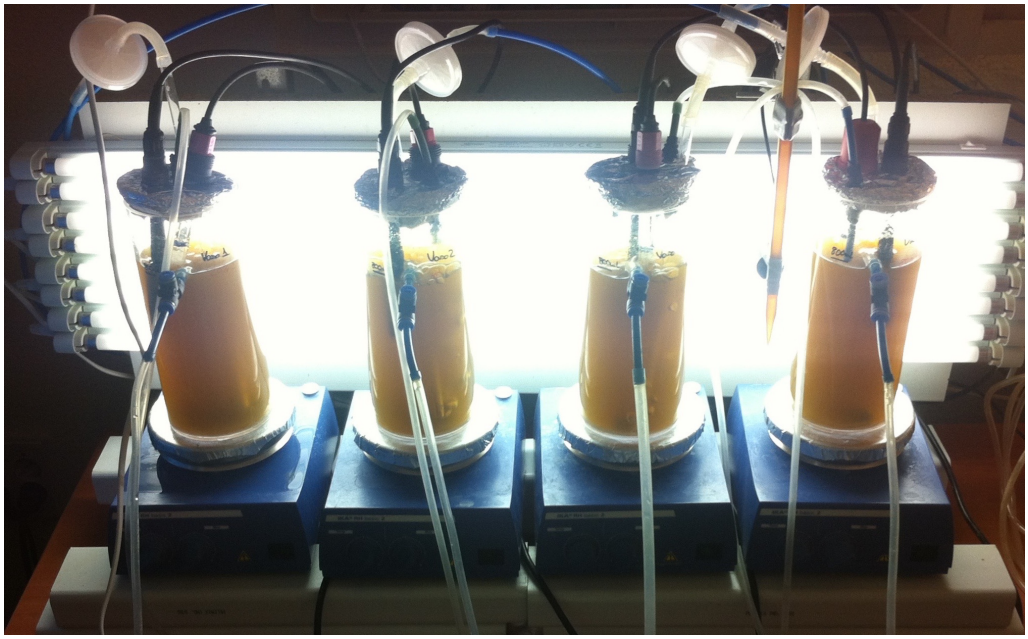

Fig. 7 – Cultivation of *Isochrysis galbana* in laboratory photobioreactors (Estación Experimental Las Palmerillas, Almeria)

The culture temperature modifies along the day, ranging from 30°C during the simulated noon to 25°C during the simulated night, average daily temperature of the culture being 28±3ºC. On the reactor the dissolved oxygen concentration was maintained lower than 200 %Sat. by the on-demand injection of air into the reactors, also to simulate the conditions prevailing in outdoor T-PBRs.

3.3. Outdoor photobioreactors

Outdoor cultures were performed in industrial T-PBRs of 3.0 m^3 capacity. The microalgal production facility used is situated inside a

greenhouse at the “Estación Experimental Las Palmerillas”, which belongs to Fundación CAJAMAR (Almería, Spain).

Fig. 8 – Greenhouses and laboratory owned by Fundación CAJAMAR (Almería, Spain).

The facility consists of ten tubular fence-type photobioreactors built as previously described (Fernández et al., 2014). Each photobioreactor is made of a 400 m-long PMMA tube, 0.09 m in diameter, with a bubble column (3.5 m high and 0.4 m in diameter) for degassing and heat exchange.

Fig. 9 – Cultivation of *Isochrysis galbana* in outdoor photobioreactors. (Estación Experimental Las Palmerillas, Almeria)

The pH, temperature and dissolved oxygen at the end of the loop are measured using Crison probes (Crison Instruments, Spain), connected to an MM44 control-transmitter unit (Crison Instruments, Spain), in turn connected to a PC control unit, allowing complete monitoring and control of the facility. Each reactor is bubbled at a constant airflow rate of 200 $l \cdot min^{-1}$, whereas the pH is controlled by on-demand injection of pure CO_2 at 3 $l \cdot min^{-1}$. The culture temperature is controlled by passing cooling water at 1500 $l \cdot h^{-1}$

(when needed - as determined by the computer control) through an internal heat exchanger located in the bubble column of each photobioreactor. The reactor was operated in continuous mode by harvesting 20% of the culture volume daily, which was then replaced by fresh medium. The culture medium was prepared online using a fertigation unit with a 4 $m^3 \cdot h^{-1}$ capacity. The culture medium was sterilized prior to introduction into the PBRs during the daylight period. The pH, DO, temperature in each PBR, and radiation inside the greenhouse and inside the laboratory were recorded daily.

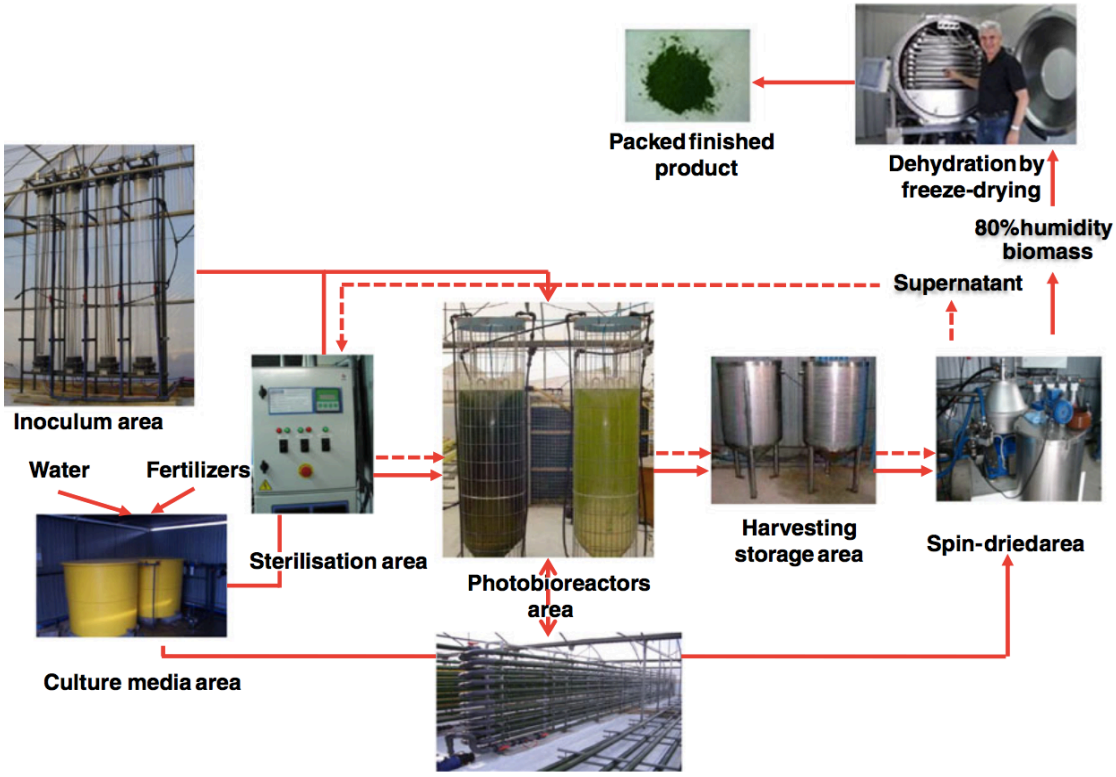

Fig. 10 - Schematic diagram of the production process for *Isochrysis galbana* biomass using recycled culture medium in a closed circuit (J. Camacho-Rodríguez et al. 2013)

The data capture system and control software (developed in DaqFactory 5.0, Azeotech Inc.) controlled and monitored all activities in the facility.

3.4. Measurement of photosynthesis and respiration rate

Photosynthesis and respiration measurements were performed in a specifically designed equipment, by measuring the variation of dissolved oxygen concentration under controlled conditions. Samples from steady-state culture on the laboratory photobioreactor were taken and diluted to 0.15 $g \cdot l^{-1}$ to achieve a diluted culture. The samples were placed in a 100 ml cylindrical stirred glass chamber (0.04 m diameter, 0.10 m height), in which irradiance (QSL-1000, Walz, Germany), temperature (pt-100), pH (Crison 5343, Barcelona, Spain) and dissolved oxygen (Crison 5002, Barcelona, Spain) probes were located. The irradiance sensor was located in the centre of the glass chamber whereas the other sensors were located close to the surface to avoid shadows into the system. To illuminate the system two sets of four fluorescent lamps (Osram 80 W) were placed at right and left of the glass chamber, the intensity of the lamps being automatically regulated to obtain the desired irradiance inside the center of the chamber once the sample was added.

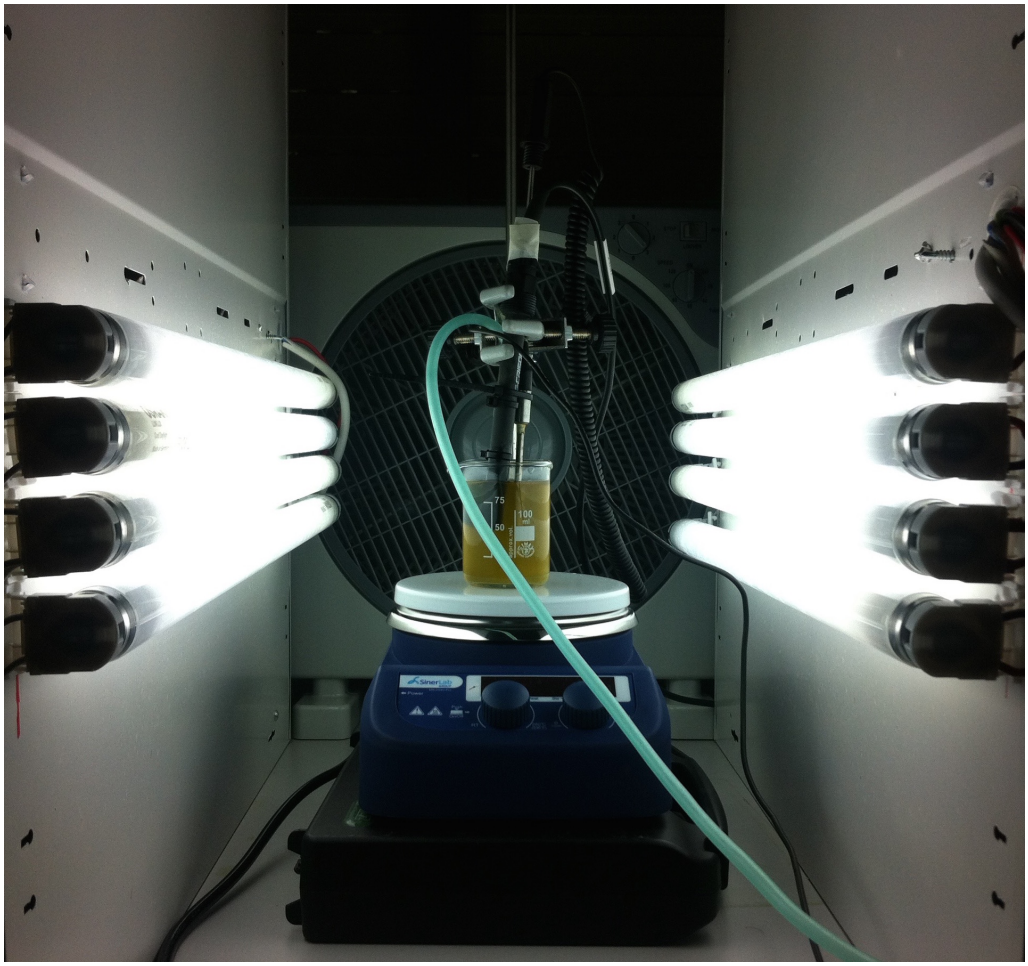

Fig. 11 - Sample of *Isochrysis galbana* during the measurement of photosynthesis and respiration rate. ((Estación Experimental Las Palmerillas, Almeria)

Due to the utilization of diluted cultures, the both side illumination and the short light path of the sample chamber used, the maximum deviation of irradiance at which the cells were exposed to into the system was 10%. Each sample was subjected to repeated 5 min light-dark periods for a maximum of 30 min to measure and register the variation of culture parameters (dissolved oxygen, pH and temperature) under whichever conditions. The entire system was computer controlled using DaqFactory software. Data allow us confirming that during measurements no relevant variations of

temperature or pH took place, whereas dissolved oxygen variations allowed calculating the photosynthesis and respiration rate. Gross photosynthesis rate was calculated from slope of dissolved oxygen accumulation during light period, whereas respiration rate was calculated from slope of dissolved oxygen accumulation during dark period, net photosynthesis rate being calculated as the sum of both gross photosynthesis and respiration rate to include respiration performed during light period. Three consecutive measurements were performed to obtain mean values of photosynthesis and respiration rates.

Photosynthesis and respiration rate of samples exposed to different irradiancies inside the glass chamber were measured. Moreover experiments were performed also modifying the pH of the samples. To obtain this the pH of fresh medium used to dilute the sample from the laboratory photobioreactor was adjusted by adding HCl or NaOH, to desired value then the photosynthesis and respiration rate of final diluted sample being measured. Concerning temperature a similar strategy was used, the temperature being adjusted by using cold or warm culture medium to dilute the sample. Finally experiments were performed modifying the dissolved oxygen by bubbling pure oxygen or pure nitrogen into the fresh medium solution used to dilute the sample. For each measurement a new sample from the laboratory photobioreactor in steady state was used, to avoid accumulation of effects.

3.5. Biomass concentration, fluorescence of chlorophylls and biochemical composition

Either for laboratory or outdoor cultures the microalgal biomass concentration was measured by dry weight measurements. 50 mL aliquots of the cell suspension were filtered through 0.7 μm Millipore filter paper, washed with 30 mL of ammonium formate, and the filters containing the algae were dried in an oven at 80°C for 24 h.

The physiological status of the cells was determined by chlorophyll fluorescence (Fv/Fm) using a fluorimeter (AquaPen-C AP-C 100; Photon Systems Instruments, Czech Republic).

The biochemical composition of the biomass was determined after collecting, centrifuging (Sigma Sartorius 4-15, Sartorius A.G., Germany) and freeze-drying (Telstar Cryodos 50, Telstar, Spain) culture samples at the steady state of each experiment. The protein content was determined following a modification of the Lowry method (López et al., 2010). The total lipid content was quantified using the method proposed by Kochert (1978). Total ash was determined by incineration of a 0.5 g sample in an oven at 450ºC for 48 h. Finally, carbohydrates were estimated as the difference (out of a hundred) after subtracting the lipid, protein and ash content. To avoid interferences due to ash content the biochemical composition is expressed on ash free basis.

3.6. Light utilization by the cultures

The biomass extinction coefficient (Ka), was calculated from the average absorption value in the visible range (400-700 nm), measured in a double beam Helios Alpha spectrophotometer. The extinction coefficient was calculated by dividing the average absorption by the biomass concentration (Cb) and the cuvette light path (p) (Equation 1).

$$Ka = \frac{\overline{Abs}_{400-700}}{C_b \cdot p} \qquad \text{Equation 1}$$

The average irradiance at which cells are exposed inside a culture (Iav) is a function of irradiance in the absence of cells (Io), the biomass extinction coefficient (Ka), the biomass concentration (Cb) and the light path inside the reactor (p). It can be approximated using Equation 2 (Molina et al. 1997).

$$Iav = \frac{I_o}{K_a C_b \cdot p} \cdot \left(1 - \exp(-K_a C_b \cdot p)\right) \qquad \text{Equation 2}$$

Quantum yield (Ψ_E) is defined in microalgal cultures as the amount of biomass generated by the unit of radiation (usually a mole of photons) absorbed by the culture. Since it represents the ratio between the biomass generated and the absorbed photon flux, it can be calculated using Equation 3 (Molina et al. 1997) as a function of the volumetric biomass productivity (Pb) and the photon flux absorbed in the volume unit (Fvol). The photon flux absorbed through the reactor volume can be obtained from the average irradiance (Iav) on a culture volume; this can be calculated using Equation 4 (Molina et al. 1997).

$$\Psi_E = \frac{P_b}{F_{vol}}$$ Equation 3

$$F_{vol} = Iav \cdot K_a \cdot C_b$$ Equation 4

Photosynthetic efficiency was calculated as the ratio of energy stored in the biomass produced to energy impinging on the reactor surface (Equation 5). The combustion heat of the biomass (H) was calculated considering the specific caloric value of the lipids (38.9 kJ/g), proteins (24 kJ/g) and carbohydrates (16.6 kJ/g), and by knowing the biochemical composition of the biomass. This equation uses the volume to surface ratio (V/S) of the reactor, and the PAR to global ratio of light, which was 2 $E \cdot MJ^{-1}$.

$$PE = \frac{P_b \cdot H \cdot V}{I \cdot S}$$ Equation 5

3.7. Software and statistical analysis

DaqFactory (Azeotech, USA) was used to control the photobioreactors. Statistical analysis of the data was carried out using the Statgraphics Centurion XVI software package. Non-linear regression was used to fit experimental data to the proposed models and to determine characteristic parameter values. Linear regression was used to evaluate the influence of the dilution rate relatively to the variables studied. Microsoft Excel was used to perform simulations using the developed models.

4. RESULTS AND DISCUSSIONS

4.1. Photosynthesis and respiration rate models

Microalgae productivity is influenced by various factors but under unlimited nutrient conditions, the most important are irradiance, temperature, pH and dissolved oxygen. To optimize the productivity of whichever strain in real-scale reactors, the most accurate method is to expose it to different conditions directly in industrial-scale photobioreactors. However, this would require high costs and a long time. Alternatively, the influence of these factors can be studied in the laboratory simulating outdoor conditions to develop models that must subsequently be verified outdoors; most of these models analyse the production of oxygen (photosynthesis and respiration) as the first step in the biomass production process (Costache et al., 2013; Yun and Park, 2003; Vejrazka et al., 2013), this was the method used in the present work. Therefore, to model the response of *Isochrysis galbana* cells to environmental factors, experiments were performed measuring the oxygen production rate under light conditions and the respiration rate under dark conditions, with controlled environmental variables. These measurements allowed us to calculate the cells' net photosynthesis from the difference between these two measurements. Samples were collected from laboratory cultures operated in indoor continuous mode but simulating outdoor conditions to maximize the applicability of the developed models. Thus, experimental values of biomass concentration and productivity measured indoors were close to those measured outdoors, ranging from 0.6 to 0.9 $g_{biomass} \cdot l^{-1}$

and from 0.2 to 0.3 $g_{biomass} \cdot l^{-1} \cdot day^{-1}$. These values are higher than those previously reported for this strain under outdoor conditions - being 0.075 $g \cdot l^{-1} \cdot day^{-1}$ in tubular photobioreactors (van Bergeijk, Salas-Leiton and Cañavate, 2010) and 0.13 $g \cdot l^{-1} \cdot day^{-1}$ in flat panels (Zhang and Richmond, 2003), thus verifying the adequacy of the culture conditions. Moreover, this strategy validates the experiments themselves and their applicability in simulating real outdoor cultures.

Concerning irradiance, it was observed that the net photosynthesis rate increased from zero at zero irradiance up to values of 565 $mgO_2 \cdot g_{biomass} \cdot h^{-1}$ at an irradiance of 600 $\mu E \cdot m^{-2} \cdot s^{-1}$, then decreased due to photo-inhibition to 394 $mgO_2 \cdot g_{biomass} \cdot h^{-1}$ at an irradiance of 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Figure 12A). According to these data, the photosynthesis rate is maximal at an irradiance ranging from 500 to 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$. Furthermore, it is confirmed that this strain is less tolerant to high irradiance than other commercial strains such as *Haematococcus pluvialis*, *Chlorella vulgaris* and *Scenedesmus almeriensis* (Costache et al., 2013; Yun and Park, 2003; Jeon, Cho and Yun, 2006). Nevertheless, the maximum daily irradiance to which the T-PBRs were exposed during this research (April to June) ranged from 600 to 900 $\mu E \cdot m^{-2} \cdot s^{-1}$, within the experimentally-determined optimal value range. Concerning respiration, at zero irradiance the respiration rate was 37 $mgO_2 \cdot g_{biomass} \cdot h^{-1}$, increasing with irradiance up to 111 $mgO_2 \cdot g_{biomass} \cdot h^{-1}$ at an irradiance of 400 $\mu E \cdot m^{-2} \cdot s^{-1}$, then remaining constant whatever the irradiance in the range tested (Figure 12B). This upward trend in the respiration rate is also confirmed by other strains such as *Coelastrum sphaericum* and *Scenedesmus falcatus* (Grobbelaar and Soeder, 1985). The

oxygen production rate is the difference between these two parameters, representing the production of oxygen under light conditions, which is proportional to the biomass production rate. The data demonstrated that the oxygen production rate is negative under dark conditions, with an irradiance level equal to 15 $\mu E \cdot m^{-2} \cdot s^{-1}$ being necessary to achieve an oxygen production rate equal to zero (the compensation point) (Figure 12C).

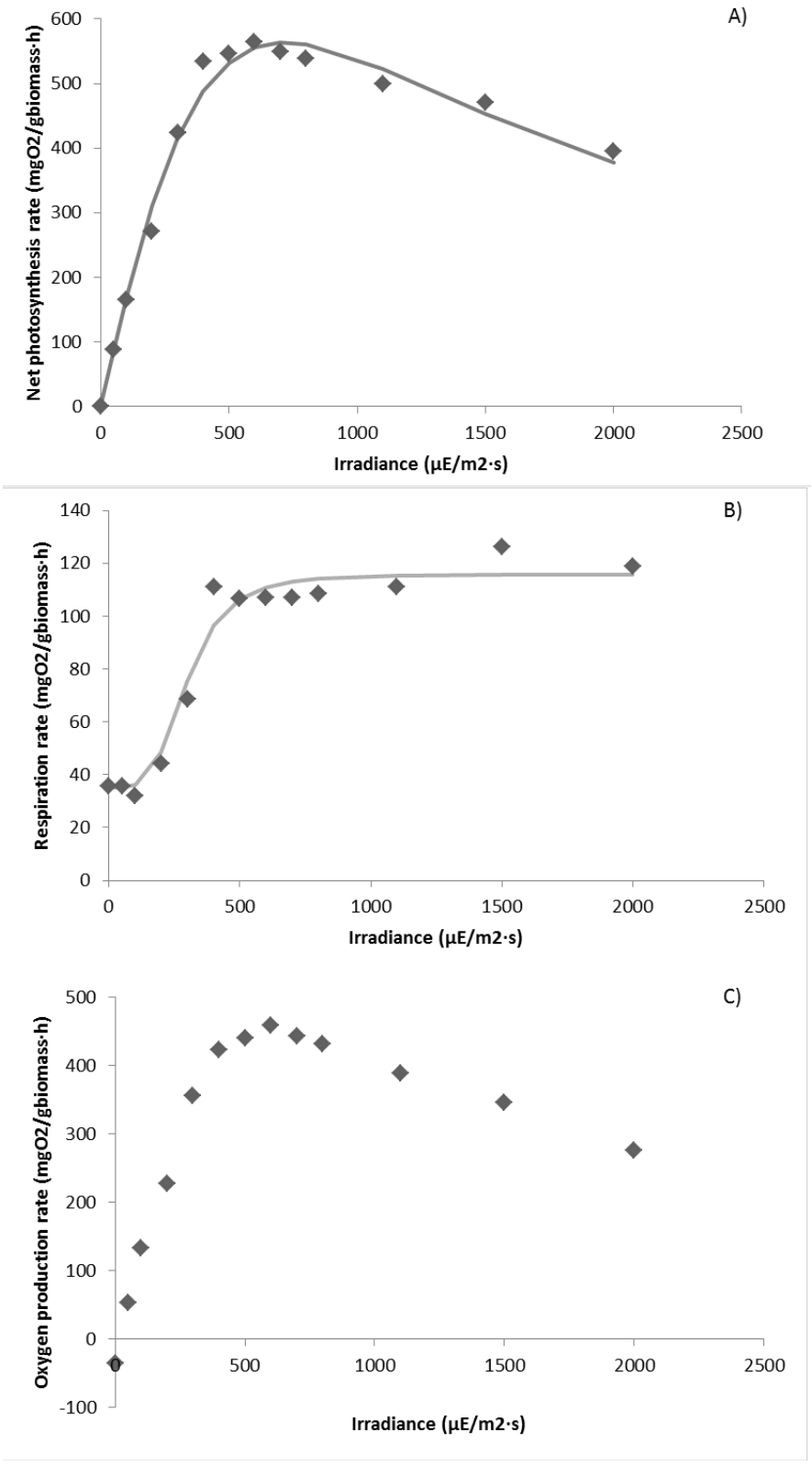

Fig. 12 - Influence of irradiance on the net photosynthesis (A), respiration (B) and oxygen production rate (C) of *Isochrysis galbana* at 29ºC, pH=7.5 and DO_2=7.6 mg/L. Lines correspond to fit the proposed models (Equation 6, Equation 7).

At an irradiance higher than 15 $\mu E \cdot m^{-2} \cdot s^{-1}$, the net photosynthesis rate is much higher than the respiration rate, thus the oxygen production rate fits to the behaviour observed for the net photosynthesis rate. According to these results, the maximal respiration rate is 20% of the maximal photosynthesis rate; this ratio being strain specific (Geider and Osborne, 1989). From these data it could be concluded that the net photosynthesis rate can be fitted to the Eiler and Peters model (Eq. 6) (Eilers and Peeters, 1988) whereas the respiration rate can be fitted to the hyperbolic model with no inhibition (Eq. 7).

$$PO2(I) = \frac{I}{a \cdot I^2 + b \cdot I + c} \qquad \text{Equation 6}$$

$$RO2(I) = RO2min + \frac{RO2max \cdot I^n}{Ik^n + I^n} \qquad \text{Equation 7}$$

The response of the net photosynthesis rate to irradiance is modulated by other environmental conditions such as temperature, pH and dissolved oxygen. The experiments carried out allow us to calculate the normalized net photosynthesis rate as a function of these culture conditions. Data showed that temperature and pH exhibit similar behaviour, with the net photosynthesis rate being zero at 12ºC and pH 3, increasing to a maximum of 36ºC and pH 7.5, then decreasing to zero at maximum values of 45ºC and pH 10 (Figure 13A,B). It was unexpected that the optimal temperature for maximizing the photosynthesis rate was close to 36ºC. This temperature is high when compared to previously reported optimal temperatures for this strain, ranging from 25ºC to 30ºC (Marchetti et al., 2012; Renaud et al., 2002; Claquin et al., 2008). Regarding the pH, tolerance to this parameter is different for each strain. For most microalgae growth it is optimal at pH values between 7.0 and

8.0; however for others such as *Spirulina,* alkaline pH is recommended; pH has even been reported as useful in controlling contaminants in mixed cultures (Goldman et al., 1982). Regarding the dissolved oxygen concentration, the net photosynthesis rate is maximal at dissolved oxygen concentrations from zero to 11 $mg \cdot l^{-1}$, then exponentially decreases to zero at 20 $mg \cdot l^{-1}$ due to oxygen inhibition (Figure 13C).

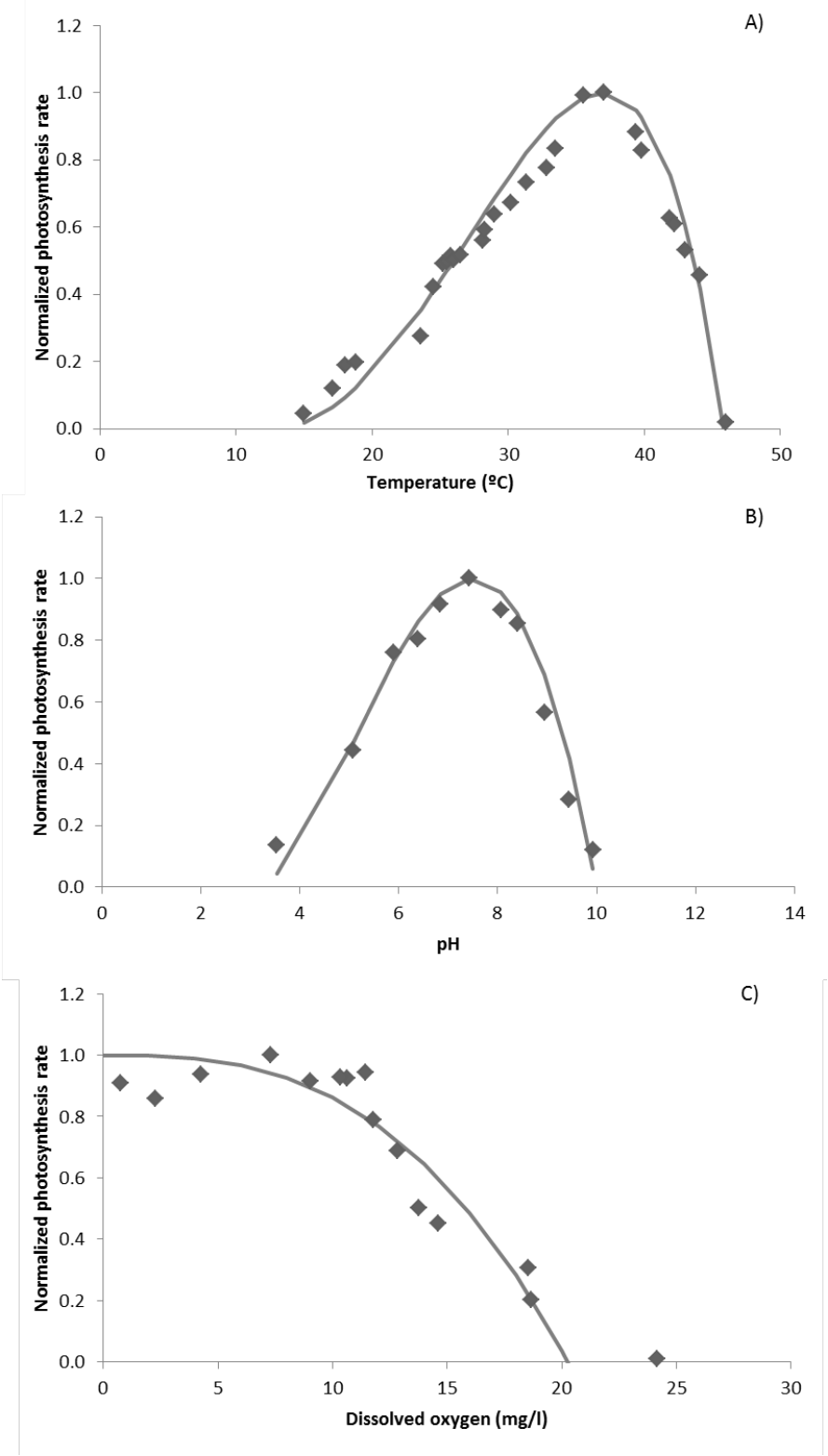

Fig.13 - Influence of temperature (A), pH (B) and dissolved oxygen concentration (C) on the normalized photosynthesis rate of *Isochrysis galbana* at 600 $\mu E/m^2 \cdot s$. Experiments performed under standard conditions for other culture conditions (29ºC, pH=7.5 and DO2=7.6 mg/L). Lines correspond to fit the proposed models (Equation 8, Equation 9, Equation 10).

It’s important to note that this is a relevant parameter because oxygen is produced during photosynthesis and can accumulate in high concentrations in closed photobioreactors, reducing the photosynthesis rate and favouring culture photorespiration (Mendoza et al., 2013). Therefore, in high dissolved oxygen concentrations, photorespiration takes place; the oxygen binds to the Rubisco enzyme and modifies its role from carboxylase to oxygenase, reducing carbohydrate synthesis (Badger et al., 2000). Variation in the normalized net photosynthesis rate with temperature and pH can be fitted to the cardinal model developed for bacteria (Rosso et al., 1993) and validated for microalgae (Bernard and Rémond, 2012). According to this model, the net photosynthesis rate is a function of the difference between the variables (temperature, pH) and the characteristic values of the strain (maximal, minimal and optimal), defined only in the range of tolerable values. Other authors proposed models based on the Arrhenius equation (Costache et al., 2013; Pérez et al. 2008) or a non-linear correlation (Blanchard et al., 1996; Moisan, Moisan and Abbott, 2002) to consider the influence of temperature on the microalgae cultures. Thus, equation 8 and equation 9 allow us to model the response of the net photosynthesis rate to temperature and pH according to the cardinal model (Bernard and Rémond, 2012). A model considering inhibition by product, as previously reported (Costache et al., 2013) can be used to model the response to dissolved oxygen concentration (Eq. 10).

$$\overline{PO2}(T) = \frac{(T - Tmax)(T - Tmin)^2}{(TPopt - Tmin)[(TPopt - Tmin)(T - TPopt) - (TPopt - Tmax)(TPopt + Tmin - 2T)]}$$

Equation 8

$$\overline{PO2}(pH) = \frac{(pH - pHmax)(pH - pHmin)^2}{(pHopt - pHmin)[(pHopt - pHmin)(pH - pHopt) - (pHopt - pHmax)(pHopt + pHmin - 2pH)]}$$

Equation 9

$$\overline{PO2}(DO2) = 1 - \left(\frac{DO2}{DO2max}\right)^m$$

Equation 10

An analogous study was performed concerning the respiration rate. The variation in the normalized respiration rate with the temperature, pH and dissolved oxygen shows the same behaviour (Figure 14), which was analogous to that previously observed for the variation in the net photosynthesis rate with temperature and pH.

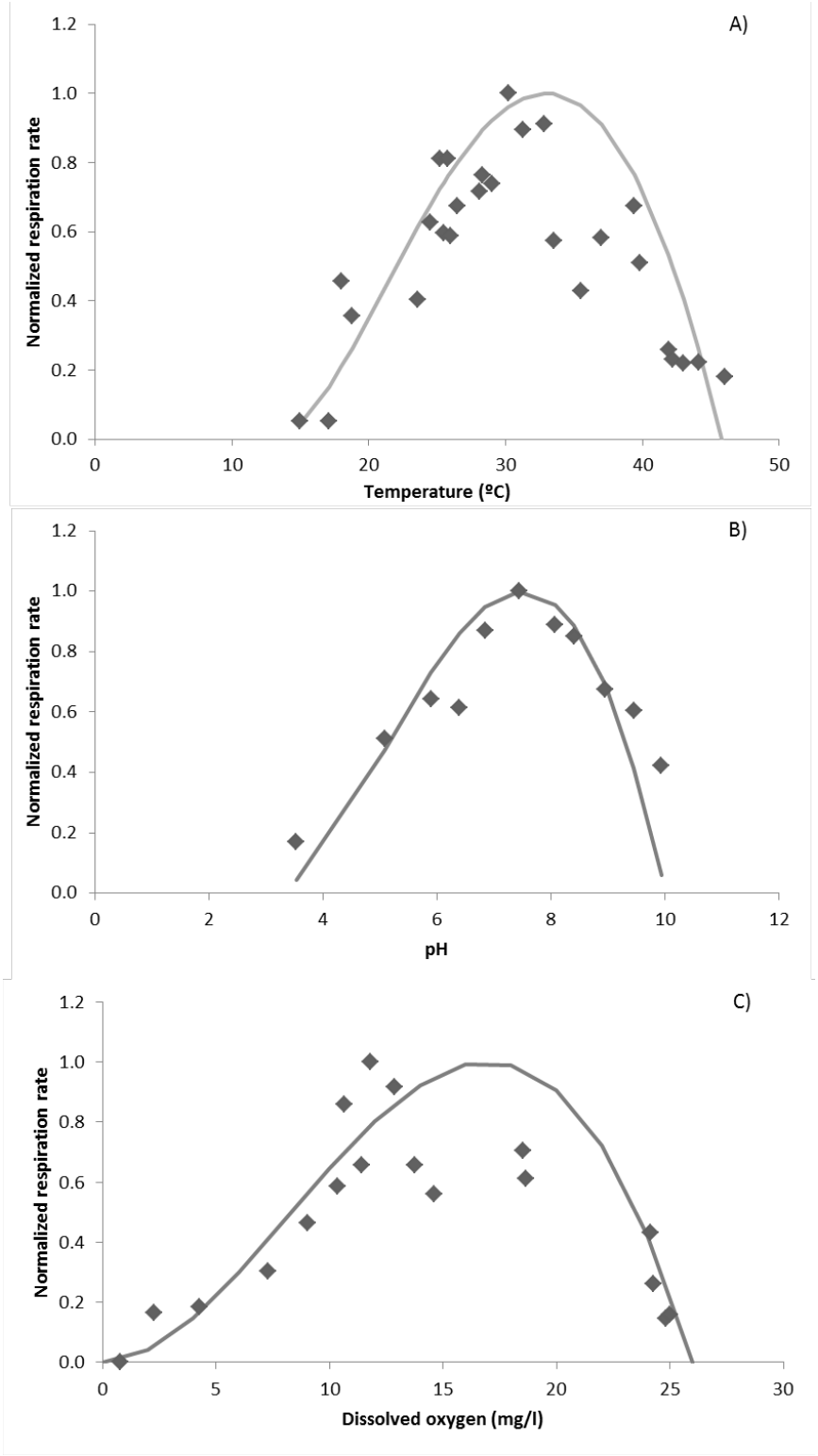

Fig.14 - Influence of temperature (A), pH (B) and dissolved oxygen concentration (C) on the normalized respiration rate of *Isochrysis galbana*. Experiments performed under standard conditions for other culture conditions (29ºC, pH=7.5 and DO2=7.6 mg/L). Lines correspond to fit the proposed models (Equation 11, Equation 12, Equation 13).

The respiration rate was zero at values of 12ºC, pH 3 and 0 $mg \cdot l^{-1}$, but it increased with the temperature, pH and dissolved oxygen to be maximal at 32ºC, pH 7.5 and 16 $mg \cdot l^{-1}$, then decreased to zero at maximal values of 46ºC, pH 10 and 26 $mg \cdot l^{-1}$. The influence of temperature and pH on the respiration rate is related to the adequacy of the culture conditions to the optimal ones required by the strain, whereas the influence of dissolved oxygen concentration is related to the nutrient availability or excess; in this case, the dissolved oxygen required for respiration. According to these results, the variation in the normalized respiration rate to changes in temperature or pH agree with the previously-reported cardinal model, whereas the influence of dissolved oxygen can be modelled using nutrient limitation-inhibition models. However, because the cardinal model also reproduces the observed pattern, and to reduce the variety of the equations used, this cardinal model has also been employed to model the influence of dissolved oxygen on the respiration rate. Thus, the following equations are used to model the respiration rate's response to temperature (Eq. 11), pH (Eq. 12) and dissolved oxygen (Eq. 13).

$$\overline{RO2}(T) = \frac{(T - Tmax)(T - Tmin)^2}{(TRopt - Tmin)[(TRopt - Tmin)(T - TRopt) - (TRopt - Tmax)(TRopt + Tmin - 2T)]}$$

Equation 11

$$\overline{RO2}(pH) = \frac{(pH - pHmax)(pH - pHmin)^2}{(pHopt - pHmin)[(pHopt - pHmin)(pH - pHopt) - (pHopt - pHmax)(pHopt + pHmin - 2pH)]}$$

Equation 12

$$\overline{RO2}(DO2) = \frac{(DO2 - DO2max)(DO2 - DO2min)^2}{(DO2opt - DO2min)[(DO2opt - DO2min)(DO2 - DO2opt) - (DO2opt - DO2max)(DO2opt + DO2min - 2DO2)]}$$

Equation 13

The proposed equations allows us to model the photosynthesis and respiration rates as a function of the culture conditions the cells are exposed to inside the culture over a short period, but possibly conditions that are not supported over long periods, or whose performance modifies as a function of exposure time. To determine the "validity time" of the model, it is necessary to evaluate the tolerance of *Isochrysis galbana* cells to more adverse culture conditions. From the experimental data, the most unexpected value was the optimal temperature of 36ºC. It is possible that although the photosynthesis rate increases with temperature over a short period, over longer periods other adverse effects occur at such a temperature and, thus, the overall performance of the cells diminishes. To study the model validity time, a culture was continuously exposed to a temperature of 36ºC, samples being taken at different times to study the variations in the photosynthesis and respiration rates over time. Data show that the net photosynthesis rate was stable for at least 100 minutes, then slightly decreased after 150 minutes (Figure 15A), thus confirming the tolerance of this strain to high temperatures for a maximal two-hour period. Regarding the respiration rate, it was constant the whole time, there was no observable tendency for exposure time to high temperature (Figure 15B). Because the net photosynthesis rate was much higher than the respiration rate under the standard conditions used, the oxygen production rate behaved similarly to that discussed for the net photosynthesis rate (Figure 15C).

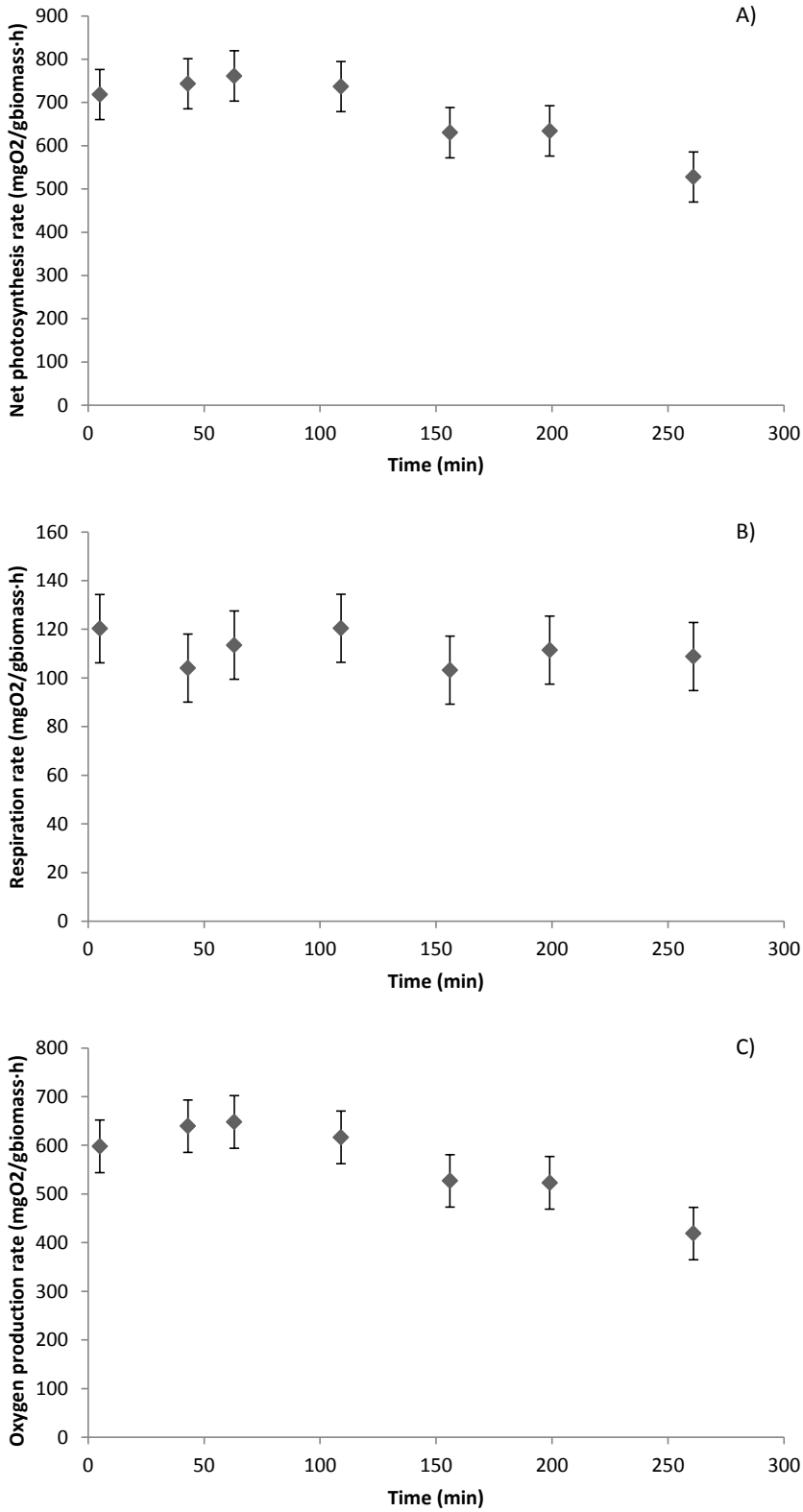

Fig.15 - Variation in the net photosynthesis rate (A), respiration rate (B) and oxygen production rate (C) with time for *Isochrysis galbana* cells exposed to high temperature (35ºC) for a long period. Measurements performed under the standard culture conditions (I=600 µE/m^2·s , pH=7.5, DO2=10 mg/L).

According to these results, the net photosynthesis and respiration rates can be modelled by combining these equations to obtain a general equation representing the overall strain behaviour based on the observed patterns. Thus, equation 14 and equation 15 allow us to modelled the net photosynthesis and respiration rates as a function of the culture conditions (irradiance, temperature, pH and dissolved oxygen) to which the cells are exposed.

$$PO2 = PO2(I)\cdot\overline{PO2(T)}\cdot\overline{PO2(pH)}\cdot\overline{PO2(DO2)} \quad \text{Equation 14}$$

$$RO2 = RO2(I)\cdot\overline{RO2(T)}\cdot\overline{RO2(pH)}\cdot\overline{RO2(DO2)} \quad \text{Equation 15}$$

In order to determine the optimal values of the characteristic parameters included in these models, we performed a non-linear regression of the entire data to the experimental data set.

Table 1.- Values for the proposed model's parameter characteristics (Equations 6-15) obtained by non-linear regression of the experimental values of the net photosynthesis rate and respiration rate under the experimental conditions tested.

Net photosynthesis rate			**Respiration rate**		
Parameter	**Value**	**Units**	**Parameter**	**Value**	**Units**
a	3.42E-07		RO2min	52.17	$mgO_2/L\cdot h$
b	9.30E-04		RO2max	153.00	$mgO_2/L\cdot h$
c	2.90E-01		Ik	1152.00	$\mu E/m^2\cdot s$
PO2max	641.01	$mgO_2/L\cdot h$	n	1.90	
Ik	186.05	$\mu E/m^2\cdot s$	Tmin	12.84	ºC
Im	921.23	$\mu E/m^2\cdot s$	Tmax	45.82	ºC
alfa	3.45		Topt	33.00	ºC
Tmin	11.88	ºC	pHmin	3.00	
Tmax	46.15	ºC	pHmax	10.00	
Topt	35.73	ºC	pHopt	7.50	
pHmin	2.24		DO2min	0.70	mgO_2/L
pHmax	10.00		DO2max	23.27	mgO_2/L
pHopt	7.34		DO2opt	11.96	mgO_2/L
KO2	19.99	mgO_2/L			
z	2.90				

The value of characteristic parameters obtained is shown in table 1 whereas the correlation between experimental and simulated values obtained using the proposed model (Equation 6-15) and the characteristic parameter values obtained is shown in figure 16.

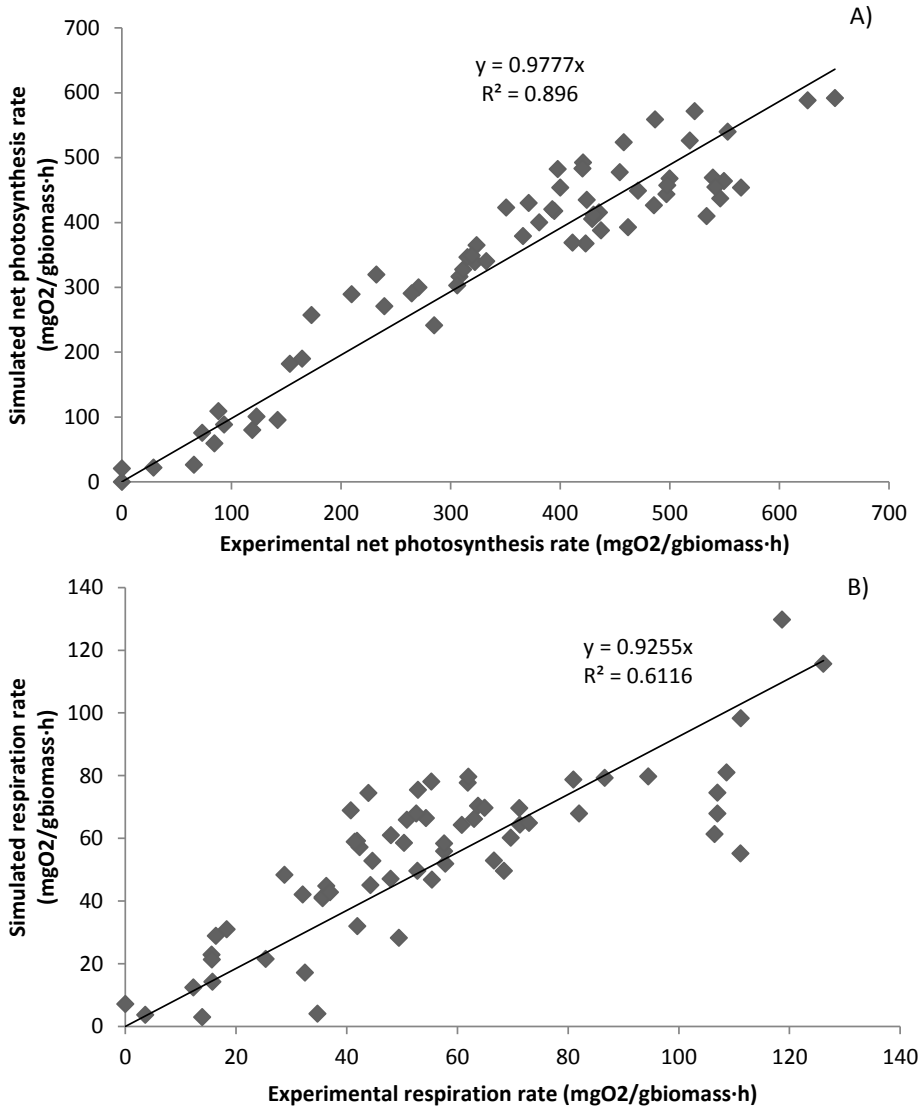

Fig.16 - Correlation between experimental and simulated values of the net photosynthesis rate (A) and respiration rate (B) of *Isochrysis galbana*. Simulated values obtained using the proposed models (Equation 6-15) and the parameter characteristic values shown in table 1.

One can observe how the proposed model simulates the experimental values of the net photosynthesis and respiration rates; with the correlation for the net photosynthesis rate being even

higher than that for the respiration rate. According to these results, it is demonstrated that the developed model reproduces the photosynthesis and respiration rates of *Isochrysis* not only for short time periods (minutes) but also for long periods (up to two hours); thus making them sufficiently robust to be exposed to real outdoor conditions where changes in the culture conditions, especially temperature, take place slowly in line with the solar cycle.

4.2. Application of the photosynthesis rate model to industrial-scale T-PBRs

The model has been validated to study the performance of *Isochrysis galbana* cultures grown in an industrial-scale tubular photobioreactor. For this, experimental data of the average irradiance, temperature, pH and dissolved oxygen measured on-line in the reactor during a solar cycle in steady-state has been used (Figure 17A). Data show that the average irradiance inside the culture ranges daily from zero to 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$ confirming that the cells are mainly light-limited, even at noon when there is high solar irradiance (up to 1600 $\mu E \cdot m^{-2} \cdot s^{-1}$). Moreover, during the solar cycle, the temperature ranges from 19.0ºC to 29.7ºC, whereas the pH varies from 7.6 to 8.1, and the dissolved oxygen from 5.1 to 14.4 $mg \cdot l^{-1}$. These values are close to those previously reported for outdoor cultures of *Isochrysis* in outdoor photobioreactors (van Bergeijk, Salas-Leiton and Cañavate, 2010; Zhang and Richmond, 2003). Concerning temperature variation, this modifies by up to 10ºC, according to the solar cycle, because the control system only allows us to avoid overheating of the culture inside the reactor.

Furthermore, the average value was 24.3ºC, far from the optimal photosynthesis rate value determined at 36ºC (Figure 13A) but close to the previously-reported optimal rate, at 21ºC (van Bergeijk, Salas-Leiton and Cañavate, 2010). The temperature effect is very important on outdoor microalgal cultures subject to daily culture condition variations (particularly irradiance and temperature) for which simultaneous adverse conditions can take place (van Bergeijk, Salas-Leiton and Cañavate, 2010). The negative effect of low temperature and high irradiance has already been demonstrated when occurring in the first hours of the morning in outdoor raceway ponds, with these conditions enhancing the photoinhibition phenomena. The utilization of closed photobioreactors rather than open raceways allows us to improve temperature control, making it possible to increase the night-time temperature and to avoid overheating at noon - but this involves a higher production cost (Acién et al., 2013). The variation in pH is minimal, up to 0.5 pH units, due to the control system's adequacy in supplying CO_2 and controlling pH. The average pH value is 7.9, close to the optimal value determined for photosynthesis rate of *Isochrysis* T-iso, of 7.5 (Figure 13B). Microalgae culture productivity can be influenced not only by the mean pH value but also by local pH gradients that take place when pure carbon dioxide is supplied (Fernández et al., 2014). The data obtained show the optimal pH range is between 7.0 and 8.0 - therefore to optimize productivity in the T-PBRs, it was enough to use the on-demand injection of pure CO_2 to keep the pH lower than 8.0. Finally, the average dissolved oxygen concentration was 9.1 $mg \cdot l^{-1}$, close to air saturation, but at noon, values up to 14.4 $mg \cdot l^{-1}$ were measured due to the system's inability to remove all the

oxygen produced by photosynthesis. According to the photosynthesis model, dissolved oxygen concentrations above 11 $mg \cdot l^{-1}$ reduce cell performance (Figure 13C), and therefore represent a relevant reduction in productivity.

From these experimental values of temperature, pH and dissolved oxygen, the normalized photosynthesis rate can be calculated by Equation 8 to Equation 10 (Figure 17B). Results confirm that pH was adequately controlled and did not reduce the photosynthesis rate, whereas temperature and dissolved oxygen varied greatly through the solar cycle, values being far from optimal, and thus the normalized photosynthesis rate was less than maximal. A larger contribution to a reduced photosynthesis rate came from the inadequacy of the culture conditions because of temperature and dissolved oxygen deviations taking place during the light period. The integral of values during the light period was 0.97 for PO2(pH), whereas it was 0.61 for PO2(T) and 0.77 for PO2(DO2). These results indicate that the photosynthesis rate can be increased 39% by optimizing the temperature control and 27% by optimizing the dissolved oxygen concentration inside the culture - thus photosynthesis can be more than doubled by optimizing both parameters at the same time (PO2(T-pH-DO2) being 0.44).

To better understand these phenomena, figure 6C shows the expected photosynthesis rate based on average irradiance inside the culture (considering optimal values of temperature, pH, and dissolved oxygen) (Eq. 6), and calculated considering all the culture conditions (irradiance, temperature, pH and dissolved oxygen) (Eq. 14). It can be observed that net photosynthesis, as a function solely of irradiance (PO2(I)), is much higher than that calculated when

considering all the culture parameters (PO2(I-T-pH-DO2)), especially at noon when light availability is maximal. Thus, the integral of PO2(I) values allows us to calculate the maximal oxygen production, 0.92 $gO_2 \cdot l^{-1} \cdot day^{-1}$, whereas the integral of PO2(I-T-pH-DO2) allows us to calculate the real oxygen production, 0.42 $gO_2 \cdot l^{-1} \cdot day^{-1}$. In this way, it is confirmed that the photosynthesis rate can be doubled by optimizing the control of the culture conditions in the reactors used.

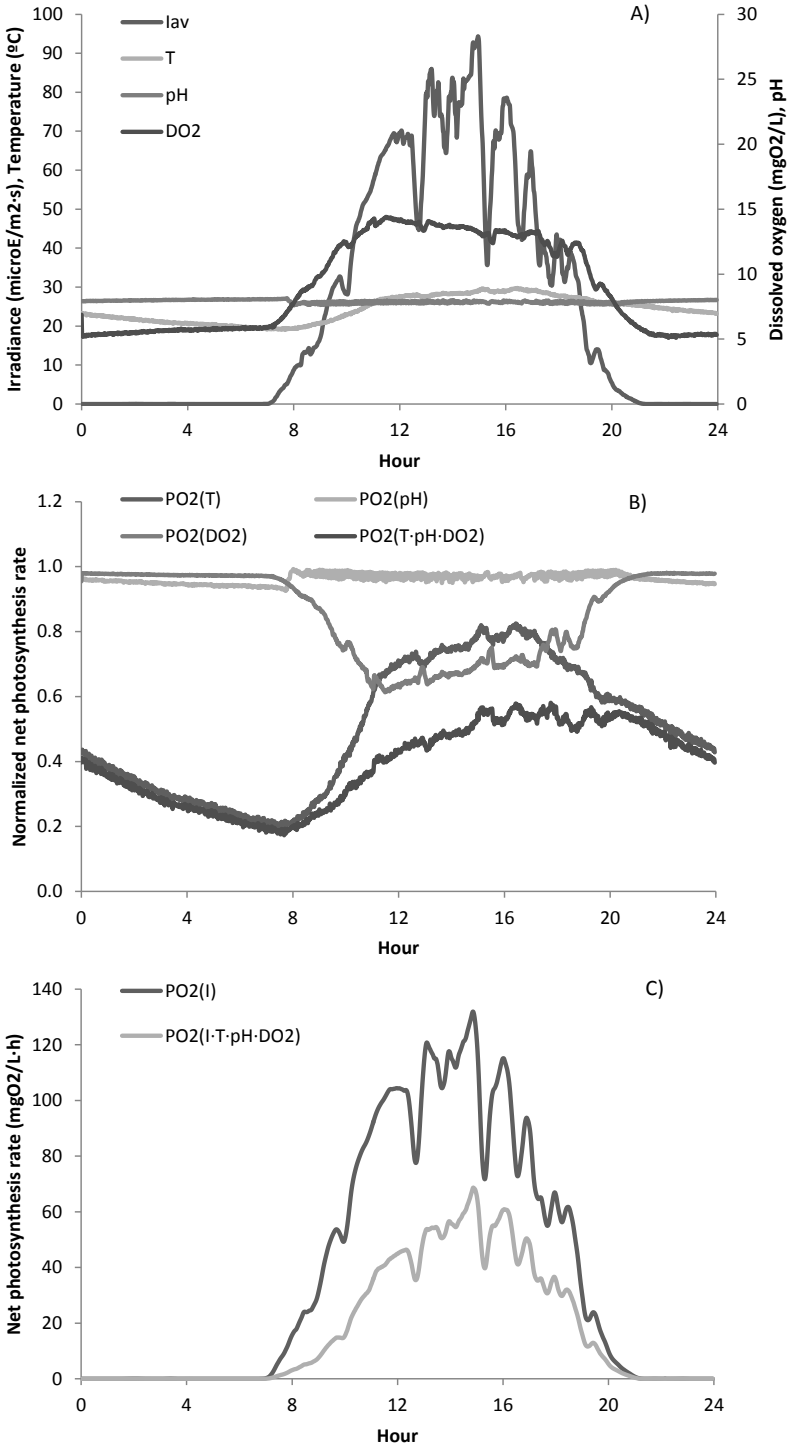

Fig.17 - Daily variation in culture parameters of *Isochrysis galbana* culture in an industrial-scale tubular photobioreactor operated in continuous mode at 0.4 1/day. A) Experimental values of average irradiance, temperature, pH and dissolved oxygen to which the cells are exposed inside the culture; B) Influence of culture conditions (T, pH, DO2) in the normalized photosynthesis rate according to the proposed equations (Equation 13-15); C) Net photosynthesis rate as a function of irradiance (Equation 6) and of all the variables (I, T, pH, DO2) (Equation 14).

Moreover, by comparing the real oxygen production, calculated using the proposed model, with the experimental biomass productivity measured, of 0.22 gbiomass·l^{-1}·day^{-1}, the oxygen to biomass ratio can be calculated a value of 1.94 $gO_2 \cdot g_{biomass}^{-1}$. In outdoor cultures of *Scenedesmus almeriensis* carried out in open raceway reactors, an oxygen-to-biomass ratio of 1.46 gO_2 $g_{biomass}^{-1}$ was measured when using flue gas to control pH and to supply CO_2; whereas a ratio of 0.99 gO_2 $g_{biomass}^{-1}$ was measured when using pure CO_2 (Mendoza et al., 2013). The oxygen yield value reported here agrees with that previously reported, thus confirming the proposed model's validity in determining oxygen production as a function of culture conditions; and, moreover, loss of productivity due to deviation in the culture conditions. It is therefore a useful tool for taking decisions regarding the implementation of different control strategies.

4.3. Outdoor production of *Isochrysis galbana* (T-iso)

To determine the feasibility of producing *Isochrysis galbana* T-iso in outdoor industrial scale tubular photobioreactors experiments were performed by modifying the dilution rate under standard conditions. Experiments were performed in April-May (2015), the cultures performing adequately at the dilution rates tested. Subsequently, to evaluate the influence of the various factors linear correlations were made between the various parameters and the dilution (Table 2).

Tab. 2 – P-Values obtained by linear correlations (Pearson) between the parameters and the rate of dilution

Parameter	P-Value	Parameter	P-Value
Biomass concentration	**--0.003528**	pH minimum	0.238610
Fluorescence of chlorophylls	0.253081	Temperature average	0.547727
Volumetric productivity	**0.006219**	**Temperature maximum**	**-0.037913**
Areal productivity	**0.006219**	Temperatura minimum	0.138590
Extinction coefficient	**0.005737**	**Average irradiance**	**0.007631**
Dissolved oxygen average	0.097486	**Protein content**	**0.002218**
Dissolved oxygen maximum	0.142337	Lipid content	-0.070122
Dissolved oxygen minimum	**0.042923**	Carbohydrate content	-0.082813
pH average	**0.045145**	**Photosynthetic efficiency**	**0.015367**
pH maximum	0.090452	**Quatum yield**	**0.006739**

Results show as increasing the dilution rate from 0.15 to 0.35 1/h the biomass concentration on steady state diminishes from 1.1 to 0.7 g/L (P-Value < -0.05: significant difference, Tab. 2), but the chlorophyll fluorescence remained constant, at values of 0.58 to 0.65 (P-Value > 0.05: no significant difference, Tab. 2) , in all the experiments (Figure 18A). The reduction of biomass concentration with the increase of dilution rate represents the behaviour expected from light limited cultures, whereas the maintenance of fluorescence of chlorophylls with the increase of dilution rate indicates that this variable has not influence on the status of the photosynthetic apparatus. Regarding biomass productivity, it increased with the increase of dilution rate (P-Value < 0.05: significant difference, Tab. 2), thus confirming that the cultures were light limited under the imposed culture conditions, and that higher biomass productivity could be reached by operating at higher dilution rates (Figure 18B). However, the dilution rate was not

increased over 0.35 1/day because the biomass concentration in steady state was lowered below a critical value of 0.8 g/L. In this case the T-iso cultures in outdoor conditions can be unstable. The maximal biomass productivity achieved was 0.25 g/Lday equivalent to 20 $g/m^2 \cdot day$ due to the V/S ratio of the photobioreactors utilized. With respect to the light availability, results shows as increasing the dilution rate the extinction coefficient of the biomass increased, at the same time the average irradiance inside the culture also increased (both P-Values < 0.05: significant difference, Tab. 2), in spite of constant solar radiation (Figure 18C).

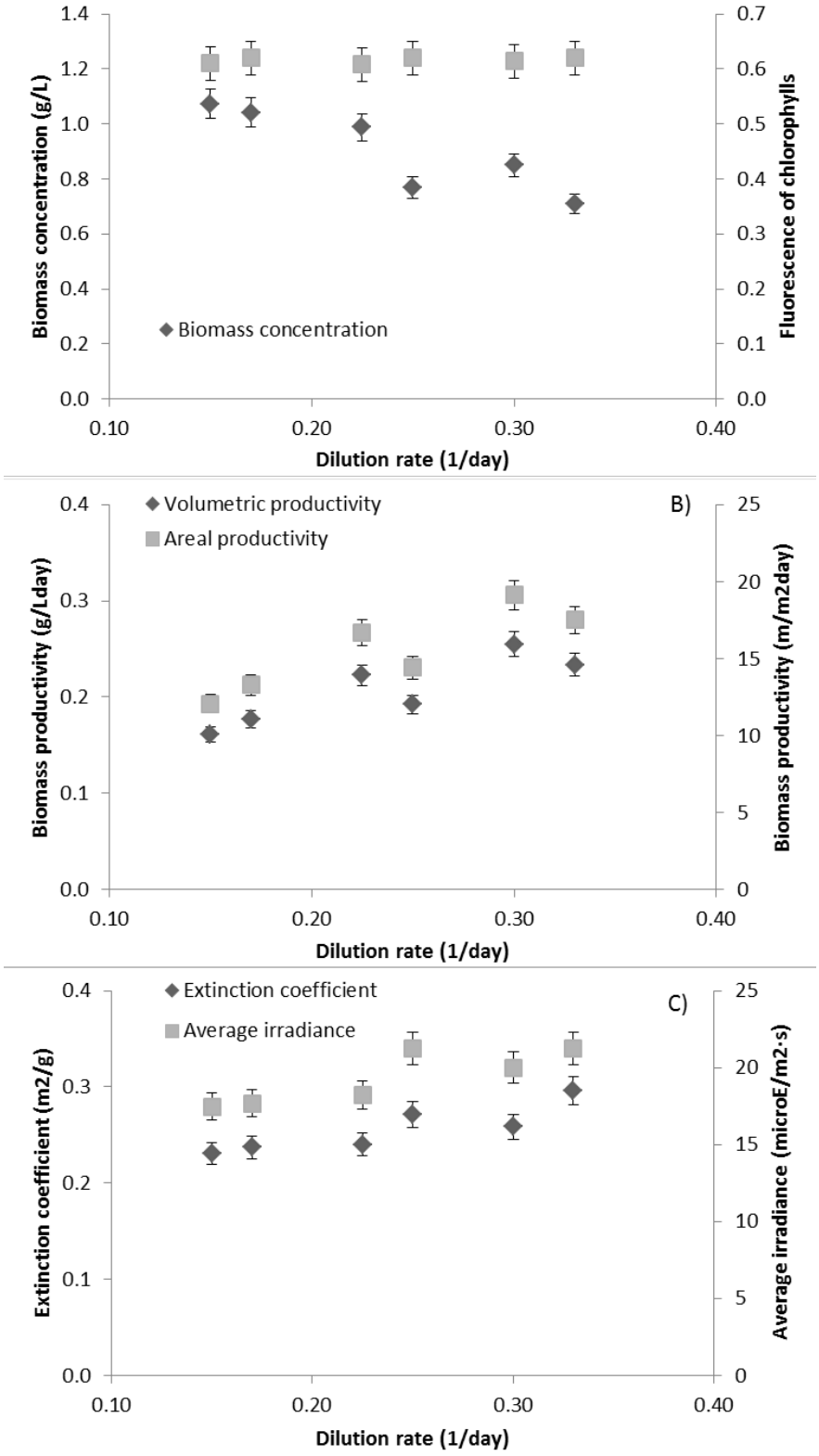

Fig.18 - Variation of (A) biomass concentration and fluorescence of chlorophylls, (B) volumetric and areal productivity, and (C) extinction coefficient and average irradiance inside the culture, with the dilution rate, of *Isochrysis galbana* T-iso cultures performed in 3.0 m^3 industrial scale tubular photobioreactors.

Thus, on the days when the experiments were performed the solar radiation was stable, daily average radiation ranging from 350 to 360 W/m^2. These results confirmed that the cultures were not photo-limited, and that photo-limitation was not present or was negligible. Thus, the extinction coefficient increased from 0.23 to 0.30 m2/g, whereas the average irradiance increased from 17 to 22 μE/m^2·s when the dilution rate was increased from 0.15 to 0.35 1/day.

To determine if the productivity of the cultures was negatively affected by the culture conditions, the experimental dissolved oxygen, pH and temperature values were registered and analysed. Maximum, minimum and average daily values are shown in Figure 19.

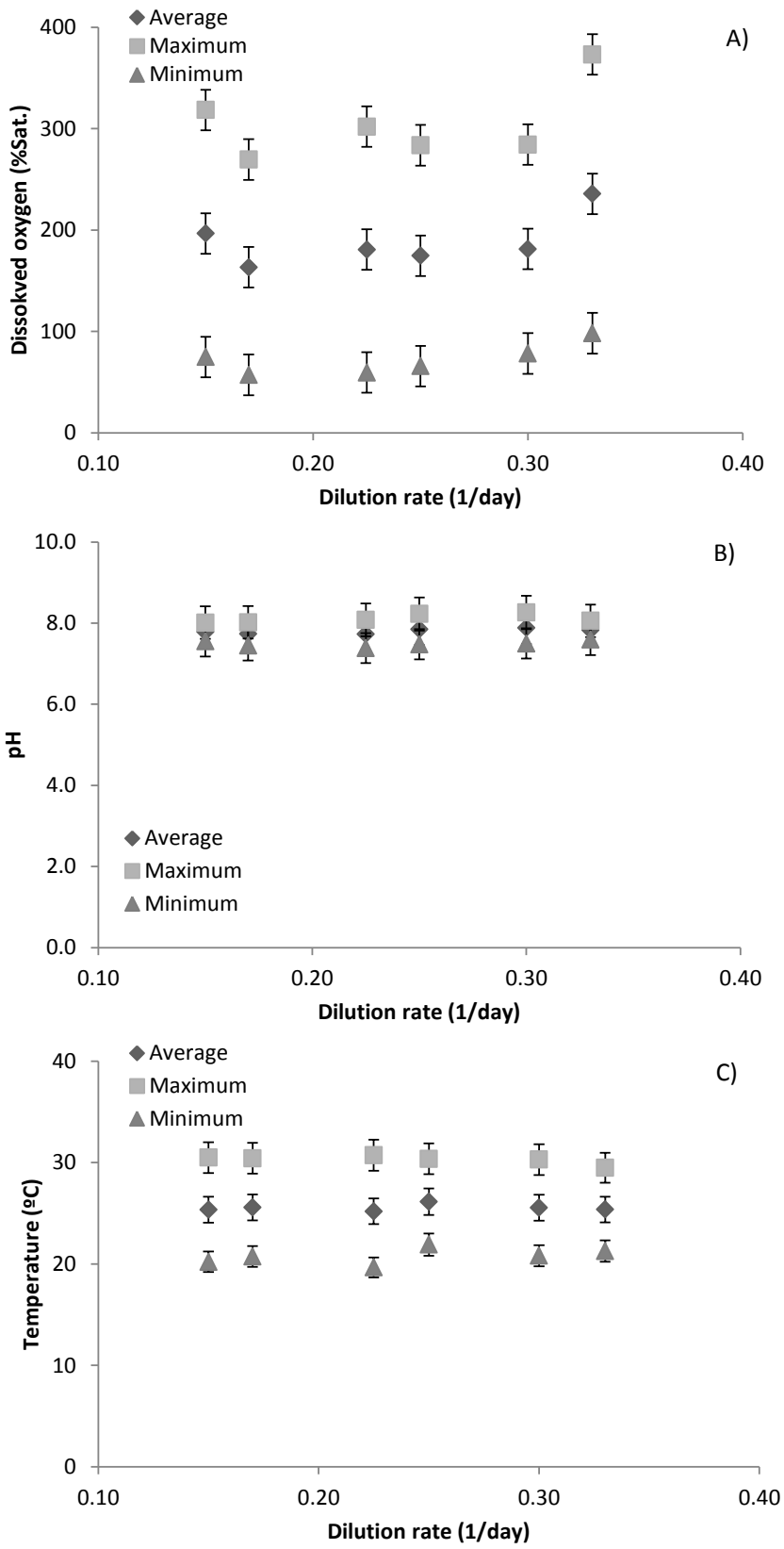

Fig. 19 - Variation of (A) dissolved oxygen, (B) pH, and (C) temperature into the culture, with the dilution rate, of *Isochrysis galbana* T-iso cultures performed in 3.0 m^3 industrial scale tubular photobioreactors.

Data show as the dissolved oxygen largely modifies along the day, ranging from minimum values of 70%Sat during the night to more than 300%Sat at noon, the average daily value being 170%Sat. However, no clear influence of imposed dilution rate was observed (Tab. 2). According to these results the reactors mass transfer capacity was not enough to remove all the oxygen produced at noon, thus photo-respiration phenomena can occur and diminish the productivity of the cultures. Regarding the pH, variations along the day resulted really low, lower than 0.5, with no differences being observed as a function of dilution rate (Tab. 2). Finally, regarding temperature, data shows as the temperature modifies along the day from 20ºC during the night to maximum of 31ºC at noon. No heating systems were used but the cultures increased its temperature with the solar radiation due to the absorption of heat by radiation. This heat absorption could increase the temperature over 40ºC, but the reactors had a cooling system to prevent overheating and death of the cultures, and the system worked adequately according to the results. These experimental values are close to those previously reported for cultures of *Isochrysis* in outdoor photobioreactors (van Bergeijk et al., 2010; Zhang et al., 2003).

The biochemical composition of the biomass was analysed. To avoid the ash content influence the data are expressed in ash free basis. Results show as the dilution rate modifies the biochemical composition of the biomass: the higher the imposed dilution rate the higher was the protein content (P-Value <0.05 : significant difference, Tab.2). It was also found a correlation with the decrease of the lipid content, although not statistically significant (P-Value = -0.07 : no significant difference, Tab. 2) (Figure 20).

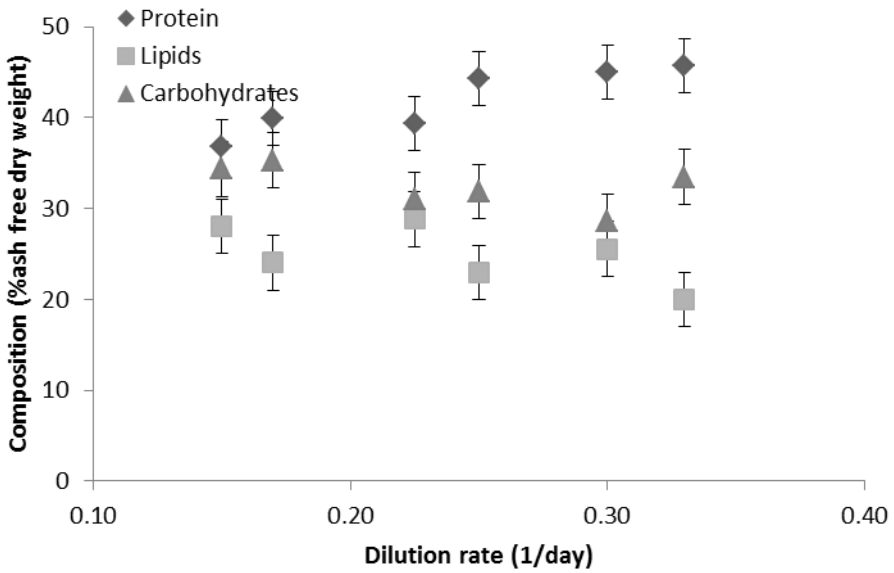

Fig. 20 - Variation of biochemical composition (protein, lipids and carbohydrates) of the biomass with the dilution rate, of *Isochrysis galbana* T-iso cultures performed in 3.0 m^3 industrial scale tubular photobioreactors.

Protein content increased from 35 to 45% when the dilution rate increased from 0.15 to 0.35 1/day, the lipids content reducing from 28 to 20%. Carbohydrate content did not show a clear tendency with the dilution rate variation, it represents between 28 to 30% of the biomass. The protein and lipid content variation with increasing the dilution rate is an expected behaviour due to the faster growth rate of the cells, thus requiring more proteins and storing less energy as lipids. However, it is very important to be capable to produce whatever strain at stable dilution rate, as this allows to maintain a stable biochemical composition of the biomass.

Tubular photobioreactors are recommended as the most suitable for the outdoor production of microalgae strains which can be easily contaminated, such as *Isochrysis galbana* (T-iso). On these reactors it is possible to provide a better control of culture conditions, thus being reliable to produce microalgae in continuous mode. However,

the tolerance of whatever strain to grown in this type of reactors must be tested under real conditions. Thus, although on these reactors different mechanisms can be used to control the pH, dissolved oxygen and temperature, the daily variation of these parameters along the solar period and including along the reactor due to the existence of gradients, can diminish the productivity or simply kill the cells.

The performed experiments demonstrated that *Isochrysis galbana* (T-iso) can be produced in industrial size tubular photobioreactors in continuous mode. The results indicate that the cultures mainly perform as light limited cultures, thus the biomass productivity increase by increasing the dilution rate. The chlorophyll fluorescence remained constant whatever the imposed dilution rate, therefore it was not observed photosynthetic apparatus stress. The biomass showed an adequate and stable composition, being rich in proteins and lipids, the most valuable components when used as feed in aquaculture.

The productivity measured during experiments, of 0.16-0.23 g/L·day, is in the range previously reported for this strain or even higher. Thus, at indoor conditions biomass productivities from 0.2 to 0.3 $g_{biomass} \cdot l^{-1} \cdot day^{-1}$ has been reported (in this study) whereas at outdoor conditions a value of 0.075 $g \cdot l^{-1} \cdot day^{-1}$ has been reported in tubular photobioreactors (van Bergeijk et al. 2010), and 0.13 $g \cdot l^{-1} \cdot day^{-1}$ in flat panels (Zhang,C.W. 2003). However, these productivities are much lower than those reported in tubular photobioreactors using different strains. This can be due to the lower growth rate of *Isochrysis galbana* T-iso versus other strains or to the higher influence of culture deviation conditions versus the

optimal one for this microalga. By using the proposed model and considering the culture parameters variation (dissolved oxygen, pH and temperature) inside the reactors along the day, the normalized photosynthesis rate can be calculated. Results demonstrate that the pH was adequately controlled in the reactor, the normalized photosynthesis rate being 0.97 at whatever dilution rate (Figure 21).

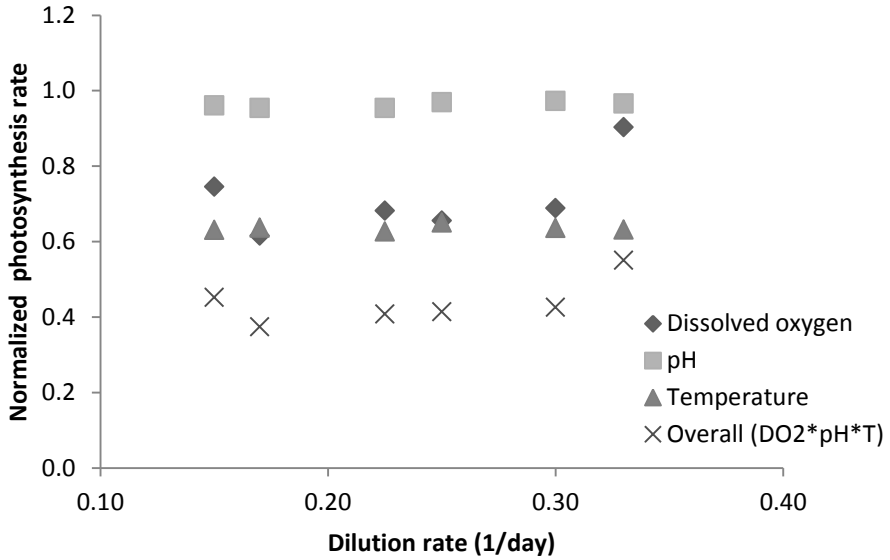

Fig. 21 - Influence of deviation of culture conditions versus the optimal reported for *Isochrysis galbana* T-iso into the normalized photosynthesis rate at different dilution rates tested. Data from experiments performed in 3.0 m^3 industrial scale tubular photobioreactors.

The optimal pH for this strain was reported to be 8.0, although the tolerance to deviations of this parameter are different according to the strain. For most microalgae the growth is optimal at pH values between 7.0 and 8.0; however for others as *Spirulina* alkaline pH is recommended, considering that the high pH has been reported to be useful for the control of contaminants in mixed cultures (Goldman 1982). However, dissolved oxygen has a larger variation during the day, reaching values higher than 150 %Sat. that has been reported as maximal tolerable for this strain without reduction of

photosynthesis capacity. On this way the normalized photosynthesis rate due to dissolved oxygen concentration was in the range of 0.7-0.8, thus indicating that inadequate dissolved oxygen concentration reduces the performance of the cultures between 30 and 20%. It has been reported that the net photosynthesis rate is maximum at dissolved oxygen concentration from zero to 11 $mg \cdot l^{-1}$, then exponentially decreasing to reach zero at 20 $mg \cdot l^{-1}$ due to inhibition by oxygen. It's worth noticing that this is a relevant parameter because oxygen is produced during photosynthesis and it can accumulate in high concentrations in closed photobioreactors, reducing the photosynthesis rate and favouring the photorespiration of the cultures (Mendoza et al., 2013). Therefore, in high dissolved oxygen concentrations photorespiration takes place; the oxygen binds to the Rubisco enzyme and modifies its role from carboxylase to oxygenase, reducing carbohydrate synthesis (Badger et al., 2000). Regarding temperature, a similar trend is observed due to low temperature during the morning, with the normalized photosynthesis rate due to temperature ranging from 0.65 to 0.63. The optimal temperature for *I. galbana* T-iso has been reported to be close to 36ºC (in this study), although other authors reported values ranging from 25ºC to 30ºC (Renaud et al., 2002; Claquin et al., 2008; Marchetti et al., 2012). Because the unfavourable dissolved oxygen concentration, pH or temperature has a multiplicative influence into the overall behaviour of the culture, the final normalized photosynthesis rate can be calculated by multiplying individual factors. Results shows as the overall normalized photosynthesis rate range from 0.37 to 0.55, thus

indicating that biomass production achieved is approximately half than the maximum achievable under optimal conditions.

To verify these phenomena the cultures light utilization efficiency has been calculated on the basis of solar energy received and energy stored into the biomass. Results show as the dilution rate has a positive effect into the light use efficiency of the cultures by the improvement of light availability however measured values are lower than previously reported for other strains (Figure 22).

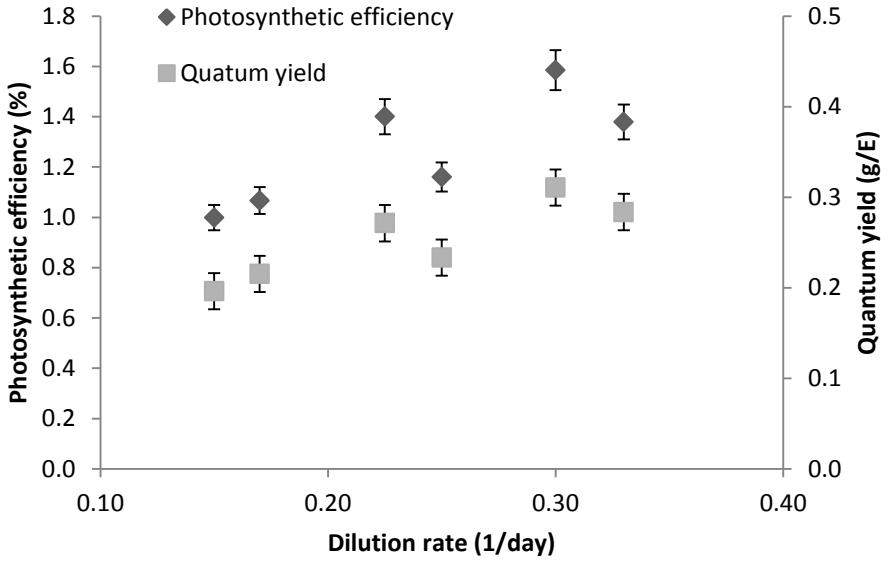

Fig. 22 - Variation of photosynthetic efficiency and quantum yield of *Isochrysis galbana* T-iso cultures with the dilution rate from experiments performed in 3.0 m^3 industrial scale tubular photobioreactors.

Thus the photosynthetic efficiency ranged from 1.0 to 1.6 %, whereas the quantum yield ranged from 0.2 to 0.3 g/E. Regarding the photosynthetic efficiency, microalgae can conserve a maximum of 10% of solar energy (photosynthetic efficiency) but outdoor microalgal production systems rarely exceed 6% as yet (Carvalho et al., 2006). The values reported here are lower than the maximal values reported for outdoor cultures, 6.94% to 7.05% previously

reported for *Chlorella* sp. cultures in thin-layer reactors (Doucha et al., 2006; Doucha et al., 2009), or 3.6% reported for *Scenedesmus* cultures in large tubular photobioreactors (Acién et al., 2012). Regarding the quantum yield, measured values are lower than the values of 0.5 g/E reported for *Muriellopsis* sp., 0.43 g/E reported for *P. subcapitata* and 1.3 g/E reported for *N. gaditana* (Morales-Amaral, et al., 2015; Sepulveda et al., 2015). For *Isochrysis galbana*, a maximal value of 0.62 g/E was reported indoors although this reduced to 0.1 g/E when calculated from outdoor cultures (Molina et al., 1997).

5. CONCLUSIONS

Irradiance, temperature, pH and dissolved oxygen are relevant variables determining the performance of *Isochrysis galbana* cultures. In collaboration with the researchers at the "Universidad de Almeria" a complete photosynthesis and respiration rate model for *Isochrysis galbana* based on these variables was developed. The model's validity was verified outdoors using industrial-scale tubular photobioreactors. The model allowed us to determine that inadequate temperature and dissolved oxygen in the outdoor tubular photobioreactors could reduce productivity by half that of the maximal level according to light availability. The developed model is a power tool for the design and management of *Isochrysis galbana*-based outdoor processes, to take decisions about the implementation of profitable control strategies. In addition, the method used to construct this model is applicable to other strains allowing us to optimize microalgae-based processes. Concerning the production, the data confirm that *Isochrysis galbana* has a growth rate lower than other strains. However, it is confirmed that the photosynthesis rate can be doubled by optimizing the control of the culture conditions in the reactors used.

Furthermore, this study showed that the productivity increases with increasing dilution rate, so future studies considering higher rate could be useful.

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