

# Feedstocks for Second-Generation Biodiesel: Microalgae's Biology and Oil Composition

Patricia I. Leonardi, Cecilia A. Popovich and M. Cecilia Damiani  
*Laboratorio de Estudios Básicos y Biotecnológicos en Algas y Hongos,  
Centro de Recursos Naturales Renovables de la Zona Semiárida  
(CERZOS) – CONICET, Camino La Carrindanga, Bahía Blanca;  
Departamento de Biología, Bioquímica y Farmacia,  
Universidad Nacional del Sur, Bahía Blanca;  
Argentina*

## 1. Introduction

The solar energy is an inexhaustible source, while other energy reserves, like fossil and nuclear fuels, are limited in quantity and are depleted as years go by. Renewable energy is necessary to replace petroleum-derived fuels. The first generation biofuels, which are produced from oil seeds and crops, are a possible alternative, but they are limited in their capacity to provide all the energy demanded in the world. Therefore, new sources for the sustainable production of renewable energy are being looked for. This concern has promoted the keen interest in developing second generation biofuels, which are produced from other feedstocks, such as microalgal oils (Schenk et al., 2008; Mata et al., 2010). Some microalgal species are capable of producing biomass yields containing high percentages of oils (Aaronson et. al., 1980). In addition, microalgal systems can use low value natural resources, such as arid lands and saline water, thus offering the potential for large biomass energy contributions without competing for prime agricultural or forest land. Most microalgae grow photoautotrophically by using solar energy and mainly carbon dioxide as carbon source. Alternatively, some species can grow heterotrophically or mixotrophically using organic compounds as energy and carbon sources (Kitano et al., 1997; Hu & Gao, 2003; Xu et al., 2006; Liang et al., 2009).

Some microalgae are called oleaginous because they synthesize and accumulate substantial amounts of neutral lipids, mainly as triacylglycerol (TAG), under diverse stress conditions (Bigogno et al., 2002; Hu et al., 2008; Gardner et al., 2010; Damiani et al., 2010). TAGs as storage lipids are the best substrate to produce biodiesel (Xu et al., 2006; Schenk et al., 2008). This biofuel is obtained by transesterification of oil or fat with a monohydric alcohol, yielding the corresponding mono-alkyl esters (Knothe, 2005). Since transesterification maintains the relative ratio of fatty acids present in the feedstock (Costa Neto et al., 2000), the profile of the fatty acid methyl esters is a reflection of the feedstock fatty-acid composition (Lang et al. 2001; Ferrari et al. 2005). Biodiesel production from microalgae is technically feasible (Xu et al., 2006; Patil et al., 2008; Francisco et al., 2010), but for an effective use of this renewable resource as biofuel, it is necessary to be able to modify microalgal growth conditions in order to obtain high biomass productivity and the desired lipid quantity and quality. Those interested in

microalgal biomass production and lipid productivity are referred to recent reviews by Griffiths & Harrison (2009), Rodolfi et al. (2009) and Pruvost et al. (2011). In addition, it is important to have information about the various fatty-acid profiles of diverse microalga oils in order to evaluate their suitability as feedstocks for fuel-conversion processes (Gouveia & Oliveira, 2009; Damiani et al., 2010; Sobczuk & Chisti, 2010; Francisco et al., 2010; Popovich et al., 2011). Unlike land plants, oils of some microalga species have a significant amount of polyunsaturated fatty acids with four and more double bonds (Belarbi et al., 2000; Harwood & Guschina, 2009), which are valuable oils. This is a feature that limits the microalgal species that may be used for biodiesel production.

This chapter aims to provide an overview of the current status of research on microalgal feedstocks as regards biodiesel production. Since there are many species of microalgae with varied biological characteristics and lipid composition, a diversity of approaches for biodiesel production have been analysed. In this review the following relevant topics will be considered: 1) diagnostic characteristics of some microalgal main groups, such as Chlorophyceae, Eustigmatophyceae and Bacillariophyceae classes 2) triggering of lipid production, and 3) oil composition, i.e. lipid fractions, content of each lipid class and fatty acid composition of each fraction. In this context, how the latter might affect the biodiesel quality will be discussed. We hope this information provides a framework for future screening of oleaginous microalgae employed as feedstock for biofuel production.

## 2. Diagnostic characteristics of some main microalgal groups

In recent years plenty of research has been focused on promising microalgal species aiming at the development of sustainable, commercially feasible and economic processes for biodiesel production (Rodolfi et al., 2009; Mandal & Mallick, 2009; Mata et al., 2010). The first step for these studies includes the species selection, essential for a reliable analysis. This step requires knowledge of diagnostic characteristics of different microalgal groups to achieve correct species identification. Thus, contradictory or erroneous information about fatty acid profiles and other important features as reported by Zhukova & Aizdaicher (1995) and Goldberg & Boussiba (2011) can be avoided.

Algae are (with numerous exceptions) aquatic organisms that (with frequent exceptions) are photosynthetic and oxygenic autotrophs. They are typically smaller, except for the seaweeds, and less structurally complex than land plants (Graham & Wilcox, 2000). Microscopic algae are commonly named microalgae that live as solitary cells or as colonies. They vary a great deal with respect to their cell sizes, pigments, storage products, cell wall compositions and life cycles (van den Hoek, 1995). This highly specialised group of microorganisms has the potential to adapt to diverse habitats as well as the ability to efficiently modify its lipid metabolism in response to changes in environmental conditions (Guschina & Harwood, 2006). Oleaginous microalgae can be found in diverse taxonomic groups and their total lipid content and fatty acid composition may vary noticeably among individual species or strains within and between taxonomic groups (Hu et al., 2008).

According to van den Hoek (1995) algae can be classified in ten major algal groups (Divisions). A number of characteristics has been traditionally used to distinguish these algal groups. The most prominent features are the types of photosynthetic pigments, storage reserves and the nature of the cell covering. One of the greatest groups is represented by Chlorophyta Division, commonly known as green algae because they look bright grass green. This colour is because the chlorophylls are usually unmasked by large amounts of

accessory pigments. However, chlorophytes may not always have green colouring. Widely encountered examples include the flagellates *Haematococcus* and *Dunaliella*, whose deep red to purple colouring is due to astaxanthin and  $\beta$ -carotene pigments, respectively (Borowitzka & Borowitzka 1988; Boussiba & Vonshak, 1991; Ben-Amotz, 1995). Features that are common to nearly all green algae include: chloroplasts enclosed by a double membrane, chlorophylls a and b, and starch storage ( $\alpha$ -1,4-linked polyglucans) inside the chloroplasts. The light-harvesting systems of green algae resemble those of land plants. Hence, they are relatively well characterized (Larkum & Howe, 1997). In addition to chlorophylls and proteins, light-harvesting complexes also include carotenoids (Demming-Adams & Adams, 1992).

Ultrastructural and molecular evidences obtained within the past few decades have demonstrated the existence of several distinct green algal lineages (classes). Each lineage is characterized by specific differences in cellular features and primary habitat (Graham & Wilcox, 2000).

In Chlorophyta Division, Chlorophyceae Class represents the largest taxonomic group where oleaginous candidates have been identified (Hu et al., 2008). The Chlorophyceae Class includes some very familiar green algal genera. For example, *Chlamydomonas* is an important laboratory model system, while *Dunaliella*, *Haematococcus* and *Chlorella* can be valuable in production of industrially useful products (Borowitzka, 1992, 1995, 1997; Spolaore et al., 2006). Members of this class may occur as flagellate or non-flagellate unicells, either as individuals or colonies. Flagellate organisms inhabit fresh (or in a few cases, brackish or marine) waters. Non-flagellate forms occur in freshwaters or on soils. The genera differ in their types of asexual reproductive cells, i.e. formation of one or more zoospores, aplanospores or autospores within individual parental vegetative cells (Bold & Wynne, 1985). The chemical composition of cell walls varies greatly within the class. However, the cell wall of the most oleaginous species, like *Chlorella*, *Scenedesmus* and *Haematococcus* cysts, consists of fibrillar polysaccharides and an outer coat of algaenan substance (Pickett-Heaps, 1975; Allard & Templier, 2000; Damiani et al., 2006). Algaenan walls are considered to be the single most decay-resistant biopolymer (Gelin et al., 1997), together with land plants' sporopollenin. It is noteworthy that this biopolymer's high resistance hinders oil extraction in these microalgae. Oleaginous green microalgae vary widely in cell sizes, ranging from 3 to 75  $\mu$ m.

Heterokontophyta Division includes nine classes (van den Hoek, 1995). In this review some oleaginous species included in Eustigmatophyceae and Bacillariophyceae classes will be reviewed. The chloroplasts of all members of Heterokontophyta are enclosed by four membranes instead of two, as found in green algae and land plant chloroplasts (van den Hoek, 1995; Bozarth et al., 2009).

The Eustigmatophyceae Class includes small (2-32  $\mu$ m) unicellular, coccoid microalgae. Cells have one or more yellow-green chloroplasts that only contain chlorophyll a. Violaxanthin is the major accessory pigment, which is also the main pigment involved in light harvesting (Whittle & Casselton, 1975). The storage product's chemical structure is unknown. Polysaccharide walls were indicated by van den Hoek (1995). Non-hydrolysable macromolecular constituents, i.e. algaenans, were also isolated from two species (Gelin et al., 1996, 1997). Asexual reproduction occurs by autospores production or in some cases by zoospores. Because of the similarities in morphology, reproduction, cell colour and chloroplast structure, eustigmatophyceans are commonly mistaken for coccoid green microalgae at the light-microscopy level. Its identification requires cell examination by transmission electron microscopy and/or pigment analysis by chromatography (Graham & Wilcox, 2000). There are about seven genera, most occurring in freshwater or in soil, but

there are also some marine forms (van den Hoek, 1995). The oleaginous microalga *Nannochloropsis* is a marine coccoid form that resembles *Chlorella* (Santos & Leedale, 1995). This genus does not produce zoospores.

The Bacillariophyceae Class includes diatoms. They occur only as single cells or chains of cells. These microalgae are ubiquitous, occurring in marine and freshwaters, where they may be principally planktonic (they live suspended or growing in a fluid environment) or benthic (they live in the lowest level of a water body, often attached to the substrate bottom). There are diatoms in an immense variety of shapes. Circular, triangular, and modified square shapes are common. These diatoms are known as centric. Other diatoms, especially benthic forms, display varying types of bilateral symmetry and are termed pennate diatoms. Cell sizes range from less than 15  $\mu\text{m}$  to 1 mm in length. Some species have been indicated as oleaginous microalgae (McGinnis, et al., 1997; Hu et al., 2008; Matsumoto et al., 2010; Yu et al., 2009; Popovich et al., 2011). The chloroplasts are usually golden-brown, because the chlorophylls a and c are masked by the accessory pigment fucoxanthin. The reserve polysaccharide is chrysolaninaran, a  $\beta$ -1,3 linked glucan that is formed outside the chloroplast. They also store carbon in the form of natural oils (Bozarth et al., 2009). The cell wall is siliceous and is termed frustule (van den Hoek, 1995). Diatoms normally reproduce asexually by cell division. In terms of contributions to global primary productivity, diatoms are among the most important aquatic photosynthesizers. They dominate the phytoplankton of the oceans and recently circulated in lake waters.

### 3. Triggering of lipid production and oil composition

As it is usual in photosynthetic cells, microalgae contain polar and neutral lipids. Polar lipids include glycolipids and phospholipids. The glycolipids (monogalactosyl-, digalactosyl- and sulphoquinovosyldiacylglycerol) and phosphatidylglycerol have been attributed to chloroplast membranes, while the phospholipids (phosphatidylcholine and phosphatidylethanolamine) are considered more characteristic of extrachloroplastic membranes. Neutral lipid fraction is very diverse and compounds as different as sterols, free-fatty acids and acyl lipids (mono-, di- and triacylglycerols) can be found (Harwood & Jones, 1989; Berge, et al., 1995). TAG accumulation specifically occurs in oil droplets distributed in the cytoplasm. Nile red fluorescence (Figs 1- 2) is a technique that has been used in some microalgae as a rapid screening method to detect TAG presence (Damiani et al. 2010; Popovich et al., 2011), as well as to determine the relative neutral lipid content (McGinnis et al., 1997; Yu et al., 2009).

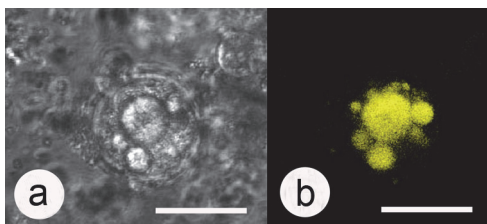


Fig. 1. Light micrographs of a *Haematococcus pluvialis* cyst. (a) Phase contrast microscopy. (b) Epifluorescent microscopy. Numerous yellow-gold neutral lipid droplets under stress condition (high light intensity) after 11-day growth are shown. Scale bars = 20  $\mu\text{m}$ . Source: Damiani et al. (2010).

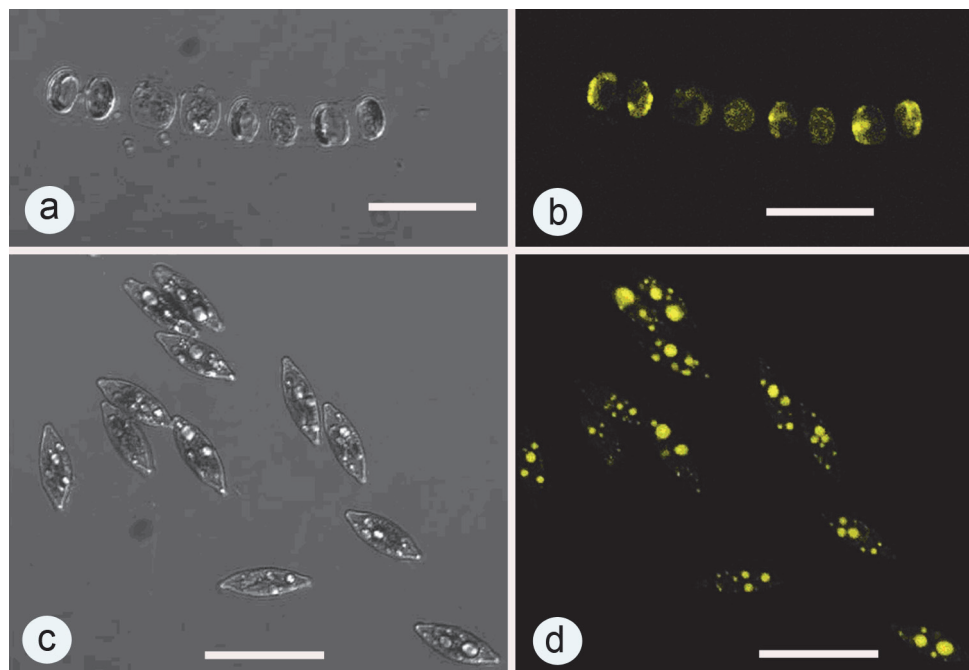


Fig. 2. Light micrographs of *Skeletonema costatum* (a, b) and *Navicula gregaria* (c, d). Phase contrast microscopy (a, c). Epifluorescent microscopy (b, d). Yellow-gold neutral lipid droplets after 15-day growth are shown. Lipid droplets are in a marginal position in *S. costatum*, while multiple lipid droplets homogenously distributed in the cytoplasm appear in *N. gregaria*. Scale bars = 25  $\mu\text{m}$ . Source: Popovich et al. (2011).

Even though it is known that the intrinsic ability to produce large quantities of lipids is species- and strain specific (Hu et al., 2008), the energy storage capacity can be maximized by controlling the organism's metabolism. Tuning the microalgae's metabolism can lead to enhanced production of energy-rich compounds, such as fatty acids and glycerol. A single microalgal species may show remarkable variation in its metabolism, according to the conditions to which it is exposed, such as carbon dioxide supply, light intensity, temperature, nutrient concentrations and salinity (Shifrin & Chisholm, 1981; Roessler, 1990). Synthesis and accumulation of large amounts of TAGs accompanied by considerable alterations in lipid and fatty acid composition occur in the cell when oleaginous microalgae are placed under stress conditions imposed by chemical or physical environmental stimuli (Hu et al., 2008). Nutrient starvation (as nitrogen, phosphorus, and silicate), salinity and growth-medium pH are the major chemical stimuli employed, whereas light intensity and temperature are frequently used as physical stimuli (Hu et al., 2008). In addition, it is well known that certain levels of carbon dioxide supplementation in microalgal cultures can increase lipid content, especially TAG fraction (Gordillo et al., 1998; Huntley & Redalje, 2007; Tang et al., 2010; Francisco et al., 2010). Besides, the lipid content and fatty acid composition also depend on the age of culture and different life-cycle stages (Siron et al., 1989; López Alonso et al., 2000; Spolaore et al., 2006; Hu et al., 2008).

From a chemical point of view, oils from different sources have different fatty acid compositions. The fatty acids vary in their carbon chain length and in their number of unsaturated bonds. The fatty acids in land plant oils are very well studied and stearic acid (18:0), palmitic acid (16:0), oleic acid (18:1n9c), linoleic acid (18:2n6c) and linolenic acid (18:3n3) are commonly found (Durrett et al., 2008; Singh & Singh, 2010). As regards microalgae, there is a greater diversity of fatty-acid profiles of oils among different classes. Moreover, their information is very limited at present and most of the analyses of fatty acid composition have used total lipid content rather than the examination of individual lipid fractions (Hu et al., 2008). As previously indicated, the fatty acid composition can widely vary both quantitatively and qualitatively with the microalgae's physiological status and the environmental conditions (Hu et al., 2008; Rodolfi et al., 2009), making it difficult to compare microalgal species/strains across experimental conditions (Molina Grima et al., 1994).

Fatty-acid composition was used to predict the quality of fatty acid methyl esters of oils for use as biodiesel (Knothe, 2005). The most important characteristics include the ignition quality (i.e. cetane number), cold-flow properties and oxidative stability. For example, saturated oils produce a biodiesel with superior oxidative stability and a higher cetane number, but rather poor low-temperature properties. On the other hand, the biodiesel produced from feedstocks rich in polyunsaturated fatty acids (PUFAs) has good cold-flow properties. However, these fatty acids are particularly susceptible to oxidation (Knothe, 2005; Hu et al., 2008). Among PUFAs, some fatty acids should be taken into account. European standard EN 14214 limits linolenic acid's methyl ester for vehicle use to 12% and methyl esters with four and more double bonds to a maximum of 1%, (CEN EN-14214, 2003).

### 3.1 Chlorophyceae class

#### 3.1.1 Triggering of lipid production

Nitrogen is the most commonly reported nutritional-limiting factor that triggers total lipid accumulation, mainly TAG in green microalgae (Hu et al., 2008; Pruvost et al., 2009, 2011). When nitrogen deprivation is imposed upon a culture exposed to suitable irradiances, photosynthesis continues, albeit at a reduced rate, and the flow of fixed carbon is diverted from protein to either lipid or carbohydrate synthesis (Shifrin & Chisholm, 1981). Some oleaginous microalgae seem to have the capacity for synthesizing *de novo* lipids when grown under nitrogen-deficiency, channelling the excess of carbon and energy into storage lipids, mainly TAGs (Shifrin & Chisholm, 1981; Rodolfi et al., 2009). The effect of nitrogen deficiency has been demonstrated a long time ago in numerous chlorophycean (Iwamoto et al., 1955; Fogg, 1959; Zhukova et al., 1969; Thompson, 1996). Moreover, a study of fifteen chlorophycean species by Shifrin & Chisholm (1981) showed that lipid content doubled or tripled when cells from exponential-phase growth were transferred to nitrogen-free conditions. In this way total lipid fractions of 30% to 50% dry weight (dw) were measured. In addition, at low nitrogen level, *Chlorella vulgaris* and *Scenedesmus obliquus* contained high percentage of total lipids (45% of the biomass); more than 70% of these were neutral lipids (Piorreck et al., 1984). Recently, an increase of total lipid content (from 12.7 to 43% dw) was obtained when stationary phase cultures of *S. obliquus* were transferred to media deficient in nitrate for 7 days (Mandal & Mallick, 2009). Higher lipid content values were reported under nitrogen-deficient conditions in *Chlorella emersonii* (63% dw) and *C. minutissima* (56% dw), but no changes were observed in *C. sorokiniana* under the same culture conditions

(Illman et al., 2000). Up to now, lipid contents as high as 85%, as reported earlier for *Chlorella pyrenoidosa* and *C. ellipsoidea* (Spoehr & Milner, 1949; Iwamoto et al., 1955), have been reported in no other chlorophycean species at all. It is important to remark that lipid content and lipid composition also vary according to the exposition time at nitrogen starvation. For example, a long time of nitrogen starvation (17 days) resulted in higher lipids and TAG accumulation (74%) than that obtained after 7 days of starvation in *Chlorella vulgaris*. In addition, lipid composition gradually changed from free fatty acid-rich lipid to TAG-rich lipids (Widjaja et al. 2009).

*Chlorella protothecoides* is a microalga that can grow photoautotrophically or heterotrophically under different culture conditions. Xu et al. (2006) reported that with the addition of organic carbon source (glucose) of the medium and the decrease of the inorganic nitrogen source, the heterotrophic *C. protothecoides* reached up to 55.2% dw lipid content, which was about four times higher than that in photoautotrophic cells (Miao & Wu, 2004).

In recent years special attention has also been given to *Neochloris oleoabundans* for its ability to accumulate high lipid content, especially TAGs. Nitrogen-starved cultures of this species showed 36-54% dw total lipid content, where more than 80% of these were TAGs (Tornabene et al., 1983). Similarly, 56% dw of lipid content after 6 days of nitrogen starvation was reported in the same species by Gouveia et al. (2009).

Moreover, formation of chloroplastic and extraplastidial lipid bodies containing both TAGs and carotenoids under nitrogen starvation was reported in *Dunaliella bardawil*, *Chlorella zofingiensis* and *Haematococcus pluvialis* (Thompson, 1996; Boussiba, 2000). In the latter species nitrogen starvation induced a sharp increase in TAG content (Zhekisheva et al., 2002).

There are few studies in green microalgae regarding phosphorus deficiency. According to Reitan et al. (1994), phosphorus deprivation results in decreased lipid content in *Nannochloris atomus*. However, phosphorus deprivation leads to a significant increase in the total fatty acid content of *Dunaliella tertiolecta* (Siron et al., 1989). In addition, a significant increase in lipid content (29.5% dw) was also obtained in *Scenedesmus obliquus* when stationary phase cultures were transferred to medium deficient in phosphate for 3 days (Mandal & Mallick, 2009).

On the other hand, Liu et al. (2008) reported that high iron concentration stimulated lipid storage in *Chlorella vulgaris*. Lipid content increased up to 56.6% dw when cells in late-exponential growth phase were re-inoculated into new medium containing  $1.2 \times 10^{-5}$  mol L<sup>-1</sup> iron concentration.

Regarding physical stimuli, it is extensively known that low light intensity induces the formation of polar lipids, whereas high light intensity decreases total polar lipid content with a concomitant increase in the amount of neutral lipids (Hu et al., 2008 and cites therein). In *Haematococcus pluvialis* high light intensity (300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) doubled the total lipid content (34.85% dw). In addition, the neutral lipid fraction also increased about 2-fold compared to the control (Table 1) (Damiani et al., 2010).

The effects of temperature on the total lipid content have only been reported for a few species in green microalgae; though a general trend cannot be established. A decrease in the growth temperature from 30 to 25°C led to an increase in the lipid content of *Chlorella vulgaris* from 5.9 to 14.7 % dw (Converti et al., 2009). However, no significant change in the lipid content was observed in *Chlorella sorokiniana* grown at various temperatures (14°, 22° and 38°C) (Patterson, 1970). On the other hand, maximum lipid content (56% dw) was

<i>H. pluvialis</i>	Total Lipids (% dw)	Neutral Lipids (% dw)	Glycolipids (% dw)	Phospholipids (% dw)
Control	15.61 ± 1.46 (b)	9.20 ± 0.67 (1)	3.70 ± 0.38(3)	1.87 ± 0.05(4)
High light intensity	34.85 ± 0.78 (c)	19.80 ± 0.14 (2)	7.85 ± 1.77(3)	9.50 ± 0.00 (3)

Table 1. Lipid content (percentage of dry weight biomass = % dw) and fractions (neutral, glycolipid and phospholipid) in *Haematococcus pluvialis* under control (90  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and high light intensity (300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) culture conditions. Values are means  $\pm$  standard deviations of three replicates. Identical superscripts indicate non-significant ( $\alpha = 0.05$ ) differences. Adapted from Damiani et al. (2010).

reported in *Neochloris oleoabundans* grown under nitrogen starvation and 30°C. Temperature has a major effect on the fatty acid composition of microalgae (Roessler, 1990; Hu et al., 2008). The general trend towards an increase in fatty acid unsaturation with decreasing temperature observed in land plants and other organisms (Raison, 1986) also occurs in many microalgae. Patterson (1970) reported a greater degree of fatty acid unsaturation in *Chlorella sorokiniana* cells grown at 22°C relative to cells grown at 38°C, although cells grown at 14°C had an intermediate degree of unsaturation. On the other hand, Widjaja et al. (2009) have noticed that the drying temperature during lipid extraction from algal biomass not only affects lipid composition but also lipid content. For example, heating at higher than 60°C resulted in a decrease of total lipid content and TAG content in *Chlorella vulgaris*.

### 3.1.2 Lipid composition

Although variations have been reported in the fatty acid composition of some representatives of Chlorophyceae Class, in general the most abundant fatty acids saturated and mono-unsaturated are palmitic acid (C16:0) and oleic acid (C18:1n9c), respectively. In turn, the major polyunsaturated fatty acids found in green algae are linoleic acid (C18:2n6c) and linolenic acid (C18:3n3). PUFAs above C18 are not usually present as majority fatty acids (Hu et al., 2008; Gouveia & Oliveira 2009; Gouveia et al., 2009; Damiani et al., 2010; Ho et al., 2010). Some examples of fatty acids profiles of green microalgae are shown in Table 2. As to saturated fatty acids (SFAs), a significant percentage (25-17%) of palmitic acid was indicated in *Chlorella vulgaris* INETI 58, *Scenedesmus obliquus* FCTU Coimbra, *Neochloris oleoabundans* UTEX # 1185 and *Dunaliella tertiolecta* (IPIMAR) by Gouveia & Oliveira (2009). Similarly, high palmitic acid contents were reported in *Haematococcus pluvialis* (19%) (Leonardi et al., 2008) and *S. obliquus* CNW-N (15%) (Ho et al., 2010). Two strains of *S. obliquus* are shown in Table 2; however, high content of stearic acid (15 %) has only been found in the strain CNW-N. This SFA is commonly found in land plant oils (Ramos et al., 2009; Singh & Singh, 2010). The monounsaturated oleic acid was well represented in all species studied, showing *D. tertiolecta* the lowest percentage. Among PUFAs, linoleic acid was also well represented in all species. Regarding linolenic acid, except for the oils extracted from *S. obliquus* FCTU Coimbra and *S. obliquus* CNW-N, the other oils showed contents above 12%. The length of dominant fatty acids in all species was intermediate with a maximum of 18 carbons and the maximum degree of chain unsaturation was three (Table 2).

As to stress conditions, a marked increase in the level of SFAs and MUFAs with a concomitant decrease in PUFAs is usually associated with nitrogen deficiency (Piorreck et al., 1984). For example, *Neochloris oleoabundans* UTEX # 1185 grown under nitrogen starvation showed oleic acid as the main fatty acid present, followed by palmitic and stearic



Fatty acids (% of total fatty acids)	<i>Chlorella vulgaris</i> (1)	<i>Scenedesmus obliquus</i> FCTU (1)	<i>Scenedesmus obliquus</i> CNW-N (2)	<i>Dunaliella tertiolecta</i> (1)	<i>Neochloris oleoabundans</i> (1)	<i>Haematococcus pluvialis</i> (3)
C12:0	n.d.	n.d.	0.99	n.d.	n.d.	0.07
C13:0	n.d.	n.d.	1.43	n.d.	n.d.	n.d.
C14:0	3.07	1.48	0.91	0.47	0.43	0.60
C15:0	n.d.	n.d.		n.d.	n.d.	0,17
C16:0	25.7	21.78	15.05	17.70	19.35	18,88
C17:0	n.d.	n.d.	2.36	n.d.	n.d.	0,25
C18:0	0.63	0.45	17.16	n.d.	0.98	2,93
C20:0	0.09	n.d.		n.d.	n.d.	0,35
C22:0	n.d.	n.d.	0.33	n.d.	n.d.	0.18
C14:1	n.d.	n.d.	0.36	n.d.	n.d.	n.d.
C16:1	5.25	5.95	2.30	0.88	1.85	0,52
C17:1	n.d.	n.d.	0.37	n.d.	n.d.	n.d.
C18:1	12.64	17.93	15.55	4.87	20.29	
C18:1 n9c						15,98
C18:1 n9t						0,17
C20:1	0.93	n.d.	0.75	n.d.	n.d.	0,09
C16:2	n.d.	3.96		3.03	1.74	n.d.
C16:3	1.27	0.68		1.24	0.96	n.d.
C16:4	4.06	0.43		10.56	7.24	n.d.
C18:2	7.19	21.74	13.39	12.37	12.99	
C18:2 n6c						24,82
C18:2 n6t						7,25
C18:3	19.05	3.76	3.97	30.19	17.43	
C18:3 n3						16,06
C18:3 n6						1.40
C18:4	n.d.	0.21		n.d.	2.10	n.d.
C20:2	n.d.	n.d.		n.d.	n.d.	0.25
C20:3	0.83	n.d.		n.d.	n.d.	n.d.
C20:4	0.23	n.d.		n.d.	n.d.	1.25
C20:5	0.46	n.d.		n.d.	n.d.	0.50
SFA (%)	28.56	23.71	37.9*	18.17	20.76	23,59
MUFA (%)	18.82*	23.88*	19.33*	5.75*	22.14*	16,76
PUFA (%)	33.09*	30.78*	17.36*	57.39*	42.46*	51,73
Unsaturated	51.91	54.66		63.14	64.60	68.49
Source	Gouveia & Oliveira 2009	Gouveia & Oliveira 2009	Ho et al. 2010	Gouveia & Oliveira 2009	Gouveia & Oliveira 2009	Leonardi et al. 2008

Table 2. Fatty acid profiles of green microalgae. (1) *Chlorella vulgaris* INETI 58, *Scenedesmus obliquus* FCTU Coimbra, *Neochloris oleoabundans* UTEX # 1185 and *Dunaliella tertiolecta* IPIMAR cultivated in an appropriate growth medium with bubbling air, under 150  $\mu\text{mol E m}^{-2}\text{s}^{-1}$  of light intensity and finally, outdoors during 4 months. (2) *Scenedesmus obliquus* CNW-N cultivated in a nutrient-rich medium with 10%  $\text{CO}_2$ , under 60  $\mu\text{mol E m}^{-2}\text{s}^{-1}$  of light intensity. (3) *Haematococcus pluvialis* cultivated in Basal Bold medium with bubbling air, under 150  $\mu\text{mol E m}^{-2}\text{s}^{-1}$  of light intensity. \*In order to compare total SFAs, MUFAs and PUFAs, the sums were performed according to published data.

acids (Gouveia et al., 2009). In contrast, linolenic acid proportion was below 12%, which was lower than the content (17.43%) reported for the same strain grown under sufficient nitrogen (Gouveia et al., 2009; Gouveia & Oliveira, 2009). In a similar way, Mendoza Guzmán et al. (2011) reported in *Dunaliella salina* ITC-5.003 a significant decrease in relative PUFA content and unsaturation index in cultures exposed to nitrogen starvation when compared to control conditions (Table 3). This variation occurred primarily related to variations in MUFA and PUFA contents. The MUFA content increased in the nitrogen-starved cultures, while PUFA had a higher relative content in control cultures. Conversely, in *Chlorella pyrenoidosa* BNA-10-013 no significant variation was observed in the content of fatty acids in control and nitrogen-starvation conditions (Table 3). This species' culture probably required a more prolonged exposure to nitrogen deficiency in order to be able to observe significant variations in the fatty acid composition (Mendoza Guzmán et al., 2011).

Fatty acids (% of total fatty acids)	<i>Dunaliella salina</i> Control exponential growth phase	<i>Dunaliella salina</i> Nitrogen-starved	<i>Chlorella</i> <i>pyramimosa</i> Control exponential growth phase	<i>Chlorella</i> <i>pyramimosa</i> Nitrogen-starved
C13:0	0.68	0.68		2.95
C14:0	0.92	1.08	0.85	0.85
C16:0	24.99	34.33	20.54	21.13
C18:0	15.92	12.60	0.24	0.38
C14:1	0.06	0.06	0.05	0.13
C16:1	3.03	2.62	3.29	2.48
C18:1 n9c	3.59	15.52	2.30	3.45
C18:1 n9t	3.22	1.95	0.90	0.80
C16:2	0.08	1.20	7.59	10.39
C16:3	0.52	0.62		
C16:4	2.17	1.21	19.36	13.00
C18:2 n6	8.59	6.64	17.75	18.10
C18:3 n3	34.86	20.81	26.36	24.91
C18:3 n6			1.37	0.68
C22:2			0.27	1.43
SFA (%)	42.51*	48.69*	21.63*	25.31*
MUFA (%)	9.9*	20.15*	6.54*	6.86*
PUFA (%)	46.22*	30.48*	72.70*	68.51*

Table 3. Fatty-acid profiles of *Dunaliella salina* ITC-5.003 and *Chlorella pyramimosa* BNA-10-013 cultivated under two experimental conditions. Control at exponential growth-phase and nitrogen starved. Adapted from Mendoza Guzmán et al. (2011). \*In order to compare total SFAs, MUFAs and PUFAs, the sums were performed according to published data.

High content of TAGs (more than 70% of the total lipids) containing mainly palmitic and oleic acids was reported in *Chlorella vulgaris* and *Scenedesmus obliquus* grown under low nitrogen (Piorreck et al., 1984). A detailed TAG-composition study under nitrogen starvation was performed by Zhekisheva et al. (2002) in a German strain of *Haematococcus pluvialis*. After 1 day of nitrogen starvation, the proportion of oleic acid increased sharply to 24.1%, compared with 5% in the control cells, while PUFA content decreased. In the following days, there was a further decrease in the unsaturation level that was expressed by

an increase in the proportion of linoleic acid at the expense of PUFAs (C16:4 and C18:3). On the other hand, Zhekisheva et al. (2002) found in *H. pluvialis* a response of the TAG's fatty acid composition to high light intensity (350  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), which was similar to the behaviour observed under nitrogen starvation. The most outstanding change was noted in the proportion of oleic acid that increased from 5.2 % in the control to 19.8% after 1.5 days and thereafter decreased. Recently, Damiani et al. (2010) studied the fatty acid composition of neutral fraction in an Argentinian strain of *Haematococcus pluvialis* under control and high

Fatty acids (%)	Neutral lipids	
	Control	High light intensity
C6:0	nd	1.33
C8:0	nd	0.27
C10:0	tr	0.23
C12:0	0.21	0.30
C13:0	nd	nd
C14:0	1.25	1.35
C14:1	tr	nd
C15:0	0.19	0.27
C15:1	nd	nd
C16:0	22.49	18.87
C16:1	0.64	0.58
C17:0	0.19	0.32
C17:1	tr	tr
C18:0	3.15	7.07
C18:1n9t	tr	0.67
C18:1n9c	19.36	18.25
C18:2n6t	6.67	5.37
C18:2n6c	20.23	22.06
C20:0	0.20	0.32
C18:3n6	0.86	1.02
C20:1	0.13	0.23
C18:3n3	16.18	12.01
C21:0	tr	tr
C20:2	0.32	1.15
C22:0	0.18	0.31
C22:1n9	tr	0.17
C20:4n6	0.89	1.21
C24:0	tr	0.20
C20:5n3	0.57	0.48
C22:5n3	nd	nd
SFA %	27.81 $\pm$ 0.42 <sup>(a)</sup>	30.36 $\pm$ 1.19 <sup>(b)</sup>
MUFA %	20.07 $\pm$ 0.06 <sup>(g)</sup>	19.91 $\pm$ 0.12 <sup>(g)</sup>
PUFA %	45.80 $\pm$ 0.18 <sup>(k)</sup>	43.15 $\pm$ 0.68 <sup>(l)</sup>

Table 4. Neutral fraction fatty-acid profiles (percentage of total fatty acids) of *Haematococcus pluvialis* under control and high light intensity culture conditions. Identical superscripts indicate non significant differences in the values ( $\alpha = 0.05$ ). Values are the means of four replicates. tr: trace, nd: no detected. Source: Damiani et al. (2010).

light intensity ( $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) conditions during 14 days of growth. The fatty acid profile was similar under both culture conditions, and the major components were palmitic, stearic, oleic, linoleic, linolenic and linolelaidic acids (Table 4). The percentage of SFAs was significantly higher in cultures grown under high light intensity, when compared to the control. The palmitic acid content slightly declined under stress condition. In contrast, the stearic acid's relative content increased. The MUFAs showed no significant differences between control and stress conditions. In addition, PUFAs presented a significant decrease in the stress condition compared to the control, with a concomitant decrease in linolenic acid.

Even though the concept of using microalgae as a source of biofuel is old (Sheehan et al., 1998), at present there are few studies related to lipid valorisation as biodiesel using chlorophycean and most of the microalgae tested are species currently cultivated for aquaculture or for human nutritional products. Some studies concerning to the potential use of green algae for biodiesel include the analysis of the total fatty acid quality- for example, *Chlorella vulgaris* INETI 58, *Scenedesmus obliquus* FCTU Coimbra, *Dunaliella tertiolecta* IPIMAR (Gouveia & Oliveira, 2009), *Neochloris oleoabundans* UTEX # 118 (Gouveia & Oliveira, 2009; Gouveia et al., 2009) and *Scenedesmus obliquus* CNW-N (Ho et al., 2010), while only one analyses the neutral lipid fraction: *Haematococcus pluvialis* (Damiani et al., 2010). On the other hand, some studies are focused on the analysis of fatty acid methyl esters (FAMEs)- for example, *Chorella protothecoides* (Xu et al. 2006); *Chorella vulgaris* CCAP 211 (Converti et al. 2009), *Scenedesmus obliquus* SAG 276-3a (Mandal & Mallick, 2009); *Chlorella vulgaris* UTCC90, *Scenedesmus obliquus* UTCC 5, *Dunaliella tertiolecta* UTCC 420 (Francisco et al., 2010) and *Chlorella* sp. (Rasoul-Amini et al., 2011).

Recently, Chinnasamy et al. (2010) evaluated the feasibility of producing biodiesel from microalgal consortium of fifteen isolates (consisting of chlorophycean and cyanobacterial species) grown in treated wastewater.

## 3.2 Eustigmatophyceae class

### 3.2.1 Oil composition and triggering of lipid production

The Eustigmatophyceae Class is characterized by a typical fatty acid composition that includes four abundant fatty acids: palmitic acid, palmitoleic acid (16:1n7), arachidonic acid (ARA, 20:4n6) and eicosapentaenoic acid (EPA, 20:5n3) (Volkman et al., 1993; Goldberg & Boussiba, 2011). In contrast with green algae, fatty acids with C18 chain length are present as relatively minor components. Regarding lipid fractions, there are only few studies related to TAG composition in *Nannochloropsis* (Sukenic et al., 1989; Hodgson et al., 1991). *Nannochloropsis* spp. are widely used in aquaculture and have been investigated as a potential EPA source (Sukenic, 1999). EPA is an essential fatty acid currently sourced from fish oil. No other sources are commercially available. In recent years special interest has developed in this microalga as biodiesel feedstock due to its high lipid content (Rodolfi et al., 2009) and its composition (Gouveia & Oliveira, 2009). Biodiesel was specifically obtained from two *N. oculata* strains by Umdu et al. (2009) and Converti et al. (2009) and *Nannochloropsis* sp. by Koberg et al. (2011). However, for biodiesel production approaches more related to culture conditions tending to a PUFA decrease in *Nannochloropsis* spp. should be considered. Nitrogen deprivation is a triggering factor in *Nannochloropsis* spp. The strain *Nannochloropsis* sp. Q2 grown under nitrogen deficiency was characterized by high lipid content (from 24% to 55%) and the principal lipid fraction synthesized was TAG (79%) rich in SFAs (Suen et al., 1987). In

addition, lipid content of *Nannochloropsis* sp. PP983 that was grown in low nitrogen level increased up to 62% dw (Hu & Gao, 2006). Similarly, *Nannochloropsis* sp. F&M-M24 growing in photobioreactors reached 60% lipid content after nitrogen starvation (Rodolfi et al., 2009). The effect of irradiance on lipid content has been reported for many strains of *Nannochloropsis* growing under different culture conditions and modes. For example, Greek

Fatty acids (% of total fatty acids)	<i>Nannochloropsis</i> sp. Irradiance $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$		<i>Nannochloropsis</i> sp. MFD-2 Temperature $^{\circ}\text{C}$			
	40	480	20	25	30	35
C14:0	2.14	6.35	5.60	6.45	7.75	10.85
C15:0			0.40	0.40	0.55	0.50
C16:0	25.88	40.44	26.70	20.85	24.75	42.55
C17:0			0.40	0.40	0.35	0.20
C18:0	4.00	0.84	5.50	0.40	0.55	1.15
C19:0			0.30	0.30	0.15	n.d.
C20:0			n.d.	n.d.	n.d.	0.20
C14:1n9			n.d.	n.d.	n.d.	0.25
C16:1 n7	18.25	36.15	30.05	30.35	27.20	24.90
C16:1n9	1.06	0.46				
C18:1 n9	3.05	3.33	n.d.	3.75	2.65	3.80
C18:1n7	9.92	0.35				
C20:1 n9			n.d.	n.d.	0.55	n.d.
C22:1 n9			n.d.	0.35	n.d.	n.d.
C18:2 n6	1.88	0.65	1.40	3.05	3.35	1.60
C18:3 n6	1.76	0.42	n.d.	n.d.	n.d.	0.30
C18:3 n3			1.35	0.90	n.d.	n.d.
C20:3n3			5.50	5.15	3.80	3.00
C20:3n6	n.d.	0.37				
C20:4 n6	3.19	2.31				
C20:5 n3	28.90	8.02	18.90	23.70	19.50	7.40
C22:1 n9			n.d.	0.35	n.d.	n.d.
C22:3 n3			n.d.	0.30	n.d.	n.d.
C22:6n3			n.d.	0.40	n.d.	n.d.
SFA (%)	32.57	48.78	38.90 *	28.80 *	34.10 *	55.45 *
MUFA (%)	32.30	40.06	30.05 *	34.45 *	30.40 *	28.95 *
PUFA (%)	35.13	11.16	27.15 *	33.85 *	26.65 *	12.30 *
Total lipids (%dw)			29.09	20.20	17.65	32.10
Source	Fábregas et al. 2004		James et al. 1989			

Table 5. Fatty acid profiles of *Nannochloropsis* spp. cultures. *Nannochloropsis* sp. grown in semicontinuous culture under light-limited ( $40 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ ) and light-saturated ( $480 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ ) conditions (Fábregas et al. 2004). Massive cultures of *Nannochloropsis* MFD-2 at different temperatures (Adapted from James et al. 1989). \* In order to compare total SFAs, MUFAs and PUFAs, the sums were performed according to published data.

*Nannochloropsis* sp. strain growing in continuous cultures under saturating light ( $290 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) was characterized by high lipid content with a TAG increase. This fraction contained mainly palmitic and palmitoleic acids with myristic (C:14) and stearic acids as minor fatty acids (Sukenik et al., 1989). *Nannochloropsis* sp. growing semicontinuously showed an increase of SFAs with increasing irradiance to the detriment of unsaturated fatty acids (Table 5) (Fábregas et al., 2004). A similar trend was observed in *N. oculata* CS179 growing under high irradiance ( $1100 \mu\text{E m}^{-2} \text{s}^{-1}$ ) in outdoor cultures (Renaud et al., 1991). The strain showed a decrease in the ratio of total C16 unsaturated fatty acids to saturated C16:0, and a decrease in the ratio of total C18 unsaturated fatty acids to saturated C18:0. The effect of temperature on lipid content and fatty acid composition of *Nannochloropsis* seems to be quite complex, so a general trend cannot be established. An increase in temperature from 20 to 25°C practically doubled the lipid content (from 7.90 to 14.92% dw) of *N. oculata* (Converti et al., 2009). However, in *Nannochloropsis* sp. MFD-2 an increase in temperature from 15° to 20°C increased the total lipid content (from 21.60 to 29.05% dw), while an increase from 20° to 30°C brought about a decrease of the total lipid content (from 29.05 to 17.65% dw). Maximum lipid content (32.10%) was observed at 35°C (Table 5) (James et al., 1989). Among PUFAs, EPA constituted the major component. This fatty acid showed an increasing trend with increasing temperature up to 25°C, while a declining trend in EPA was observed from 25° to 35°C (Table 5). In a similar way, PUFAs and EPA were accumulated at lower temperatures in *Nannochloropsis* sp. PP983 (Hu & Gao, 2006).

### 3.3 Bacillariophyceae class

#### 3.3.1 Oil composition and triggering of lipid production

The diatoms' fatty acids have been studied more extensively than other microalgal classes. This interest is related to the wide use of diatoms in aquaculture (Zhukova & Aizdaicher, 1995; Lebeau & Robert, 2003a, 2003b; Bozarth et al., 2009). Diatoms are characterized by an unusual distribution of fatty acids compared to green algae and land plants (Darley, 1977). The C14, C16 and C20 acids comprise the bulk of the diatom fatty acids, while unsaturated C18 acids, particularly linolenic acid, are either absent or present at very low levels. In general, SFAs and MUFAs in diatoms are myristic acid, palmitic acid and palmitoleic acid (Reitan et al., 1994; Hu et al., 2008 and cites therein). Although this composition is relatively constant, some differences have been observed in the number of fatty acids detected among species (Dunstan et al., 1994; Rousch et al., 2003), and among individual microalgal strains within a species (Johansen et al., 1990). For example, the fatty-acid numbers detected in *Skeletonema costatum* were 19 (Berge et al., 1995), 24 (Zhukova & Aizdaicher, 1995), 12 (Nahon et al., 2010) and 28 (Popovich et al., 2011). However, it is noticeable that different strains, conditions and growth phases were analysed in these studies. High ratios of C16:1n7/ C16:0 and  $\sum\text{C16}/\sum\text{C18}$  are also characteristic of diatoms (Budge & Parrish, 1999). Furthermore, this prevalence and the high level of C14:0 have been used as markers of diatoms (Zhukova & Aizdaicher, 1995; Napolitano et al., 1997). Another characteristic feature of the composition of diatoms' fatty acid is the PUFA predominance, such as EPA and ARA (Reitan et al., 1994; Belarbi et al., 2000; Molina Grima et al., 2005). Like EPA, ARA is another essential fatty acid. Chu et al. (1994) reported ARA production by *Nitzschia conspicua* in a range of 0.6-4.7% total fatty acids.

The lipid content of oleaginous diatoms of freshwater and marine origin growing under normal and stress culture conditions was summarized by Hu et al. (2008). Statistical analysis

indicated that the average lipid content of oleaginous diatoms was 22.7% dw when maintained under normal growth conditions, whereas a total lipid content of 44.6% dw was achievable under stress conditions. However, lipid content and lipid fraction composition in diatoms are subjected to variability during the growth cycle and nutrient-deficiency (Table 6). Specifically, TAG synthesis and accumulation take place naturally in the stationary growth-phase (Siron et al., 1989; Sicko-Goad & Andresen, 1991; Lombardi & Wangersky, 1995; López Alonso et al. 1998, 2000). This fact occurs when photosynthetic assimilation is carried out while cell division is blocked due to a nutritional deficiency (Siron et al., 1989; Dunahay et al. 1996). In addition, a marked increase in the level of SFAs and MUFAs (e.g. 16:0, 16:1n7 and 18:1n9), with a concomitant decrease in the levels of PUFAs (e.g. 16:3n4 and 20:5n3) with increasing culture age has been observed in *Phaeodactylum tricorutum* and *Chaetoceros muelleri* (Liang et al., 2006). Table 6 gives evidence that culture age led to a significant increase of SFAs and MUFAs in detriment of PUFAs in both the total fatty acid and the TAG profiles in different diatoms. For example, Siron et al. (1989) reported that in *P. tricorutum* during exponential-growth phase the fatty acid pattern was characterized by a large fraction of EPA, palmitic and palmitoleic acids (Table 6). Conversely, this species in stationary-growth phase showed an increase of palmitic and palmitoleic acids in detriment of EPA (Table 6). The latter pattern was related to lipid storage. López Alonso et al. (2000) showed that for the same species, culture age had almost no influence on the fatty acid content. Conversely, age had a high impact on lipid fractions, producing a TAG increase (68%) and a polar lipids' decrease. Berge et al. (1995) studied the fatty acid composition of each lipid fraction of *Skeletonema costatum* during exponential growth-phase. They showed that polar lipids were mostly represented and TAGs were less abundant (ca. 10%). The authors reported that EPA, C16:3n4 and C16:4n1 accounted for about 61% of the total fatty acids (Table 6). Popovich et al. (2011) studied the fatty acid composition of each lipid fraction of *Skeletonema costatum* and *Navicula gregaria* during stationary growth-phase under similar culture conditions to Berge et al. (1995). TAGs were the main fraction of total lipids in both species, respectively accounting for ca. 65 and 76%, at 15 days of culture. *Skeletonema costatum* predominated in SFAs and MUFAs (Table 6), while *N. gregaria* was predominant in MUFAs, followed by SFAs. In *S. costatum*, the main fatty acids in the neutral lipid fraction were myristic, palmitic, palmitoleic and oleic acids (Table 6), while the main ones in *N. gregaria* were palmitic (28.74%) and palmitoleic (50.28%) acids (Popovich et al., 2011). Regarding nutrient availability, high TAG amounts in diatoms have been related to silicon (Hu et al., 2008) and phosphorus deficiency (Siron et al., 1989; Reitan et al., 1994). Silicate is the essential compound of a diatom's cell wall. This nutrient has a positive effect on growth (Turpin et al., 1999) and affects cellular lipid metabolism (Hu et al., 2008). The response to

Fatty acids (%)	<i>P. tricorutum</i> (1)	<i>P. tricorutum</i> (2)	<i>S. costatum</i> (3)	<i>S. costatum</i> (4)	<i>P. tricorutum</i> (5)	<i>P. tricorutum</i> (6)
C6:0				0.11		
C10:0				0.08		
C12:0	1.0	1.8		0.57	0.4	
C13:0				0.16		
C14:0	5.3	6.3	5.4	20.19	5.6	9.4
C15:0				0.95		
C16:0	15.5	31.5	4.2	15.77	32.5	24.3

Fatty acids (%)	<i>P. tricornutum</i> (1)	<i>P. tricornutum</i> (2)	<i>S. costatum</i> (3)	<i>S. costatum</i> (4)	<i>P. tricornutum</i> (5)	<i>P. tricornutum</i> (6)
C17:0				0.38		
C18:0	0.4	0.4	0.3	5.21	0.8	
C20:0				tr.		
C22:0	0.1	tr.			0.3	
C14:1				0.38		
C16:1 n7	24.2	43.0	9.6	19.47	40.4	30.0
C16:1 n5			1.6			
C17:1				8.45		
C18:1 n9	0.6		1.1		0.6	3.3
C18:1 n9c				11.06		
C18:1 n7		2.1	0.2			0.3
C20:1 n9			0.2	1.6		
C22:1 n9			0.3			
C16:2	11.7	4.5			1.1	
C16:2 n4			4.5			1.9
C16:3	1.8	0.4			0.4	
C16:3 n4			13.1			1.9
C16:4 n4			0.1			
C16:4 n1			12.5			0.1
C18:2 n6			1.0		1.1	1.0
C18:2 n3			0.5			
C18:2 n6c	1.0	0.5		3.38		
C18:2 n6t				0.12		
C18:3 n3	0.3	tr.		0.19	3.4	
C18:3 n6				0.25		
C18:4 n3	0.4	1.3	4.4		0.5	
C18:n <sup>a</sup>	2.2	tr.			3.7	
C20:2 n6				2.37		
C20:3 n6			0.1			
C20:4 n6				0.22		0.1
C20:5 n3	21.3	4.0	35.3	6.63	6.9	18.5
22:1 n9			3.3			
C22:6 n3	0.9	tr.	5.6	0.58	0.3	0.6
others						8.9
SFA (%)	22.3*	40*	9.9	43.48	39.6*	33.7*
MUFA (%)	24.8*	45.1*	13.0	40.11	41*	33.6*
PUFA (%)	39.6*	10.7*	77.1	13.74	17.4*	23.1*
Source	Siron et al., 1989	Siron et al., 1989	Berge et al., 1995	Popovich et al., 2011	Siron et al., 1989	López Alonso et al., 2000

Table 6. Fatty acid profiles of diatoms. Total fatty acid profiles (TFA) of *Phaeodactylum tricornutum* growing in appropriate medium at (1) exponential growth-phase and (2) late stationary growth-phase, (3) TFA of *Skeletonema costatum* at exponential growth-phase growing in appropriate medium, and (4) Neutral lipid profile (TAGs) of Argentinian strain *S. costatum* at late stationary growth-phase growing in appropriate medium. (5) TFA of *P. tricornutum* under phosphorus-deficient condition. (6) TAGs of *P. tricornutum* under nitrogen deprivation. \* In order to compare total SFAs, MUFAs and PUFAs, the sums were performed according to published data. <sup>a</sup>The number of double bonds (n $\geq$ 3) was not determined.



stress induced by silicate starvation indicates a rapid increase in neutral lipids. For example, after 6h of silicon deprivation, the total lipid fraction in *Cyclotella cryptica* increased from 30 to 42% dw (Shifrin & Chisholm, 1981). In addition, silicon-deficient *C. cryptica* cells had higher TAG levels and higher proportions of saturated and monounsaturated fatty acids than silicon-replete cells (Roessler, 1988). The lipid content in several species of *Navicula* reached up to 49% dw under silicon deficiency conditions (Griffiths & Harrison, 2008 and cites therein). On the other hand, phosphorus deficiency resulted in increased lipid content, mainly neutral lipids, in *Phaeodactylum tricorutum* (Siron et al., 1989), *P. tricorutum* and *Chaetoceros* sp. (Reitan et al., 1994) and *C. gracilis* (Lombardi & Wangersky, 1995). In addition, the PUFA percentage decreased and the percentage of MUFAs and SFAs increased with phosphorus limitation in *Phaeodactylum tricorutum* (Table 6) and *P. tricorutum* and *Chaetoceros* sp. (Reitan et al., 1994). *Stephanodiscus minutulus* showed an increase in TAGs and a decrease of polar lipids under silicon, phosphorus or nitrogen limitation (Lynn et al., 2000). However, large variability exists in the response of diatoms to nitrogen deficiency, e.g. under such condition some strains increase in their lipid content, particularly neutral lipids (Shifrin & Chisholm, 1981; Parrish & Wangersky, 1987; McGinnis et al., 1997) and others decrease (Shifrin & Chisholm, 1981 and cites therein). TAG was the lipid fraction with the highest increase under nitrogen limitation in *Chaetoceros muelleri*, *Navicula saprophila* (Chelf, 1990) and *P. tricorutum* (Table 6) (López Alonso et al., 2000) cultures. The latter authors indicated that saturated and monounsaturated fatty acids accumulated when nitrogen was decreased (Table 6). Under nitrogen deficiency conditions the lipid content in several species of *Navicula* also reached up to 51% dw, while in *Skeletonema costatum* it increased from 16 to 25% dw (Griffiths & Harrison, 2008 and cites therein). However, other strains remain unchanged in lipid content (Roessler, 1990; Sheehan et al., 1998; Rodolfi et al., 2009). This phenomenon might be due to the fact that diatoms have relatively high lipid content during the exponential growth-phase, and they do not increase their lipid content by nutrient starvation (Shifrin & Chisholm, 1981). In addition, the response of a microalgal culture to a factor (cell age or nutrient level) is also affected by the type of culture, either batch or continuous (López Alonso et al., 2000), and by the culture step during the batch scale-up process and the semi-continuous mode (Pernet et al., 2003).

Light intensity is another factor that affects lipid composition in diatoms (Orcutt & Patterson, 1974; Roessler, 1990). For example, increased TAG levels were observed in *Cylindrotheca fusiformis* under high intensity (Orcutt & Patterson, 1974). Temperature has a major effect on the types of fatty acids produced by microalgae (Thompson et al., 1992). Many microalgal species respond to decreased growth temperature by increasing the ratio of unsaturated to saturated fatty acids (Mortensen et al., 1988; James et al., 1989; Thompson et al., 1992; Renaud et al., 1995; Oliveira et al., 1999). However, the response to growth temperature varies from species to species, with no overall consistent relationship between temperature and fatty acid unsaturation (James et al., 1989; Thompson et al., 1992; Renaud et al., 1995). Rousch et al. (2003) investigated the effect of heat stress on the fatty acid composition of thermo-intolerant (*Phaeodactylum tricorutum*) and thermo-tolerant (*Chaetoceros muelleri*) marine diatoms under laboratory conditions. They found a production of fatty acids with greater saturation during heat stress. They also observed that changes in both fatty acid composition and fatty acid saturation degree occur more quickly in diatoms in response to increased temperature than under nutrient starvation. This implies that mechanisms associated with lipid changes in response to temperature may differ from those associated with other stresses (Rousch et al., 2003) Furthermore, the opposed trend to

produce unsaturated fatty acids during cooler temperatures has also been observed in several diatoms (Blanchemain & Grizeau, 1999; Rousch et al., 2003).

The number of genera and species of diatoms is in the order of 250 and 100,000, respectively (Norton et al., 1996; van den Hoek et al., 1995). In spite of their abundance and diversity in nature, cultures of diatoms of biotechnological interest are still at the early stage of development, except for aquaculture. This is most likely due to difficulties in their cultivation. In addition to EPA, which is usually extracted on a semi-industrial scale, and biomass for feeding in aquaculture, silicon production from diatoms' frustules is the most promising application, particularly in the field of nanotechnology (Lebeau & Robert, 2003a, 2003b; Bozarth et al., 2009). Only a few authors have reported lipid valorisation as biodiesel using diatoms, for example in *Hantzschia* DI-60 (Sriharan et al., 1990), *Chaetoceros muelleri* (McGinnis et al., 1997), *Skeletonema costatum* and *Navicula gregaria* (Popovich, et al., 2011). Among these studies, only Popovich et al. (2011) analyzed TAG profiles. Others studies have focused on the analysis of FAMES. For example, there are analyses in *Navicula* JPC DA0580 (Matsumoto et al., 2010) and *Phaeodactylum tricornutum* (Francisco et al., 2010). On the other hand, aiming at the production of biodiesel from microalgae, *Cyclotella cryptica* and *Navicula saprophila* were genetically manipulated by Dunahay et al. (1996) to optimise lipid production. Yu et al. (2009) identified and compared the molecular species of TAGs in *P. tricornutum* CCAP1055/1 (CCMP632) and *Thalassiosira pseudonana* 3H (CCMP1335) under control and nutrient-limitation. This study represents an advance in the development of molecular genetic tools for the manipulation of these strains.

#### 4. Influence of oil composition on biodiesel quality

As was previously indicated, the most important properties of biofuel- i.e. cetane number (ignition quality), cold-flow properties, oxidative stability, and iodine value- are determined by the structure of fatty esters, which form essential part of the biodiesel (Knothe, 2005; Chisti, 2007). In turn, the properties of fatty esters are determined by the characteristics of fatty acid's oil- i.e. carbon chain length, its unsaturation degree, and the alcohol moieties that comprise a fatty ester (Knothe, 2005). Thus, the fatty-acid composition of different oils has a significant effect on the characteristics of the produced biodiesel. For instance, palmitic, stearic, oleic, linoleic and linolenic acids were recognized as the most common fatty acids contained in biodiesel from land plants (Miao & Wu, 2006; Knothe, 2008). These fatty acids are well represented in some green microalgae recently examined.

The selection of appropriate microalgal strains is an important factor for the overall success of biofuel production from microalgae. Rigorous selection is challenging owing to the large number of microalgal species available, the limited characterization of these organisms and their varying sets of characteristics. At present no ideal species have been found for this purpose; however, some examples of promising microalgal species are the following. Regarding chlorophycean, some research has been performed with the aim to analyze fatty acid composition so as to infer biodiesel quality. Total fatty acid profiles of *Chlorella vulgaris* INETI 58, *Scenedesmus obliquus* FCTU, *Neochloris oleabundans* UTEX # 1185 and *Dunaliella tertiolecta* (IPIMAR) (Table 2) were studied by Gouveia & Oliveira (2009). They indicated that only the oils extracted from *S. obliquus* FCTU presented linolenic acid contents within European standard EN 14214 specifications ( $\leq 12\%$ )(CEN EN-14214, 2003). Another aspect reported by these authors was the iodine value. This is a parameter that only depends on the oil origin (Mittelbach, 1996) and it is a measurement of oil unsaturation (Knothe, 2002).

*Senedesmus obliquus* and *N. oleoabundans* were the only species that presented iodine values (69 g I<sub>2</sub>/100 g and 102 g I<sub>2</sub>/100 g, respectively) below of the European standard EN 14214 allowed (maximum value of 120 g I<sub>2</sub>/100 g). They concluded that if the purpose is to produce biodiesel only from one species, *S. obliquus* presented the most adequate fatty acid profile. However, *N. oleoabundans* and *D. tertiolecta* oils may be used for good quality biodiesel if associated with other oils. Under nitrogen-starvation conditions Gouveia et al. (2009) indicated a better oil quality in *N. oleoabundans* UTEX # 1185, with linolenic acid below 12% and iodine value of 72 I<sub>2</sub>/100 g.

As was reported, lipids produced from green microalgal species usually contain fatty acid profiles of mainly C16 and C18. In *Senedesmus obliquus* CNW-N, C16 and C18 groups accounted for about 70% and 89% of total fatty acids. This fact was observed in cells growing under both nutrient-rich and nutrient-deficient media respectively (Ho et al., 2010). In both conditions the authors reported linolenic acid values below 12%, indicating that the lipid produced from *S. obliquus* CNW-N would be suitable for biodiesel production. The eustigmatophycean *Nannochloropsis* sp. also presented linolenic acid and iodine value (52 g I<sub>2</sub>/100 g) within European standard (EN 14214) specifications (Gouveia & Oliveira, 2009). However, due to the high content of EPA and ARA, the authors remarked that the oil from this species may be used for good quality biodiesel, if associated with other oils.

Even though it is important to have information about total fatty acid profiles to screen microalgae for biodiesel production, transesterification includes TAG conversion to diglycerides, monoglycerides and then esters and glycerol (Mata et al., 2010). In this context, the utility of microalgal oils as biodiesel will depend on fatty acids' quantity and composition in TAG fraction. The analysis of TAG profiles for Argentinian *Haematococcus pluvialis* strain (Table 4) showed that the fatty acid composition was in agreement with the European standard: linolenic acid was below 12 % and oil's iodine values were 110.95 g I<sub>2</sub>/100 g and 99.64 g I<sub>2</sub>/100 g under control and high light intensity culture conditions, respectively. In addition, the maximum unsaturation degree of the PUFA chains was three and the length of the main fatty acid was intermediate with a maximum of 18 carbons. These features allowed them to infer that quality *H. pluvialis*' oil would be suitable for biodiesel production (Damiani et al., 2010).

In a similar way, the TAG profiles were analyzed in the diatoms *Skeletonema costatum* (Table 6) and *Navicula gregaria* under stationary growth phase (Popovich et al., 2011). The oils extracted from these species presented linolenic-acid contents within European biodiesel's quality specifications. However, both species showed EPA levels higher than the required limit. The iodine values for *S. costatum* (35.87 g I<sub>2</sub>/100 g) and *N. gregaria* (51.93 g I<sub>2</sub>/100 g) oils were well below the allowed European biodiesel standards. On the other hand, Knothe (2010) demonstrated that fatty-acid profiles enriched in palmitoleic acid may impart overall favourable properties to a biodiesel fuel, giving especially improved cold-flow properties. This fatty acid occurs in small amounts in land plant oils; however, it is noteworthy that *S. costatum* and *N. gregaria* respectively presented 19.45% and 50.28% of palmitoleic acid. In addition, cetane number and cold-flow properties were inferred in both diatoms. Cetane number (CN) is the dimensionless descriptor of a diesel fuel's ignition quality, and it is a prime indicator of biodiesel quality. It augments with both increasing saturation and straight-chain length in the fatty acids (Knothe, 2008). The minimum limits allowed in biodiesel standards are equal to 51 in European EN 14214, and 47 in American ASTM D675 (ASTM D675, 2002). According to SFA composition in both diatoms, biodiesel derived from these oils may present an acceptable CN. On the other hand, biodiesel coming from oils with

high amounts of saturated long-chain fatty acids tends to have relatively poor cold-flow properties (Chiu et al., 2004). Thus, high SFA content found in both diatoms would likely indicate poor cold-flow properties (Popovich et al., 2011). The authors concluded that lipid quality in *S. costatum* and *N. gregaria* indicated the microalgae's potential as biodiesel feedstocks.

As to FAME analysis, there are few studies focused on biodiesel production from different microalgal species. It is well known that certain methyl esters allow us to improve biodiesel quality. For instance, methyl oleate has been suggested as a possible candidate since it exhibits a combination of improved fuel properties (Knothe, 2005). Mandal & Mallick (2009) reported a high amount of methyl palmitate (38.8%) and methyl oleate (35.4%) fatty acids in biodiesel from *Scenedesmus obliquus* SAG 276-3a, which gives its high oxidative stability. In a similar way, *Chlorella protothecoides* growing under heterotrophic conditions showed oleic acid methyl ester as the most abundant (60.84%). In addition, in this species the total content of oleic-, linoleic- and stearic-acid methyl esters was over 80%. These features resulted in the biodiesel's high quality, according to Xu et al. (2006). Moreover, it is important to remark that biodiesel properties, such as density ( $0.864 \text{ kg L}^{-1}$ ), viscosity ( $5.2 \times 10^{-4} \text{ Pas}$  at  $40^\circ\text{C}$ ), flash point ( $115^\circ\text{C}$ ), cold filter plugging point ( $-11^\circ\text{C}$ ), solidifying point ( $-12^\circ\text{C}$ ) and heating value ( $41 \text{ MJ kg}^{-1}$ ) were determined only for this heterotrophic species (Xu et al. 2006). All these physical and fuel properties studied were in agreement with the US Standard (ASTM 6751), and, specifically, its cold filter plugging point ( $-11^\circ\text{C}$ ) was much lower in comparison with the diesel fuel's. The authors concluded that the biodiesel from heterotrophic microalgal oil might be a competitive alternative to conventional diesel fuel.

Francisco et al. (2010) analyzed feedstocks of six microalgal strains for biodiesel production, taking into account important properties like ester content, cetane number, iodine value and unsaturation degree. In this study they found *Chlorella vulgaris* UTCC 90 proves to be the best strain for use as a feedstock for biodiesel production. Qualitative analysis of FAMES demonstrated the predominance of saturated (43.5%) and monounsaturated (41.9%) fatty acids. The biodiesel's quality properties were an ester content of 99.8%, a cetane number of 56.7%, an iodine value of  $65 \text{ g I}_2/100 \text{ g}$ , an unsaturation degree of 74.1 % and a cold filter plugging point of  $4.5^\circ\text{C}$ . All these parameters agreed with the limits established by the US Standard (ASTM 6751), the European Standard (EN 14214), the Brazilian National Petroleum Agency Standard (ANP 255) (ANP 255, 2003) and the Australian Standard for biodiesel quality (Fuel Standard, 2003).

As regards Eustigmatophyceae, Koberg et al. (2011) reported that the major composition of biodiesel produced from *Nannochloropsis* sp. consisted of methyl esters of palmitic and palmitoleic acids. This fact agrees with the concept indicated above, where fatty-acid profiles enriched in palmitoleic acid may impart overall favourable properties to a biodiesel fuel (Knothe, 2010). Moreover, the low percentage of methyl esters with a carbon chain of  $> 18$  carbons observed in this species would guarantee a low viscosity for the biodiesel. Similarly, the FAME profile of the marine diatom *Navicula* sp. JPCC DA 0580 mainly contained methyl palmitate and methyl palmitoleate. This oil might be suitable for biodiesel production according to Matsumoto et al. (2010).

## 5. Conclusions

The production of biodiesel from microalgae is an emergent area greatly promising for the gradual replacement of diesel fuel. This review covers only a small part of various aspects

that should be taken into account when choosing a microalgal species to produce biodiesel. The results clearly indicate the need for more research on microalgal lipids, especially TAG and fatty-acid profiles. Besides, rigorous comparisons across experiments under different conditions are impossible to carry out. As has been shown, the lipid content and fatty acid composition can vary with: a) strain/species, b) environmental conditions, c) type of stress condition, d) duration of stress condition, e) nutrient's concentration or irradiance's intensity during cultivation, f) life cycle (exponential or stationary phase), g) culture age, h) culture strategies (one- or two-stage cultivation, indoor or outdoor), among others. Even though no generalization can be strictly made, stress conditions seem to be effective in promoting lipid accumulation and improving TAG amount in many microalgae. These features are often associated with low productivity of biomass and lipids. Thus, the major challenge in process development for microalgal biodiesel production consists in choosing the best strains and defining cultivation strategies in order to simultaneously achieve three objectives: to obtain high biomass yields, high lipid contents and lipids with adequate fatty acid profiles.

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