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Evaluation of photoperiod effect on the growth and protein content of microalgae

Inês Soraia Hipólito Pedro

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Dissertação para obtenção do Grau de Mestre em Biotecnologia dos Recursos Marinhos

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Título: Evaluation of photoperiod effect on the growth and protein content of microalgae

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Resumo:

Os recursos renováveis têm recebido um especial interesse nos últimos anos e as microalgas são uma excelente fonte renovável e natural. Estes organismos são fonte de proteínas e lípidos e são aplicadas em aquacultura e na produção de biodiesel.

Neste estudo, foi avaliado o efeito do fotoperíodo (Luz: Escuro) 12:12; 18:6; 24:0) e fase de crescimento (logarítmica e estacionária) no conteúdo de proteína em três sistemas modelo biológico: *Arthrospira maxima* (Cyanobacteria) foi selecionada como espécie de água doce a estudar e para explorar microalgas marinhas foram escolhidas *Isochrysis galbana* (Haptophyta) e *Tetraselmis chuii* (Chlorophyta) devido às suas aplicações em aquacultura marinha. Diferentes métodos de rutura celular foram também testados na extração de proteína em fase aquosa.

Arthrospira maxima exibiu melhor produção de biomassa e conteúdo de proteína no fotoperíodo de 18L:6D. O mesmo fotoperíodo também atingiu melhor produção de biomassa e conteúdo de proteína em *Isochrysis galbana* quando comparado com os outros fotoperíodos em estudo. *Tetraselmis chuii* exibiu melhor produção de biomass no fotoperíodo de 24L:0D, enquanto que o fotoperíodo 18L:6D atingiu melhor conteúdo de proteína.

Palavras-chave: *Arthrospira maxima*; *Isochrysis galbana*; *Tetraselmis chuii*; Fotoperíodo; Proteína; Métodos de rutura.

Abstract:

Renewal resources have received special interest in the last years and microalgae are an excellent natural source of it. This organisms contain protein and lipid, becoming a great resource of it and can be applied in aquaculture and biodiesel production.

In this study, the effect of photoperiod regime and growth phase (logarithmic and stationary) was evaluated (Light:Dark 12:12; 18:6; 24:0) on the protein content of three biological model systems: *Arthrospira maxima* (Cyanobacteria) selected as freshwater specie to study and *Isochrysis galbana* (Haptophyta) and *Tetraselmis chuii* (Chlorophyta) – chosen to explore marine microalgae due to their application on marine aquaculture. Different cell disruptor methods were also tested on protein extractability in water.

Arthrospira maxima exhibits higher biomass production and protein content at 18L:6D photoperiod regime. The same photoperiod also achieved better production and protein content in *Isochrysis galbana* when compared with the others photoperiods in study. *Tetraselmis chuii* exhibits better biomass production at 24L:0D photoperiod, while 18L:6D photoperiod achieved better protein content.

Key-words: *Arthrospira maxima*; *Isochrysis galbana*; *Tetraselmis chuii*; Photoperiod; Protein; Disruption methods.

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Abbreviations list

ARA	Arachidonic acid
BCAA	Branched chain amino acid
BHA	Butylated hydroxyanisole
BHT	Butylatedhydroxytoluene
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
EPS	Extracellular polysaccharide
f/2	Guillard's medium
GC	Gas chromatography
GLA	γ-Linolenic acid
IgG	Immunoglobulin G
O.D.	Optical density
OP	Open system
P	Volumetric biomass productivity
PBR	Photobioreactor
PG	Propyl gallate
PHAs	Polyhydroxyalkanoates
PHB	Poly-β-hydroxybutyrate
PHR	Pulse height resolution
PUFAs	Polyunsaturated fatty acids
r^2	coefficient of determination
rpm	Rotations per minute
SFC	Supercritical fluid chromatography
sPS	Sulphate containing exopolysaccharide
t	Time
TBHQ	tert-Butylhydroquinone
TCA	Trichloroacetic acid
t_d	Doubling time
UV/Vis	Ultraviolet-visible spectral region
Δt	Time variation
μ	Specific growth rate

Chapter 1: Introduction

1.1 Microalgae

Microalgae are a diverse group with over 50.000 different species. They can be unicellular, free-living or live in symbiotic association with other organisms, motile (with flagella (Figure 1-d)) or non-motile, (figure 1-b) colourful and usually photoautotrophic (Lordan *et al.*, 2011; Richmond, 2004). They can be eukaryotic (e.g. *Chlorophyta*) or prokaryotic (*Cyanobacteria*) and can be colonial with little or no cell differentiation (Figure 1- c). (Olaizola, 2003; Khattar *et al*., 2009; Koller *et al.*, 2014; Bahadar and Khan, 2013). They can be found in rivers, lakes, glacial ice flows, hot springs, sea water or salt lakes (Lordan *et al.*, 2011).

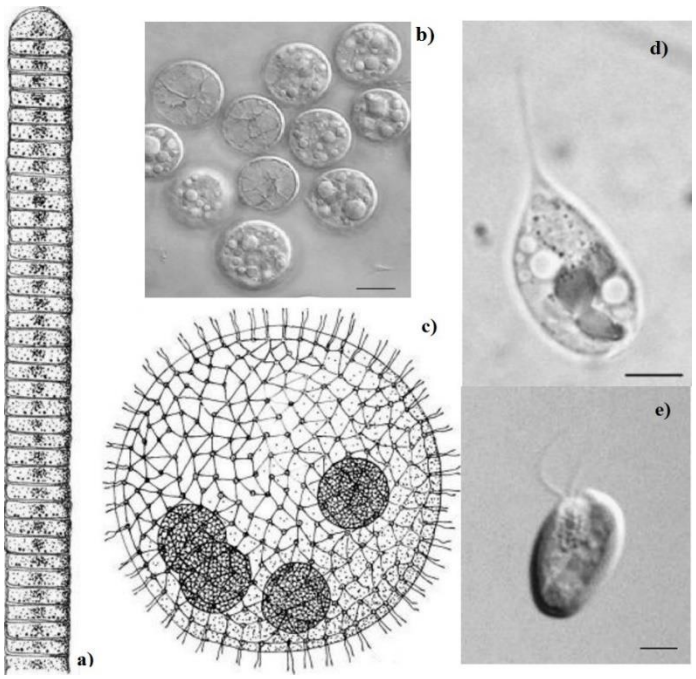

Figure 1 Examples of microalgae diversity: a) Simple filament of *Oscillatoria* sp.; b) Cells of *Prochloron* sp. (Bar: 10 µm.); c) Motile coenobium of *Volvox aureus*; d) *Ochromonas sp*., motile unicell. (Bar: 4 µm.); e) Unicell of *Cryptomonas sp*. (Bar: 6 µm.) (Barsanti and Gualtieri, 2006).

Their diversity allows them to colonize and be found in virtually any environmental condition (Table 1). The type of microalgae found in each place depends on the selective action of the chemo-physical environment and the organism's ability to colonize a particular environment (George *et al*., 2014). Water may be considered the main *habitat* of microalgae, nevertheless microalgae can also be found on the surface of all type of soils (Olaizola, 2003; Richmond, 2004). The cyanobacteria *Nostoc* sp. is an example of a terrestrial organism and is frequently found in association with lichens (Merinero *et al*., 2015).

Table 1 Distribution of microalgae divisions. n.d., not detected. (Adapted from Barsanti and Gualtieri, 2014)

Phylum	*Marine*	*Freshwater*	*Terrestrial*
Cyanobacteria	Yes	Yes	Yes
Glaucophyta	n.d.	Yes	Yes
Rhodophyta	Yes	Yes	Yes
Ochrophyta	Yes	Yes	Yes
Haptophyta	Yes	Yes	Yes
Cryptophyta	Yes	Yes	n.d.
Cercozoa	Yes	n.d.	n.d.
Euglenozoa	Yes	Yes	Yes
Chlorophyta	Yes	Yes	Yes
Myzozoa	Yes	Yes	n.d.
Euglenozoa	Yes	Yes	Yes

1.2 Growth conditions and production systems

Growth conditions of microalgae culture are extremely important to obtain high yield and to control synthesis of different metabolites. Microalgae are organisms with metabolic plasticity that allows the synthesizing of compounds with high biotechnological potential manipulating physical and chemical conditions (e.g., variations in levels of protein, carbohydrate, lipids, among others) (Guedes *et al.,* 2011; Coêlho *et al.,* 2013). The most important parameters of microalgae growth are temperature, light, pH, salinity, aeration and nutrient quality/ quantity (Barsanti and Gualtieri, 2014).

1.2.1 Culture Parameters

Most microalgae cultures support temperatures of 16-27^{o}C. Ideally the temperature should be as close as possible to the temperature that microalgae were collected. Temperatures higher than 35^{o}C could be lethal for a several species, whereas those less than 16^{o}C will slow down growth (Barsanti and Gualtieri, 2014).

Different microalgae have various pH needs, but generally the amplitude for microalgae species is between 7 and 9. Culture medium pH tends to increase when cell density and age of the culture increase too, due to the consumption of the CO_2 (Barsanti and Gualtieri, 2014; Sayegh and Montagnes, 2011).

Marine microalgae usually are tolerant to changes in salinity. Nevertheless the salinity amplitude of the majority marine microalgae should be at 20-24 according to Barsanti and Gualtieri, 2014 and according to Muller-Feuga should be at 25-27 (Støttrup and McEvoy, 2003)

Aeration is essential in microalgae cultures since it uniforms the exposition to the light and nutrients and also avoids sedimentation and thermal stratification. Aeration should be gentle to prevent damaging cells, but strong enough to produce the above effects (Gouveia, 2011; Barsanti and Gualtieri, 2014).

Nutrient are important to obtain high yield or to control synthesis of different metabolites. The culture medium should be chosen according the natural environment and species requirement (Barsanti and Gualtieri, 2014).

As photosynthetic organisms, microalgae photoautotrophic culture depends on light intensity, spectral quality and photoperiod to produce cell biomass and secondary metabolites (Anderson, 2005). Light is the main factor affecting photosynthesis kinetics and its quantity and quality determine the amount of energy available to microalgae to conduct their metabolic activities, as microalgae absorb light from the different wavelengths at unequal rates (Richmond, 2004).

Light intensity needed depends of the density and depth of culture and at higher depths and cell concentration, it must be increased. This phenomena is even more obvious in green microalgae cultures, which have higher chlorophyll content, due to the fact that this

pigment has a high extinction coefficient – leading to high light absorption. Then, in dense cultures, if light is too low its absorbed by the first cell layers, leaving the inner ones virtually in the dark (Anderson, 2005). To prevent this effects, light intensities frequently variety between 100 and 200 μE s^{-1} m^{-2} and light is usually provided by fluorescent lamps or by natural source (Laing, 1991).

Microalgae's growth and photosynthetic ability can be conditioned by the amount of light provided. Cells have a light compensation point that must be balanced with their needs – when in light deficit, microalgae growth can slow down, or even cease, due to respiratory loss, however, when in light excess, growth can be so rapidly increased that the culture becomes light-saturated and a photoinhibition effect occurs. For example, under supra-optimal irradiance, pigments are reduced but under light-limiting conditions, microalgae increase the number of photosynthetic units (Richmond, 2004). To even light effects on cultures, usually microalgae are grown under different photoperiod. In this conditions, illuminations is provided in light:dark cycles, simulating what occurs in the natural environment (Laing, 1991). The changes of size of light-harvesting complexes occur at a timescale of days (Barsanti and Gualtieri, 2014; Khoeyi *et al*., 2012), and can actively modify the biochemical composition of the cells, since light is a source of stress (Richmond, 2004).

Reactor configuration is also an important parameter to be control. To produce microalgae at industrial scale, the photobioreactors type must be selected, depending on the type of algae in culture and on the biomass target, from two main sorts: outdoor ponds (e.g. in raceways) and closed systems (e.g. tubular reactors) (Borowitzka, 1999).

1.2.2 Outdoor Ponds

An open system (OP) is composed of a race track shaped shallow canal that is typically circulated by a paddle wheel and thus OP systems are usually referred to as raceway ponds. OP system requires minimal maintenance to operate, and has low investment costs, however has many disadvantages in relation to performance (e.g. when compared with photobioreactors, OP systems have lower productivities). As OP systems are open to the

environment, they permit significant contamination and experience a typical evaporative loss of approximately 1 litre of water per gram of biomass produced (Johnson *et al*., 2015).

1.2.3 Closed Systems

A photobioreactor is a closed system used for culturing photosynthetic cells, which uses natural light source or artificial light. It permits control temperature, pH, carbon dioxide, oxygen and minimizes contaminations (Barsanti and Gualtieri, 2014).

There are different categories of photobioreactors, with diverse geometries, dimensions, materials and type of operation (Figure 2) (Barsanti and Gualtieri, 2014).

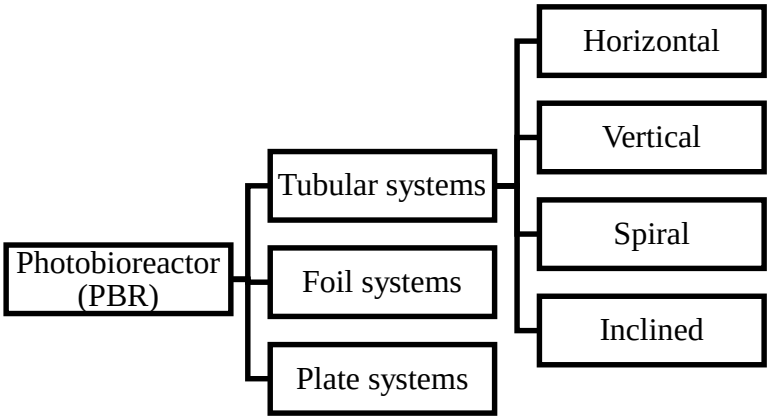

Figure 2 Different types of photobioreactors (Barsanti and Gualtieri, 2014).

The tubular photobioreactors are the most used systems that are constituted by tubes arranged in multiple possible orientations. Thus, there are horizontal, vertical, spiral or inclined tubular photobioreactors. This type of reactor is built with rigid transparent material, typically acrylic. The biggest problem of the tubular photobioreactors is the luminous efficiency, which is prejudiced by the curvature of the tubes´s surface. It makes the sunlight being reflected, not being utilized for growth of the biomass. However, the horizontal tubular photobioreactors allow a better use of light (comparing with the vertical ones), but need a large area installed. Spiral tubular photobioreactors are more efficient in terms of sunlight

use without requiring much installation area. Inclined tubular photobioreactors gather some of the advantages of the horizontal and vertical reactors, such as luminous efficiency, gas exchange and a good ratio volume/area installed (Fernandez *et al*., 1999; Ugwu *et al*., 2008).

1.3 Harvesting of microalgae biomass

Harvesting of biomass is a necessary process and contributes to 20-30% of the total of the biomass cost production. There are various recovery methods and does not exist a suitable method to every case. Biomass can be recovery by filtration, centrifugation and gravity sedimentation.

Filtration under pressure or vacuum are satisfactory for harvesting microalgae with a large size such as *Arthrospira maxima* and *Coelastrum proboscideum* but don't result to recover microalgae with a small size, for instance *Chlorella* sp., *Scenedesmus* sp. and *Dunaliella* sp.. Membrane microfiltration and ultrafiltration are another way to conventional filtration for fragile cells (Grima *et al*., 2003).

Centrifugation can harvest the majority of microalgae and is the preferred method of recovery microalgae cells. This method can be rapid, but expensive. Conditions for centrifugation are dependent on the residence time of the cell slurry in the centrifuge, on the settling characteristics of the cells and the settling depth (Grima *et al*., 2003).

1.4 Cell disruption methods

Cell disruption is very a important procedure for isolation and purification of intracellular products from microalgae (Grima *et al*., 2003). The choice of the most appropriate method determines, not only the yield but the downstream processes success. Thus, it is desirable a complete cell disintegration and simultaneously obtaining high yields and integrity of the bioproduct (Lima and Mota, 2003). Some microalgal cell disruption methods are in table 2 and their advantages and disadvantages.

Table 2 Microalgae cell disruption methods: advantages and disadvantages.

Methods	*Advantages*	*Disadvantages*	*References*
Chemical treatment	Allow a selective delivery of a product depending on its location	Generally not appropriate for sensitive products (e.g. proteins); Caution must be taken for Human consumption.	(Grima *et al*., 2003) (Lima and Mota, 2003)
Enzymes	Low impact on the environment	High cost of the enzymes; Caution must be taken for Human consumption	(Olaizola, 2003) (Lima and Mota, 2003) (Sari *et al*., 2013)
Bead mill	Applicable to large-scale use; Continuous operating;	High energy consumption. Coupled cooling system;	(Lima and Mota, 2003)
High-pressure cell disruptor	Applicable to large-scale use.	Not appropriate for enzymes; Coupled cooling system.	(Lima and Mota, 2003)
Ultrasonication/ Ultrasound bath	Produces cavitation in cells; and facilitates cell disruption.	High cost; Increases the heat.	(Grima *et al*., 2003) (Safi *et al*., 2014)
Supercritical fluid	Fast extraction yields; Use of solvents generally recognized as safe; Low extraction times; Possibility of direct coupling with analytical chromatographic techniques (e.g.GC and SFC)	High cost.	(Herrero *et al*., 2006)

1.5 Microalgae metabolites with high biotechnological potential

The idea to explore microalgae at industrial level appeared for the first time in Germany during World War II, as a cheap source of proteins. In the 50s and 60s were made efforts on optimizing algal cultures in US and Japan but unsuccessfully. Only in the 70s occurred major improvements with the cultures use for pigments production, food supplements and vitamins for the pharmaceutical industry (Soeder, 1986).

However, microalgae still remain an uncharted resource. Of the tens of thousands of microalgae species believed to exist, merely a few thousand strains are preserved in collections, and only a few hundred have been studied for metabolites. This represents a unique opportunity to discover new compounds and produce known metabolites at lower costs (Olaizola, 2003; Guedes *et al.*, 2011).

Pigments, lipids, proteins and polysaccharides are some of microalgae metabolites, with high biotechnological interest due to their wide application range (Table 3) (Spolaore *et al.*, 2006). They can act as antioxidant, antibacterial, antiviral, anti-inflammatory, anticarcinogenic compounds, etc (Choochote *et al.*, 2014).

Antioxidants are known for scavenging active oxygen products (e.g. Singlet oxygen, superoxide radicals, hydroxyl radicals, hydroxylperoxide) and for increasing cells' natural defences. They are also used to prolong the storage stability of food. Some synthetic antioxidants (Butylatedhydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary tert-Butylhydroquinone (TBHQ), propyl gallate (PG)) are authorized for use in food. However, this compounds can be accumulated in body organs and tissues. For that reason, use of natural antioxidants are essential (Yingying *et al.*, 2014).

Table 3 Products synthesized by microalgae and areas of application. * Microalgae species with high relevance for biotechnological applications.

Class of compounds	*Type of compounds*	*Areas of application*	*Species**	*References*
Pigments	Chlorophylls; Carotenoids; Phycobilins.	Cosmetics; Human nutrition; Animal feeding; Pharmaceutical application.	*Dunaliella salina* *Haematococcus pluvialis* *Arthrospira platensis* *Dunaliella tertiolecta* *Botriococcus braunii* *Nannochloropsis* sp. *Eustigmatos cf. polyphem* *Scenedesmus almeriensis* *Chlorella zofingensis* *Chlorella ellipsoidea*	(Koller *et al.*, 2014) (Pulz and Gross 2004) (Herrero *et al.*, 2006) (Mendiola *et al.*, 2007*)* (Li *et al.*, 2012*)* (Granado-Lorencio *et al.*, 2009*)* (Del Campo *et al.*, 2004*)* (Jaime *et al.*, 2010*)* (Plaza *et al.*, 2009)
Lipids	Polyunsaturated fatty acids; Glycerol; Hydrocarbons	Human nutrition; Pharmaceutical application; Animal feeding; Energy creation (Biodiesel; Biogasoline)	*Isochrysis galbana* *Phaedactylum tricornutum* *Odontella aurita* *Nannochloropsis gaditana* *Arthrospira* sp.	(Koller *et al.*, 2014) (Pulz and Gross 2004) (Pedro et al., 2013).
Proteins	Enzymes; Hormones; Amino acid; Peptides.	Human nutrition; Animal feeding.	*Lyngbya majuscula* *Chlamydomonas reinhardtii* *Phaeodactylum tricornutum*	(Koller *et al.*, 2014) (Pulz and Gross, 2004)

Polysaccharides	*β-1,3-glucan;* *Carrageenan;* *Starch; Agar;* *Alginates;* *Cellulose;* *EPS;* *sPS;*	*Human nutrition; Pharmaceutical application;* *Energy creation (Bioethanol);* *Industrial sector*	*Porphyridium cruentum* *Rhodella reticulate* *Arthrospira platensis* *Chlorella stigmatophora* *Chlorella ellipsoidea* *Rhodella maculate* *Rhodella reticulata* *Dunaliella salina* *Schizochytrium sp.* *Isochrysis galbana*	*(Koller et al., 2014)* *(Markou and Nerantzis, 2013)* *(Pulz and Gross 2004)* *(Herrero et al., 2005)* *(Shi, 2007)* *(Murthy et al., 2005)* *(Chen et al., 2010)* *(Wang et al., 2011)* *(Yingying et al., 2014)* *(Ko et al., 2012);*
Bioplastics	Polyhydroxyalkanoates (PHA); poly-β-hydroxybutyrate (PHB)	"Green Plastics"	*Arthrospira maxima* *Nostoc muscorumn* *Synechocystis* sp.	(Koller *et al.*, 2014) (Markou and Nerantzis, 2013)
Other compounds	Silver; Gold; Biometallic; Silicon–germanium; Silica	Human nutrition; Pharmaceutical application; Industrial sector;	*Navicula atomus* *Diadesmis gallica* *Arthrospira platensis*	(Markou and Nerantzis, 2013) (Asmathunisha and Kathiresan, 2013)

1.5.1 Pigments

The main groups of pigments found in microalgae are chlorophylls (e.g. *a*, *b* and *c*), carotenoids and phycobilins (Koller *et al.*, 2014).

Chlorophylls (green coloration) have applications in food industry, for example as food additive (E140 dye). Chlorophyllin, a chlorophyll derivative, has a high effectiveness as a chemo-preventive agent on colon cancer cells when supplied as dietary supplement (Díaz *et al*., 2003). Chlorophylls also have deodorant capacity, therefore is used as an ingredient of pastilles (against bad breath) and body deodorants (Montgomery and Nachtigall, 1950).

Carotenoids (orange and yellowish colour) are strong antioxidants and therefore have important applications on human metabolism avoiding the negative consequences of free radicals, which are commonly associated with the induction of certain cancers. This way, carotenoids are used for "functional food" products (Koller *et al.*, 2014). "Functional food" is a natural or processed food that was demonstrated to has biologically-active compounds with good benefit in the body when consumed in a regular diet. A functional food mustn't be in the form of capsules or pills (Plaza *et al.*, 2009).

β-carotene (Figure 3) is used as vitamin supplement (provitamin A) and also furnish an orange colour to egg yolk as wish by the customer (food colouring agent). *Dunaliella salina* (Chlorophyceae) is cultivated for β-carotene biosynthesis because reaches levels of up to 100g.kg^{-1} dry weight (Hu *et al*., 2008; Koller *et al.*, 2014; Ben-Amotz, 1993).

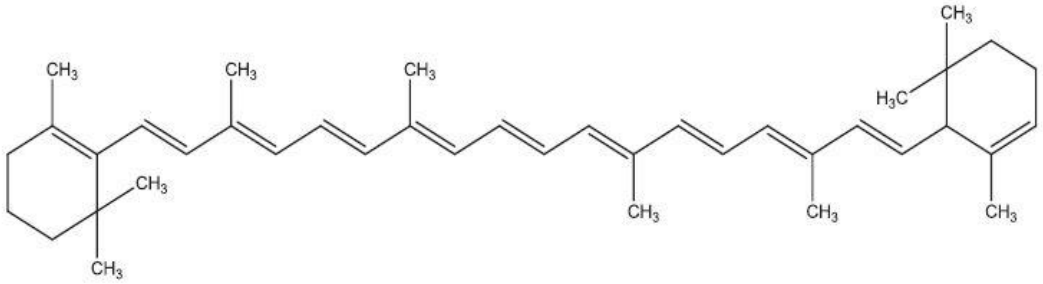

Figure 3 Chemical structure of β-carotene (Dewapriya and Kim, 2014).

Astaxanthin (Figure 4) is the most powerful known natural antioxidant with several applications. Cosmetic industry utilizes astaxanthin on sunscreen creams mainly because of its UV-protecting action but also because its waterproofness. In aquaculture sector, is common the addition of astaxanthin (E161j) on salmon and trout feed to give them a reddish colour with higher consumer acceptance, and has also an important function on the immune-system and a benefit impact in their fertility (Cardozo *et al*., 2007). *Haematococcus pluvialis* (Chlorophyceae) is used for the large production of astaxanthin (Durmaz, 2007).

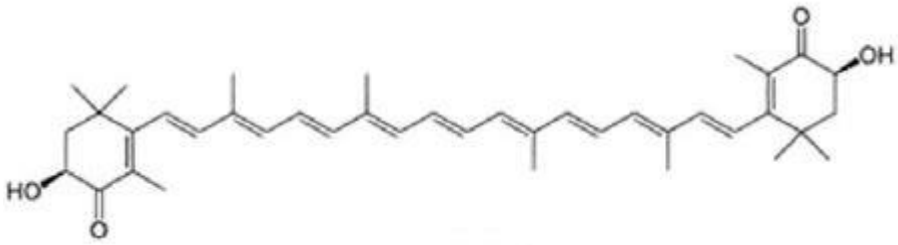

Figure 4 Chemical structure of Asthaxanthin (Koller *et al.*, 2014).

Fucoxanthin (Figure 5), which is available in diatoms and brown algae, has anti-inflammatory, antidiabetic, antioxidant and anti-obesity properties and can inhibit cell growth and induce apoptosis in human cancer cells (Maeda *et al*., 2005).

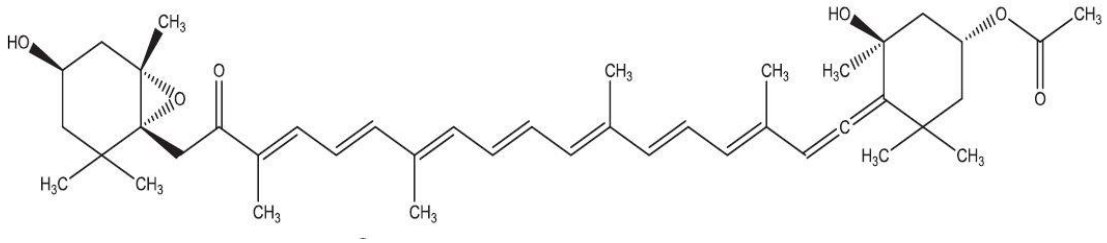

Figure 5 Chemical structure of fucoxanthin (Dewapriya and Kim, 2014).

However, there are some pigments that are still not approved for use as food additive in some countries. Violaxanthin is a food colorant (E161e) that has an orange colour and it is not allowed to use as food additive in USA and EU. Though, in Australia and New Zealand, it is already permitted. *Dunaliella tertiolecta*, *Botryococcus braunii* and *Chlorella ellipsoidea* are potential producers of this pigment. On the other hand, canthaxanthin is not allowed to use as food additive (E161g) in Australia, New Zealand and some countries of

EU, however USA approves this pigment as food additive. *Nannochloropsis salina*, *Nannochloropsis oculata* and *Nannochloropsis gaditana* can produce canthaxanthin (Plaza *et al*., 2009; Koller *et al.*, 2014).

Phycobilins (eg. phycocyanin and phycoerythrin) are mostly found in stroma of chloroplasts. Phycocyanin is a blue pigment mainly found in cyanobacteria, namely *Arthrospira* is the major source for this pigment with up to 20% of its dry weight. Whereas phycoerythrin is a red pigment found in red algae. Phycobilins are linked to determined water-soluble proteins, the phycobiliproteins (Parmar *et al.*, 2011). Phycobiliproteins are the algal-derived products the highest market values and have been utilized commercially as natural colorant. They are used as chemical tags, by linking phycobiliproteins to antibodies, in immunofluorescence techniques, due to its fluoresce at a particular wavelength. *Aphanizomenon flos-aque* can be cultivated to produce phycobiliproteins. (Arad and Yaron, 1992; Yaakob *et al*., 2011).

1.5.2 Lipids

Polyunsaturated fatty acids (PUFAs) from microalgae have shown advantages above fish oils, since they have no unpleasant odour, have lower risk of chemical contamination and better purifying potential. Thus, there is a large potential biotechnological market for microalgae PUFAs. For example, purified PUFAs are added to infant milk in EU with proved benefit. Other example is the production of "ω" eggs, hens are feed with microalgae (Pulz and Gross, 2004). It also, there is an increasing request for so called "vegan health food" wealthy in PUFAs for with microalgal biomass could play here an important role as well (Koller *et al.*, 2014).

Eicosapentaenoic acid (EPA) (Figure 6), a ω-3 fatty acid, was established by the European Food Safety Authority (EFSA) as an important nutrient supplement. It is responsible for a proper order of the blood pressure, blood clotting, immune system (protective effects on the development of several cancers). Furthermore, EPA is applied in aquaculture as dietary constituents (e.g. in marine fish). *Nannochloropsis sp.* and *Phaeodactylum tricornutum* are possible microalgal EPA large scale-producers (Karmali, 1996; Støttrup and McEvoy, 2003).

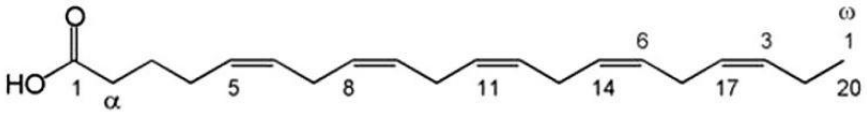

Figure 6 Chemical structure of Eicosapentaenoic acid (EPA) [(5Z,8Z,11Z,14Z,17Z)-5,8,11,14,17-icosapentaenoic acid] (Koller *et al.*, 2014).

Docosahexaenoic acid (DHA) (Figure 7) is used as dietary supplement in food and drinks due to its positive effects on developing human fetus, healthy breast milk, infantile brain and eye development. It also has anti-inflammatory healthy effects, cardiovascular and nervous system (Fradique *et al*., 2013). DHA's health effects are stated by EFSA. In addition, DHA is used in fish farming. *Crypthecodinium cohnii*, *Schizochytrium*, or *Pavlova lutheri* are cultivated to produce DHA (Yaakob *et al.*, 2011).

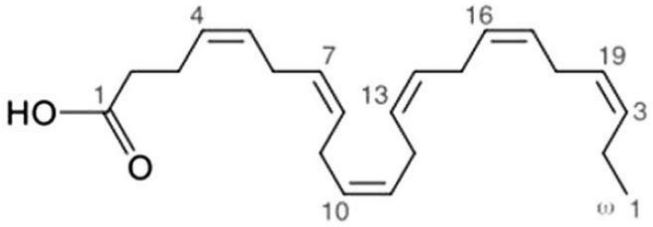

Figure 7 Chemical structure of Docosahexaenoic acid (DHA) [(4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,6,19-hexaenoic acid] (Koller *et al.*, 2014).

Arachidonic acid (ARA) (Figure 8), a four-fold unsaturated ω−6 fatty acid, is used for nutrient supplements once it's an important component of membrane phospholipids, has anti-inflammatory effects, behaves as a vasodilator and is essential for the repair and growth of skeletal muscle tissue. ARA is also applied to aquaculture (Koller *et al.*, 2014).

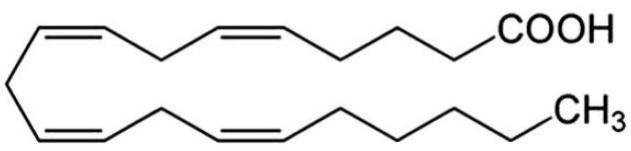

Figure 8 Chemical structure of Arachidonic acid (ARA) [(5Z,8Z,11Z,14Z)-5,8,11,14-Eicosatetraenoic acid] (Koller *et al*., 2014).

γ-Linolenic acid (GLA) (Figure 9), an ω−6 unsaturated fatty acid, is used as food additive, since it has therapeutic applications due to its anti-inflammatory effects. It helps people suffering from diabetes, rheumatoid arthritis, multiple sclerosis, breast cancer, skin allergies, obesity, heart disease, high blood pressure, attention deficit hyperactivity disorder (ADHD), neurological problems related to diabetes and premenstrual syndrome. GLA is mostly present in cyanobacterial representatives (Fan and Chapkin, 1998).

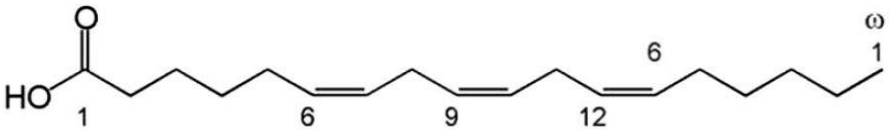

Figure 9 Chemical structure of γ-Linolenic acid (GLA) [all-cis-6,9,12-octadecatrienoic acid] (Koller *et al*., 2014).

Nowadays, biodiesel production from microalgae is the most explored technology in development for lipids usage. In the past, two oil crises occurred (1973 and 1979) and leading the US Department of Energy began in 1978 a research program of biofuel production from microalgae in open fields. However, the program was abandoned in 1996 due to lack of finance, without achieving the purpose for which it was created (Sheehan et al., 1998). Nevertheless interest in microalgal biomass kept growing, since it is considered as one of the most promising feedstock for the large-scale production of biofuels, mainly due to its high lipid and carbohydrate content, fast generation of biomass and high photosynthetic efficiency. In order for biodiesel from microalgae production to be profitable it is necessary to generate revenues from other co-products of microalgae. This could be possible in a biorefinery setting. In a biorefinery, lipids are fractionated for biodiesel and the residual biomass from biodiesel production can be used as livestock feeds, or be fermented to produce bioethanol. However, cell disruption and lipid extraction process needs to be explored to guarantee that the functionalities of different cell components are kept (Pereira *et al.,* 2005; Halim *et al*., 2012). High levels of lipids have been reported mainly in green algae (George *et al*., 2014). *Nannochloropsis gaditana* represents an attractive alternative as a renewable biofuel feedstock (Pedro *et al.,* 2013).

1.5.3 Polysaccharides

Polysaccharides are macromolecules formed by the union of several monosaccharides. They play an important role on structural function and store energy, which why is essential to living beings (Raposo *et al.*, 2013).

Sulphate polysaccharides released by marine microalgae have demonstrated the ability to avoid the accumulation, diminish the activity of free radicals and reactive chemical species, thus, acting as protecting systems against oxidative and radical stress agents. Sulphate containing exopolysaccharide (sPS) from *Porphyridium cruentum* and *Rhodella reticulata* was demonstrated that has antioxidants and are dose-dependent, correlating positively with sulphate content of the sPS (Tannin-Spitz *et al*., 2005; Sun *et al*., 2009).

The antiviral bioactivity of sPS on different host cell-lines had been already highlighted. *Arthrospira platensis* produces an intracellular polysaccharide, named calcium spirulan that it is an inhibitor of enveloped virus replication (Hayashi *et al*.. 1996). Without any toxicity to the host cells, sPs can inhibit the attachment/absorption or replication while the early phases of the virus cycle (Martinez *et al.*, 2005; Hasui *et al*., 1995; Kim *et al*., 2012).

Anti-inflammatory activity and immunomodulatory agents from polysaccharides have been studied. From *Chlorella stigmatophora*, extract of sPS showed immunosuppressant effects. Positive phagocytic activity *in vitro* or *in vivo* of *Phaeodactylum tricornutum* is an evidence of direct stimulatory effect on the immune cells. Another example comes from *Gyrodinium impudicum*, a marine dinoflagellate, that produces sPS p-KG03 and also stimulates the production of nitric oxide and immunostimulates the production of cytokines in macrophages. The homopolysaccharide of this dinoflagellate has immunomodulatory properties, suppressed tumour cell growth, stimulating the innate immune system *in vivo* and *in vitro* (Guzman *et al.*, 2003;Bae *et al.*,2006).

As a tumour cell growth prevention, calcium spirulan of *A. platensis* prevents also pulmonary metastasis, adhesion and proliferation of tumour cells. From *Porphyridium* sp., was demonstrated that high molecular weight oversulphated EPS (extracellular polysaccharide) inhibited neoplastic mammalian cell growth. In addition, EPS could be a good candidate as an antitumoral agent, due to its immunostimulating properties (Geresh *et al.*, 2002).

1.5.4 Bioplastics

Bioplastic, as its names implies, is a biodegradable plastic produced from biopolymers. Polyhydroxyalkanoates (PHAs) are linear polyesters produced typically in prokaryotic organisms. Thus, several cyanobacteria synthesize PHAs. These organisms store the carbon and energy in response to adverse growth conditions. (Markou and Nerantzis 2013).

Poly-β-hydroxybutyrate (PHB) is the simplest member of PHA, a natural thermoplastic polyester with similar properties to petroleum-based plastics but with the great advantage of complete biodegradability. *Arthrospira maxima* accumulates PHB under some adverse growth conditions. Under nitrogen starvation, *A. maxima* amasses 0,7% of dry weight and, under phosphorus starvation, up to 1,2% of dry weight. Furthermore, phosphorus and nitrogen starvation makes to a significant accumulation of carbohydrates that amounts up to 23% and up to 60-70% for phosphorus and nitrogen, respectively (De Philippis *et al*., 1992). Although, mixotrophy (microalgae can obtain energy from both organic carbon and light (Richmond, 2004)) increases accumulation of PHB in several species. In mixotrophic cultures of *Synechocystis* sp. PCC 6803, PHB accumulation amounts are up to 38% of dry weight when combined phosphorus starvation and gas-exchanging limitation. The supplement of organic carbon, such as acetate, fructose or glucose allows higher intracellular PHA accumulation (Panda and Mallick, 2007). For example, *Nostoc muscorumn* when grows photoautotrophically, produces PHB up to 8% of dry weight but when grows mixotrophically with 0,4% (w/v) glucose and acetate, amounted up to 35% (Markou and Nerantzis 2013).

1.5.5 Proteins

Microalgae are considered as a potential proteins source with high quality and can be used as direct food supplements or as nutraceuticals (“A nutraceutical is a food or a part of a food for oral administration with demonstrated safety and health benefits beyond the basic nutritional functions to supplement diet, presented in a non-food matrix or non-conventional food formats, in such a quantity that exceeds those that could be obtained from normal foods

and with such frequency as required to realize such properties, and is labeled as a 'nutraceutical'." (Palthur *et al.,* 2010). Microalgal proteins includes essential amino acids, particularly lysine and branched chain amino acid (BCAA) leucine, valine and isoleucine that count for 35% of the essential amino acids in human muscle proteins (Dewapriya and Kim, 2014).

Microalgae can produce high yields of recombinant proteins faster and with lower cost than cell culture (Specht *et al*., 2010). Additionally, microalgae have the capacity of post-transcriptional and post-translational modifications of biosynthesized proteins. This capacity and its intrinsic easiness of cultivation, confers a potential candidate of protein bioreactor (Himaya *et al*., 2013). As example, recombinant proteins produced in *Chlamydomonas reinhardtii* chloroplasts, gives advantages over other protein biosynthesizing systems due to its ability to fold proteins correctly and the capacity of assemble more complex proteins easily. Further, microalgae can be produced in full containment reducing the problems about environmental contamination, moreover, microalgae do not have the risk of gene flow because algae do not reproduce by a powder that spreads, like pollen (Mayfield *et al*., 2007).

Another example is the diatom *Phaeodactylum tricornutum* that was used to express a full-length IgG antibody against the Hepatitis B surface protein and the respective antigen with average expression levels of 9% of total soluble protein (Hempel *et al.,* 2011).

Bioactive peptides, peptides with therapeutic potentials, are 2-20 amino acids long protein fragments and have potential to be used as nutraceuticals and functional food ingredients for therapeutic activities, such as antioxidant, antimicrobial, antihypertensive, immunomodulatory, anticancer and cholesterol-lowering effects. Studies demonstrated that cultivation of *Pavlova lutheri* could provide small peptide with high anti-oxidative activity. Further, *Chlamydomonas* sp. produce an active peptide witch potently suppresses *Helicobacter pylori* induced carcinogenesis (Ryu *et al*., 2012; Dewapriya and Kim, 2014).

1.5.6 The market of microalgae metabolites

Microalgae are possibly one of the most useful organisms, however microalgal biotechnology is the younger branch of algal biotechnology. The biomass of microalgae is the predominant product in microalgal biotechnology and its market is about 5000 t per year

of dry matter and has a turnover of ca. U.S $1.25x10^9 per year (2004). Biomass is collected from cultures in artificial ponds or photobioreactor (PBR) or from natural waters. The final product of biomass production is usually a powder and it is sold manly in human health food market. An example is the production of *Haematococcus pluvialis*. This microalgae is cultivated for astaxanthin production and there are culture techniques well developed. In Japan and Israel there are two different types of industrial-scale closed PBR for *H. pluvialis*. In Japan, a special spherical thin layer PBR and in Israel, a glass tube PHR. In China and Hawaii *H. pluvialis* is cultivated in open ponds (Pulz and Gross, 2004). Another examples of innovative processes and products that are produced are described on table 4.

Table 4 Microalgae as healthy ingredients and companies that produces them. Adapted from Pulz and Gross, 2004.

Country	*Company*	*Alga*	*Product*	*Activity*
USA	Martek/Omegatec	*Crypthecodinium*	DHA	Brain development
USA	Cyanotec	*Haematococcus*	Astaxanthin	Treating carpal tunnel syndrme
USA	MERA	*Haematococcus*	Astaxanthin	Anti-inflammatory, treats mucle soreness
Canada	OceanNutrition	*Chlorella*	Carbohydrate extract	Immune system, anti-flu
France	InnovalG	*Odontella*	EPA	Anti-inflammatory
Austria	Panmol/Madaus	*Spirulina*	Vitamin B_{12}	Helps immune system

One of the most important products that can be produce from microalgae are pigments. Carotenoids market is estimated at about $1200 million per annum in 2010 with market value of β-carotene estimated at $261 million per annum in the same period. In 2010, the global use of bioplastics was 0,64 Kt and in 2011 increased to 0,85 Kt, while glycerol use was 1995,5 Kt. In the same period, vitamin and supplement, derived from microalgae, market was around $68 milion per annum (Table 5) (Markou and Nerantzis 2013). However, the majority of the microalgal high-value compounds are either not established in the market or are still not commercialized (Borowitzka, 2013).

Table 5 Global market of added value compounds from microalgae (1- Pulz and Gross, 2004; 2 -Markou and Nerantzis, 2013

Product group	*Product/ Added-value compound*	*Global market (x10^6 U.S. $/annum)*	*Production kt/annum*	*Price $/kg*	*References*
Biomass	Health food	1250-2500 (2004)	-	-	1
Pigments	Carotenoids	1200 (2013)	-	-	2
	β-Carotene	261 (2013)	-	300-700	2
	Astaxanthin	240 (2013)	-	2000-7000	2
	Phycobilins	>60 (2013)	-	-	2
Bioplastics		-	64 (2013)	-	2
Fatty acids	(omega-3)	>700 (2013)	85 (2013)	0.88-3.83 (2013)	2
Vitamins and supplement		68 (2013)	-	-	2
Antioxidants	β-Carotene	>280 (2004)	-	-	1
	ARA	20 (2004)	-	-	1
	DHA	1500 (2004)	-	-	1
	PUFA extracts	10 (2004)	-	-	1
Lipids	Glycerol	-	1995.5	0.3-1.0	2
Other Products	Toxins	1-3 (2004)	-	-	1
	Isotopes	>5 (2004)	-	-	1

1.6 Microalgae species with biotechnological potential

There are many species that have their biotechnological potential well recognized (Tables 3 and 4). The majority of species that are already produced industrially are from fresh water (e.g. *Haematococcus pluvialis* and *Chlorella* sp.). Therefore, it would be interesting to explore more marine microalgae.

In the present study, the following species were selected as biological model systems: (1) *Arthrospira* genus is widely used and was selected *Arthrospira maxima* as freshwater representative specie to study; (2) to explore marine microalgae, were selected *Isochrysis galbana* and (3) *Tetraselmis chuii,* due to their world wide application on marine aquaculture.

1.6.1 *Arthrospira maxima*

Arthrospira maxima Setchell & N.L.Gardner in N.L.Gardner 1917 is a blue green aquatic mesophilic (grows best in moderate temperature) microalgae that belongs to the phylum Cyanobacteria (Table 6). This algae is an oxygenic phototroph which means that doesn't have heterocysts to fix nitrogen and use it as a source of energy. Its photosystem II and water oxidizing complex can be up to five times faster than higher plant photosystem II enzymes and it also can put up with high light fluxes (1400 $\mu Em^{-2}s^{-1}$) (Carrieri *et al*.. 2008).

Table 6 Taxonomy of *Arthrospira maxima* (Guiry and Guiry, 2015).

	Classification
Empire	Prokaryota
Kingdom	Eubacteria
Subkingdom	Negibacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Subclass	Oscillatoriophycideae
Order	Oscillatoriales
Family	Microcoleaceae
Genus	*Arthrospira*

A. maxima flourish in alkaline (pH 9.5 to 11) carbonate and bicarbonate water with high concentrations of sodium, so it propagates with low contamination in the wild environment (0.4-1.4 M) (Carrieri *et al*., 2008; Carrieri *et al*., 2011; Cheng *et al*., 2011). Therefore, Zarrouk's medium is successfully used as a standard medium for *A. maxima* cultures, since its rich in bicarbonate (Raoof *et al*., 2006; Göksan *et al*., 2007).

It presents a perfect open spiral of 3-8 turns and 40-60µm of diameter with a length of 5-7µm (Setchell and Gardner, 1917) (Figure 10). It has a genome size of 6 Mb, with a guanine-cytosine content of 44.8% (U.S. Department of Energy, Walnut Creek, CA 2008). *Arthrospira maxima*, as others cyanobacterias, do not have the DNA organized in chromosomes. The genetic information is on the cytoplasm together with the photosynthetic membranes (Richmond, 2004).

Figure 10 *Arthrospira maxima* (Adapted from Setchell and Gardner, 1917)

The *A. maxima* biomass is mostly composed of protein (72%) and was demonstrated its toxicological and nutritional adequacy for human food complement (Dismukes *et al*., 2008; Torres-Durán *et al*., 2006).

It is widely used as nutraceuticals due the fact of the content of its protective antioxidants, vitamins, PUFA's, immunomodulatory, antiviral and hypolipidemic effects (Rodríguez-Sánchez *et al*.. 2012; Torres-Durán *et al*.. 2006).

1.6.2 *Isochrysis galbana*

Isochrysis galbana Parke 1949 is a brown marine unicellular microalgae that belongs to the Haptophyta phylum (Sadovskaya *et al.*. 2014; Kaplan *et al.*. 1986) (Table 7). This microalgae is small (length 5-6 µm; breadth 2-4µm), motile cell with two equal flagella (Figure 11). It has a capacity to change shape so, for that reason, under microscope visualization, individuals shape are quite different. The movement is normally slow, with rotation of the body around the long axis (about one rotation per second) and in a forward direction. It also shows a slight phototactic reaction (Parke, 1949).

Table 7 Taxonomy of *Isochrysis galbana* (Guiry and Guiry, 2015).

	Classification
Empire	Eukaryota
Kingdom	Chromista
Phylum	Haptophyta
Class	Coccolithophyceae
Subclass	Prymnesiophycidae
Order	Isochrysidales
Family	Isochrysidaceae
Genus	*Isochrysis*

I. galbana grows optimally at 18-24ºC of temperature, 20-24 of salinity, 2500-5000 lux pf light intensity, 8,2-8,7 of pH and a maximum photoperiod of 24 hours of light (UMDU 2012). It is known for its fast growth rate and absence of toxins (Sadovskaya *et al.*, 2014).

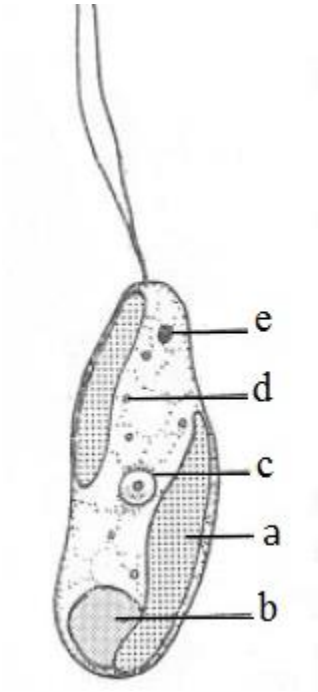

Figure 11 *Isochrysis galbana* (x 5000.): a) chromatophore; b) leucosin; c) nucleus; d) oil globule; e) stigma (Adapted from Parke, 1949).

It has a high content of proteins, fatty acids and soluble and insoluble polysaccharides. It has a complex mixture of biopolymers with different proportions of galactose, glucose, xylose, mannose, arabinose, rhamnose and fucose. *Isochrysis galbana* is usually used to feed mollusc larvae stages, fish and crustaceans in their early stages of growth in aquaculture (Dewapriya and Kim, 2014; Sadovskaya *et al.*, 2014; Yingying *et al*., 2014).

1.6.3 *Tetraselmis chuii*

Tetraselmis chuii Butcher 1959 is a green marine unicellular microalgae that belongs to the Chlorophyta phylum (Table 8).

Table 8 Taxonomy of *Tetraselmis chuii* (Guiry and Guiry, 2015).

	Classification
Empire	Eukaryota
Kingdom	Plantae
Phylum	Chlorophyta
Class	Chlorodendrophyceae
Order	Chlorodendrales
Family	Chlorodendraceae
Genus	*Tetraselmis*

This microalgae has 12-14µm in length and 9-10µm in breadth. It is distinguished by a curved body when viewed in different sideways and an ovoid body shape with four flagella (Figure 12), which gives high mobility (Mohammadi *et al.*, 2015; Nunes *et al.*, 2005).

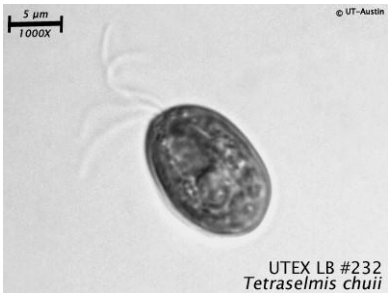

Figure 12 *Tetraselmis chuii* (x 1000) (Adapted from UTEX – University of Texas at Austin).

T. chuii has high content of proteins, essential fatty acids, lipids and sterols (Ghezelbash *et al.*, 2008). It is used in aquaculture to feed several species of crustaceans as *Artemia* and molluscs as oysters (Nunes *et al.*, 2005; Meseck *et al.*, 2005). It also has been vastly used to estimate the toxicity of chemical agents of marine ecosystems on producers and primary consumers (Ferreira *et al.*, 2007).

Chapter 2: Objectives

2.1 Objectives

The objective of the present study was to evaluate the effect of photoperiod regime and growth phase on the protein content of three different microalgae, *Arthrospira maxima, Isochrysis galbana* and *Tetraselmis chuii*. To achieve such objective, the microalgae were subjected to different photoperiod regimes. The biomass was collected at logarithmic and stationary growth phase. For each situations it was estimated the doubling time, the specific growth rate, the biomass level production, the most suitable cell disruptor method and the crude protein content.

Chapter 3: Materials and Methods

3.1 Growth and biomass production

Arthrospira maxima, *Isochrysis galbana* and *Tetraselmis chuii* strains were supplied by the School of Tourism and Maritime Technology. Culture conditions were monitored and controlled during all experimental setup.

Microalgae were cultured in carboys and in batch mode (final volume=5L), with constant and gentle aeration, under different photoperiods – light:dark 24:0, 12:12 and 18:6. The temperature of culture room was kept constant and equal to 23±1 ^{o}C. The light was provided by a white fluorescent lamp with 1641 lux of illuminance [Illuminance meter T-10. (Konica Minolta Sensing, Inc)].

Freshwater, for *A. maxima*, and natural seawater for *I. galbana* and *T. chuii* were filtered (Whatman glass microfiber filter, grade GF/C, 47 mm) and sterilized (121^{o}C, 15 min, 1 atm). Water was enriched with Zarrouk's medium (Appendix 1) ((Zarrouk, 1966), following Redfield's ratio (N:P= 5:1; pH=9) for the first strain and with f/2 medium (Appendix 2) (Gillard, 1975) (N:P= 15:1) for the other considered microalgae. At day 0, microalgae volume corresponded to 10% of total volume.

3.1.1 Growth measurement

All the experiments were conducted in triplicate (n=3), to ensure statistical relevance. Growth parameters was followed every day for *Isochrysis galbana* and *Tetraselmis chuii* and every other day for *Arthrospira maxima*. For *I. galbana* and *T. chuii* were estimated specific growth rate, doubling time, and volumetric biomass productivity. For *A. maxima* was estimated volumetric biomass productivity.

3.1.1.1 Cell counting and calibration curve

The cells of *Isochrysis galbana* and *Tetraselmis chuii* were counted in a particle counter (Beckman coulter – Z1 S). For *I. galbana* were considered particles from 3 to 6 µm and for *T. chuii* from 8 to 13 µm. Previously, a 1% NaCl solution was prepared and filtered (glass microfiber filter, grade 0,45µm, 47 mm), to ensure no particles were added to the solution. The blank count was done using 100 mL of 1% NaCl for each cell diameter considered. Sample counting was performed with a 1:10 dilution (1 mL of sample: 99 mL of 1% NaCl), to ensure equipment sensibility. Sampling cup was washed between samples with 100 mL of NaCl 1% solution.

Specific growth rate was calculated according to Equation 1, relating number of cells per millilitre and days in culture. For *A. maxima*, it is not possible to determine specific growth rate due the fact that has helical shape and forms filaments. This characteristic does not permit cell counting in a particle counter.

$$\ln(x) = \ln(x_0) + \mu t$$

Equation 1 Specific growth rate equation, where **x** refers to the cell density (cell.ml^{-1}), $\mathbf{x_0}$ initial cell density (cell.ml^{-1}), **µ** the specific growth rate (day^{-1}) and **t** the time (days).

The time period defined for logarithmic phase for *I. galbana* and *T. chuii* for each photoperiod are described on Table 10.

Table 9 Days in culture of logarithmic phase for *Isochryisis galbana* and *Tetraselmis chuii*.

	Days of Logarithmic phase	
Photoperiod	*Isochryisis galbana*	*Tetraselmis chuii*
12L:12D	4-11	4-11
18L:6D	6-10	8-16
24L:0D	3-6	3-6

Doubling time was calculated from the Equation 2, relating the specific growth rate with natural logarithm.

$$t_d = \frac{ln2}{\mu}$$

Equation 2 Cultures doubling time ($\mathbf{t_d}$), where $\boldsymbol{\mu}$ represents the specific growth rate.

A linear equation between optical density and biomass dry weight ($g.L^{-1}$) was defined for *Arthrospira maxima*. For *Isochrysis galbana* and *Tetraselmis chuii* was defined two linear equation 1) between optical density and cell density ($cell.mL^{-1}$) 2) between optical density and biomass dry weight ($g.L^{-1}$).

A wavelength screening in the VIS- spectrum (400 nm to 800 nm) was performed for each microalgae, to ensure that the wavelength used was the optimal one. Table 9 summarizes the wavelength in which each microalgae showed higher absorbance.

Table 10 Wavelenght (nm) in which each microalgae presented higher absorbance.

Microalgae	*Wavelenght (nm)*
Arthrospira maxima	441
Isochrysis galbana	440
Tetraselmis chuii	433

Optical density was measured every day using the wavelength which had the major absorbance on the screening, using an UV/VIS spectrophotometer Thermo Electron Corporation [Helios Aquamate]. Samples with absorbance higher than 1 were diluted, to ensure Beer-Lambert linearity (Griffiths *et al*., 2011).

3.1.2 Biomass quantification

Dry weight and productivity were gravimetrically calculated, according to Zhu and Lee (1997). All the filters (Whatman glass microfiber filter, grade GF/C, 47 mm) were previously dried at 60ºC for 72 hours and then weighed (**Xi)**. For biomass productivity measuring for *Isochrysis galbana* and *Tetraselmis chuii*, every three days, 10 mL of culture was filtered and filter dried at 60ºC for 72 hours and then weighed, **Xf**. For *Arthrospira maxima* was made the same method but to follow growth parameters was taken every other day. Dry weight was calculated from the Equation 3 and volumetric biomass productivity (**P**) was calculated from Equation 4.

$$X = \frac{Xf - Xi}{VF}$$

Equation 3 Dry weight ($g.L^{-1}$) estimate, where **Xf** is dry weight after a 72h drying (g), **Xi** is filter weight prior filtration (g) and **VF** refers to volume filtered (mL).

$$P = \frac{Xf - Xi}{\Delta t}$$

Equation 4 Volumetric biomass productivity ($g.L^{-1}.day^{-1}$), where **Xi** refers to initial dry biomass concentration, **Xf** refers to final dry biomass concentration and **Δt** refers to time variation.

Microalgae were grown in different photoperiod (Light:Dark - 12L:12D; 18L:6D; 24L:0D), biomass was collected at logarithmic and stationary growth phases.

3.1.3 Sample collection and storage

Total culture volume were harvested during logarithmic and stationary growth phase. Both *Isochrysis galbana* (v=3000 rpm, t=10 minutes, T=4ºC) and *Tetraselmis chuii* (v= 5000 rpm, t=5 minutes, T=4ºC) were harvested by centrifugation. *A. maxima* was recovered through vacuum filtration with filter glass fibre (Whatman glass microfiber filter, grade GF/C, 47 mm). Biomass was kept frozen at -80ºC until further analysis.

3.2 Cell disruption methods

Cell disruption was tested using homogenizer (Ystral X10/25 Ballrechten-Dottingen) and ultrasound bath (Ultrasonic clearer – VWR). The experiments were performed in triplicate with 0.5 mL of distillate water and 2% of biomass. Control samples were vigorously vortexed for 1 minute. For cell disruption time optimization with homogenizer, two different times were tested: 30 and 90 seconds. Cell disruption using ultrasound bath (45kHz / 130W) was taken continuously for 15 minutes and then another 15 minutes with 30 seconds stops on ice, each 5 minutes to avoid overheating.

3.3 Protein content analysis

3.3.1 Protein Precipitation

After cell disruption, samples were centrifuge (15000g) 5 minutes at 4ºC. Supernatant was collected and kept at 4ºC. 1 mL 0,1 NaOH was added to pellet and incubated for 60 minutes, mixing from time to time. After that, samples were centrifuge (15000g) 5 minutes at room temperature. Next, the supernatant was collected and added to the supernatant previously collected. Pellet was discarded.

To the supernatants was added 25% of trichloroacetic acid (TCA) and kept on ice for 30 min. After that was centrifuge (15000g) 10 min at 4ºC. Supernatant was discarded. 1 mL 0.1 NaOH was added to pellet and mixed to homogenize. 5% of TCA was added and kept on ice for 30 min. Next was centrifuge (15000g) 10 min at 4ºC. Supernatant was discarded. Pellet was suspended with 1 mL of NaOH.

3.3.2 Lowry Method (Lowry et al. 1951)

Lowry method was modified (Department of Biochemistry and Molecular Biology - Michigan State University) and performed on 96 well plate. A calibration curve was prepared using a concentration range of bovine serum albumin (Sigma) from 0 to 1 $mg.mL^{-1}$. In order to measure the protein content, 40 µL of each standard or samples containing the crude protein extract were withdrawn into a separate well, and 200 µL of modified Lowry reagent was added to each sample at nearly the same moment using a multi-channel pipette. Immediately microplate was mixed with spectrophotometer for 30 seconds and incubated, protected from light, at room temperature for exactly 10 minutes. After incubation, 20 µL of Folin–Ciocalteu Reagent (1:2) were added each well using a multi-channel pipette and again mixed immediately for 30 seconds and incubated, protected from light, for 30 minutes. The absorbance was measured at 750 nm absorbance on a plate reader (Synergy H1 Hybrid Reader – BioTek).

3.4 Statistical analysis

In order to evaluate the existence of significant differences between the growth parameters obtained at different experimental conditions it was applied One-Way ANOVA with pairwise multiple comparison procedures. To evaluate the existence of significant differences between protein content at different cell disruption methods it was applied One-Way ANOVA with pairwise multiple comparison procedures. In order to evaluate the existence of significant differences between the protein content at different photoperiod regimes and growth phase it was applied Two-Way ANOVA with pairwise multiple comparison procedures. All statistical analysis were performed by the use of the software Sigmaplot for Windows Version 12.0.

4.1 Biomass production

4.1.1 *Arthrospira maxima*

The growths of *Arthrospira maxima* was followed by measuring the biomass dry weight and optical density along the culture period.

Figure 13 describes the dry weight variation along the culture period for *Arthrospira maxima*. The logarithmic phase was verified between day 2 and day 12. The stationary phase was achieved at day 14.

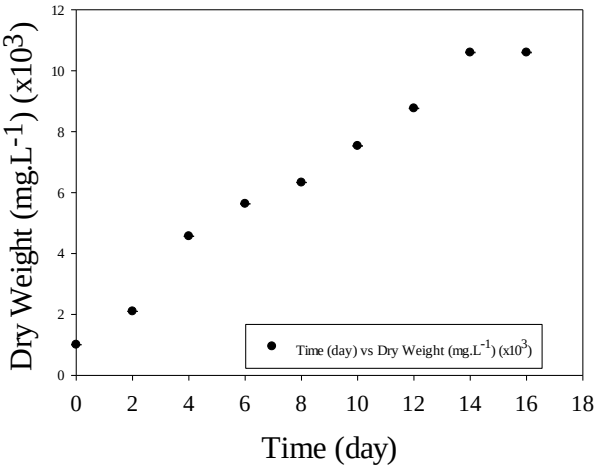

Figure 13 Dry weight (mg.L^{-1}) variation of *Arthrospira maxima* along the culture period (symbols and bars represents average value ± standard deviation).

The relationship between dry weight and optical density for *Arthrospira maxima* is on figure 14. This calibration curve was used to define the different growth phase of the cultures subjected to different photoperiod regimes (Figure 15).

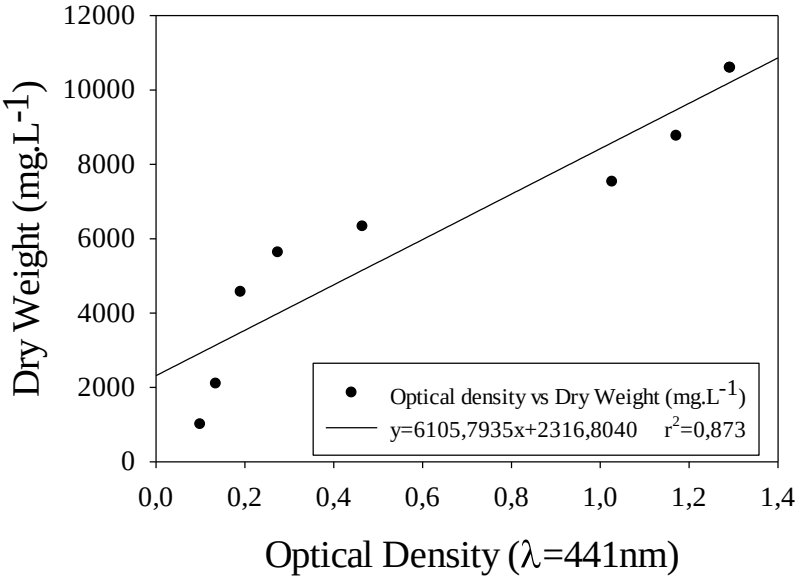

Figure 14 Calibration curve for *Arthrospira maxima* obtained from the linear regression between biomass dry weight and optical density (λ=441nm).

Arthrospira maxima exhibits different growth patterns when exposed to different photoperiods. Between the three studied photoperiods, 12L:12D allowed cells to grow for a longer time period (23 days), being also the photoperiod in which *A. maxima* exhibit longer logarithmic phase (14 days). Photoperiods with shorter or absent periods of darkness (18L:6D and 24L:0D) grew for fewer days, presenting also fewer days in logarithmic phase (Table 11). Lag phase was similar in the three studied photoperiods, suggesting that *A. maxima* adapts quickly to different photoperiod conditions.

Table 11 Growth parameter ***P*** daily biomass productivity of *Arthrospira maxima* when exposed to different photoperiods (12L:12D, 18L:6D and 24L:0D). Letters represent statistical differences between treatments: [a] statistically significant difference [(p<0,050) (Holm-Sidak)].

Arthrospira maxima	***P*** *(mg.L-1.day-1)*	*Days in Lag Phase*	*Days in Log Phase*	*Days in Culture*
12L:12D	0.128[a]	3	14	23
18L:6D	0.435[a]	2	4	15
24L:0D	0.294[a]	2	2	8

Regarding daily productivity (Figure 15), *A. maxima* when exposed to 18L:6D photoperiod, achieved the highest value (0.435 $mg.L^{-1}.day^{-1}$), on the other hand, the lowest productivity value was obtained with 12L:12D photoperiod. There was statistically significant difference between all the photoperiods daily productivity [(p<0,050) (Holm-Sidak)] (Table 11).

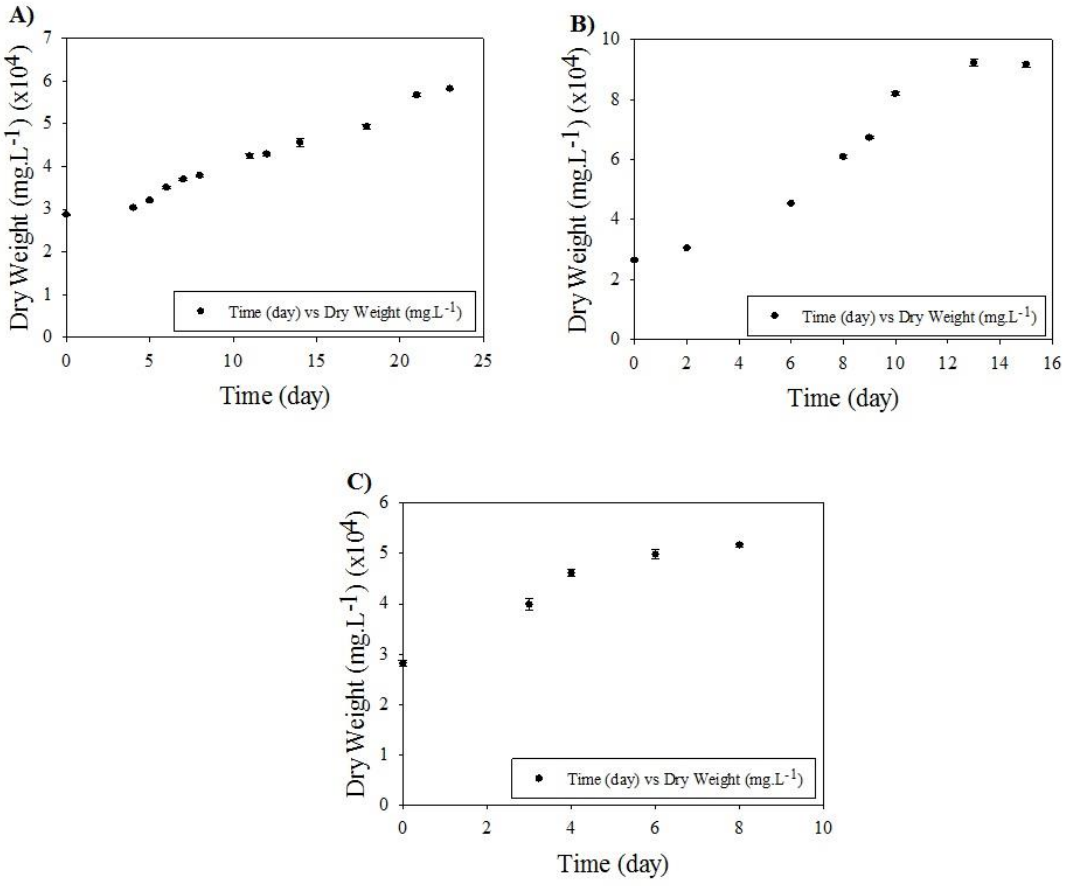

Figure 15 *Arthrospira maxima* biomass dry weight ($mg.L^{-1}$) evolution along the cultivation period (symbols and bars represents average value ± standard deviation): **A)** 12L:12D photoperiod; **B)** 18L:6D photoperiod; **C)** 24L:0D photoperiod.

In terms of fresh biomass extraction of *A. maxima* when exposed to different photoperiods the results are consistent with daily productivity. The 18L:6D photoperiod achieved the highest biomass quantity, with 5.337g at logarithmic phase and 7.418g at stationary phase (Table 12).

Table 12 Wet biomass production of *Arthrospira maxima* when exposed to different photoperiods (12L:12D, 18L:6D and 24L:0D). Letters represent statistical differences between treatments: [a] statistically significant difference [(p<0,050) (Dunn's method)].

Photoperiod	*Logarithmic Phase (g)*	*Stationary Phase (g)*
12L:12D	3.485	5.770
18L:6D	5.337[a]	7.418
24L:0D	3.607[a]	5.834

4.1.2 *Isochrysis galbana*

The growths of *Isochrysis galbana* growth was followed by measuring cell density, optical density and biomass dry weight.

Figure 16 describes the cell density variation along the culture period for *Isochrysis galbana*. The logarithmic phase was verified between day 1 and day 5. The stationary phase was achieved at day 6.

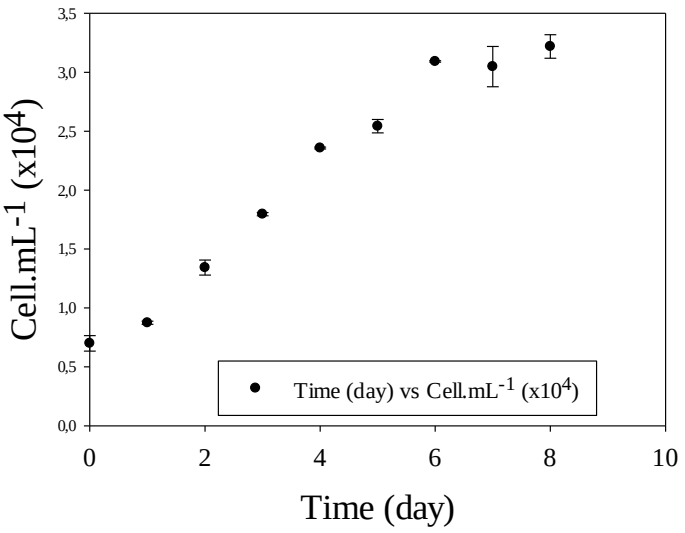

Figure 16 Cell density (cell.mL^{-1}) variation of *Isochrysis galbana* along the culture period (symbols and bars represents average value ± standard deviation).

The relationship between cell density and optical density for *Isochrysis galbana* is on figure 17-A. This calibration curve was used to define the different growth phase of the cultures subjected to different photoperiod regimes (Figure 19). The relationship between dry weight and optical density for *I. galbana* is on figure 17-B. This calibration curve was used to estimate the biomass dry weight along the culture period subjected to the different photoperiod regimes (Figure 20).

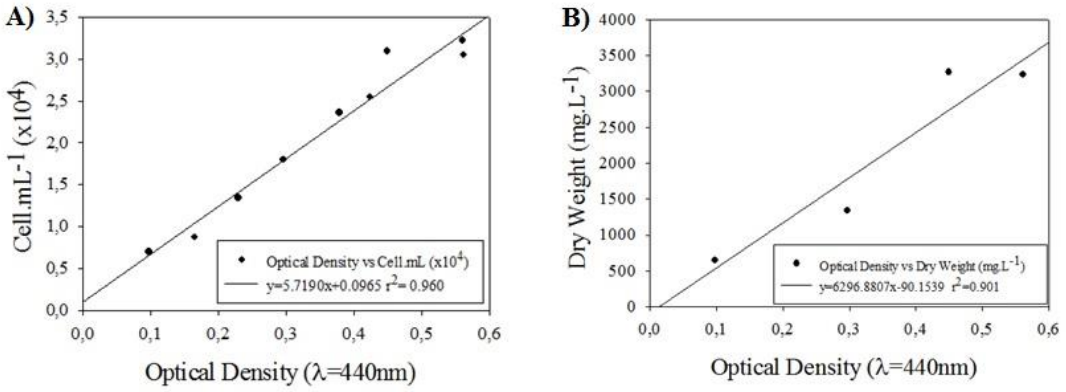

Figure 17 **A)** Calibration curve for *Isochrysis galbana* given by the linear regression between cell density and optical density (λ=440nm). **B)** Calibration curve for *Isochrysis galbana* given by the linear regression between biomass dry weight and optical density (λ=440nm).

Isochrysis galbana also exhibits different growth patterns when exposed to different photoperiods. Between the three studied photoperiods, 12L:12D allowed cells to grow for a longer time period (16 days), being also the photoperiod in which *I. galbana* exhibit longer logarithmic phase (7 days). Photoperiods with shorter or absent periods of darkness (18L:6D and 24L:0D) presenting fewer days in logarithmic phase (Table 13). Lag phase was more evident in 18L:6D photoperiod, suggesting that culture took a longer period to adapt to the given growth conditions.

Table 13 Growth parameters (specific growth rate, μ; doubling time, t_d; P daily biomass productivity) of *Isochrysis galbana* when exposed to different photoperiods (12L:12D, 18L:6D and 24L:0D). Letters represent statistical differences between treatments: [a] statistically significant difference [(p<0,050) (Tukey)]; [b] statistically significant difference [(p<0,050) (Holm-Sidak)].

Isochryisis galbana	μ (day^{-1})	t_d (day)	P ($mg.L^{-1}.day^{-1}$) ($x10^4$)	*Days in Lag phase*	*Days in Log Phase*	*Days in Culture*
12L:12D	0.109[a]	6.348[a]	0.139[b]	3	7	16
18L:6D	1.028[a]	0.674[a]	0.498[b]	5	4	15
24L:0D	0.512	1.354	0.373[b]	2	3	10

Regarding to cell density, *I. galbana* achieved higher densities when exposed to photoperiod 18L:6D than cells grown in other photoperiods (Figure 18 – B). When grown in 12L:12D, cells achieved the lowest recorded value of this assay (Figure 18-A).

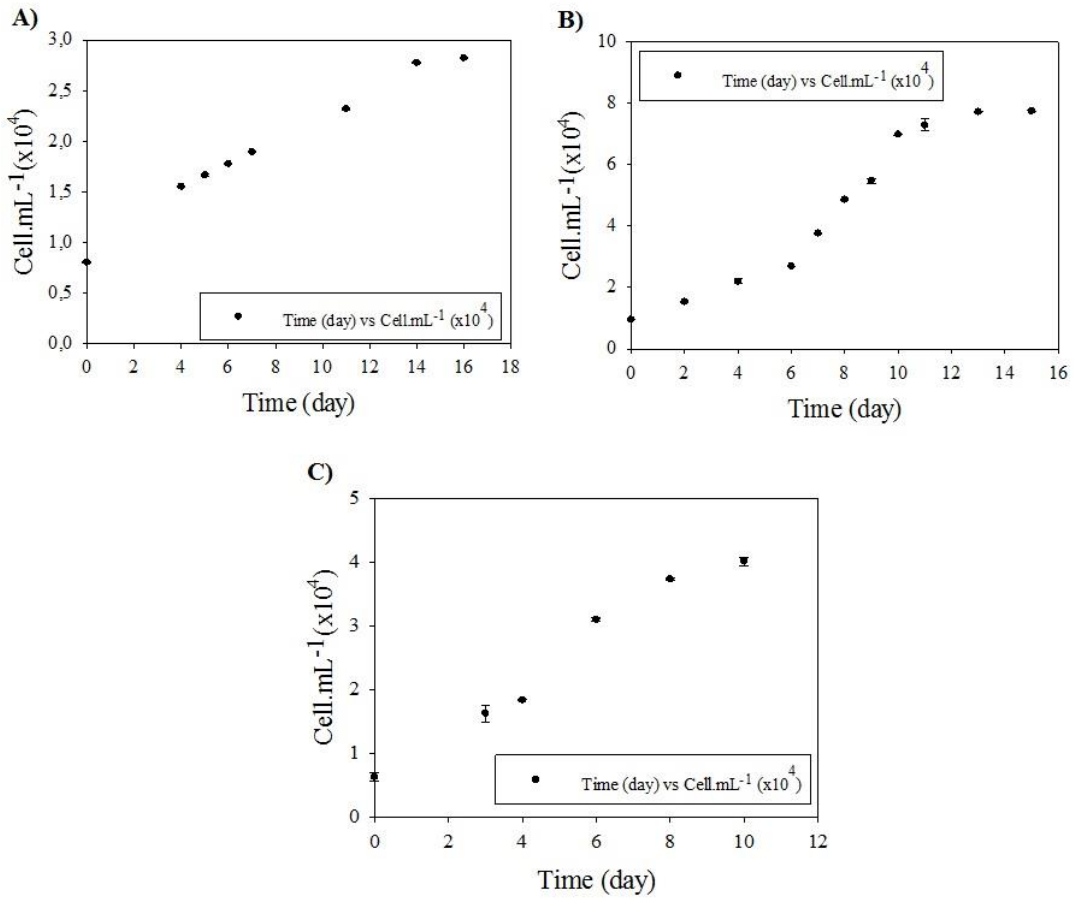

Figure 18 Cell density (cell.mL^{-1}) variation of *Isochrysis galbana* along the cultivation period (symbols and bars represents average value ± standard deviation): **A)** 12L:12D photoperiod; **B)** 18L:6D photoperiod; **C)** 24L:0D photoperiod.

According to this results, the specific growth rate for 12L:12D was the lowest value. There was statistical differences between photoperiods 12L:12D and 18L:6D but statistical differences between 12L:12D and 24L:0D and between 18L:6D and 24L:0D (Table 13) were not detected. The same tendency was observed in, doubling time, since 12L:12D had the higher values than in other photoperiods and 18L:6D had the lowest value. There was statistical differences between photoperiods 12L:12D and 18L:6D but there wasn't statistical differences between 12L:12D and 24L:0D and between 18L:6D and 24L:0D.

Being directly influenced by cell density, daily productivity was also higher in 18L:6D photoperiod than in the other two tested, achieving 0.498 $mg.L^{-1}.day^{-1}$. Productivity was statistically different ($p<0,050$) between the three treatments (Table 13). In terms of dry weight, when exposed to photoperiod 18L:6D *I. galbana* achieved higher values than other photoperiod (Figure 19-B).

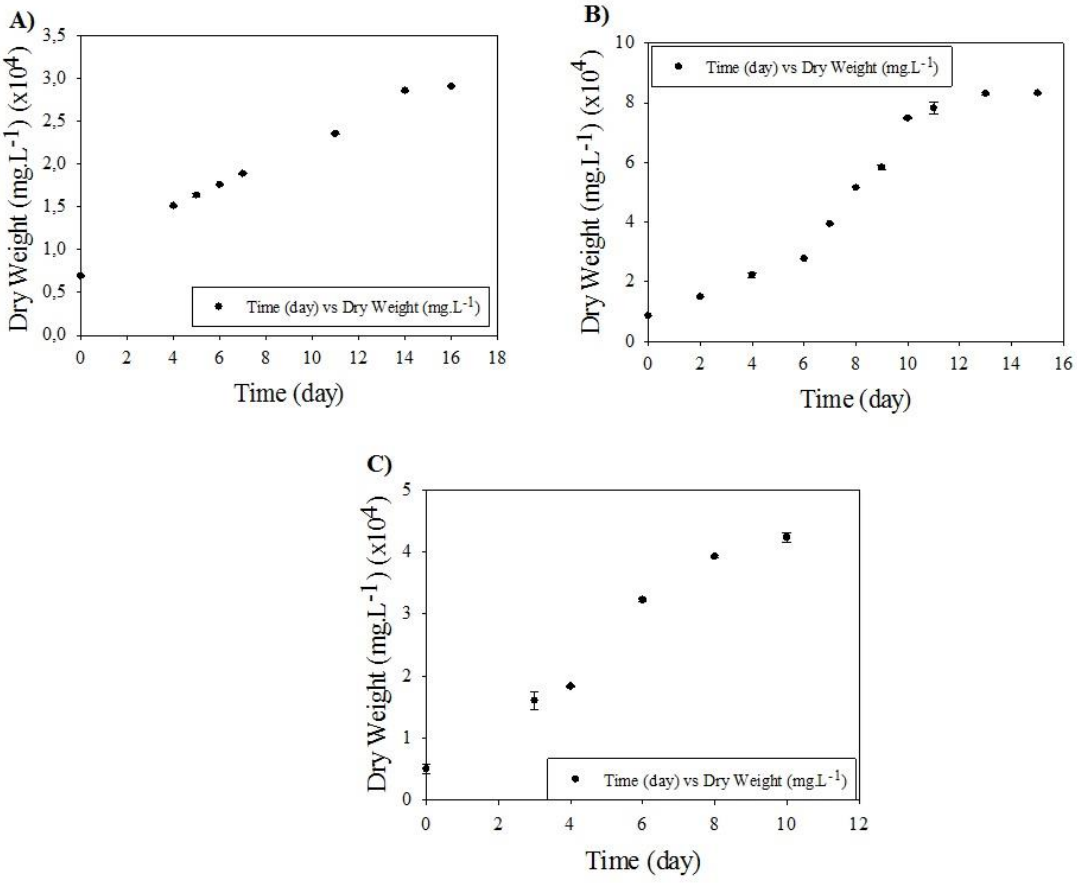

Figure 19 *Isochrysis galbana* biomass dry weight ($mg.L^{-1}$) evolution along the culture period (symbols and bars represents average value ± standard deviation): **A)** 12L:12D photoperiod; **B)** 18L:6D photoperiod; **C)** 24L:0D photoperiod.

In terms of wet biomass extraction of *I. galbana* when exposed to different photoperiods the results are consistent with the previously results. The 18L:6D photoperiod achieved the highest biomass quantity, with 8.575g at stationary phase (Table 14).

Table 14 Fresh wet production of *Isochrysis galbana* when exposed to different photoperiods (12L:12D, 18L:6D and 24L:0D). Letters represent statistical differences between treatments: [a];[b] statistically significant difference [(p<0,050) (Holm-Sidak)]; [c];[d] statistically significant difference [(p<0,050) (Tukey)].

Photoperiod	*Logarithmic Phase (g)*	*Stationary Phase (g)*
12L:12D	3.46[b]	4.718[c;d]
18L:6D	2.219[a;b]	8.575[c]
24L:0D	2.610[a]	7,466[d]

4.1.3 Tetraselmis chuii

The growths of *Tetraselmis chuii* growth was followed by measuring cell density, optical density and biomass dry weight.

Figure 20 describes the cell density variation along the culture period for *Tetraselmis chuii*. The logarithmic phase was verified between day 2 and day 7. The stationary phase was achieved at day 8.

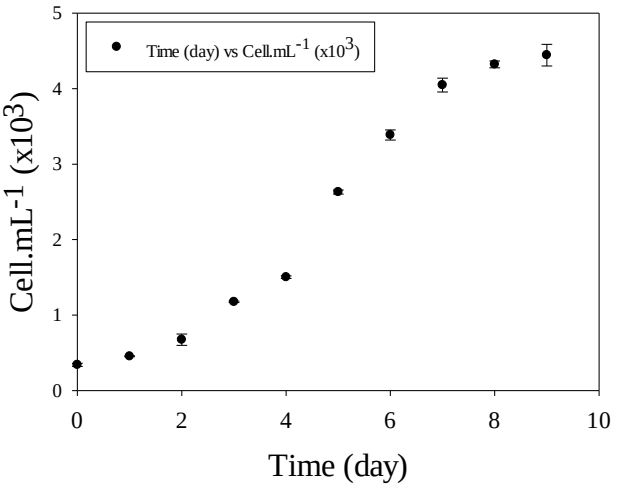

Figure 20 Cell density (cell.mL-1) variation of *Tetraselmis chuii* along the culture period (symbols and bars represents average value ± standard deviation).

The relationship between cell density and optical density for *Tetraselmis chuii* is on figure 21-A. This calibration curve was used to define the different growth phase of the cultures subjected to different photoperiod regimes (Figure 22). The relationship between dry weight and optical density for *T. chuii* is on figure 21-B. This calibration curve was used to define the dry weight evolution of the cultures subjected to each photoperiod studied (Figure 23).

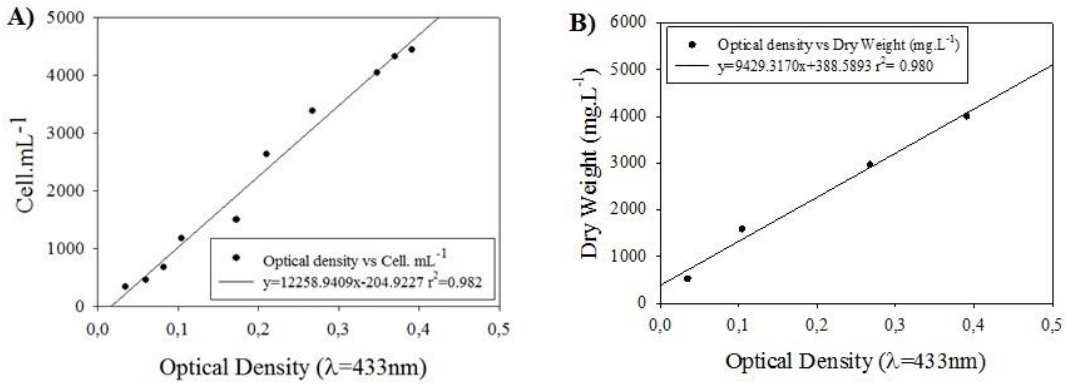

Figure 20 **A)** Calibration curve for *Tetraselmis chuii* given by the linear regression between cell density and optical density (λ=433nm). **B)** Calibration curve for *Tetraselmis chuii* given by the linear regression between biomass dry weight and optical density (λ=433nm).

Tetraselmis chuii shows different growth patterns when exposed to the photoperiods under study. The photoperiod 18L:6D allowed cells to grow for a longer time period (20 days), being also the photoperiod in which *A. maxima* exhibit longer logarithmic phase (8 days). Photoperiod with absent period of darkness (24L:0D) grew for fewer days, presenting also fewer days in logarithmic phase (Table 15). Lag phase was more evident in 18L:6D photoperiod, suggesting that culture took a longer period of adaptation to the given growth conditions.

Table 15 Average growth parameters (specific growth rate, μ; doubling time, t_d; **P** daily biomass productivity) of *Tetraselmis chuii* when exposed to different photoperiods (12L:12D, 18L:6D and 24L:0D). Letters represent statistical differences between treatments: a statistically significant difference [(p<0,050) ((Holm-Sidak))]; b statistically significant difference [(p<0,050) (Tukey)].

Tetraselmis chuii	μ *(day^{-1})*	t_d *(day)*	**P** *(mg.L^{-1}.day^{-1})*	*Days in Lag Phase*	*Days in Log Phase*	*Days in Culture*
12L:12D	0.316[a]	2.195[b]	0.163[b]	3	7	16
18L:6D	1.3[a]	0.533[b]	0.517[b]	6	8	20
24L:0D	0.365[a]	1.9	0.294	2	3	11

Regarding cell density *T. chuii* when exposed to photoperiod 18L:6D achieved higher densities than cells grown in other photoperiods (Figure 22 – B). When grown in 12L:12D, *T. chuii* achieved the lowest recorded value of this assay (Figure 22-A).

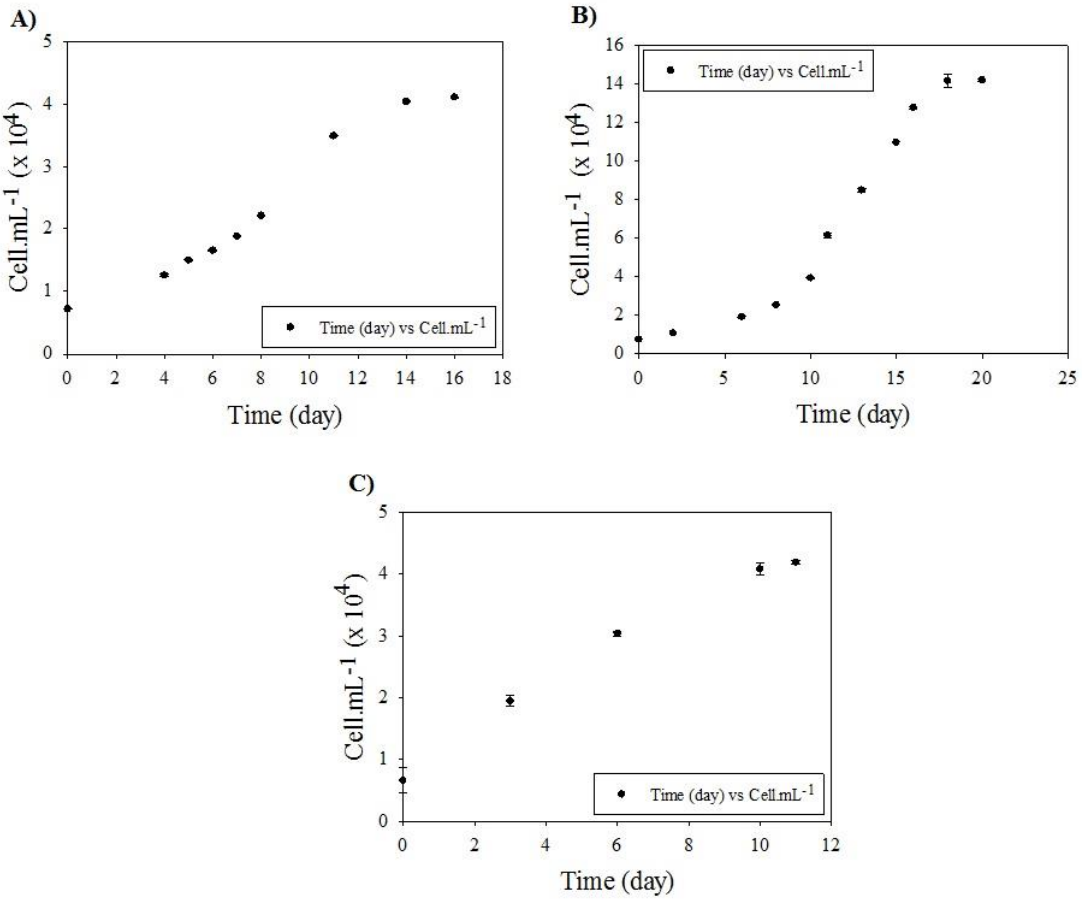

Figure 22 Cell density (cell.mL^{-1}) variation of *Tetraselmis chuii* along the culture period (symbols and bars represents average value ± standard deviation): A) 12L:12D photoperiod; B) 18L:6D photoperiod; C) 24L:0D photoperiod.

According with this results, specific growth rate for 12L:12D was the lowest value. There was statistically significant difference between all photoperiods [(p<0,050) ((Holm-Sidak))] (Table 15). In the same way, doubling time had the highest value in 12L:12D than in other photoperiods and 18L:6D had the lowest value. There was statistical differences between photoperiods 12L:12D and 18L:6D [(p<0,050) (Tukey)] but there wasn't statistical differences between 12L:12D and 24L:0D and between 18L:6D and 24L:0D.

Being directly influenced by cell density, daily productivity was also higher in 18L:6D photoperiod than in the other two tested, achieving 0.517 mg.L-1.day-1. There was statistical differences between photoperiods 12L:12D and 18L:6D [(p<0,050) (Tukey)] but there wasn't statistical differences between 12L:12D and 24L:0D and between 18L:6D and 24L:0D (Table 15). *Tetraselmis chuii* when exposed to 18L:6D photoperiod achieved higher values of dry weight than other photoperiod (Figure 23-B).

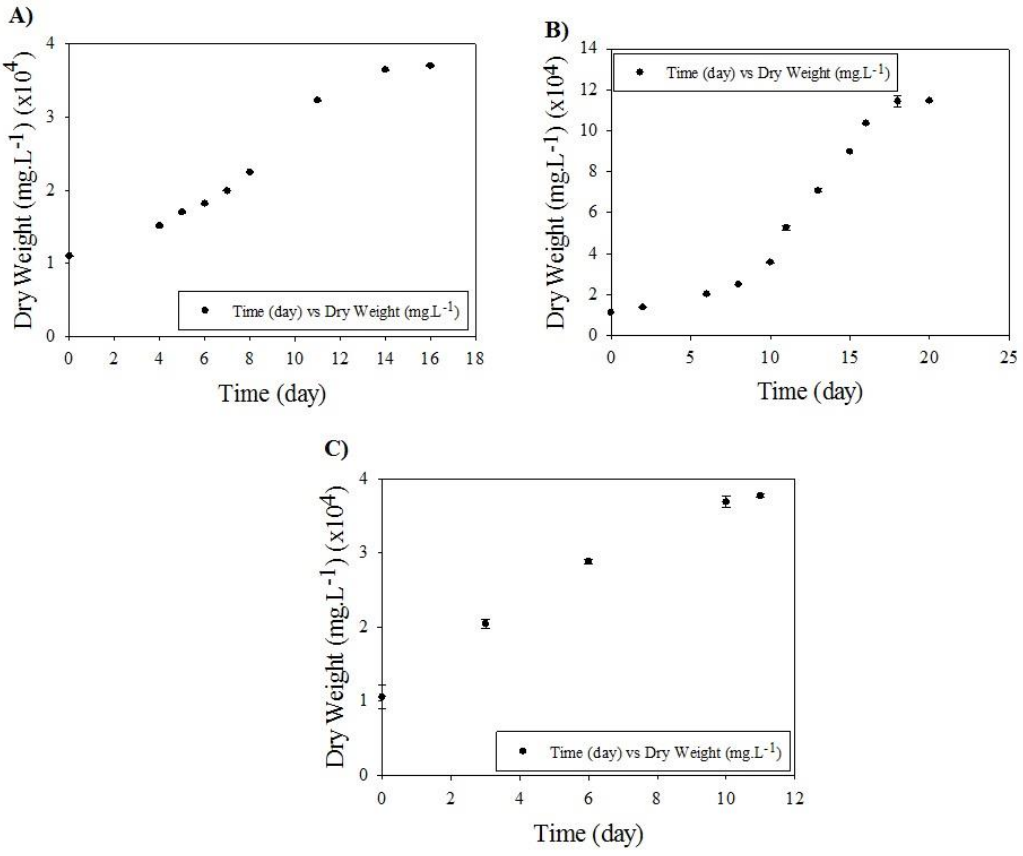

Figure 21 Biomass dry weight (mg.L^{-1}) variation of *Tetraselmis chuii* along the culture period (symbols and bars represents average value ± standard deviation): **A)** 12L:12D photoperiod; **B)** 18L:6D photoperiod; **C)** 24L:0D photoperiod.

In terms of wet biomass extraction of *T. chuii* when exposed to different photoperiods the results are consistent with the previously results. The 18L:6D photoperiod achieved the highest biomass quantity, with 9.788g at stationary phase and 12L:12D photoperiod the lowest biomass quantity with 5.686g at stationary phase (Table 16).

Table 16 Wet biomass extraction of *Tetraselmis chuii* when exposed to different photoperiods (12L:12D, 18L:6D and 24L:0D). Letters represent statistical differences between treatments: [a];[b] statistically significant difference [(p<0,050) (Holm-Sidak)].

Photoperiod	*Logarithmic Phase (g)*	*Stationary Phase (g)*
12L:12D	3.892[a]	5.686[b]
18L:6D	7.025[a]	9.788[b]
24L:0D	5.486[a]	7.491[b]

4.2 Cell disruption methods

Protein content obtained from each cell disruption methods tested are shown at figure 24 and One-Way ANOVA analysis results are in appendix 4.

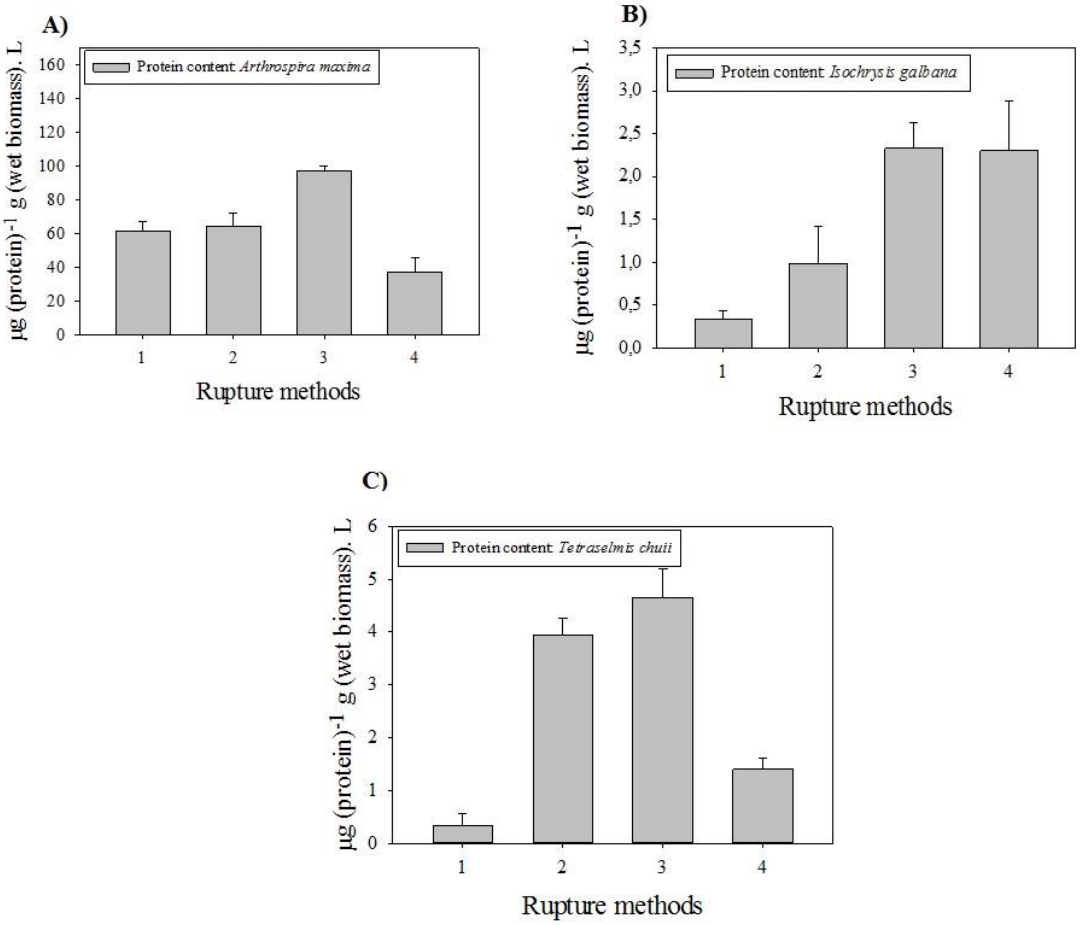

Figure 22 Protein content (µg (protein)$^{-1}$ g (wet biomass). L) in each rupture method: 1) Control method; 2) homogenizer 30 seconds; 3) homogenizer 90 seconds; 4) ultrasound bath. **A)** *Arthrospira maxima* **B)** *Isochrysis galbana* **C)** *Tetraselmis chuii*. Values represent an average value ± standard deviation through time in culture.

With statistically significant differences [p<0.050) Holm-Sidak method] between all the methods (except 30 seconds of homogenizer with control method), the best rupture method for *A. maxima* was the 90 seconds of homogenizer (97.327 [µg(protein)$^{-1}$ g(wet biomass)]) (Table 17) (Appendix 4).

For *I. galbana* the 90 seconds of homogenizer was selected too as the best rupture method (2.329 [µg(protein)$^{-1}$ g(wet biomass)]) (Table 17). Although there were not

statistically significant differences [p<0.050) Holm-Sidak method] (Appendix 4) between 30 and 90 seconds of homogenizer and ultrasound bath and 90 seconds of homogenizer, there were biological differences of protein content.

In the same way, for *T. chuii* the 90 seconds of homogenizer was selected as the best rupture method (4.651 [µg(protein)$^{-1}$ g(wet biomass)]) (Table 17). There was not statistically significant difference [p<0.050) Holm-Sidak method] between 30 and 90 seconds of homogenizer but was biological difference of protein content (Appendix 4) (Table 17).

Table 17 Protein content [µg(protein)$^{-1}$ g (wet biomass)] in each cell disruption method.

Species	*Rupture method*	*Protein Content [µg(protein)$^{-1}$ g (wet biomass)]*
Arthrospira maxima	Control	61.4[a]
	Homogenizer 30s	64.152[a]
	Homogenizer 90s	97.327[a]
	Ultrasound bath	37.251[a]
Isochrysis galbana	Control	0.334
	Homogenizer 30s	0.990
	Homogenizer 90s	2.329
	Ultrasound bath	2.304
Tetraselmis chuii	Control	0.335
	Homogenizer 30s	3.939
	Homogenizer 90s	4.651
	Ultrasound bath	1.402

4.3 Protein content

Protein content obtained from each photoperiod regime and growth phase tested are shown on figure 25. Two-Way ANOVA analysis results are listed on appendix 5.

Arthrospira maxima had a maximum value of protein content in 18L:6D photoperiod with statistically significant difference between all the photoperiods [p<0.050) Holm-Sidak method] (Appendix 5). Although, the maximum value of protein was obtained at stationary phase with 125.642 µg(protein)$^{-1}$ g(wet biomass) (Table 18). In the same way, *Isochrysis galbana* had a maximum value of protein content in 18L:6D photoperiod with statistically significant difference between all the photoperiods [p<0.050) Holm-Sidak method] (Table 18).

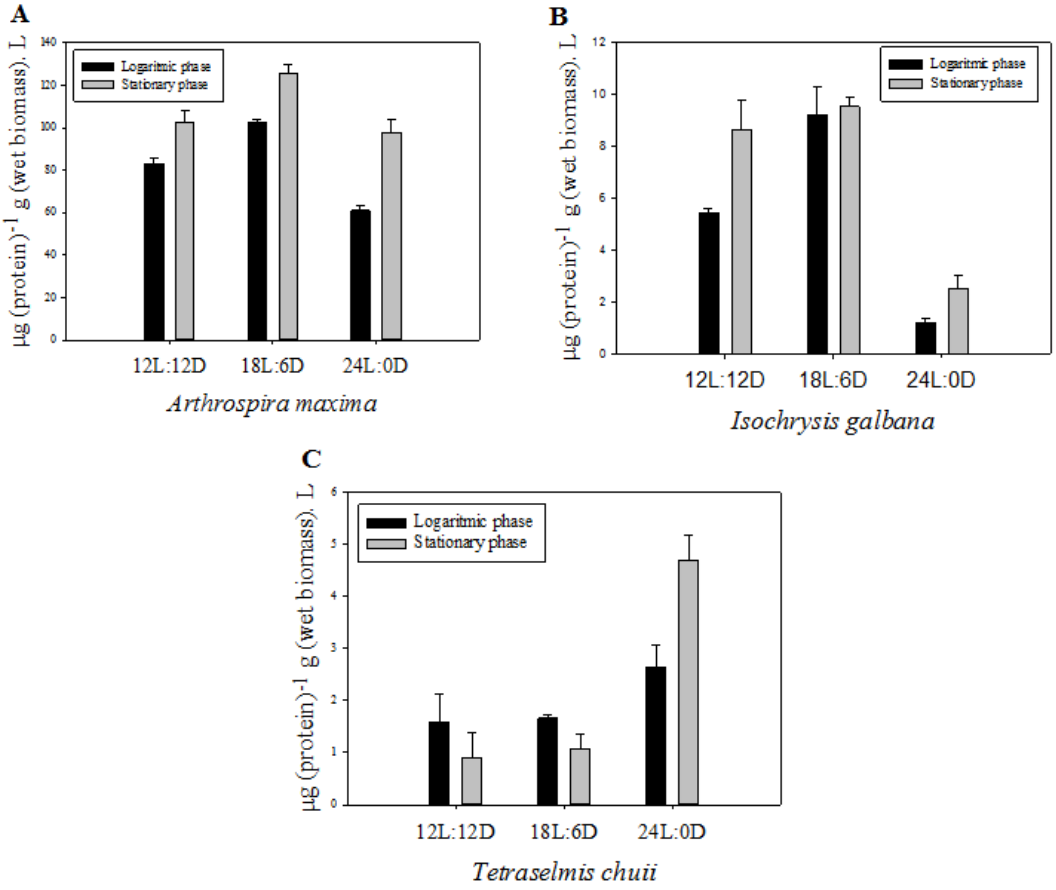

Figure 23 Protein content [µg(protein)$^{-1}$ g (wet biomass)] for each photoperiod and growth phase in **A)** *Arthrospira maxima*; **B)** *Isochrysis galbana*; **C)** *Tetraselmis chuii*. Values represent an average value ± standard deviation through time in culture.

However, there was not significant difference between growth phases at 18L:6D photoperiod (logarithmic growth phase with 8.652 [µg(protein)$^{-1}$ g(wet biomass)] and stationary growth phase with 9.180 [µg(protein)$^{-1}$ g(wet biomass)]) (Appendix 5). Otherwise, *T. chuii* had a maximum value of protein content in 24L:0D with statistically significant difference between 12L:12D and 24L:0D photoperiods and 18L:6D and 24L:0D photoperiods. There was not statistically significant difference between 12L:12D and 18L:6D photoperiods. [$p<0.050$) Holm-Sidak method] (Appendix 5). This maximum value was obtained at stationary growth phase with 4.702 µg(protein)$^{-1}$ g(wet biomass) (Table 18). There was not statistically significant difference between logarithmic and stationary growth phase at 12L:12D and at 18L:6D photoperiod.

Table 18 Protein content [µg(protein)$^{-1}$ g (wet biomass)] in each photoperiod and growth phase.

Species	*Photoperiod*	*Growth Phase*	*Protein Content [µg(protein)$^{-1}$ g (wet biomass)]*
Arthrospira maxima	12L:12D	Logarithmic	83.026
		Stationary	102.253
	18L:6D	Logarithmic	102.228
		Stationary	125.642
	24L:24D	Logarithmic	60.918
		Stationary	97.715
Isochrysis galbana	12L:12D	Logarithmic	5.444
		Stationary	8,652
	18L:6D	Logarithmic	9,180
		Stationary	9,519
	24L:24D	Logarithmic	1,209
		Stationary	2,511
Tetraselmis chuii	12L:12D	Logarithmic	1.599
		Stationary	0.902
	18L:6D	Logarithmic	1.660
		Stationary	1,074
	24L:24D	Logarithmic	2,644
		Stationary	4,702

Chapter 5: Discussion

In the present study, it was selected as biological model system, three different microalgae species with biotechnological potential. *Arthrospira maxima* was selected as freshwater representative specie from *Arthrospira* genus and to explore marine species were selected *Isochrysis galbana* and *Tetraselmis chuii*, due to their applications in aquaculture.

To study the differences of metabolites along the growth period, experiments were conducted in logarithmic and stationary growth phase, beyond under different photoperiod regime.

Arthrospira maxima growth at 18L:6D photoperiod shows better daily productivity (0.435 $mg.L^{-1}.day^{-1}$) than the other photoperiod in study. This result is in concordance with wet biomass production. At 18L:6D photoperiod *A. maxima* achieved the highest biomass quantity, with 5.337g at logarithmic phase and 7.418g at stationary phase. These results demonstrated that light regime is an important factor on biomass production in *A. maxima*.

Isochrysis galbana growth at 18L:6D photoperiod also shows better daily productivity (0.498 $mg.L^{-1}.day^{-1}$) and achieved the highest wet biomass production with 8.575g at stationary phase. This results are supported with specific growth rate (1.028 day^{-1}) and doubling time (0.674 day), which had better results than the other photoperiods. These results demonstrated that photoperiod is an important factor on biomass production in *I. galbana*. It was reported that the growth of *Isochrysis affinis galbana* was better when was under discontinuous light regime than under continuous one (Tzovenis *et al*., 2003). This is in concordance with the obtained results when compared 18L:6D with 24L:0D photoperiods but not when compared 12L:12D with 24L:0D, where specific growth rate was better at 24L:0D (0.512 day^{-1}) than 12L:12D photoperiod (0.512 day^{-1}) (Table 13). However, for *Isochrysis affinis galbana*, was obtained the same growth pattern as *I. galbana* in this study, with constant irradiance (24L:0D photoperiod) (Figure 18 – C) (Bougaran *et al*., 2012) For mixotrophic culture for *I. galbana* the 12L:12D photoperiod is recommend as a suitable photoperiod for this specie (Alkhamis and Qin, 2013).

Tetraselmis chuii growth also shows better daily productivity (0.517 $mg.L^{-1}.day^{-1}$) at 18L:6D photoperiod and had the highest wet biomass production with 7.025g at logarithmic

phase and 9.788g at stationary phase. This results are in concordance with specific growth rate (1.3 day^{-1}) and doubling time (0.533 day), that also are the better results when compared with the others photoperiods in study. These results, also demonstrated that photoperiod is an important factor on biomass production in *T. chuii*. Meseck *et al*., 2005, reported that with the photoperiod increasing the specific growth rate also increases. However, this is not concordant with the present results. The specific growth rate at 24L:0D photoperiod (0.365 day^{-1}), was lower than in 18L:6D photoperiod (1,3 day^{-1}). Such fact could be justified by the different culture conditions used on the present study (Meseck *et al*., 2005 used E/4 medium, 18ºC, photoperiod regime tested were 24:0, 16:8, 12:12, and 8:16 L:D, and light intensities were 220, 110, and 73 µEinst. m^{-2} s^{-1}).

Since cell wall differs for each genre (e.g *Arthrospira* has a relatively fragile cell wall (Safi *et al.*, 2014)) was necessary evaluating the effect of different cell disruption methods on protein extractability. 90 seconds of homogenizer was the method that achieved better protein extractability for each specie. Tibbetts *et al.*, 2015 used a laboratory hammer mill as disruption method on several species. For *Arthrospira platensis* was achieved 55.8% of crude protein and for *Tetraselmis chuii* (PLY-429) was obtained 46,5% of crude protein.

Alterations in the growth medium have a significant effect on the growth characteristics and chemical composition of microalgal cells (Fidalgo *et al.*, 1998). Particularly, protein synthesis capacity is lower when cells are under salinity-stress (Zeng and Vonshak, 1998). Piorrec *et al.*, 1984, reported that with the increasing nitrogen concentration, the protein content increased.

Protein content of *A. maxima* achieved the highest result on stationary phase of 18L:6D photopriod (125.642 [µg(protein)$^{-1}$ g (wet biomass)]). This result is concordant with biomass production.

Protein content in *I. galbana* obtained was also better on 18L:6D photoperiod. However, there were not statistically significant difference between growth phases (logarithmic growth phase with 9.180 [µg(protein)$^{-1}$ g (wet biomass)] and stationary growth phase with 9.519 [µg(protein)$^{-1}$ g (wet biomass)].

Protein content in *T. chuii* had better result at stationary growth phase in 24L:0D photoperiod with 4.702 [µg(protein)$^{-1}$ g (wet biomass)].

Microalgae of different origins typically decrease protein content when they enter at stationary phase (Gatenby *et al.,* 2003). Nitrogen starvation affects negatively protein content and on the other hand increases lipid concentration and carbohydrates (Fogg, 1959; Phatarpekar *et al.*, 2000). Nitrogen is essential in amino acids, genetic material and other cell components and it is mandatory for cell growth and division (Roopnarain *et al.*, 2015). However protein content obtained in this study do not follow the tendency. Pandey and Tiwari, 2010 reported in *A. maxima* that after 25 days of culture the protein content did not decrease, but increased. Nitrogen deficiency on *Cyanobacteria* has a particular effect of changing colour to green. This phenomenon occurs because phyconcyanin is used as nitrogen source and this pigment is responsible for the blue-green colour characteristic of *Cyanobacteria* (Göksan *et al*., 2007). This change of colour did not occur on *A. maxima* cultures in this study leading us to believe that nitrogen starving did not occur. Various studies with *Isochrysis* genus showed a decrease of total protein content at stationary growth phase (Kaplan *et al.*, 1986; Phatarpekar *et al.,* 2000; Brown *et al*., 1993). Lourenço *et al.*, 1997 studied the influence of different growth media on protein content in *Tetraselmis gracilis*. They obtained different results when harvested at mid-logarithmic growth phase, late logarithmic/ early stationary growth phase and stationary phase. In some growth media there was an increase protein content from mid-logarithmic to late logarithmic/ early stationary growth phase and then a decrease on stationary phase. In this present study, biomass was not harvested at a late stationary phase but in the early stationary growth phase. This can possibly be the reason for these results of protein content.

Chapter 6: General conclusions and final remarks

The objective of the present study was to evaluate the effect of different photoperiod and growth phase on the metabolites production. Due to experimental and temporal limitations it was only possible evaluate the protein content. However, this unique metabolite already allows conclusions concerning the influence of photoperiod for each specie. For *Arthrospira maxima* and *Isochrysis galbana* the photoperiod that achieved better results was 18L:6D both for protein content and biomass production. For *Tetraselmis chuii* the photoperiod that achieved better result on the protein content was 24L:0D and biomass production obtained the best result in 18L:6D photoperiod.

It was obtained biomass quantity from logarithmic and stationary growth phase that allows to explore these present results studying others metabolites, namely lipids and pigments. It would be interesting connect these metabolites with the protein content in logarithmic, early stationary and late stationary phase.

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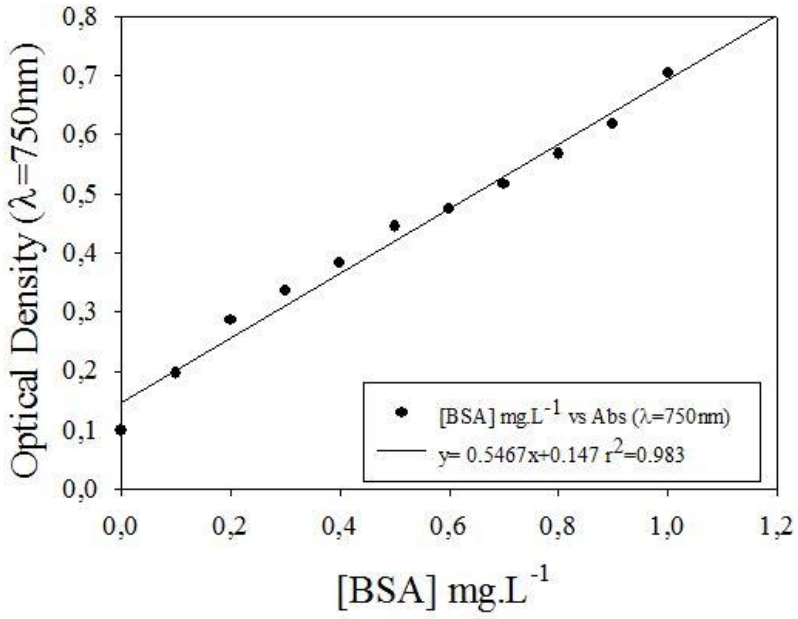
Appendix 1 - Zarrouk's Medium Composition (Zarrouk, 1966)

Reagents	*Per Litre*
$NaHCO_3$	16.8 g
K_2HPO_4	0.5 g
$NaNO_3$	2.5 g
K_2SO4	1.0 g
$NaCl$	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.04 g
$Fe_2SO_4 \cdot 7H_2O$	0.01g
$EDTA$	0.08 g
Microelement Stock Solution	**Per Litre**
H_3BO_3	2.86 g
$MnSO_4 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 4H_2O$	0.222 g
Na_2MoO_4	0.0177 g
$CuSO_4 \cdot 5H_2O$	0.079 g
pH 9	

Appendix 2 - f/2 Medium Composition (Gillard, 1975)

Reagents	*Per litre Seawater*
$NaNO_3$	0.075 g
$NaH_2PO_4 \cdot H_2O$	0.005 g
Microelement stock solution	1 mL
Vitamin solution	1 mL
Microelement Stock Solution	**Per litre**
$FeCl_3 \cdot 6H_2O$	3.150 g
$Na_2 \cdot EDTA$	4.160 g
$MnCl_2 \cdot 4H_2O$	0.180 g
$CoCl_2 \cdot 6H_2O$	0.010 g
$CuSO_4 \cdot 5H_2O$	0.010 g
$ZnSO_4 \cdot H_2O$	0.022 g
$Na_2MoO_4 \cdot 2H_2O$	0.006 g
Vitamin Solution	**Per litre**
Biotin (Vitamin H)	0.5 mg
Thiamine HCl (Vitamin B1)	100 mg
Cyanocobalamin (Vitamin B12)	0.5 mg
pH ¼ adjust to 8.0 with 1 M NAOH or HCl	

Appendix 3 – Bovine serum albumin standard curve



Appendix 4 - Cell disruption method. One Way Analysis of Variance. All Pairwise Multiple Comparison Procedures (Holm-Sidak method): Overall significance level = 0.05

Specie	*Comparison*	*Diff of Means*	*t*	*P*	*P<0,050*
Arthrospira maxima	Control and Homogenizer 30	2.753	0.380	0.712	No
	Control and Homogenizer 90	35.927	4.953	0.003	Yes
	Control and Ultrasound bath	24.149	3.329	0.015	Yes
	Homogenizer 30 and Homogenizer 90	33.174	4.573	0.004	Yes
	Homogenizer 30 and Ultrasound bath	26.901	3.709	0.012	Yes
	Homogenizer 90 and Ultrasound bath	60.076	8.282	<0.001	Yes
Isochrysis galbana	Control and Homogenizer 30	0.656	1.354	0.498	No
	Control and Homogenizer 90	1.995	4.118	0.021	Yes
	Control and Ultrasound bath	1.969	4.066	0.020	Yes
	Homogenizer 30 and Homogenizer 90	1.339	2.764	0.149	No
	Homogenizer 30 and Ultrasound bath	1.313	2.712	0.143	No
	Homogenizer 90 and Ultrasound bath	0.0254	0.0523	0.959	No
Tetraselmis chuii	Control and Homogenizer 30	3.604	9.283	<0.001	Yes
	Control and Homogenizer 90	4.316	11.115	<0.001	Yes
	Control and Ultrasound bath	1.066	2.746	0.061	No
	Homogenizer 30 and Homogenizer 90	0.711	1.831	0.097	No
	Homogenizer 30 and Ultrasound bath	2.538	6.537	<0.001	Yes
	Homogenizer 90 and Ultrasound bath	3.249	8.368	<0.001	Yes

Appendix 5 - Protein content Two Way Analysis of Variance. All Pairwise Multiple Comparison Procedures (Holm-Sidak method): Overall significance level = 0.05

Specie	*Factor*	*Comparison*	*Diff of Means*	*t*	*P*	*P<0,050*
Arthrospira maxima	Photoperiod	18L:6D vs. 24L:0D	34.619	11.867	<0.001	Yes
		18L:6D vs. 12L:12D	21.296	7.300	<0.001	Yes
		12L:12D vs. 24L:0D	13.323	4.567	<0.001	Yes
	Growth phase	Logarithmic vs. Stationary	26.479	11.117	<0.001	Yes
	Growth Phase within 12L:12D	Logarithmic vs. Stationary	19.227	4.661	<0.001	Yes
	Growth Phase within 18L:6D	Logarithmic vs. Stationary	23.414	5.675	<0.001	Yes
	Growth Phase within 24L:0D	Logarithmic vs. Stationary	36.797	8.919	<0.001	Yes
	Photoperiod within Logarithmic growth phase	18L:6D vs. 24L:0D	41.311	10.013	<0.001	Yes
		12L:12D vs. 24L:0D	22.108	5.359	<0.001	Yes
		18L:6D vs. 12L:12D	19.203	4.655	<0.001	Yes
	Photoperiod within Stationary growth phase	18L:6D vs. 24L:0D	27.928	6.769	<0.001	Yes
		18L:6D vs. 12L:12D	23.390	5.669	<0.001	Yes
		12L:12D vs. 24L:0D	4.538	1.100	0.293	No
Isochrysis galbana	Photoperiod	18L:6D vs. 24L:0D	6.772	13.536	<0.001	Yes
		12L:12D vs. 24L:0D	4.470	8.935	<0.001	Yes
		18L:6D vs. 12L:12D	2.302	4.601	<0.001	Yes
	Growth phase	Logarithmic vs. Stationary	1.138	2.786	0.016	Yes
	Growth Phase within 12L:12D	Logarithmic vs. Stationary	3.207	4.533	<0.001	Yes
	Growth Phase within 18L:6D	Logarithmic vs. Stationary	0.339	0.479	0.641	No
	Growth Phase within 24L:0D	Logarithmic vs. Stationary	0.133	0.187	0.854	No
	Photoperiod within Logarithmic growth phase	18L:6D vs. 24L:0D	6.536	9.238	<0.001	Yes
		18L:6D vs. 12L:12D	3.736	5.281	<0.001	Yes
		12L:12D vs. 24L:0D	2.800	3.958	0.002	Yes
		18L:6D vs. 24L:0D	7.008	9.905	<0.001	Yes

	Photoperiod within Stationary growth phase	12L:12D vs. 24L:0D	6.140	8.678	<0.001	Yes
		18L:6D vs. 12L:12D	0.868	1.226	0.244	No
Tetraselmis Chuii	Photoperiod	24L:0D vs. 12L:12D	2.423	8.457	<0.001	Yes
		24L:0D vs. 18L:6D	2.306	8.048	<0.001	Yes
		18L:6D vs. 12L:12D	0.117	0.409	0.690	No
	Growth phase	Logarithmic vs. Stationary	0.259	1.105	0.291	No
	Growth Phase within 12L:12D	Logarithmic vs. Stationary	0.697	1.719	0.111	No
	Growth Phase within 18L:6D	Logarithmic vs. Stationary	0.586	1.446	0.174	No
	Growth Phase within 24L:0D	Logarithmic vs. Stationary	2.058	5.080	<0.001	Yes
	Photoperiod within Logarithmic growth phase	24L:0D vs. 12L:12D	1.045	2.580	0.071	No
		24L:0D vs. 18L:6D	0.983	2.427	0.063	No
		18L:6D vs. 12L:12D	0.0619	0.153	0.881	No
	Photoperiod within Stationary growth phase	24L:0D vs. 12L:12D	3.800	9.380	<0.001	Yes
		24L:0D vs. 18L:6D	3.628	8.954	<0.001	Yes
		18L:6D vs. 12L:12D	0.172	0.426	0.678	No