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The potential role of some microalgae in biotechnological fields

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To my family

Table of Contents

List of Abbreviations

ARA	*Arabidopsis thaliana*
ATP	adenosine triphosphate
ATPase	ATP synthase
BM	brackish medium
BN	Blue Native
BP	biomass productivities
BSA	bovine serum albumin
C	freshly prepared BM medium (Control)
Car	carotenoids
Chl	chlorophyll
CO_2	carbox dioxide
CP	*Auxenochlorella protothecoides*
CV	*Chlorella vulgaris*
Cyt	Cytochrome
DD	diadinoxanthin
DHA	dodecahexaenoic acid
div	division
DMSO	dimethyl sulphoxide
DNS	3,5-dinitrosalicylic acid assay
DT	diatoxanthin
DW	dry weight
E	autotrophic exhausted medium;
E+	autotrophic exhausted medium replenished with nitrate and phosphate concentrations as for BM medium
E_2+	2^{nd} run of autotrophic exhausted medium replenished with nitrate and phosphate concentrations as for BM medium
EDTA	ethylenediaminetetraacetic acid
EG	mixotrophic exhausted medium after cultivation with glucose
EG+	mixotrophic exhausted medium after cultivation with glucose and replenished with nutrients as in E+
EG_2+	2^{nd} run of mixotrophic exhausted medium after cultivation with glucose and replenished with nutrients as in E_2+
EPA	eicosapentaenoic acid
ESAW	Enriched Seawater, Artificial Water
F_0	minimal fluorescence
FAME	fatty acid methyl esters
FCP	fucoxanthin-chlorophyll protein complexes
Fd	ferrodoxin
FFA	free fatty acids
F_M	maximum fluorescence
FNR	Fd-$NADP^+$ oxidoreductase
F_V	variable fluorescence
F_V/F_M	maximum quntum yield of PSII in the dark adapted state
Fx	fucoxanthin

Glu	glucose
Gly	glycerol
H_2O	water
k	division rate
LHC	light-harvesting complexes
N	nitrogen
NaAc	sodium acetate
NADP+	nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
Nile Red	9-diethylamina-5Hbenzo[α]phenoxazine-5-one
NO	*Neochloris oleoabundans*
OD	optical density
P	phosphate
PA	polyamine
PAGE	polyacrylamide gel electrophoresis
PAM	pulse amplitude modulation
PAR	photosynthetically active radiation
PBR	photobioreactor
PHB	poly-3-hydroxybutyrate
Pheo	pheophytin
PQ	plastoquinone
PSI	photosystem I
PSII	photosystem II
PUFA	polyunsaturated fatty acids
PVDF	polyvinylidene fluoride
Q_A	primary quinone acceptor
Q_B	secondary quinone acceptor
RuBisCO	ribulose-1,5-biphosphate carboxylase/oxygenase
SA	*Scenedesmus acutus*
SDS	sodium dodecyl sulphate
T1-15% (v/v)	15 mL of *C. vulgaris* in 100 mL water
T2- 30% (v/v)	30 mL of *C. vulgaris* in 100 mL water
TAG	triacylglycerol
TEM	transmission electron microscope
TIC	dissolved inorganic carbon
TN	total nitrogen
TOC	dissolved organic carbon
Tyr_z	tyrosine
Tyr_z^+	tyrosine reduced
Vx	violaxanthin
WOC	water oxidizing complex
X	biomass concentrations
μ	growth rate
1D	first dimension
2D	second dimension

Chapter 1

1. General introduction

During the last decades, growing world population and increasing energy demand create an enormous competition for the scarce resources of the planet. At the present, 90% of global energy demand is generated from fossil fuels and only 10% is fulfilled by renewable energy sources (Ho et al. 2011; Maity et al. 2014; Shen 2014). Besides this, the anthropogenic carbon dioxide (CO_2) emissions, mainly come from the intensive burning of fossil fuels, has contributed for over 50% of the increase in the atmospheric CO_2 levels, from 280 ppm in pre-industrial age to 400 ppm currently (Blasing et. al 2009; Shen 2014; Cheah et al. 2015). Carbon dioxide, which is the main greenhouse gas, is now widely considered the major responsible of the global warming (Shen 2014; Cheah et al. 2015). The negative effects caused by the excessive exploitation of fossil fuels on climate and ecosystems, together with diminishing natural resources, call for renewable energy sources and sustainable production processes (Kumar et al. 2010; Chisti 2013; Razzak et al. 2013; Maity et al. 2014). The competition for food, water and energy is perhaps the greatest challenge in modern time. In this perspective, microalgae have gained considerable attention as a relevant source for applied societal purposes, due to their rich biodiversity and potential use in a wide range of applications (Mata et al. 2010; Borowitzka 2013a; Markou and Nerantzis 2013; Odjadjare et al. 2015).

1.1 Exploring the richness of microalgal biodiversity

Microalgae comprise a large group of eukaryotic photosynthetic microorganisms, which are characterized by remarkable biological, ecological and functional traits, certainly determined by their evolutionary trends (Metting et al. 1996; Tomaselli 2004; Quigg et al. 2011; De Clerck et al. 2012; Andersen 2013). The richness of microalgal biodiversity is estimated varying from roughly 70,000 to more than one million species, which are classified in 15 phyla and 54 classes (Guiry 2012). It is evaluated that approximately 40,000 of the existing microalgal species have been described, 30,000 of these included into the online taxonomic database AlgaeBase and another 30,000 species yet to be described (Guiry 2012).

Microalgae have evolved to be practically ubiquitous throughout the globe, and their varied distributions and evolutionary histories are reflected in extremely different metabolic capabilities (Fig. 1) (Andersen 2013). These microorganisms can be found in all existing Earth ecosystems, both aquatic and terrestrial, and include a big variety of species living in a

wide range of environmental conditions (Hu et al 2008; Mata et al. 2010; Andersen 2013). Moreover, microalgae can thrive in non-arable lands being able to grow in waters having different salinity levels and chemical composition (Smith et al. 2010; Borowitzka and Moheimani 2013). They have also the ability to easily modify their metabolism in response to changes in environmental conditions (Gushina and Harwood 2006; Leonardi et al. 2011; Popovich et al. 2012a; Juneja et al. 2013). These microorganisms have numerous key features, which potentially make them a more appreciable renewable source than terrestrial plants (Richmond 2004; Hu et al 2008; Ndimba et al. 2013). They have faster growth potential coupled with their relatively synthesis of valuable products, higher photosynthetic efficiencies, higher rates of carbon dioxide fixation and higher biomass productivities as compared to plants (Richmond 2004; Gouveia et al. 2009; Harun et al. 2010; Mata et al. 2010). Due to this great taxonomical diversity and an exceptional range of adaptability, there has been steady increasing interest in microalgae as a potential source to be exploited in food, pharmaceutical, bio-remediation, and recently bio-energy sectors (Mata et al. 2010; Borowitzka and Moheimani 2013; Borowitzka 2013a; Markou and Nerantzis 2013; Odjadjare et al. 2015). However, a deeper exploration of the biodiversity richness and ecophysiological contraits of microalgae is crucial for enhancing their biotechnological use.

Among microalgae, species belonging to the Trebouxiophyceae and Chlorophyceae classes (Chlorophyta phylum) and Bacillariophyceae class (Heterokontophyta phylum) are widely considered among the best candidates to be exploited in several applications including human and animal nutrition, cosmetics, pharmaceuticals, wastewater treatments or renewable energy source (Pulz and Gross 2004; Bozarth et al. 2009; Mata et al. 2010; Hildebrand et al. 2012; Borra et al. 2014; Fu et al. 2015; Odjadjare et al. 2015).

For this reason, members of these three classes of microalgae are used as objects of this Thesis and described in greater details in the following sections with special attention on their photosynthetic machinery in terms of light harvesting and photosynthetic electron transport mechanisms.

1.1.1 The Chlorophyta phylum: the Trebouxiophyceae and Chlorophyceae classes

The division Chlorophyta includes the majority of described species of green algae (almost 2000 species), whose members are common inhabitants of aquatic environments and exhibit great morphological and cytological diversity (Fig. 2) (de Duve 2007; Proschold and Leliaert 2007; Leliaert et al. 2012). Green algae belong to the green lineage or Viridiplantae,

which includes also land plants, and is one of the major groups of oxygenic photosynthetic eukaryotes (Archibald 2009; Keeling 2010; Leliaert et al. 2012). The green lineage originated following an endosymbiotic event, which was occurred approximately 1,8 billion years ago, in which a heterotrophic eukaryotic host cell captured a cyanobacterium that became stably integrated and ultimately turned into a plastid (Fig. 1) (Falkowski et al. 2004; Gould et al. 2008; Archibald 2009; Keeling 2010). Green algae are characterized by a number of distinct features, many of which are also shared with the land plants (van den Hoek et al. 1995). The chloroplasts are enclosed by a double membrane with thylakoids grouped in lamellae, and contain chlorophyll *a* and *b* along with some accessory pigments including carotenes and xanthophylls. Pyrenoids, when present, are embedded within the chloroplast and are surrounded by starch (α-1,4-linked polyglucan, with α-1,6 ramifications), which is the main reserve polysaccharide. Most green algae have firm cell walls with a fiber matrix generally composed of cellulose. Many green algae are flagellates or have flagellate cells in some stage of the life cycle. The flagella are isokont, which means that they are similar in structure, although they may differ in length. The region between the flagellar axoneme and the basal body is characterized by a stellate structure (van den Hoek et al. 1995). Despite their many unifying ultrastructural and biochemical features, green algae exhibit a remarkable variation in morphology and ecology reflecting their evolutionary diversification. Morphological diversity ranges from microscopic to macroscopic organisms. Four classes are recognized within the Chlorophyta: Prasinophyceae, Ulvophyceae, Trebouxiophyceae and Chlorophyceae (Pröschold and Leliaert 2007). Recently, modern molecular studies revealed that the green algal classes Chlorophyceae and Trebouxiophyceae, which are characterized by similar morphological shape but by remarkable diversity of physiological and biochemical constraints, evolved in independent phylogenetic lineages as a result of convergent evolution within the Chlorophyta phylum (Leliaert et al. 2012; Krienitz et al. 2015). Among the division Chlorophyta, species belonging to the Trebouxiophyceae and Chlorophyceae class are widely used in biotechnological fields. For example, *Chlamydomonas* sp. is widely explored as a photosynthetic model organism (Leonardi et al. 2011), whereas *Dunaliella* sp., *Haematococcus* sp., *Scenedesmus* sp., *Neochloris* sp. and *Chlorella* sp. are considered natural sources of industrially useful products (Pulz and Gross 2004; Spolaore et al. 2006; Raja et al. 2008).

1.1.2 The Heterokontophyta phylum: the Bacillariophyceae class

The Bacillariophyceae, generally called diatoms, are unicellular eukaryotic microalgae, with a wide distribution in sea and fresh water. They belong to the Stramenopiles or Heterokontophyta phylum, which includes, among the others, brown algae and oomycetes (Kooistra et al. 2007; Gould et al. 2008; Archibald 2009; Keeling 2010). It is estimated that the Bacillariophyceae class accounts for approximately 200,000 different diatom species, but only 8000-10,000 of these have been described (Guiry et al. 2012). These microorganisms have considerable ecological significance, estimated to be the dominant component of phytoplankton (Armbrust 2009) and to be responsible for up to 25% of the global CO_2 fixation and to contribute up to 40% of marine primary production (Dugdale and Wilkerson 1998; Falkowski et al. 1998; Field et al. 1998; Granum et al. 2005; Hildebrand et al. 2012). Characterized by complex evolutionary history and recurrent genetic rearrangements, diatoms developed a unique metabolism and subcellular organization, allowing them to greatly adapt to changes in environmental stress conditions (Kooistra et al. 2007; Armbrust 2009). Diatoms evolved from two different endosymbiotic events, which significantly shaped their genetic setup and separated them from red, green algae and land plants (Becker et al. 2008; Armbrust 2009). Common to all photosynthetic eukaryotes was a primary endosymbiosis event, which caused a progressive gene transfer from the cyanobacterium's genome to the host nucleus, giving origin to the ancestor of red algae, green algae and land plants (Falkowski et al. 2004; de Duve 2007; Keeling 2010; De Clerck et al. 2012). It has been suggested that at this stage, a chlamydial endosymbiosis might have occurred in diatoms, significantly contributing to the gene transfer (Fig. 3) (Armbrust et al. 2009). Approximately 500 millions of years later, a red alga that evolved from such primordial organism, was subsequently incorporated by another eukaryotic cell, causing the consequent gene transfer towards the nucleus and progressive reduction of the primitive alga to the present plastid of Stramenopiles (Kooistra et al. 2007; Gould et al. 2008; Archibald 2009; Keeling 2010). As indelible sign of the particular series of endosymbiotic events, four membranes surround the plastid of Stramenopiles (Fig. 3).

The most conspicuous feature of diatoms is their silica-based cell wall, also called the "frustule". Their rigid cell wall has constrained them to acquire an unusual mode of cell division, with mitotically-derived daughter cells becoming progressively smaller until they reach a critical size threshold, following which they must undergo sexual reproduction or size restoration (Armbrust 2009). Diatoms are phylogenetically divided into four groups:

radial centrics, multipolar centrics, araphid pennates and raphid pennates diatoms, reflecting morphological differences but also substantial genetic separation (van den Hoek et al. 1995). The storage product is chrysolaminarin (β-1,3-linked glucan), but also lipids can be highly accumulated inside the cytoplasm. Due to their peculiar evolution evolutionary origin, the plastid ultrastructure and the organization of the photosynthetic apparatus of diatoms differ greatly respect from that of higher plant and other groups of algae (as reviewed by Wilhelm et al. 2006; Lavaud 2007; Finazzi et al. 2010; Grouneva et al. 2013). Diatom thylakoids, the site of light harvesting and the photosynthetic electron transport chain, are organized in bands of three with no differentiation into stacked and unstacked regions (Lepetit et al. 2012; Grouneva et al. 2013). The chloroplast contains chlorophyll (Chl) *a* and *c* and Car such as β-carotene and xantophylls, which confer the characteristic "gold-green" colour to the cell (van den Hoek et al. 1995). In addition, the available genome sequences of the marine centric diatom *Thalassiosira pseudonana* (Armbrust et al. 2004) and the marine pennate diatom *Phaeodactylum tricornutum* (Bowler et al. 2008) established these strains as model organisms for genomic, transcriptomic and metabolic studies in diatoms researches (Poulsen et al. 2007; Trentacoste et al. 2013). In spite of these attractive attributes, only a few diatom species and within the same phylum the marine eustigmatophyte *Nannochloropsis* sp. have been employed for biotechnological applications (Lebeau and Robert 2003a; Bozarth et al. 2009; Rodolfi et al. 2009; Hildebrand et al. 2012; Borra et al. 2014; Fu et al. 2015).

1.2 The organization of the photosynthetic apparatus in organisms performing oxygenic photosynthesis

During photosynthesis, plants, algae and certain bacteria transform the photon energy of the sunlight to chemical energy, which is essential in the synthesis of biomolecules necessary for energy storage in the metabolic processes. Photosynthesis occurs in two chloroplast compartments. In the thylakoid membranes, biophysical processes, called light reactions, allow the synthesis of ATP and NADPH. In the stroma, the Calvin-Benson cycle utilizes ATP and NADPH to assimilate the atmospheric carbon dioxide for synthesis of carbohydrates.

The overall reaction catalysed during the photosynthetic process is described as follows:

$$6\ CO_2 + 6\ H_2O + \text{light energy} \rightarrow C_6H_{12}O_6 + 6\ O_2$$

O_2, ATP and NADPH are produced as a consequence of the photolysis of water (Nelson and Ben-Sherm 2004; Taiz and Zeiger 2010).

Comparison of the plastid ultrastructure

In plants and green algae, the chloroplast is delimited by a double layer of membranes, mainly composed by phospholipids, and this is a remnant of the primary endosymbiosis (Fig. 4). Inside the organelle, a very complex system of membranes forms the thylakoids. In green algae and higher plants, the thylakoid system, the site of light harvesting and the photosynthetic electron transport chain, is distinctly segregated into stacked regions called grana and unstacked regions called stroma lamellae (Anderson 1986). The chloroplast thylakoid network constitutes a membrane-surrounded internal continuum, called lumen. The liquid space between thylakoids and the envelope is denoted as "stroma" (Taiz and Zeiger 2010; Nelson and Ben-Sherm 2004). The most fundamental difference between green algae and diatom chloroplasts is the number of chloroplast envelope membranes. Diatom plastids are engulfed by four membranes, a consequence of their origin in a secondary endosymbiosis event (Fig. 4). Also, the thylakoid organization in diatom as well as the composition of the pigment-protein complexes displays several differences compared to those of other algae groups and higher plants. Diatom thylakoids are organized into loosely appressed bands of three lamellae, they so-called "girdle stack", with no differentiation into stacked and unstacked regions.

The role of pigments in photosynthetic membranes

The first step of the photosynthetic processes is the absorption of a photon by a photosynthetic pigment. The pigmentation of the diatoms, and consequently, their absorption capabilities, differs considerably from one of green algae and higher plants. Among photosynthetic pigments, which are associated with proteins to give pigment-protein complexes, Chls are the most important as they are specifically involved in light-energy harvesting (Taiz and Zeiger 2010). These molecules derive from the δ-aminolevulinic acid and are composed by a tetrapyrrolic ring, with one atom of Mg bound in the centre. Moreover, a long hydrophobic chain composed by phytol allows the molecule fixation inside the thylakoid membrane bilayer. In green algae and higher plants Chl*a* and Chl*b* are present (Fig. 5). These two molecules differ from each other only for the group bound at carbon atom 3: in the former it is a methyl group, while the latter has a formil group. Chl*a* is the main chlorophyll of green algae and higher plants. It is contained in all reaction centres, but it is also found in light-harvesting complexes. Conversely, Chl*b* is an accessory pigment and it is part of the antenna system. Car can be distinguished in

carotenes, which are hydrocarbons containing only carbon and hydrogen, and xanthophylls, their oxygenated derivatives. They are organised in long polyene chains which present up to 15 carbons conjugated with double bonds (Fig. 6). This feature is responsible for their characteristic absorption spectra and their specific photochemical properties (Naik et al. 2003). The main carotene in photosynthetic membranes of plants and green algae is β-carotene, which is mainly found inside the reaction centres, whereas lutein, violaxanthin, zeaxanthin and neoxantin are the main xanthophylls, localized in the antenna systems. Car are accessory pigments, which absorb light between 400 and 500 nm and transfer the excitation energy to Chl. Similarly to the majority of photosynthesizing organisms, diatoms contain Chl*a*, Chl*c* (present as Chlc_1, c_2, and/or c_3) instead of Chl*b* (Wilhelm et al. 2006; Lavaud 2007). Chl*c* does not possess aliphatic phytol chain and is based on the porphyrin ring rather than a chlorin ring (Fig. 5). Different forms of Chl*c* exist; in diatoms the most abundant are Chlc_1 and c_2. These two Chl*c* forms exhibit small structural differences, Chlc_1 has an ethyl group present at position C4 in ring of the Chl macrocycle, whereas Chlc_2 has a vinyl group in this position (Larkum 2003). Diatoms are only able to synthesize carotenoids derived from β-carotene pathway and not from α-carotene, so they contain fucoxanthin (β -carotenoid) as their main auxiliary pigment instead of lutein (α-carotenoid), which is the main auxiliary pigment in green algae and plants (Wilhelm et al. 2006; Lavaud 2007). In diatoms, carotenoids contain a linear polyene chain, with C_{2h} symmetry (Fig. 6). The main light-harvesting carotenoid in diatoms is fucoxanthin (Fx). Fx is an unusually asymmetric molecule, which possesses an allene group at one of the ionone rings. This molecular structure lends to Fx unique spectral properties, absorbing light in extremely wide spectral range, e.g. between 460 and 570 nm (Zigmantas et al. 2004). Other carotenoids occurring in diatoms are the diadinoxanthin (DD), diatoxanthin (DT) and β–carotene (Wilhelm et al. 2006; Lavaud 2007) (Fig. 6). DD and DT are also asymmetric molecules, containing an acetylenic group at one of the ionone rings. Car play an important role for the photoprotection of the photosynthetic membrane, scavenging reactive species of oxygen and limiting the damage caused by photo-oxidation (Szabò et al. 2009; Taiz and Zeiger 2010). Among the different photoprotective mechanisms, xanthophyll cycle-related dissipation of excessively absorbed energy is of major importance for the prevention of photoinhibitory damage to the photosynthetic machinery in algae (Goss and Jakob 2010). The violaxanthin (Vx) cycle is found in vascular plants and in the green (Chlorophyta) and brown (Phaeophyceae) algae (Goss and Jakob 2010). The algal classes Bacillariophyceae, Xanthophyceae, Haptophyceae, and Dinophyceae present the diadinoxanthin (DD) cycle

(Lohr and Wilhelm 1999). It is important to note that algae containing the DD cycle also have the ability to synthesize the xanthophylls of the Vx cycle, although the pigments of the Vx cycle serve mainly as intermediate products in the biosynthesis of the DD cycle pigments (Lohr and Wilhelm 1999; Goss and Jakob 2010). The DD cycle represents the most important photoprotection mechanism of diatoms and leads to an efficient dissipation of excessively absorbed excitation energy as heat (Lavaud et al. 2007; Goss and Jakob 2010).

Plants are able to use only a small fraction of the solar emission spectrum for photosynthesis. This is comprised in the visible region, between 400 and 700 nm, and is called photosynthetically active radiation (PAR). Pigments are indeed able to adsorb only specific wavelengths:

- Chl*a* presents the most intense absorption peak at 440 nm, whereas a second peak is found at 660 nm;
- Chl*b* has a similar absorption spectra, with the first absorption peak at 470 nm and the second at 645 nm;
- Chl*c* produces a strong Soret (blue) absorption band in comparison with a weak band in the red region of the visible light spectrum at approximately 630 nm;
- Car have characteristic absorption spectra depending on the considered molecule, however all the absorption peaks overlap in the blue region, and so their main absorption can be generalised to be at 470 nm.

Pigments are found in an excited state after they have absorbed a photon. This state in unstable, so the energy is promptly transferred to another molecule in the following ways: i) electron transfer to an acceptor molecule; ii) electron transfer to another pigment by Förster resonance energy transfer process. Chl excitation energy can also be dissipated by i) fluorescence release or ii) energy release as heat (Ruban et al. 2012). Chl*a* is the first electron acceptor in all oxygenic photosynthetic organisms, whereas Chl*b* or *Chlc* and Car harvest and transfer light energy to Chl*a* by resonance. Conversely, energy released by heat or fluorescence is considered a side dissipative effect during the photosynthetic process.

The "light-driven" reaction of photosynhtesis

The basic function of photosynthetic light reactions is carried out mainly by the protein complexes that are embedded in the thylakoid membranes: photosystem II (PSII) and photosystem I (PSI), their respective light harvesting complexes (LHCII and LHCI), cytochrome b6f (Cyt b6f) and ATP synthase (Fig. 7). Between PSII and cyt b6/f the mobile

plastoquinone (PQ) serves as electron carrier. The electron transfer processes in the thylakoid membranes of higher plants are oriented as shown in Fig. 7, so that the water oxidation occurs at the lumen side, whereas the NADPH and ATP produced are released in the stroma (Eberhard et al. 2008; Dekker and Boekema 2005; Taiz and Zeiger 2010).

Photosystems are divided in two main structural and functional units: the antenna complexes, which bind the major parts of the light-harvesting pigments and where the light harvesting processes occur, and the core complexes, where the photochemical reactions and the initiation of the electron transport processes take place. During the light-reactions of photosynthesis, the electron transfer can be represented in a "Z-scheme" (Fig. 8). The photons are absorbed mainly by the antenna complexes of the photosystems and the excitation energy is transferred rapidly to the reaction centers' special Chl molecules within the core complexes where the charge separation occurs. After charge separation, rapid electron-transfer processes create a stabile charge-separated state. The oxidized primary donor, the Chl+ molecule absorbing at around 680 nm in PSII (P680) is re-reduced by a tyrosine residue, the Tyr_Z and the Tyr_Z+ is re-reduced by the Mn-cluster of the water oxidizing complex (WOC). The most important innovation of oxygen-producing photosynthesis is that its definitive electron donor is water, the most abundant chemical compound on Earth. The electron originating from the photo-oxidation of P680 will reduce the primary electron acceptor, the pheophytine (Pheo), from which -through the primary and secondary quinone acceptor molecules, the D2 protein-bound Q_A and the D1 protein-bound Q_B, respectively - the electron is transferred to the plastoquinone (PQ) pool. The PQ pool is oxidized by the cyt b6/f complex, which in the next step then reduces the plastocyanine (PC) or a soluble cytochrome c (cyt c) from which the electrons are transferred to PSI. In PSI, the primary photochemical reaction involves the oxidation of the Chl in the P700 reaction center thus producing P700+. The liberated electron is transported towards NADP+ through the primary acceptor A_0 (a Chl*a* molecule), A_1 (a phylloquinone molecule), ferredoxin (Fd) and the enzyme Fd-NADP+-oxidoreductase (FNR), which then catalyzes the reduction of NADP+ to NADPH. The vectorial electron transport chain is coupled to proton transfer processes, which generate a transmembrane electrochemical potential gradient. The transmembrane subunits of the core complexes (the intrinsic proteins) of PSII are highly conserved among the photosynthesizing organisms (Fig. 9). The main transmembrane subunits are PsbA (D1) and PsbD (D2) proteins which constitute the photochemical reaction centers and bind 6 chlorophyll a (Chl *a*) and 2 pheophytine (Pheo) molecules, and the PsbB (CP47) and PsbC (CP43)

proteins, which serve as core light-harvesting antennae and bind 16 and 14 Chl molecules, respectively (Nelson and Ben-Sherm 2004). The PSII subunits localized in the lumenal side (the extrinsic proteins) form the WOC and show large variations between the photosynthesizing organisms. The PsbO protein ensures the activity and stability of WOC and it can be found in all organisms. The other subunits responsible for the availability of Cl^- and Ca^{2+} cofactors for the water oxidation are disperse in different taxonomical groups. The structure of PSI core complex is also highly conserved in different photosynthesizing organisms (Fig. 9). In higher plants, PSI is a large membrane protein complex consisting of 14 subunits. The PsaA and PsaB proteins form the reaction center of PSI. Three extrinsic subunits (PsaC, PsaD, and PsaE) and several small intrinsic subunits can be found in the core of PSI. The PSI core binds about 100 Chl*a* and 20 β-carotene in higher plants (Nelson and Ben-Sherm 2004). The composition, structure and organization of antenna complexes of the photosystems display large variations in the photosynthesizing organisms. In higher plants and green algae, both PSI and PSII possess their own peripheral light-harvesting antenna complexes (LHCs), the LHCI and LHCII, respectively. The core complexes of the photosystems and the LHC complexes are assembled to supercomplexes. The core complexes of PSII and the attached peripheral LHCII are located in the stacked granal thylakoid membranes, while the core complexes of PSI and the attached peripheral light harvesting complexes reside in the unstacked stroma lamellae (Dekker and Boekema 2005). Such segregation of photosystems requires specific regulatory mechanisms to guarantee equal distribution of excitation energy to both photosystems under naturally fluctuating light intensities. This occurs via rapid reversible phosphorylation-triggered redistribution of light harvesting antennae between PSII and PSI, also called state transitions. This process is present in green algae, higher plants, and red algae but not in diatoms (Lavaud 2007; Grouneva et al. 2013). The PSI-LHCI supercomplex consists of the PSI core and four LHCI monomers composed of Lhca1-4 polypeptides. The PSII-LHCII supercomplex consists of a dimeric PSII core, the monomeric "minor" antenna complexes, which are called Lhcb6 (CP24), Lhcb5 (CP26) and Lhcb4 (CP29), and the most abundant "major" LHCII complexes, which are trimeric and composed of various combinations of the Lhcb1-3 proteins (Dekker and Boekema 2005). The PSII-LHCII supercomplexes are also denoted as $C_2S_2M_2$ supercomplex, where C means the PSII core, S and M mean the LHCII trimers strongly or moderately bound to cores, respectively, and the subscript indicates the number of the components. The PSII-LHCII supercomplexes are assembled into megacomplexes, which probably represent the native associations of PSII in thylakoid

membranes and form well ordered semi-crystalline layers (Dekker and Boekema 2005). LHCII trimers are also arranged into LHC-only macrodomains, lacking PSII core complexes. These are usually heptamers, and probably represent a naturally occurring aggregation state of part of the LHCII trimers in the thylakoid membranes (Dekker and Boekema 2005).

Despite the components of the photosynthetic electron transport chain are highly conserved in eukaryotes, therefore they are quite similar in higher plants and diatoms. However, several differences exist (Fig. 9) (Wilhelm et al. 2006; Grouneva et al. 2011; 2013. The PSII reaction centers isolated from diatoms contain different extrinsic proteins, the PsbU, PsbV, PsbQ` proteins and a novel protein Psb31 (whose function is not known yet), as compared to higher plants, which contain the PsbP and PsbQ proteins (Grouneva et al. 2011; 2013). Diatoms do not contain PC, instead cyt c is the mobile electron carrier between cyt b6/f and PSI (Grouneva et al. 2011; 2013). Beyond PsaA and PsaB, the PSI core of diatoms contains PsaE, PsaL and PsaM as specific PSI proteins (Fig. 10) (Grouneva et al. 2011; 2013). It has been shown that PSI of diatoms exists in monomeric form (Grouneva et al. 2011, 2013). The lateral distribution of the pigment-protein complexes in diatoms also displays several differences as compared to higher plants. In contrast to higher plants, due to the absence of granal stacks and stroma lamellae in the diatom thylakoid membranes the distribution of PSI and PSII is homogeneous, no lateral heterogeneity can be observed; however, in some cases in the outer, stromal side the PSI is more abundant (Grouneva et al. 2011, 2013). However, specific thylakoid membrane domains might exist in diatoms as proposed by Lepetit et al. (2010), where the thylakoid membrane may be differentiated based on lipid composition. The organization of the light-harvesting pigment protein complexes also exhibit differences as compared to LHCs of higher plants. For diatoms, the fucoxanthin-chlorophyll protein (FCP) complexes are the main light-harvesting antennae. The distribution of the FCP complexes is also homogeneous on both the lumenal and stromal side of the thylakoid membranes (Grouneva et al. 2011, 2013) and they transfer excitation energy equally to PSI and PSII (Grouneva et al. 2011, 2013).

1.2.1 Methods involved in studies of the photosynthetic membranes

PAM fluorometry has become one of the most common, rapid, non-invasive techniques to measure the variability of photosynthetic performance in microalgae (Kruskop and Flynn 2006; White et al. 2011; Malapascua et al. 2014; Schuurmans et al. 2015). It concerns the study of the PSII photochemical efficiency based on the amount of light emitted as

fluorescence following excitation (Lichtenthaler 2005; Baker. 2008: Kalaji et. al 2014).

Two cases are considered for the study of PSII with PAM fluorimetry: i) dark-adapted samples and ii) light-adapted samples. Dark-adapted samples are incubated in darkness before analysis. In these conditions, Q_A is completely oxidised and PSII is non-saturated, i.e. in the so-called "open" state. If a weak red (650 nm) measuring light is applied, the minimal fluorescence value F_0 is detected. If samples are then exposed to a short pulse of saturating light (typically less than one second at several thousand μmol photons m^{-2} s^{-1}), Q_A is completely reduced and PSII enters the "closed" state. Under these conditions, the maximum fluorescence value F_M is measured (Baker 2008). The difference between F_M and F_0 is defined the "variable fluorescence", F_V, whereas the ratio F_V/F_M is defined the maximum quantum yield of PSII in the dark-adapted state. Among PAM parameters, the PSII maximum quantum yield (indicated by F_V/F_M) is widely used to estimate the physiological state of microorganisms under stressful conditions (Parkhill et al. 2001; Kruskop and Flynn 2006; White et al. 2011; Schuurmans et al. 2015). Despite of this, the parameter is also employed to follow and optimize microalgae growth performance in a large-scale cultivation system (Kruskop and Flynn 2006; Malapascua et al. 2014; Schuurmans et al. 2015). In order to obtain information on native interactions of photosynthetic protein complexes, thylakoid membranes could be separated by blue native PAGE (BN-PAGE) (Rokka et al. 2005). In BN-PAGE electrophoresis charge is introduced to proteins by incubation with the dye Coomassie blue instead of the highly denaturating detergent sodium dodecyl sulphate (SDS), commonly used in SDS gel electrophoresis, which allows the proteins separation according to their size (Kügler et al. 1997; Eubel et al. 2005). The resolution capacity of BN-PAGE is very high and allows the separation of PSII and PSI, Cyt b6f complex, ATPase and LHC complexes within a single gel. This technique represents a very reliable method for analysis of protein interaction and assembly, mainly used for analysis of higher plants thylakoids but recently also for microalgae (Kügler et al. 1997; Hippler et al. 2001; Eubel et al. 2005). BN-PAGE electrophoresis is often coupled with an SDS-gel system for a two-dimensional separation in the so-called 2D BN-SDS PAGE (Eubel et al. 2005). This technique requires the incubation of native gel strips obtained from BN-PAGE with urea, β-mercaptoethanol and SDS for denaturation. After that, strips are placed horizontally on an SDS-gel system for the separation of the individual subunits that compose the protein complexes (Eubel et al. 2005). After that, SDS-gel can be stained for the visualisation of the proteins, or blotted onto nitrocellulose or polyvinylidene fluoride (PVDF) membranes for the detection of a specific protein subunit. Alternatively,

individual subunits can be cleaved from the gel and used for their identification by mass spectrometry (Eubel et al. 2005).

1.3 Biotechnological and industrial applications of microalgae

In recent decades, the idea of using microalgae for different commercial purposes has gained significant importance, and the microalgal biotechnology has started to be highly developed (Harun et al. 2010). These microorganism have received much attention as promising high-potential feedstock for biodiesel (Chisti 2007; Li et al. 2008; Brennan and Owende 2010; Sharma et al. 2012; Borowitzka and Moheimani 2013; Maity et al. 2014) and as an attractive raw material for the production of a wide range of high-value bioproducts (Molina Grima et al. 2003; Mata et al. 2010; Borowitzka 2013a; Markou and Nerantzis 2013; Odjadjare et al. 2015). Currently commercial interests are bound to the metabolic capabilities of specific microalgal strains with the aim to increase and modify the accumulation of desired compounds (Markou and Nerantzis 2013). In this perspective, recent advances in microalgal genetic engineering have opened up the possibility to use these organisms as "green cell-factories", for the over-production of natural or engineered synthesis of valuable compounds (Hempel et al. 2011; Cadoret et al. 2012; Trentacoste et al. 2013).

1.3.1 Microalgae as potential sources of bioenergy

During the last years, the increasing of global climate change and prices of petroleum and other fossil fuels has led to an urgent need to develop renewable, carbon-neutral, cost-effective alternative feedstock to displace petroleum (Scott et al. 2010; Stephens et al. 2010; Murray and King 2012). At the present, 90% of global energy demand is generated from fossil fuels and only 10% is fulfilled by renewable energy sources (Ho et al. 2011; Maity et al. 2014; Shen 2014). Among them, biomass-derived fuels have been developed as a sustainable and environmentally friendly alternative energy sources for decades (Sheehan et al. 2009; Demirbas and Demirbas 2011; Ndimba et al. 2013). "First-generation biofuels" were focused on ethanol and biodiesel fuels, and were developed primarily from terrestrial plant crops such as corn, soy, rapeseed and palm (Naik et al 2010). "Second-generation biofuels" evolved away from undesirable competition with the agricultural sector and focused on plant-derived lignocellulosic alcohols (Naik et al 2010). "Third-generation biofuels" have focused on lipid-rich microalgal biomass as promising renewable source as

compared to biomass used for first and second-generation biofuels (Wijffels and Barbosa 2010; Suali and Sarbatly 2012; Borowitzka and Moheimani 2013; Chisti 2013). Currently, a vast number of microalgal species are object of extensive research designed to identify the best oleaginous microalgae strains, since the high lipid productivity is one of the main desirable characteristics for biofuel and bioenergy production (Hu et al. 2008; Griffiths and Harrison 2009; Hempel et al. 2012; Sharma et al. 2012). Several microalgal species, mainly Chlorophytes and Bacillariophytes, have the ability to produce large amount of lipids, particularly neutral lipids in the form of triacylglycerol (TAGs), which can be converted to fatty acid methyl esters (FAMEs), the best substrate for the production of biodiesel, through transesterification, or refined into other fuel constituents (Griffiths and Harrison 2009; Brennan and Owende 2010; Suali and Sarbatly 2012). Total lipids and other biomass constituents can be converted into crude oil alternatives through thermochemical processes such as hydrothermal liquefaction (Brennan and Owende 2010; Gao et al. 2012a; Suali and Sarbatly 2012). Beside the microalgal lipids for biodiesel production, carbohydrates are also a preferable feedstock for several biological biomass conversion technologies and especially for the technology of fermentation of sugars for the production of bioethanol (Ho et al. 2013). Moreovers, some species can produce biohydrogen (Radakovits et al. 2010). Today the main focus of the microalgal biofuel research is given in the area of optimization of microalgal culture conditions for increasing the suitability of lipids for biodiesel in terms of the type and amount produced by an algal species, such as chain length, degree of saturation and proportion of total lipid made up by TAGs, since these characteristics influenced the quality of biodiesel (Hu et al. 2008; Griffiths and Harrison 2009; Francisco et al. 2010). It is widely accepted that microalgae increased the synthesis and accumulation of TAGs in response to stress culture conditions, such as high-light intensities, temperature, salinity, pH or nutrient depletion (Hu et al. 2008; Rodolfi et al. 2009; Hildebrand et al. 2012; Sharma et al. 2012; Popovich et al. 2012a; Santos et al. 2014; d'Ippolito et al. 2015). Unfortunately, these conditions of growth do not allow achieving the high biomass productivity requested for a profitable exploitation of these microorganisms in bioenergetic fields (Pruvost et al. 2009; Heredia-Arroyo et al. 2011; Adams et al. 2013). Biomass productivity and lipid content need to be consider simultaneously and are often inversely correlated, as the storage of TAGs inside cells occurs at the expense of energy used for growth (Wijffels and Barbosa 2010; Breuer et al. 2012; Hempel et al. 2012; Klok et al. 2013). However, the bioenergy processes from microalgae is technically feasible, but not yet economically competitive (Gao et al. 2012a; Chisti 2013). The production of biofuels from

algal biomass is more expensive than the current production cost of fossil-oil derived fuels. Currently, the cost for producing algal biofuels is estimated to range from $1.68 to $75 for liter and will need to be lowered to less than $1 for liter to compete with fossil oil-derived fuels production costs (Chisti 2013). On this regard, for an effective use of renewable resource as biodiesel, it is necessary to reduce the cultivation and production costs (Sheehan 2009; Solomon 2010; Wijffels and Barbosa 2010; Suali and Sarbatly 2012; Chisti 2013). Lately, some studies reported that genetic modification could boost the neutral lipid accumulation in microalgae (Poulsen et al. 2006; Radakovits et al. 2010; Trentacoste et al. 2013). These promising results suggest that targeted metabolic manipulation can be also applied greatly to improve the economic feasibility of biodiesel production.

1.3.2 Microalgae as potential sources of bioactive compounds and value-added products

Microalgae are important source of commercially bioactive compounds including pigments, polyunsaturated fatty acids (PUFAs), phytosterols, and toxins that are widely exploited in pharmaceutical, cosmetic and nutritional sectors (Pulz and Gross 2004; Spolaore et al. 2006; Raja et al. 2008; Borowitzka 2013a; Markou and Nerantzis 2013; Odjadjare et al. 2015).

Pigments

Among these molecules, microalgae and diatoms have been explored as sources of carotenoids. β-Carotene produced by microalgae is directed mainly at the natural food colorant, nutraceutical or health food market (Raja et al. 2008; Borowitzka 2013a; Markou and Nerantzis 2013; Odjadjare et al. 2015). *Dunaliella salina* is the first green alga to be commercialised as a source of a high-value product (Borowitzka 2013a). This alga, which is known to be a highly halotolerant species, is widely exploited for its ability to produce a large amount of β-Carotene (up to 14% of its dry weight), consisting of all-trans and 9-cis isomers (Ben-Amotz 2007; Leonardi et al. 2011). Unlike the synthetic β-Carotene, which is only in the form of all-trans isomer, the *Dunaliella* β-Carotene 9-cis-isomer represents an additional advantage in the marketing of the algal product. It was reported that 9-cis-isomer of β-Carotene plays a major role in quenching free oxygen radicals and preventing oxidative damage to the cell (Ben-Amotz et al. 1988). Astaxanthin is another carotenoid that can be derived from algae and is principally used as a feed additive in aquaculture and as a dietary supplement or anti-oxidant (Spolaore et al. 2006; Del Campo et al. 2007; Raja et al. 2008).

It is commercially derived from the freshwater green alga *Haematococcus pluvalis*, which is capable to synthetize a large amount of astaxanthin (up to 3% of its dry weight) under stressful conditions (Raja et al. 2008; Markou and Nerantzis 2013). Other microalgae, such as *Chlorella* (Del Campo et al. 2004), *Chlorococcum* (Ma and Chen 2001) and *Scenedesmus* (Qin et al. 2008), are also known to be potential producers of astaxanthin. Microalgae are also potential source of other pigments of interest. For examples, the green algae *S. almeriensis* and *Auxenochlorella protothecoides* are known to produce lutein (Fernández-Sevilla et al. 2010), whereas the marine diatom *P. tricornutum* is a rich source of fucoxanthin (Kim et al. 2012). In particular, fuxoxanthin has received much attention for its pharmaceutical role as an anti-oxidant, anti-inflammatory and anticancer molecule (Peng et al. 2011; Chung et al. 2013; Fu et al. 2015). Moreover, the phycobilin pigments (phycocyanin, phycoerythrin and allophycocyanin), which are mainly found in cyanobacteria such as *Spirulina* (*Arthrospira*) *platensis,* red algae such as *Porphyridium* sp., and in some Cryptophyta and Glaucophyta, have gained increasing interest (Borowitzka 2013a; Markou and Nerantzis 2013). These hydrophilic pigments have a wide range of applications such as food and cosmetic colorant, as a fluorescent agent used in flow cytometers and immunoassay, as photosensitisers in photodynamic therapy for treatment of cancers and for other therapeutic benefits (Sekar and Chandramohan 2008; Borowitzka 2013a; Markou and Nerantzis 2013).

Long-chain polyunsaturated fatty acids

Microalgae are also excellent accumulators of fatty acids, especially the long-chain polyunsatured fatty acids (PUFAs) such as ϒ-linoleic acid, arachidonic acid, eicosapentaenoic (EPA) and dodecahexaenoic acids (DHA), which can be purified to provide a high value food supplement. In addition, microalgae are the first producers of PUFA, in fact animals and higher plants are unable to synthetize these molecules (Spolaore et al. 2006). For this reason, these microorganisms are mainly used as a nutritionally food in aquaculture (Lebeau and Robert 2003a; Lopez et al. 2005; Guedes et al. 2011) but also as additive in human food nutrition (Spolaore et al. 2006). The diatoms *Phaeodactylum* sp. and *Odontella aurita* are rich in EPA, *S. platensis* is known to be the best algal source of ϒ-linoleic acid, while *Pavlova lutheri* has been shown to contain large amounts of DHA (Guedes et al. 2011). The thraustochytrids *Cryptothecodinium cohni* is known to be a highly producer of DHA and EPA (up to 30-50% of its constituent fatty acids) (Guedes et al. 2011). The

docosahexaenoic acid extracted from this alga represents the only algal PUFA that is commercially available, since the other algal extracts are not yet competitive sources of eicosapentaenoic, ϒ-linoleic and arachidonic acids against other primary sources (Brennan and Owende 2010; Yaakob et al. 2014).

Other bioactive compounds and applications

Microalgae produce a wide range of other bioactive compounds which having antibiotic, antiviral, and anticancer activity (Borowitzka 1995). It was demonstrated that phytosterols derived from algae had positive effects on human health as cholesterol lowering agents and for the maintenance of normal prostate size and function (Volkman 2003). *Chlorella* sp. represents the main source of β-1,3-glucan, which is an active immunostimulator and has many other activities such as free radical scavenger and a reducer of blood lipids (Spolaore et al. 2006; Markou and Nerantzis 2013; Yaakob et al. 2014). Microalgal proteins might be also of interest because their nutrition quality is comparable to other referenced food proteins, due to their good profile and their good proportion of their amino acids (Becker 2007). Recently, proteins with particular applications in the food industry have been isolated from *Tetraselmis* sp. (Schwenzfeier et al. 2013). Engineered microalgae have shown their potential for the production of therapeutics and recombinant enzymes antibodies (Tran et al. 2013; Hempel et al. 2011). Many microalgae, especially the dinoflagellates, are also known to produce potent toxins. Among these toxins, ciguatoxin from the dinoflagellate *Gambierdiscus toxicus*, okadaic acid from the dinoflagellates *Prorocentrum lima* and genus *Dinophysis* sp., and domoic acid from the diatom *Pseudonitzchia* sp., could be potentially useful in health industries as lead compounds for the development of drugs (Camacho et al. 2006). The above mainly have considered algal products with pharmaceutical applications; however, other applications of microalgae are possible. For example, microalgal biomass is proposed as renewable, carbon-neutral, and sustainable alternative to chemical fertilizers in agricultural sector (Shaaban 2001 a,b; Abdel-Raouf et al. 2012; Trentacoste et al. 2015). Recently, researchers have succeeded in producing relevant polymers such as poly-3-hydroxybutyrate (PHB), precursor of bioplastic, in the diatom *P. tricornutum* (up to 10% of its dry weight), designing diatoms as more desirable source of PHB for the production of biodegradable plastics (Hempel et al. 2011). Moreover, extensive researches have been explored the potential applications of diatom frustules in nanotechnology (Lebeau and Robert 2003a; Kroth 2007; Gordon et al. 2009). Among

which engineered biosensors (Bismuto et al. 2008), drug delivery systems (Zhang et al. 2013), molecular filtration (Kroth 2007), solar cells, conductive electronic devices (Jeffryes et al. 2011) and enzyme immobilizers (Poulsen et al. 2007) has been examined. Finally, after the extraction of oils and other compounds, residual biomass can be used as animal feed, fertilizer and as substrate for anaerobic digestion, anaerobic fermentation, liquefaction for the production of biofuels (Pulz and Gross 2004; Chisti 2007; Zamalloa et al. 2012; Nobre et al. 2013).

1.3.3 Microalgae as promising bioremediation agents

Due to their higher rates of CO_2 fixation and ability to grown in various wastewater systems, microalgae are widely considered as promising bioremediation agents to be exploited in order to reduce the impact on the environment derived from the growing global industrialization (Razzak et al. 2013; Maity et al. 2014; Shen 2014; Singh et al. 2016). It is currently accepted that the indiscriminate and excessive use of nutrients in wastewater has significantly contributed to the global eutrophication (Cai et al. 2013). To overcome this problem, there is an urgent need to find an eco-friendly approach for reducing the impact of nutrients and other potentially toxic compounds in wastewater. By comparing the conventional wastewater technologies that are based on chemical and physical methods, microalgae represent a potential biological treatment method for a wide variety of wastewater streams (Markou and Georgakakis 2011; Cai et al. 2013; Abinandan and Shanthakumar 2015). These microorganisms are known to efficiently remove diverse type of pollutants and toxic chemical compounds from agricultural (Mulbry et al. 2008; Wang et al. 2010), municipal (Wang and Lan 2011; de Alva et al. 2013), domestic (Cabanelas et al. 2013) and industrial (Chinnasamy et al. 2010; Levine et al. 2011) wastewaters. Among microalgae, the species *Chlorella*, *Scenedesmus* and *Neochloris* could be efficiently used for the removal of nitrogen (N) and phosphate (P) from different source of wastewater (Cai et al. 2013; Abinandan and Shanthakumar 2015; Singh et al. 2016). In addition, due to their high bioabsorption ability diatoms and green algae have been frequently used for the phytoremediation of heavy metals contamination (Lebeau and Robert 2003a; Mehta and Gaur 2005; Monteiro et al. 2012).

Global warming resulting from extensive CO_2 emissions due to human activities has become of increasing concern as an environmental issue (Shen 2014; Cheah et al. 2015). The anthropogenic emission of CO_2 from thermoelectric power plants is responsible for up

to 7% of global CO_2 emissions (de Morais and Costa 2007). Among the various approaches for mitigating CO_2, the biological CO_2 biofixation has received much attention as an alternative strategy to chemical reaction-based approaches (Wang et al. 2008; Lam et al. 2011; Kumar et al. 2010; Maity et al. 2014). In this perspective, the CO_2 bio-fixation by microalgae offers numerous advantages, since these microorganisms have faster growth rates, higher rates of carbon dioxide fixation and higher photosynthic efficiencies as compared to plants (Chisti 2007; Li et al. 2008 Singh and Singh 2014). Moreover, the microalgal biomass produced in the process of CO_2 bioremediation can be used for making high-value products (e.g. pigments and nutraceuticals; Borowitzka 2013a) or low-value commodities (e.g. food and biofuel; Wijffels and Barbosa 2010). For CO_2 bio-mitigation coupled with biodiesel production, several strains of microalgae were recently found to be highly tolerant to CO_2 because they can grow under high CO_2 concentrations and simultaneously produce a significant amount of lipids suitable for biodiesel production (Lam et al. 2012). These include the freshwater green algae *C. vulgaris* (Sydney et al. 2010; Yoo et al. 2010), *Scenedesmus obliquus* (de Morais and Costa 2007; Tang et al. 2011); *Botryococcus braunii* (Sydney et al. 2010; Yoo et al. 2010), the marine eustigmatophyceae *Nannochloropsis oculata* (Chiu et al. 2009), the marine planktonic diatoms *Chaetoceros muelleri* (Wang et al. 2014) and *Thalassiosira weissflogii* (Ishida et al. 2000) and the benthic diatoms *Nitzschia ovalis* and *Amphora coffeaeformis* (Tew et al. 2014). Recently, industrial flue gases, which usually contain between 5-15% CO_2 and other contaminants such as oxides of nitrogen and sulphur, can be used directly without any pre-treatment as carbon dioxide source in the microalgal cultivation (Lizzul et al. 2014; Mortensen and Gislerød 2014; Moheimani 2015; Singh et al. 2016).

1.4 The large-scale production of microalgae and the bio-refinery algal strategy

The environmental and economical impact of large-scale production of microalgae has been widely discussed, with the aim of making the process increasingly sustainable (Sheehan 2009; Solomon 2010). Nevertheless, significant obstacles still need to be solved before microalgal production becomes a cost-effective process (Solomon 2010; Wijffels and Barbosa 2010; Suali and Sarbatly 2012; Chisti 2013). In order to obtain that, a number of bottlenecks need to be removed and a multidisciplinary approach is required, such as the integration of the engineering with discoveries in algal biology, within a bio-refinery algal strategy (Scott et al. 2010; Wijffels and Barbosa 2010; Vanthoor-Koopmans et al. 2013).

The term "biorefinery" is an integrated facility, which combines various processes and equipment to produce biofuels, power, and high-value products from biomass (Fig. 11). The biorefinery strategy aims to a sustainable utilization of biomass feedstock into a range of commercial products and energy in order to maximizing its value (Demirbas 2009; Subhadra 2010; Vanthoor-Koopmans et. al 2013). In this perspective, currently studies have demonstrated that the improvements of the economic feasibility of the microalgae production could be attained by the cultivation of microalgae for simultaneous production of biofuels and specific high-value compounds combined in a biorefinery concept (Singh et al. 2011; Gao et al. 2012a; Nobre et al. 2013).

The efforts to enhance the production process of microalgae can be achieved by improving the knowledge of the physiological metabolism of the microalgal cell, and through the development of an effective and economic microalgal culture system in terms of engineering and design aspects (Brennan and Owende 2010; Harun et al. 2010; Scott et al. 2010; Chen et al. 2011; Gao et al. 2012a; Suali and Sarbatly 2012).

Among the physiological approaches, the choice of the best microalgae strains is an important first step for the commercial-scale production of microalgae (Pulz and Gross 2004; Borowitzka 2013b). The diversity of the microalgae represents both an opportunity and a challenge in the selection process, since each microalgal species has developeded a kind of a unique metabolic framework, certainly determined by their phylogenetic and environmental constraints (Falkowski et al. 2004; Leliaert et al. 2012; Andersen 2013). The selection of fast-growing, productive microalgae species, also well suited to the biorefinery approach and optimised for the local climatic conditions has a fundamental importance to the success of any large scale-production of microalgae contributing to both economic and environmental sustainability of the process (Griffiths and Harrison 2009). However, it is also known that the growth performance and biochemical composition of a microalgal strain is significantly depended on culture conditions (Hu et al. 2004; Juneja et al. 2013). In fact, microalgae have the ability to modify their biomass composition under stress culture conditions and accumulate along with the lipids and carbohydrates, which could be used for biofuel production (Hu et al. 2008; Mata et al. 2010), other specific high-value secondary metabolites (such as pigments, PUFA, vitamins etc.), which have several commercial applications (Spolaore et al. 2006; Markou and Nerantzis 2013). However, a serious issue about the cultivation of microalgae under stress conditions is the decrease of growth and yield rates that consequently affected the total productivity (Markou and Nerantzis 2013). For example, the accumulation of the desired compounds (such as

pigments, lipid, carbohydrates) is induced as an energy storage mechanism, when cells are cultivated under stress conditions such as high–light intensities, temperature, salinity, pH or nutrient depletion (Markou and Nerantzis 2013). Unfortunately, these conditions of growth do not allow achieving the high biomass densities requested for a profitable exploitation of these microorganisms in biotechnological fields (Pruvost et al. 2009; Heredia–Arroyo et al. 2011; Adams et al. 2013). However, this negative effect might be mitigated applying various techniques. Recently, several studies have suggested that the selection of the most adequate microalgal culture conditions could be also another way to achieve the productivities required for a profitable industrial scale-up production of microalgae (Chojnacka and Marquez-Rocha 2004; Scott et al. 2010; Chen et al. 2011). One of the most suggested techniques is the cultivation of microalgae in multiple-stage process, in which in the first stage optimum conditions are applied aiming the maximization of biomass production, while in the second stage, stress conditions are applied aiming the accumulation of the desired compounds (Rodolfi et al. 2009). Nevertheless, the cultivation in multiple-stages might consume more energy in comparison to the one-stage systems (Aflalo et al. 2007). Heterotrophy and mixotrophy have been recently proposed as alternative and efficient cultivation modes that showed several advantages over the autotrophic one (Perez-Garcia et al. 2011; Heredia-Arroyo et al. 2010; Chandra et al. 2014). Most of the microalgal cells can be cultured under autotrophic, heterotrophic and mixotrophic conditions (Heredia-Arroyo et al. 2010). Autotrophy is the normal condition of growth, where light is used as energy source in order to fix CO_2 in organic macromolecules. Conversely, heterotrophic cells utilise only organic compounds as carbon and energy source in darkness (Mata et al. 2010; Chen et al. 2011, Perez-Garcia et al. 2011), whereas mixotrophy is termed the condition in which organic carbon is supplied in the culture medium together with light, so microalgae can benefit from coupling photosynthetic activity with the organic carbon assimilation for growth (Ogbonna et al. 2002, Chandra et al. 2014). Recently, mixotrophic and heterotrophic growth have been considered alternative methods for achieving higher biomass densities and lipid production as compared to autotrophic cultivation (Brennan and Owende 2010; Heredia-Arroyo et al. 2011; Perez-Garcia et al. 2011; Cheirsilp and Torpee 2012; Chandra et al. 2014). Moreover, several studies performed on several mixotrophic microalgal species have shown a strong increase in biomass and lipid yields, not only with respect to autotrophic cultures, but also compared to heterotrophic cultures (Yang et al. 2000; Brennan and Owende 2010; Giovanardi et al. 2013; Silva et al. 2016; Giovanardi et al. *In Press*). Microalgae can assimilate a variety of organic carbon sources

including glucose, acetate, fructose, glycerol and sucrose, which can be used for growing algae in mixotrophy (Cerón García et al. 2006; Heredia-Arroyo et al. 2010,2011; Wang et al. 2012; Giovanardi et al. 2014; Baldisserotto et al. *In Press*). However, the cost of organic carbon source represents the 50-80% of the cost of microalgal growth medium (Cheng et al. 2009). For this reason, finding cheaper organic carbon sources is becoming very important in the perspective of limiting costs and allowing feasibility of microalgal exploitation on the large scale (Brennan and Owende 2010; Heredia-Arroyo et al. 2010; Cheng and Walker 2011; Chen et al. 2011). In addition, further researches in genetic and metabolic engineering could offer the possibility to improve the performance of a microalgae strains and, at the same time, to optimize the production of a desirable compound (Hu et al. 2008; Wijffels and Barbosa 2010; Cadoret et al. 2012; Trentacoste et al. 2013).

Among the engineering and design aspects, the microalgal cultivation systems are crucial factors to be taken into account in order to reduce the operative cost of microalgal production (Rodolfi et al. 2009; Smith et al. 2010; Stephens et al. 2010; Chen et al. 2011; Borowitzka and Moheimani 2013). A wide variety of systems has been described in literature (Carvalho 2006; Harun et al. 2010; Mata et al. 2010). Microalgae can be cultivated in open-culture systems such as lakes or ponds and in highly controlled closed-culture systems called photo-bioreactors (PBRs), depending on algal strain, issue of research, type of desired products and environmental conditions (Fig. 12) (Chisti 2007; Grobbelaar 2009; Brennan and Owende 2010; Harun et al. 2010; Mata et al. 2010). Open ponds have been used since the 1950s and represent the less expensive cultivation system for large-scale production of microalgae (Borowitzka 1999). The types of ponds that are currently used in research and industry include raceway ponds, shallow big ponds, circular ponds tanks and closed ponds (Borowitzka 1999; Lee 2001). The location in which the pond is situated is a critical factor in determining the type of pond selected, algal strain, and amount of light for photosynthesis (Grobbelaar 2009; Harun et al. 2010). The most commonly used open pond system is the raceway pond, which consists of a pond in the shape of a raceway, and the liquid is circulated around the pond by a paddle wheel (Fig. 12a) (Chisti 2007; Brennan and Owende 2010; Mata et al. 2010). They are typically made of a closed loop, oval shaped recirculation channels, generally between 0.2 and 0.5m deep, in order to allow light penetration through the water. A paddlewheel is usually placed in order to promote mixing and circulation of both microalgal biomass and nutrients, which are usually introduced in front of it, whereas microalgal biomass is harvested after the whole cycle, behind the paddlewheel (Chisti 2007; Brennan and Owende 2010). CO_2 is instead provided by the

atmosphere. Due to scalability and low cost of these building, these systems represent the most popular way for a large-scale cultivation of microalgae. However, several disadvantages affect these systems. Generally ponds are more susceptive to weather conditions, not allowing control of water temperature, which fluctuated within a diurnal cycle and seasonally, evaporation, CO_2 and lighting (Brennan and Owende 2010; Harun et al. 2010; Mata et al. 2010) The biomass concentration generally remains low between 0.1-1.5 gL^{-1} (Borowitzka 1999; Lee 2001; Mata et al. 2010). This is mainly because raceways are inefficient mixed and optimum light intensity cannot be achieved. In addition to the low cell density, open ponds are often subjected to significant contamination by other undesired algal species and protozoa. Thus, these systems are suitable for only a limited range of microalgae species that could grow in highly selective environments such as the halotolerant chlorophyceae *D. salina*, the high resistant trebouxiophyceae *Chlorella* sp., and the high-alkalinity cyanobacterium *Spirulin*a sp. (Borowitzka 1999; Lee 2001; Harun et al. 2010). Although open ponds are the most commonly used culture systems, due to their low cost of production and operation, recently closed PBRs are receiving much attention for the possibility of producing valuable compounds (Pulz 2001; Grobbelaar 2009; Smith et al. 2010). Closed photobioreactor technology allows to overcome some of the major problems associated with the open pond system (Brennan and Owende 2010; Harun et al. 2010). These culture systems offer better control over culture conditions and growth parameters, prevent evaporation, reduce CO_2 losses, allow to attain higher microalgae productivity and offer a more safe and protected environment, preventing contamination by other microorganisms (Chisti 2007; Grobbelaar 2009; Mata et al. 2010). Basically, closed photobioreactor comes in a different range of designs: tubular and plate-types (Carvalho 2006; Chisti 2007; Brennan and Owende 2010). Those reactor types are based on the same principles and offer an higher efficiency of mixing, higher volumetric mass transfer rates, higher productivity and the best controllable growth condition as compared the open pond system (Fig. 12b) (Chisti 2007; Brennan and Owende 2010). Both types of photobioreactor configurations comprise: a light-harvesting unit, which employs small diameter tubing that favors high photosynthetic activity, and a gas exchange unit, in which air or CO_2 is supplied (Fig. 12b) (Carvalho 2006). The culture is circulated between those two units by a pump, which needs to be carefully designed and operated in order to prevent shear forces from disrupting cell integrity. The most widely used PBR is of tubular design and most configurations of tubular reactors are one of the following three types: (i) simple airlift and bubble column, which are composed of vertical cylinder made out of transparent glass or

plastic (in the form of a vertical tubular reactor) that is aerated from the flow, usually by gas bubbling, and illuminated through transparent walls by external or internal lights; (ii) horizontal tubular reactor, which is composed of horizontal transparent tubing, usually bearing gas transfer systems attached to the connections; and (iii) helical tubular reactor, which is composed of a flexible plastic tube coiled in a circular framework (Carvalho 2006). Flat panels, also known as flat plate PBRs, are essentially rectangular boxes composed of translucent glass or plastic. Air is bubbled from the bottom, which provides sufficient mixing and gas transfer. The main advantages of flat plate are their high productivity, their low power energy consumption and uniform distribution of light (Carvalho 2006; Harun et al. 2010). The major difference between the configurations of the reactors already described (vertical, horizontal or inclined plane) is that the vertical design allows greater mass transfer and a decrease in energy usage, while the horizontal reactor is more scaleable, but requires a large area of land (Harun et al. 2010). Despite their advantages, PBRs suffer from several drawbacks that need to be considered and solved. Their main limitations include: overheating, bio-fouling, oxygen accumulation, difficulty in scaling up, the high cost of building, operating and of algal biomass cultivation, and cell damage by shear stress and deterioration of material used for the photo-stage (Mata et al. 2010; Harun et al. 2010). However, algal cultures require large amounts of water in both open and closed culture systems (Lam and Lee 2012; Borowitzka and Moheimani 2013). Considering that water is becoming a scarce natural resource, water demand represents an important factor when performing large-scale cultivation (Chisti 2013; Batan et al. 2013; Farooq et al. 2014). In this perspective, the mass cultivation of halotolerant strains in brackish and seawater media can be considered an interesting alternative to moderate the freshwater consume (Popovich et al. 2012a). In order to lower the process costs and, at the same time, to make microalgal production more environmentally sustainable, recycling culture medium has been proposed as a possible solution (Yang et al. 2011; Hadj-Romdhane et al. 2012; Sabia et al. 2015), especially in large-scale culture systems (Borowitzka 2005; Lam and Lee 2012; Zhu et al. 2013).

Tables and Figures

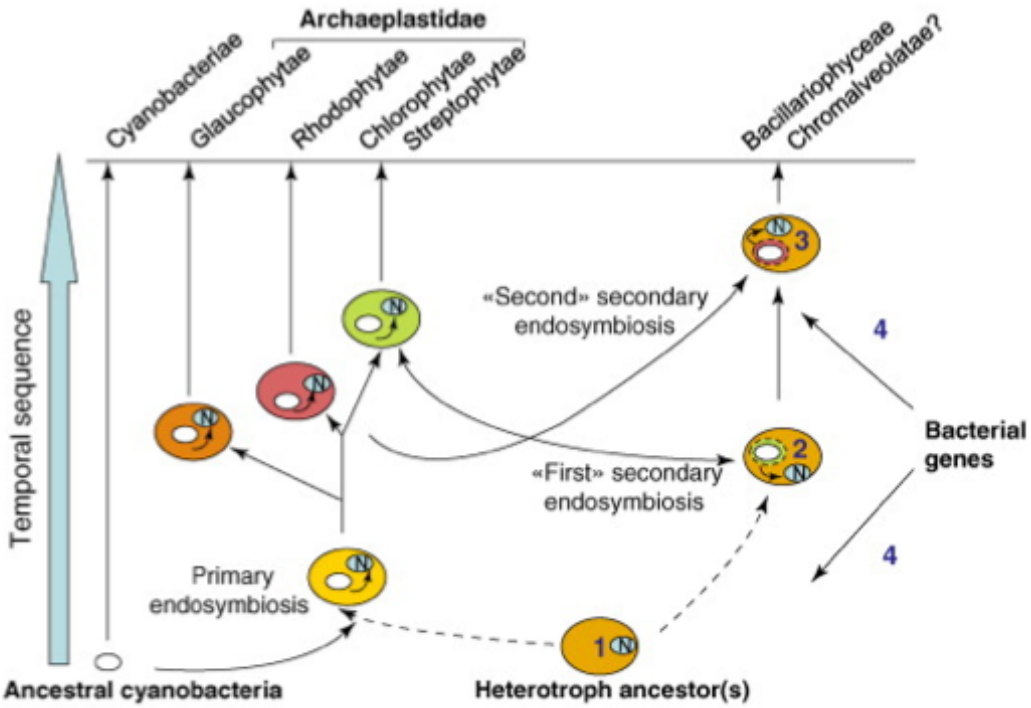

Fig. 1: Proposed origins of primary and secondary endosymbiosis in eukaryotic phytoplankton. The basic mechanisms of photosynthesis were invented in ancestral cyanobacteria and are essentially unchanged from this early proterozoic state. A unique primary endosymbiotic event between an ancestral cyanobacteria and a heterotroph is thought to be at the origin of Archaeplastidae, including Glaucophytae, Rhodophytae (red algae), Chlorophytae (green algae) and Streptophytae (land plants). Bacillariophyceae (diatoms) genomes are chimeras of genes derived from at least four different sources, including (1) the heterotrophic ancestor(s), (2) a ''first''green algal secondary endosymbiont, (3) a ''second'' red algal secondary endosymbiont, and (4) a range of bacteria via multiple lateral gene transfer events. It is not known whether these features are specific to diatoms or are common to all chromalveolates. Phylogenetic relationships between heterotrophic ancestors which gave rise to Archaeplastidae and to Chromalveolatae (including Bacillariophyceae) are unknown. N: nucleus. The small arrows inside cells represent gene transfer events from endosymbionts towards the nuclei of host cells. (*From: Finazzi et al. 2010*)

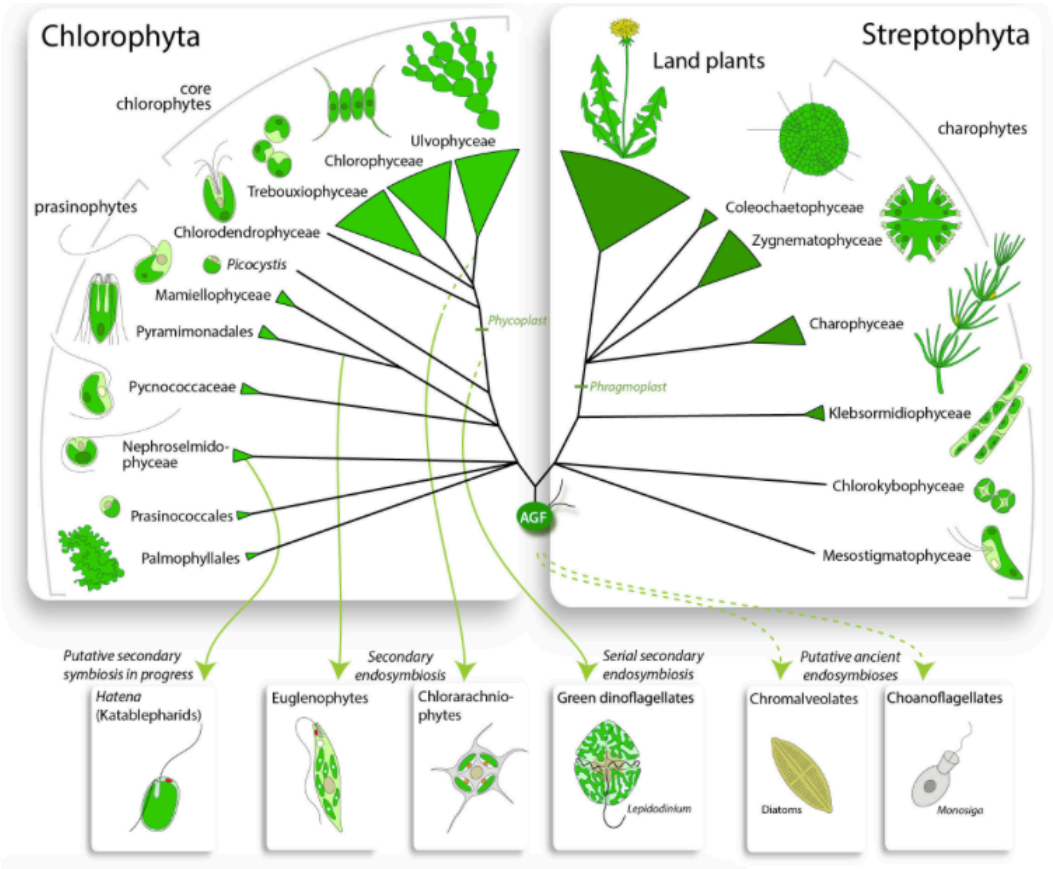

Fig. 2: Overview phylogeny of the green lineage (top) and spread of green genes in other eukaryotes (bottom). Current hypotheses on green algal evolution posit the early divergence of two discrete lineages: the Chlorophyta and Streptophyta. The Chlorophyta includes the majority of described species of green algae. The Streptophyta are comprised of the charophytes, a paraphyletic assemblage of freshwater algae, and the land plants. (*From: Leliaert et al. 2012*)

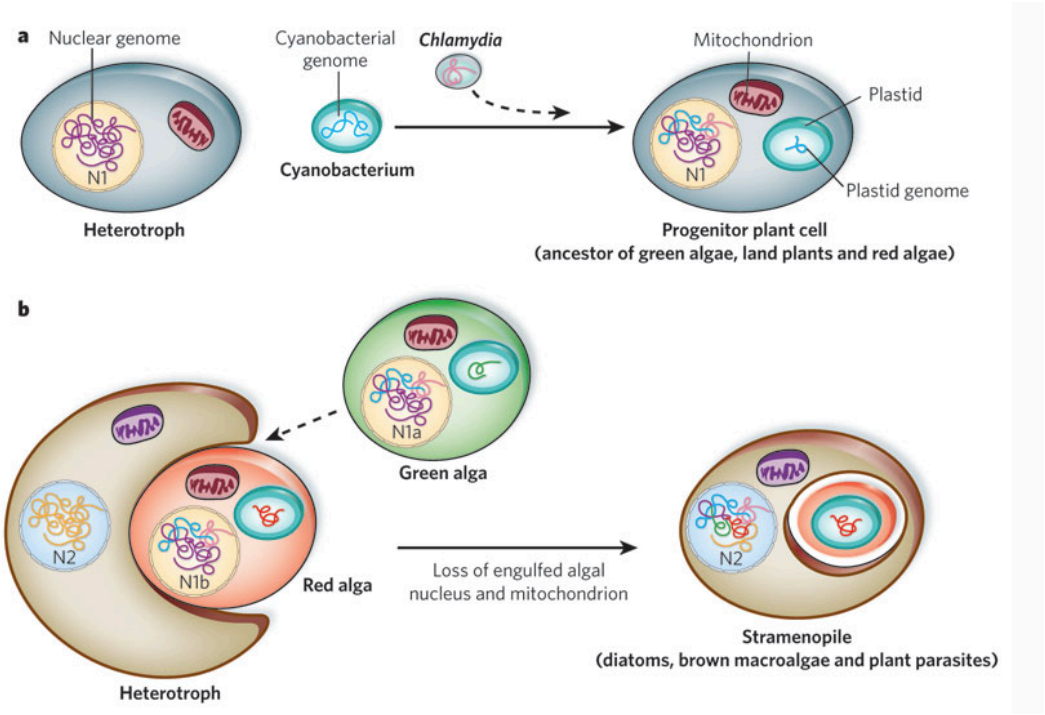

Fig. 3: Endosymbiotic origin of Stramenopiles. **(a)** Primary and **(b)** secondary endosymbiotic event. **(a)** Origin of the ancestor of plant and green/red algae, through the engulfment of a cyanobacterium and possibly a chlamydia. Genes from both invaders were progressively transferred to the host nucleus (N1). The cyanobacterium conserved some genes and became the progenitor of plant and green/red algal chloroplasts. **(b)** Origin of diatoms and Stramenopiles: the photosynthetic eukaryote, presumably differentiated as a red alga and was engulfed by a eukaryotic heterotroph. The algal nucleus (N1) progressively disappeared, genes were transferred to the host nucleus (N2) and the red algal symbiont was reduced to the present chloroplast of Stramenopiles, characterized by four membranes. (*From: Armbrust 2009*)

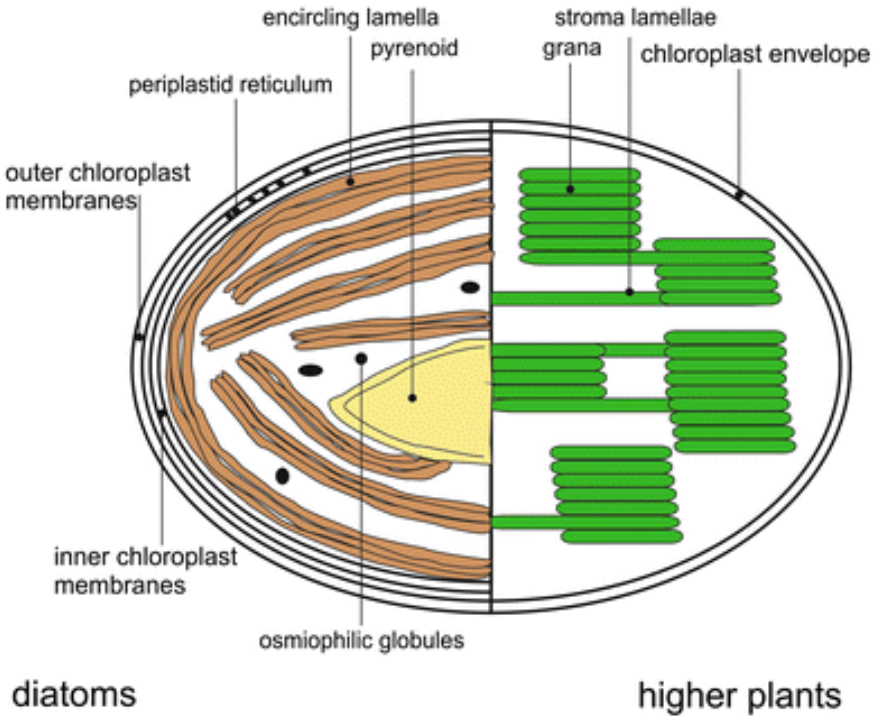

Fig. 4: Schematic overview of chloroplast and thylakoid structure in diatoms (left) and higher plants (right). Chloroplasts of higher plants have two envelope membranes. They are the product of a primary endosymbiosis in which a prokaryotic photosynthetic organism was engulfed by a heterotrophic eukaryote and reduced to an organelle. Diatom plastids are engulfed by four membranes, a consequence of their origin in a secondary endosymbiosis event, where a heterotrophic eukaryote acquired a photosynthetic eukaryote. The thylakoids of diatoms form triple lamellae. One of those encircles the chloroplast stroma. The pyrenoid is a RubisCO-enriched structure within algal chloroplasts dedicated to CO_2-fixation. (*From: Grouneva et al. 2013*)

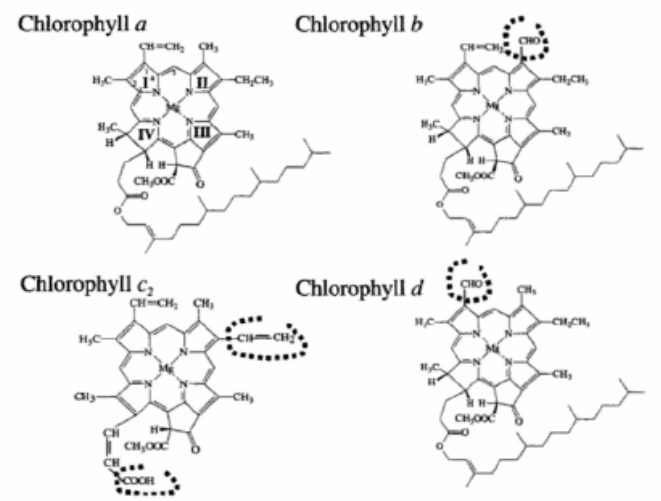

Fig. 5: Chemical structure of the different chlorophylls *(From Larkum 2003)*

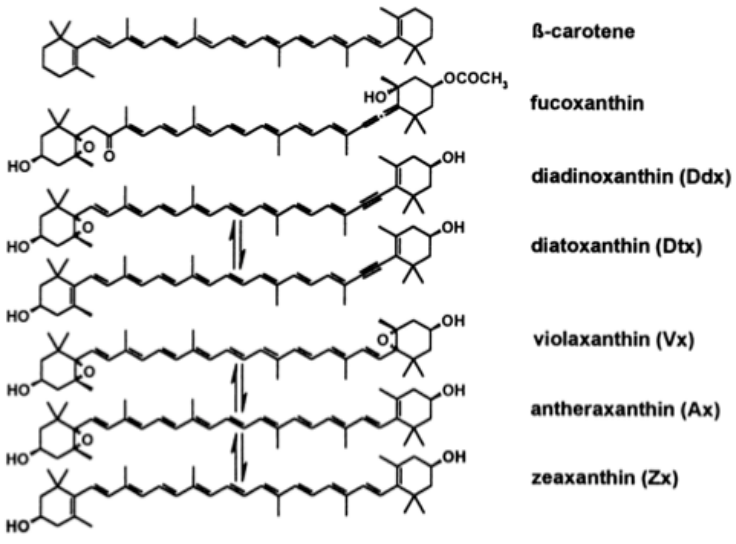

Fig. 6: Molecular structures of the carotenoids mentioned in the text (*From Lohr and Wilhelm 1999)*

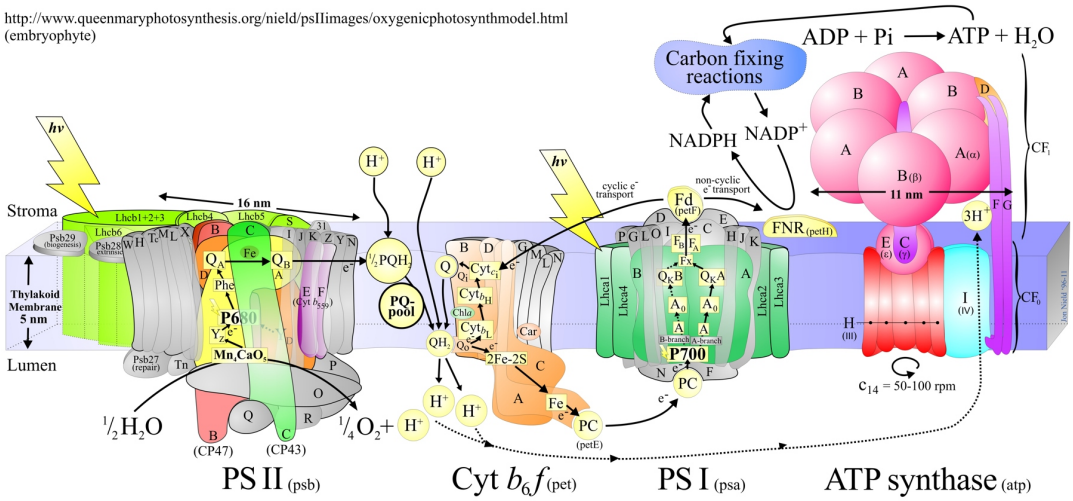

Fig. 7: A detailed model of the organisation of the protein complexes involved in electron (e-) and proton (H+) transport within the thylakoid membrane of the photosynthetic apparatus in organisms performing oxygenic photosynthesis. PSII = photosystem II; PQ = plastoquinone; PQH = plastoquinol; Cyt b6f = cytochrome b6f complex; PC = plastocyanin; PSI = photosystem I; Fd = ferredoxin; FNR = ferredoxin-NADP+reductase; NADPH = reduced nicotinamide adenine dinucleotide phosphate; ADP = adenosine diphosphate; Pi = inorganic phosphate; ATP = adenosine triphosphate. *(Figure downloaded from the official web site: http://www.queenmaryphotosynthesis.org/nield/psIIimages/oxygenicphotosynthmodel.html)*

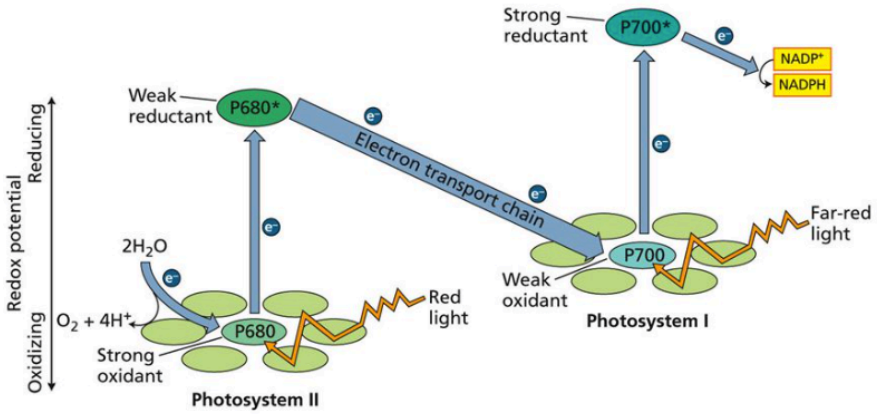

Fig. 8: Rappresentation of the «Z-scheme» of electron transfer during light-reactions in photosynthesis. *(From Taiz and Zeiger 2010)*

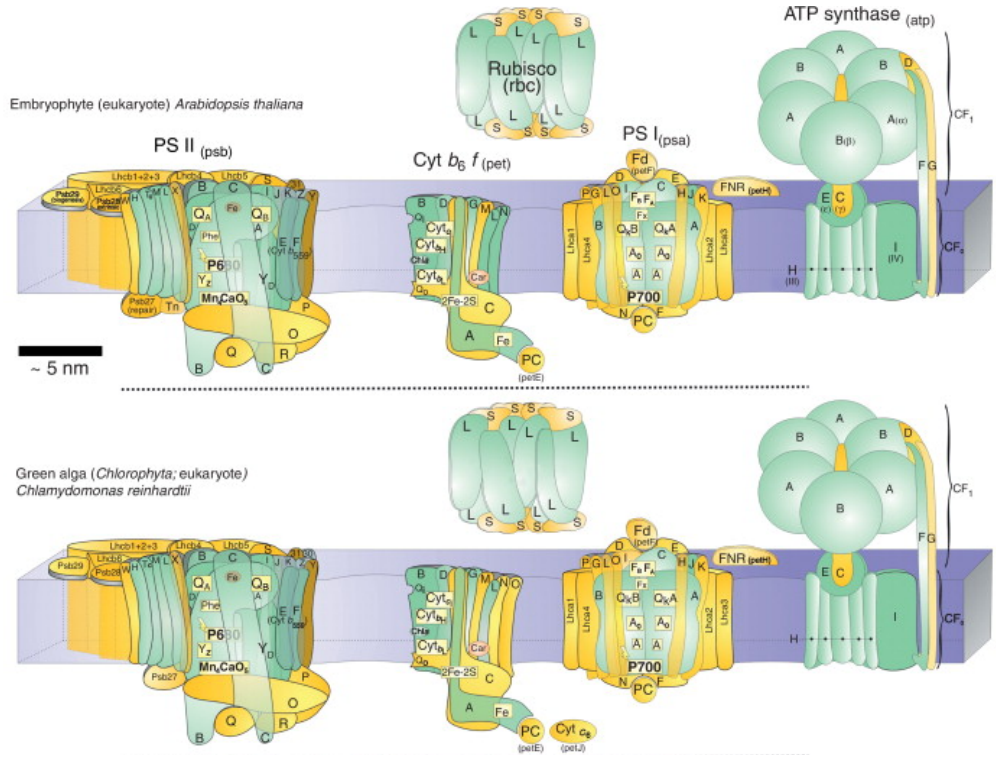

Fig. 9: Major proteins and protein complexes of the photosynthetic apparatus of a higher plant *Arabidopsis thaliana* (above) and the green model alga *Chlamydomonas reinhardtii* (below). Photosystem II (PS II); cytochrome b6f (Cyt b6f); photosystem (PS I); ATP synthase; and Rubisco. (*From Allen et al. 2011)*

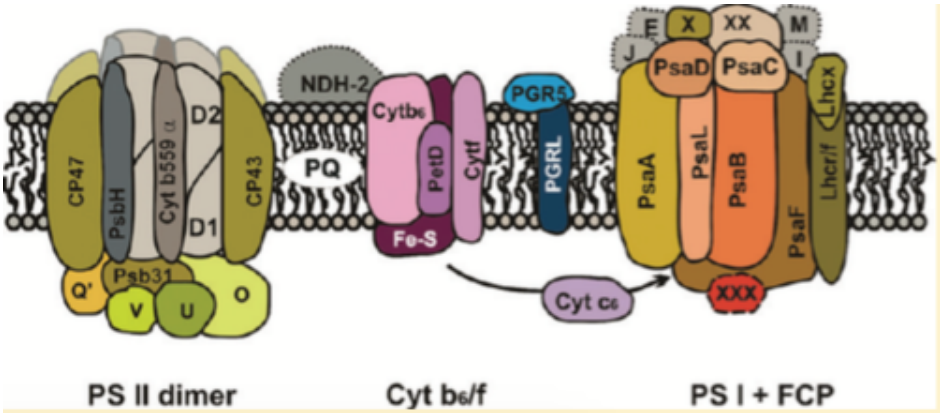

Fig. 10: Schematic overview of the main photosynthetic electron transport complexes in diatom. PS II forms dimers, PSI is a monomer. PS I subunits in dotted lines (PsaE, PsaJ, PsaM, and PsaI). (*From Grouneva et al. 2011)*

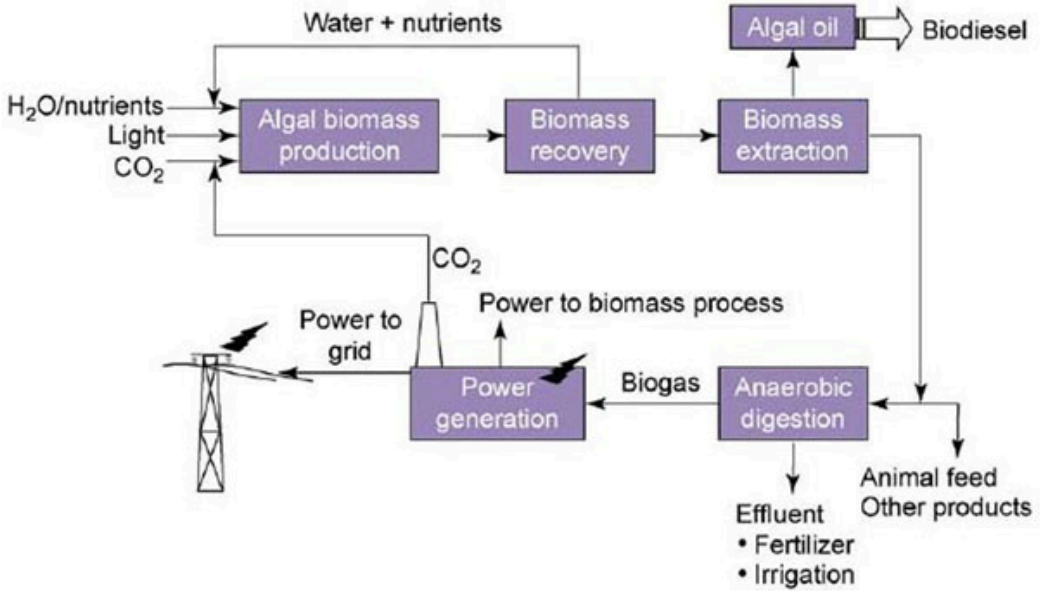

Fig. 11: Summary of the integrated process of algal biodiesel production. Water, inorganic nutrients, light, and CO_2 are provided tothe algal cultures in the algal 20 biomass production stage. Biomass is then separated from the water and nutrients in the biomass recovery stage, as the latter are recycled back into the algal cultures. The biomass then undergoes extraction to remove its lipid content. This lipid content is converted into biodiesel. Spent biomass can be used as animal feed or digested anaerobicly to generate gas for electricity while recycling CO_2 emissions back into the biomass production stage. *(From Gao et al. 2012a)*

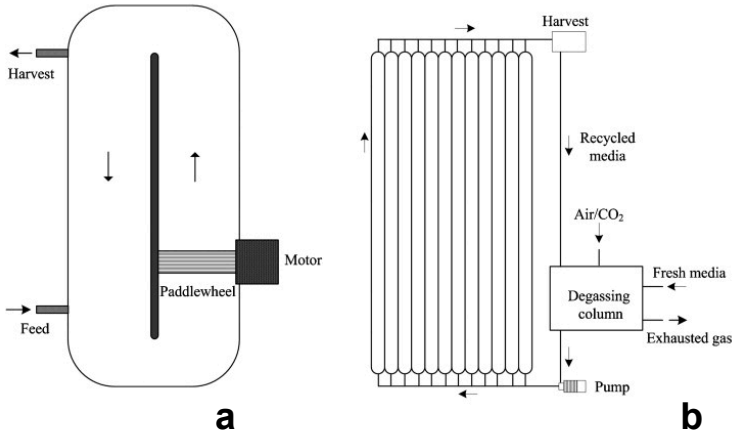

Fig. 12: An illustration of an example of an open and closed system. **(a)** The open pond design pictured represents a simplified and fairly common design for a raceway open pond. **(b)** The tubular photobioreactor system shows one possible flowpath by which harvesting, degassing, and nutrient replenishment might occur in a closed system. *(From Suali and Sarbatly 2012)*

Purposes of the Thesis

The aim of this Thesis entitled *"The potential role of some microalgae in biotechnological fields"* is to gain further knowledge on the morpho-physiological and biochemical aspects of some microalgae known to be used for biotechnological applications, in order to improve their growth performance, photosynthetic efficiency and biomass composition in the perspective of their exploitation on a large scale cultivation.

The topics of this Thesis are organized and subdivided in chapters as follows:

- **Chapter 2** is a study on the scaling-up of the cultivation and on the recycling of growth media for the re-cultivation of the green microalgae *Neochloris oleoabundans*, with the aim of gaining further knowledge on the potential behaviour of this algal strain in a large-scale cultivation system. With this purpose, this chapter has been developed in two main sections:

    1) Scaling-up of the cultivation of *N. oleoabundans* in a 20-L PBR: effect on cell growth, biomass concentration, pigment content, photosynthetic efficiency and lipid accumulation in autotrophic and mixotrophic conditions;
    2) Re-cultivation of *N. oleoabundans* in exhausted autotrophic and mixotrophic media: the potential role of polyamines and free fatty acids.

- **Chapter 3** concerns a comparative study on growth performances, photosynthetic parameters, with special attention to protein content and pattern in four Chlorophyta species, in order to obtain useful information for potential biotechnological applications of these algae in various fields, including nutraceuticals and food industry.

- **Chapter 4** is focusing on different cultivation strategies for enhancing the biomass productivity and lipid content of the marine diatom *Thalassiosira pseudonana*, with the aim of gaining further knowledge on its biotechnological potential use in the bioenergetics field. With this purpose, the chapter has been developed in two main sections:

    1) Effect of mixotrophy on cell growth, lipid accumulation and photosynthetic performance of the marine diatom *T. pseudonana*;
    2) Effect of different CO_2 concentrations on biomass, pigment content and lipid production of the marine diatom *T. pseudonana.*

This work was supported by a research fellowship "Bando Giovani Ricercatori 2015" granted by the University of Ferrara and by a fellowship granted by the University Institute

of High Studies (IUSS) – 1931 of Ferrara for PhD student's mobility. The experiments were performed in collaboration with Prof. Joan Salvadò (Dept. of Chemical Engineering, Universitat Rovira I Virgili, and Bioenergy and Biofuel Area at the Catalonia Institute for Energy Research (IREC), Tarragona, Spain).

- **Chapter 5** is a study on the application of the green microalga *Chlorella vulgaris* as biofertilizer on plant growth, yield and physiological parameters of garden pea plants (*Pisum sativum* L. var. Paladio nano), in order to improve the use of microalgal biomass as renewable, carbon-neutral, and sustainable alternative to chemical fertilizers in agricultural sector.

As tangible results of the work completed during the three years spent for my PhD, a number of publications and presentations to conferences have been produced, as listed below.

Articles on peer reviewed journals:

Baldisserotto C, Popovich C. Giovanardi M, **Sabia A**, Ferroni L, Constenla D, Leonardi PI, Pancaldi S "Photosynthetic aspects and lipid profiles in the mixotrophic alga *Neochloris oleoabundans* as useful parameters for biodiesel production". Algal Research, ***in press***, doi, 10.1016/j.algal.2016.03.022

Sabia A, Baldisserotto C, Biondi S, Marchesini R, Tedeschi P, Maietti A, Giovanardi M, Ferroni L, Pancaldi S **(2015)** "Re-cultivation of *Neochloris oleoabundans* in exhausted autotrophic and mixotrophic media: the potential role of polyamines and free fatty acids". Applied Microbiology and Biotechnology, 99:10597-10609

Giovanardi M, Baldisserotto C, Daglia M, Ferroni L, **Sabia A**, Pancaldi S "Morpho-physiological aspects of *Scenedesmus acutus* PVUW12 cultivated with a dairy industry waste and after starvation". Plant biosystems, ***in press***, doi, 10.1080/11263504.2014.991361

Conference presentations:

Sabia A, Clavero E, Salvadó J, Pancaldi S **(2015)** "Effect of different CO_2 concentrations on biomass, pigment content and lipid production of the marine diatom *Thalassiosira pseudonana*". Joint Congress of "Società Italiana di Biologia Vegetale" (Italian Vegetal Biology) and "Società Italiana di Genetica Agraria" (Italian Genetic Agrarian). *Poster presentation*. Milan (Italy), 8-11 September 2015, isbn 978-88-904570-5-0

Baldisserotto C, Popovich C, Giovanardi M, **Sabia A**, Ferroni L, Leonardi P, Pancaldi S **(2015)** "Morpho-physiological aspects, biomass and lipid production in mixotrophic *Neochloris oleoabundans*: relations between photosynthesis and lipid synthesis". Conference of the "Biotecnology and Differentiation" and "Cell and Molecular Biology" groups of the "Società Italiana di Botanica" (Italian Botanical Society). *Oral presentation*. Rome (Italy), 10-12 June 2015, pp. 26-27

Sabia A, Ferroni L, Giovanardi M, Baldisserotto C, Pancaldi S **(2014)** "A comparison of protein content of four species of Chlorophyta microalgae". Conference of the "Società Italiana di Botanica" (Italian Botanical Society). *Poster Presentation*. Firenze (Italy), 3-5 September 2014, pag. 9

Sabia A, Baldisserotto C, Giovanardi M, Ferroni L, Pancaldi S **(2014)** "Recycling of exhausted growth media for the re-cultivation of *Neochloris oleoabundans*". 2nd Edition Young Algaeneers Symposium. *Poster presentation*. Montpellier-Narbonne (France), 3-5 April 2014, pag. 101

Sabia A, Baldisserotto C, Giovanardi M, Ferroni L, Pancaldi S **(2013)** "Recycling of exhausted growth media for the cultivation of *Neochloris oleoabundans*". Conference of the "Società Italiana di Botanica" (Italian Botanical Society). ***Award received for the best poster presentation***. Baselga di Pinè (Italy), 18-20 September 2013, pag. 58

Sabia A, Baldisserotto C, Giovanardi M, Ferroni L, Pancaldi S **(2013)** "Recycling of exhausted growth media for the cultivation of *Neochloris oleoabundans*". Conference of the "Biotecnology and Differentiation" and "Cell and Molecular Biology" groups of the "Società Italiana di Botanica" (Italian Botanical Society). *Oral presentation*. Ferrara (Italy), 17-19 June 2013, pp. 24-25

Chapter 2

Scaling-up and recycling of growth media for the re-cultivation of the green microalga Neochloris oleoabundans

Adapted from the published article:

Sabia A, Baldisserotto C, Biondi S, Marchesini R, Tedeschi P, Maietti A, Giovanardi M, Ferroni L, Pancaldi S

Re-cultivation of *Neochloris oleoabundans* in exhausted autotrophic and mixotrophic media: the potential role of polyamines and free fatty acids

Applied Microbiology and Biotechnology **(2015)**, 99: 10597-10609.

DOI, 10.1007/s00253-015-6908-3

1. Introduction

During the last years, the increasing of global climate change and prices of petroleum and other fossil fuels has led to an urgent need to develop renewable, carbon-neutral, cost-effective alternative feedstock to displace petroleum (Scott et al. 2010; Stephens et al. 2010; Murray and King 2012). Biodiesel is currently receiving much attention due to its potential as a sustainable and environmentally friendly alternative to petroleum (Demirbas and Demirbas 2011; Borowitzka and Moheimani 2013). Among different biodiesel feedstocks, lipid-rich microalgal biomass is being widely proposed as promising renewable source as compared to biomass used for first and second generation biofuels (Chisti et al. 2007; Hu et al. 2008; Mata et al. 2010; Griffiths and Harrison 2009; Sharma et al. 2012).

Among these most promising oil-rich microalgae, the green unicellular microalgae *N. oleoabundans*, recently a taxonomic synonym of *Ettlia oleoabundans* (Chlorophyta, Chlorophyceae, Chlamydomonadales, Chlamydomonadales incertae sedis), has gained considerable attention due to its capability to accumulate 35-54% of lipids, which were TAGs for up to 80%, when the alga is grown under nitrogen starvation (Tornabene et al. 1983; Chisti 2007; Li et al. 2008; Gouveia et al. 2009; Pruvost et al. 2009) or 46% of cell lipid content when it is cultivated mixotrophically, in the presence of glucose (Giovanardi et al. 2014; Silva et al. 2016) or carbon-rich wastes as organic carbon sources (Giovanardi et al. 2013; Baldisserotto et al. 2014; Baldisserotto et al. *In Press*). Moreover, recent studies have focused the attention on the importance of the cell cycle of *N. oleoabundans* in the regulation of TAGs and biomass production (de Winter et al. 2013; Klok et al. 2013). *N. oleoabundans* is usually described as a freshwater organism and for this, it is often cultivated in freshwater media (Gouveia et al. 2009; Pruvost et al. 2011). However, several studies showed its capability to efficiently grown in brackish and seawater medium (Baldisserotto et al. 2012; Popovich et al. 2012a; Baldisserotto et al. 2014; Giovanardi et al. 2014). In the perspective of scaling up of microalgal cultivation for biodiesel production, only few authors have studied the culture performance of *N. oleoabundans* in large culture volume (Pruvost et al. 2011; Silva et al. 2016). It is widely known that the biodiesel production from microalgae is technically feasible, 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 achieve the productivities required for biodiesel production under a profitable cost (Suali and Sarbatly 2012). In this study, in order to improve the culture performance of the alga in large-scale cultivation system and, at the same time, to reduce its high production costs, *N.*

oleoabundans was cultivated autotrophically and mixotrophically in brackish medium in the presence of 2.5 gL^{-1} of glucose in a 20-L coaxial photobioreactor. Mixotrophy has been considered an alternative method for achieving higher biomass densities and lipid production as compared to autotrophic or heterotrophic cultivation (Brennan and Owende 2010; Cheirsilp and Torpee 2012; Chandra et al. 2014). Moreover, it has been previously demonstrated that among the different organic carbon substrates and concentrations tested, the supply of 2.5 gL^{-1} of glucose can be selected as the most optimal carbon source, allowing to obtain the best compromise between biomass production and lipid accumulation in *N. oleoabundans* (Giovanardi et al. 2013; Sabia et al. 2015; Silva et al. 2016; Baldisserotto et al. *In Press*). Nevertheless, one of the main obstacles in the biodiesel production from microalgae is that the process is technically feasible, but not yet economically competitive (Suali and Sarbatly 2012). In order to reduce the high cultivation and production costs and, at the same time, to make microalgal production more environmentally sustainable, recycling culture medium has been proposed as a possible solution (Yang et al. 2011; Hadj-Romdhane et al. 2012; Farooq et al. 2014; Sabia et al. 2015), especially in large-scale culture systems (Borowitzka 2005; Lam and Lee 2012; Zhu et al. 2013). In addition, the mass cultivation of halotolerant strains in brackish and seawater media can be considered an interesting alternative to moderate the freshwater consume (Popovich et al. 2012a). However, the feasibility of recycling growth medium to re-cultivate microalgae has been only tested in a few cases (Lívanský et al. 1996; Rodolfi et al. 2003; Hadj-Romdhane et al. 2013; Zhu et al. 2013; Sabia et al. 2015). It is reported that the recycling medium could have negative effects on biomass productivity, due to the release of inhibitory secondary metabolites (Richmond 2004). Harmful metabolites are, in fact, released during microalgal growth under physiological stress (Ikawa 2004; Moheimani and Borowitzka 2006). Free fatty acids (FFAs) and substances derived from the photooxidation of unsaturated fatty acids are the most common metabolites with inhibitory effects on microalgae (Ikawa 2004; Wu et al. 2006; Bosma et al. 2008; Stephen et al. 2010).

Aim of the work

The first chapter of this Thesis is focusing on the scaling-up and on the recycling of growth media for the re-cultivation of the green microalga *Neochloris oleoabundans*.

The aim is to gain further knowledge on the morpho-physiological and biochemical aspects of this algal strain in order to evaluate its growth performance, photosynthetic efficiency and biomass composition in a large-scale cultivation system. With this purpose, the experiments were organized in two different sections.

In the first section, *N. oleoabundans* was cultivated autotrophically and mixotrophically in a 20-L PBR, focusing on parameters that are relevant to biotechnological applications. Growth was estimated in parallel to biomass concentration, to cell morphology and to pigment content. Lipid accumulation was investigated by staining cells with the specific fluorocrome Nile Red. Pulse amplitude modulated (PAM) fluorimetry was employed in order to monitor the photosynthetic activity during growth and lipid accumulation.

In the second section, *N. oleoabundans* was cultivated in recycling autotrophic and mixotrophic growth media obtained from the previous cultivations in a 20-L PBR. The following features were analyzed in order to observe if this microalga can efficiently grow in its exhausted growth medium: growth curves in parallel to nitrate and phosphate consumption; photosynthetic pigment content; PSII maximum quantum yield; cell morphology, with special attention to intracellular lipid accumulation; free fatty acids (FFAs) accumulation in recycled growth media, and polyamine (PA) concentration both inside cells and in the corresponding culture media. PAs were determined in order to understand if these plant growth regulators could be responsible for the growth promotion of *N. oleoabundans* in recycled growth medium. It is known, in fact, that PAs, together with other plant growth regulators, have stimulatory effects on algal growth and metabolism, and are involved in mitigating various types of biotic and abiotic stress (Tate et al. 2013).

2. *Scaling-up of the cultivation of* Neochloris oleoabundans *in a 20-L PBR: effect on cell growth, biomass concentration, pigment content, photosynthetic efficiency and lipid accumulation in autotrophic and mixotrophic conditions*

2.1 Materials and Methods

2.1.1 Algal strain and culture condition

The strain used in this study was the Chlorophyta *N. oleoabundans* UTEX 1185 (syn. *Ettlia oleoabundans*) (Chlamydomonadales, Chlamydomonadales incertae sedis), obtained from the Culture Collection of Algae of the University of Texas (UTEX, Austin, Texas, USA; www.utex.org). *N. oleoabundans* was cultivated in axenic liquid brackish medium (BM) (Baldisserotto et al. 2012) in a coaxial 20-L capacity PBR (M2M Engineering, Grazzanise, Caserta, Italy). Algae were cultivated autotrophically in BM for 14 days or mixotrophically, by addition of 2.5 gL^{-1} of glucose, for 8 days, according to previously described protocols (Baldisserotto et al. 2014; Giovanardi et al. 2014; Baldisserotto et al. *In Press*). For autotrophic cultivation, cells were inoculated into the PBR to obtain an initial cell concentration of about 3 x 10^6 cells mL^{-1}, while, for mixotrophic cultivation initial cell concentration was higher (5 x 10^6 cells mL^{-1}). Culture conditions in the PBR were: 24 ± 1°C; sterile air injection at the bottom of the PBR, with 0.5/3.5 h bubbling/static cycles; irradiance 65 $\mu mol_{photons}$ m^{-2} s^{-1} of PAR (16:8 h light:dark photoperiod). Light was supplied with inner cool–white fluorescent Philips tubes. Experiments were performed in triplicate. Aliquots of cultures were collected at different times of cultivation up to 14 days for autotrophic culture and up to 8 days for mixotrophic culture, depending on the analysis. Algal growth and morphology were monitored as described below.

2.1.2 Growth and biomass evaluation

Growth evaluation was carried out on cell samples at different times of cultivation using a Thoma haemocytometer (HBG, Giessen, Germany) under a light microscope (Zeiss, model

Axiophot) and growth curves were obtained.

The growth rate (μ, number of divisions per day) during the exponential phase was calculated with the following equation:

μ (div d^{-1}) = $(\log_2 N_1 - \log_2 N_0) / (t_1 - t_0)$,

where μ is the growth rate, N_1 the cell number at time t_1, N_0 the cell number at time 0 and $t_1 - t_0$ the time interval (days) (Giovanardi et al. 2013).

The biomass productivity ($mgL^{-1}d^{-1}$) was calculated from the variation in the biomass concentration ($mgL^{-1}d^{-1}$) within a specific cultivation time (d) according to the following equation:

BP ($mgL^{-1}d^{-1}$) = $(X_1 - X_0) / (t_1 - t_0)$,

where BP is the biomass productivity, X_1 the biomass concentration at time t_1, X_0 the biomass concentration at time 0 and $t_1 - t_0$ the time interval (days) (Hempel et al. 2012).

For dry biomass determination, aliquots of samples (20 mL) were filtered through pre-dried and pre-weighed glass-fiber filters (Whatman GF/F). Filters with cell pellets were rinsed with 20 mL of distilled water and dried 72 h at 60° C, and weighted until they reached constant weight.

In parallel to growth, the pH in culture replicates was periodically monitored using a pH meter (mod. 3510, Jenway, Bibby Scientific Limited, Staffordshire, UK).

2.1.3 Photosynthetic pigment extraction and quantification

Algal cell suspensions were collected at different times of cultivation and extracted with absolute methanol for 10 min at 80°C (Baldisserotto et al. 2014). Absorption of extracts was measured at 666 (chlorophyll *a* - Chl*a*), 653 (chlorophyll *b* - Chl*b*) and 470 nm (carotenoids - Car) with a Pharmacia Ultrospec 2000 UV-Vis spectrophotometer (1-nm bandwidth; Amersham Biosciences, Piscataway, NJ, USA). Pigment concentrations were evaluated according to Wellburn (1994).

2.1.4 PAM fluorimetry

The PSII maximum quantum yield of algae was determined at the same cultivation times considered for growth curves measurements. A pulse amplitude modulated fluorometer (ADC Bioscientific Ltd, Hoddesdon, Hertfordshire, UK) was used to determine the *in vivo* chlorophyll fluorescence of PSII. The PSII maximum quantum yield is reported as F_V/F_M ratio, *i.e.* $(F_M - F_0)/ F_M$, where variable fluorescence is $F_V = (F_M - F_0)$, F_M is the maximum

fluorescence and F_0 is the initial fluorescence of samples (Lichtenthaler et al. 2005). This measurement is considered a valid method to probe the maximum quantum yield of photochemistry in PSII (Kalaji et al. 2014). Moreover, it is useful to estimate the physiological state of plants and microorganisms also under nutrient stress (White et al. 2011). Samples were prepared as reported in Ferroni et al. (2011) after 15 min of dark incubation.

2.1.5 Light and fluorescence microscopy

Cell samples were routinely observed using a microscope (Zeiss, model Axiophot) with conventional and fluorescent attachments. The light source for chlorophyll fluorescence observation was a HBO 100 W pressure mercury vapour lamp (filter set, BP436/10, LP470). Pictures of cells were taken with a Canon IXUS 110 IS digital camera (12.1 megapixels), mounted on the ocular lens through a Leica DC150 system (Leica Camera AG, Solms, Germany).

2.1.6 Lipid staining

During experiments, the intracellular presence of lipids was evaluated by staining cells with the fluorochrome Nile Red (9-diethylamina-5Hbenzo[α]phenoxazine-5-one, 0.5 mg dissolved in 100 mL acetone; Sigma-Aldrich, Gallarate, Milan, Italy), as described in Giovanardi et al. (2014). This assay is considered a quick method for identifying the presence of lipid globules via microscopic observations of stained cells (White et al. 2011; Popovich et al. 2012a; Giovanardi et al. 2013). After incubation at 37°C in darkness for 15 min, cells were observed with the microscope described above at an excitation wavelength of 485 nm (filter set, BP485, LP520). Photographs were taken with the camera described above.

2.1.7 Data treatment

Data were processed with Graphpad Prism 6 (Graph Pad Software, San Diego CA, USA). In each case, means ± standard deviations for *n* number of samples are given. Statistical analyses for comparison of means were carried out using ANOVA, followed by Student's *t*-test (significance level, 0.05).

2.2 Results

2.2.1 Growth and biomass of *N. oleoabundans* cultivated autotrophically and mixotrophically in a 20-L PBR

Fig. 1a-c reports data on both cell densities and biomass concentration of autotrophic and mixotrophic cultures. The growth and the biomass of *N. oleoabundans* was highly promoted when 2.5 gL^{-1} of glucose were supplied to BM medium. As shown in Fig. 1a, autotrophic cells showed a linear increasing trend on both cell densities and biomass concentration. In detail, cells rapidly entered the exponential phase starting on the 3rd day up to the 7thday of cultivation (Fig. 1a). After this period, cultures had a short late exponential phase and then entered the stationary phase of growth, reaching a final cell concentration value of ca. 10 × 10^6 cells mL^{-1} and a final biomass concentration value of 0.42 gL^{-1} at the end of the of cultivation time (14 days).

Conversely, during the first two days of mixotrophic growth in the PBR in the presence of glucose, cells showed an evident increase in cell concentration, reaching values of ca. 12 x 10^6 cells mL^{-1} (Fig. 1c). Interestingly, the growth rate of mixotrophic culture was found to be ca. 2.5 times higher than in autotrophic cells (mixotrophic cells 0.56 div d^{-1} *vs* autotrophic cells 0.20 div d^{-1}; $p < 0.05$). Subsequently, cells showed a short stationary phase of growth, and then on the 4th days cell concentration values started to decrease; this led to values of about 6 x 10^6 cells mL^{-1} up to the end of the cultivation period (8 days). It was noteworthy that mixotrophic cells showed an evident increase on biomass concentration during the entire experiment, reaching at 4th day of cultivation the highest value of ca. 1.45 gL^{-1} as compared to the autotrophic one. The supply of glucose allowed *N. oleabundans*-cells to achieve higher biomass productivity (282.1 ± 0.004 $mgL^{-1}d^{-1}$) as compared to the autotrophic samples (22.4 ± 0.009 $mgL^{-1}d^{-1}$).

During the experiments, aliquots of samples were periodically harvested to measure the pH of the culture media. As observed in Fig. 1b-d, the initial pH value was found to be very different in autotrophic culture medium (about 8.0) respect to the mixotrophic medium (about 9.6). During the first six days of cultivation, pH values gradually increased up to 10.50 in the autotrophic medium. After this period, the pH exhibited a slight decrease, reaching values of ca. 9.95. Conversely, during the first two days of cultivation pH values in mixotrophic culture medium decreased drastically down to 7.90; hereafter, pH maintained constant values up to the end of the experiment.

2.2.2 Photosynthetic pigment content in *N. oleoabundans* cultivated autotrophically and mixotrophically in a 20-L PBR

As reported in Fig. 2, autotrophic and mixotrophic *N. oleoabundans*-cells showed a different behaviour as regards the photosynthetic pigment content in relation to the unit of measure employed (on cell or biomass basis). About Chl*a* content, expressed on cell basis, autotrophic cells showed a strong decrease of Chl*a* during the first 7 days of growth, reaching values of ca. 17 μg 10^{-6} cells (Fig. 2a). Subsequently, cultures exhibited an evident increase until the 10^{th} day, when Chl*a* content reached and maintained the values recorded at the beginning of the experiment (0.43 μg 10^{-6} cells). Indeed, when Chl*a* content in autotrophic culture was expressed on biomass basis some difference occurred. During the first 7 days, cells showed a similar decreasing trend already described for Chl*a* (on cell basis), but starting on the 7^{th} day and up to the end of cultivation samples reached and maintained stable values (1.15 % dry weight (DW)). Conversely, independently of the unit of measure employed, mixotrophic cells shared a very similar trend of Chl*a* content (Fig. 2b). In detail, during the first 4 days of cultivation, mixotrophic cells showed an evident decrease of Chl*a*. Subsequently, cells reached and maintained constant values (0.43 μg 10^{-6} cells and 0.31%DW) up to the end of the experiment. Interestingly, some evident differences were observed on Chl*a* content between autotrophic and mixotrophic cells. At the beginning of the experiment, mixotrophic cells showed an higher Chl*a* content on cell basis as compared to the autotrophic one (mixotrophic cells 0.56 μg 10^{-6} cells *vs* 0.47 autotrophic). Conversely, when Chl*a* was expressed on biomass basis, in autotrophic cells values of Chl*a* were found to be 2.5 times higher than in the mixotrophic one. Despite no great differences on Chl*a* content reported on cell basis were observed at the end of the experiment between autotrophic and mixotrophic cells, values of Chl*a* (%DW) were found 11 times higher in autotrophic cells as compared to the mixotrophic one (autotrophic 1.43 %DW *vs* mixotrophic 0.31; $p<0.001$).

About Chl*b* content, autotrophic cells showed a linear trend of Chl*b* expressed in terms of quantity of pigment inside cells. During the entire experiment, cells showed stable values of Chl*b* (ca. 0.20 μg 10^{-6} cells) (Fig. 2c). On the contrary, a different trend on Chl*b* quantities on biomass basis was observed. In detail, autotrophic cells had constant values of Chl*b* (%DW) until the 7^{th} day when values showed an evident decrease (from about 1.22 %DW at day 7 down to 0.55 at 10 day); hereafter, cultures maintained constant values up to the end of the experiment. Conversely, independently of the unit of measure employed,

mixotrophic cells shared a very similar trend of Chl*b* content (Fig. 2d). During the first 2 days, cultures showed an evident decrease of Chl*b*, especially in terms of biomass basis (from about 0.67 %DW at day 0 down to 0.08 at 2 day). Subsequently, samples reached and maintained constant values. (0.17 μg 10^{-6} cells and 0.20 %DW) up to the end of the experiment. It was noteworthy that no so evident differences on Chl*b* content on cell basis were observed between autotrophic and mixotrophic cells at the beginning of the experiment (autotrophic 0.22 μg 10^{-6} cells *vs* 0.25 mixotrophic). Conversely, the values of Chl*b* expressed on biomass basis were found 2 times higher in the autotrophic cells compared to the mixotrophic one (autotrophic 1.33 %DW *vs* mixotrophic 0.67; $p<0.05$). Also, at the end of the cultivation the difference of Chl*b* (%DW) was very evident between autotrophic and mixotrophic cells (autotrophic 0.66 %DW *vs* mixotrophic 0.13; $p<0.05$).

In autotrophic cells, Car concentrations (on cell and biomass basis) showed similar trend (Fig. 2e). During the first 3 days, cultures had an evident decrease of Car content, reaching an average concentration of 0.10 μg 10^{-6} cells and 0.05 %DW. Subsequently, cultures exhibited a strong increase from days 7 to 10, when Car content reached the highest values observed in the experiment (0.22 μg 10^{-6} cells and 0.13 %DW). Then, samples showed a slight decrease, they reached and maintained the values recorded at the beginning of the experiment (0.16 μg 10^{-6} cells and 0.09 %DW). Conversely, mixotrophic cells had different trend of Car content in relation to the unit of measure employed (Fig. 2f). During the entire experiment, mixotrophic cells showed a linear trend of Car concentrations expressed on cell basis, with constant values (ca. 0.12 μg 10^{-6} cells). On the contrary, some evident differences occurred when Car content was expressed as a function of total biomass. During the first 2 days, mixotrophic cells showed an evident decrease of Car concentrations (from about 0.32 %DW at day 0 down to 0.05 at 2 day). Subsequently, cultures exhibited a strong increase up to 4^{th} (0.10 %DW); hereafter, cultures maintained the values recorded with only a slight decrease observed at the end of the experiment. Interestingly, at the beginning of the experiment no evident differences on Car content (on cell basis) were observed between autotrophic and mixotrophic cells (autotrophic 0.15μg 10^{-6} cells *vs* 0.13 mixotrophic). Conversely, the Car (%DW) values were found ca. 3.5 times higher in the mixotrophic cells as compared to the autotrophic one (mixotrophic 0.31 %DW *vs* autotrophic 0.09; $p<0.001$). Despite these differences observed at the beginning of the experiment between autotrophic and mixotrophic cells, it was noteworthy that at the end of the experiment all samples reached similar quantities of Car expressed on cell basis (autotrophic 0.16 μg 10^{-6} cells *vs* mixotrophic 0.12 μg 10^{-6} cells) and on biomass basis (autotrophic 0.09 %DW *vs*

mixotrophic 0.11 %DW).

The trend of the total Chls content in autotrophic and mixotrophic cells, independently of the unit of measure employed, were nearly comparable to those of Chl*a* (Fig. g, h). It was noteworthy that at the beginning of the experiment mixotrophic cells showed an higher total Chls content on cell basis as compared to the autotrophic one (mixotrophic cells 0.82 μg 10^{-6} cells *vs* 0.69 autotrophic) but in autotrophic cells values of the total Chls on biomass basis were found to be 2.5 times higher than the mixotrophic one. Despite no evident differences in total Chls, in terms of quantity of pigment inside cell, were observed in autotrophic and mixotrophic at the end of the experiment cells, the total Chls (%DW) values were found ca. 5 times higher in the autotrophic cultures as compared to the mixotrophic one (autotrophic 2.10 %DW *vs* mixotrophic 0.44; $p<0.001$).

2.2.3 Maximum quantum yield of PSII of *N. oleoabundans* cultivated autotrophically and mixotrophically in a 20-L PBR

The variations in PSII maximum quantum yield in *N. oleoabundans* cells cultivated autotrophically and mixotrophically in a 20-L PBR are shown in Fig. 3. Autotrophic cells showed stable values of the F_V/F_M ratio during the first three days of growth, reaching values of ca. 0.65 (Fig. 3a). Subsequently, cultures from the 3th day up to 7th day of cultivation showed an evident increase of the F_V/F_M ratio, reaching values of ca. 0.73. During the subsequent experimental times (10th day), the F_V/F_M ratio of autotrophic samples exhibited a slight decrease, reaching values of ca. 0.68; hereafter, samples maintained stable values of ca. 0.70 until the end of the experiment.

During the first two days of mixotrophic growth in presence of glucose, cells showed stable values of the F_V/F_M ratio, reaching values of ca. 0.67 (Fig. 3b). After this period, the F_V/F_M ratio of cultures decreased drastically down to values below 0.47 at the end of experiment. It was noteworthy that in mixotrophic cells the decrease was dramatic already from the 2nd day of cultivation. During the subsequent experimental times (4th and 6th day), the F_V/F_M ratio of mixotrophic samples started to decrease very strongly (from about 0.62 at day 2 down to 0.54 at day 6). At the of the experiment, mixotrophic cells reaching the lowest value of ca. 0.47 *vs* 0.68 for autotrophic cells, $p<0.05$.

2.2.4 Morphological observations of *N. oleoabundans* cultivated autotrophically and mixotrophically in a 20-L PBR

Light microscopy and Nile Red staining

The cell morphology of *N. oleoabundans* cultivated autotrophically and mixotrophically in a 20-L PBR is shown in Fig. 4. During the entire period, autotrophic cells showed the typical morphology of *N. oleoabundans* grown in BM medium, and cell dimensions remained similar throughout the experiment. In fact, independent of the growth period, cells had a roundish shape with a diameter variable between 3 and 4 μm μm (Fig. 4a, c). One cup-shaped bright-green chloroplast, containing a large pyrenoid, occupied most of the cell volume and emitted an intense red fluorescence due to the presence of chlorophyll (Fig. 4b, d). At the beginning of the experiment, both cell shape and size, and chloroplast features in mixotrophic cultures (Fig. 4e, f) were similar to those observed in autotrophic cultures (Fig. 4a, b). Starting on the 4th day and up to the end of cultivation, however, mixotrophic cells showed some peculiar features. The chloroplast lost its characteristic cup shape and translucent globules started to be accumulated inside the cytoplasm. After 6 days of growth, mixotrophic *N. oleoabundans* cells showed bigger dimensions and appeared bleached with respect to the autotrophic one. The droplets volume increased from the 4th day onwards to almost completely hide the chloroplast at the end of the experiment (8 day). In order to check the lipid nature of these translucent globules, cells were stained with the lipid-specific fluorochrome Nile Red. When autotrophic cells were stained with Nile Red, lipid droplets were never detected (Fig. 4d). Conversely, at the end of the experiment, lipid droplets of mixotrophic cells gave an intense positive reaction with the fluorochrome (Fig. 4h).

2.3 Discussion

N. oleoabundans is widely considered an important microalga to be potentially used as a green feedstock of lipids for biofuel production (Chisti 2007; Li et al. 2008; Pruvost et al. 2009; Popovich et al. 2012a; Giovanardi et al. 2014; Silva et al. 2016; Baldisserotto et al. *In Press*). In order to improve its culture performance in large-scale cultivation system and, at the same time, to lower its high production costs, in the first section of this Thesis, *N. oleoabundans* was cultivated autotrophically and mixotrophically in brackish medium in coaxial 20-L PBR. Most of the studies reported in literature on this alga were performed in

small cultivation volume and frequently in presence of a freshwater medium (Li et al. 2008; Gouveia et al. 2009; Pruvost el al. 2009; Klok et al. 2013). In the perspective of scaling up of microalgal cultivation, only few authors have studied the culture performance of *N. oleoabundans* in large cultivation system (Pruvost et al. 2011; Sabia et al. 2015; Silva et al. 2016). The present results clearly suggested that *N. oleoabundans* can efficiently achieved higher biomass productivity and lipid content when it was cultivated mixotrophically in a brackish medium in PBR. According to previous studies (Giovanardi et al. 2014; Silva et al. 2016; Baldisserotto et al. *In Press*), the effects of glucose supplementation on *N. oleabundans* biomass productivity, photosynthetic efficiency and lipid content were found to be very significant respect to the autotrophic cultivation. In particular, the mixotrophic cultivation allowed achieving significantly higher biomass productivity (0.28 $gL^{-1}d^{-1}$) as compared to the autotrophic one (0.02 $gL^{-1}d^{-1}$). These values were comparable or even higher than those observed in literature. In fact, studies on *N. oleabundans* grown under N-stress condition or in presence of glucose, or carbon-rich wastes, have obtained values of biomass productivity that varied from 0.15 to 0.63 $gL^{-1}d^{-1}$ (Li et al. 2008; Gouveia et al. 2009; Pruvost et al. 2009; Giovanardi et al. 2014; Silva et al. 2016; Baldisserotto et al. *In Press*). Conversely to that observed in previous studies (Giovanardi et al. 2014, Sabia et al. 2015; Baldiserotto et al. *In Press*), in the present work the cell densities achieved in mixotrophic cultivation were found to be lower. However, this is justified by characteristics of the single cell biomass and size. As shown in Fig. 5, the increase in single cell biomass in mixotrophic samples was very evident (from 54 μg_{DW} 10^{-6} at day 0 up to more 130 μg_{DW} 10^{-6} cells at day 8) respect to the autotrophic cells, which maintained stable values (ca. 40 μg_{DW} 10^{-6} cells) during the entire experiment. This result also explained the increase in biomass concentration but now in cell density in mixotrophic samples during the first 4 days of cultivation. In fact, 2-days mixotrophic cells were more but lighter, while from the 4th day onwards mixotrophic cells were less numerous but heavier. As reported by de Winter et al. (2013), the cell cycle of *N. oleoabundans* plays an important role in biomass production due to the different morphology and size of cells. Despite of this, the highest value of biomass concentration obtained in mixotrophic cultivation (1.45 gL^{-1}) was comparable to that observed by Giovanardi et al. (2013; 2014) and Baldisserotto et al. (*In Press*). Also the values found for the autotrophic biomass concentration (0.42 gL^{-1}) were in line with previous observations (Popovich et al. 2012a; Giovanardi et al. 2014; Santos et al. 2014). Few studies have investigated the correlation between the biomass productivity, photosynthetic efficiency and lipid accumulation in the mixotrophic *N. oleoabundans*-cells (Baldisserotto et al. 2014;

Giovanardi et al. 2014; Baldisserotto et al. *In Press*). However, these studies were performed in a lab-scale cultivation system. It is well known that during the process of the scaling up, the value of biomass productivity achieved is highly depended on the geometry and operating variables, mainly light intensities, of the large cultivation systems (Takache et al. 2010). Recently, closed photobioreactors are receiving much attention for the possibility of producing valuable compounds (Pulz 2001; Grobbelaar 2009; Smith et al. 2010). For photosynthetic microorganisms, such as microalgae, the supply and quality of light is one of the main parameter that influenced their growth performance. Photobioreactors offered the opportunity to optimize the light path and the extent to which the incoming light is diluted (Scott et al. 2010).

Little information about the effects of the organic carbon source on photosynthetic pigment content is available. Data in literature reported different photosynthetic pigment patterns depending on the microalgal species and on the organic carbon source employed (Cerón García et al. 2006; Liu et al. 2009a; Baldisserotto et al. 2014; Giovanardi et al. 2014). As showed in Fig. 2, the mixotrophic algal biomass contained a significantly less quantity of total Chls but a higher or similar Car content respect to the autotrophic one. In detail, total Chls (%DW) values were found ca. 5 times higher in the autotrophic one. This result was confirmed by several studies, which observed a reduction in total Chls content under mixotrophic condition (glucose supply) respect to the autotrophic (Ip et al. 2004; Liu et al. 2009a; Giovanardi et al. 2014). Moreover, Liu et al. (2009a) observed that Car content was less influenced by the addition of an organic carbon source. Concerning the autotrophic cultivation, the trend of photosynthetic pigment content on cell basis was found to be similar to that reported by Giovanardi et al. (2014). In detail, the total Chls and Car content showed a decreasing trend but, at the end of the experiment, cell pigment content reached the values recorded at the beginning. The initial decrease of photosynthetic pigment content could be attributed to the effect of the dilution. In fact, Schenk et al. (2008) reported that in diluted cultures the light penetration is usually higher than in dense culture. Whereas, the higher pigment content at the end of experiment could be linked to the increase in cell density. Also the photosynthetic activity of *N. oleoabundans* was influenced by the mixotrophic growth conditions. Accordingly, autotrophic cells showed stable and normal F_V/F_M values throughout the experiment (Giovanardi et al. 2014). Indeed, mixotrophic cells showed higher values of F_V/F_M ratio during the exponential phase of growth but, subsequently, the photosynthetic efficiency decreased drastically until the end of experiment (Giovanardi et al. 2014; Baldisserotto et al. *In Press*). As showed in Fig. 1c

and 3b, during the first 2 day of mixotrophic cultivation, the alga showed an evident increase in biomass that could be linked to its higher values of F_V/F_M ratio. It has been reported, in fact, that the increase in biomass was also influenced by a good photosynthetic efficiency. Whereas, the decrease in PSII maximum quantum yield is correlated to lipid accumulation (Baldisserotto et al. 2014; Giovanardi et al. 2014; Baldisserotto et al. *In Press*). In accordance, when the F_V/F_M ratio of the mixotrophic glucose-treated cells decreased, lipid droplets started to be accumulated inside cells. This aspect was confirmed by the Nile Red staining (Fig. 4). From analyses, it was clear that mixotrophy had a strong impact not only on the photosynthetic apparatus but also on lipid production. Present results supported the hypothesis proposed by Baldisserotto et al. (*In Press*), who observed that the lipid synthesis in mixotrophic cultures starts when the alga slows replication due to the consume of nitrogen source available in culture medium. It is well known that nitrogen is one of the most important inorganic limiting nutrients required for growth of microalgal cells (Brennan and Owende 2010). In this work, the initial rapid increase in mixotrophic biomass could be attribute to a sufficient nutrients concentration present in the medium. Subsequently, the alga entered the stationary phase, when nitrogen became limiting, and lipid accumulation was induced. As reported by Scott et al. (2010) during the mixotrophic cultivation, the decrease of N/C ratio produced similar effects to those induced by the nitrogen starvation. When nitrogen becomes limiting, since it is essential for the synthesis of proteins and Chls, a consequent decrease in the photosynthetic apparatus is observed. In this way, the nitrogen sources consumption during the stationary phase induces the lipid synthesis. Moreover, the lipid synthesis requires energy, which might be provided by the assimilation of organic carbon source (Li et al. 2008; Gouveia et al. 2009; Giovanardi et al. 2014). Another aspect highlighted the peculiar behaviour of glucose-treated cells respect to the autotrophic. As showed in Fig. 1b-d, the time-course variations in the pH in culture media of *N. oleoabundans* cells cultivated autotrophically and mixotrophically was completely different. In the autotrophic cultivation the pH gradually increase during the exponential phase (from 8 at day 1 to 10.5 at day 7), and after the pH levelled off to a high value around 10 until the end of the experiment. Conversely, in mixotrophic cultivation the starting pH is very high (around 10) but decreased rapidly during the first 2 days and maintained to a stable value of 8 until the end. This different pH trend observed between autotrophic and mixotrophic cells could be attributed to the different assimilation and release of CO_2 of the cells during photosynthesis and respiration, respectively. According to previous studies (Zhang et al. 2014, Difusa et al. 2015; Sabia et al. 2015), the increasing trend of pH observed in autotrophic cultivation was due to the

consumption of CO_2 during the photosynthetic process. Conversely, the decrease of pH value in mixotrophic medium could be attributed to the high respiratory rates of the mixotrophic-cells. Recent studies observed that, due to the production of acids during the metabolism of organic carbon source, the pH values rapidly decreased in culture (Fang et al. 2004; Andrade et al. 2007; Kong et al. 2011). The higher respiratory rates found in mixotrophic cells as compared to the autotrophic ones, suggested that mixotrophic cells could have utilized the glucose as a respiratory substrate (Fang et al. 2004).

Tables and Figures

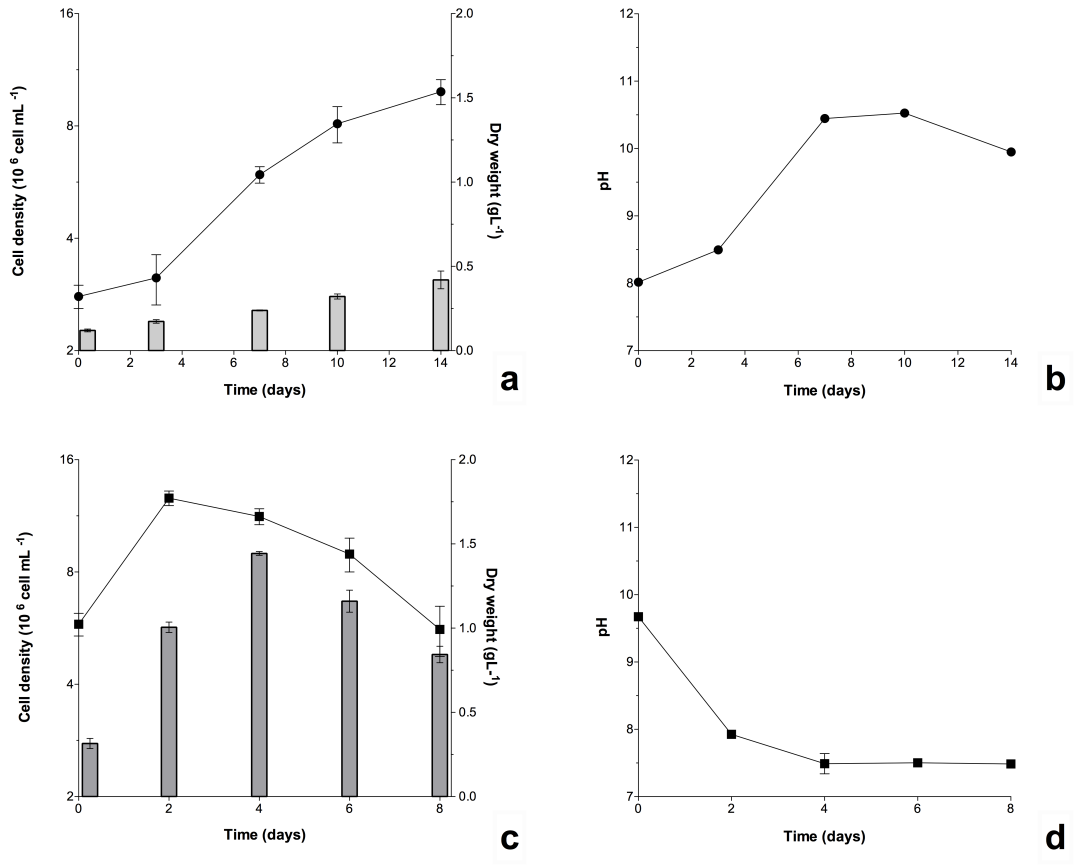

Fig. 1: **(a)** Growth curve (filled circles) and biomass concentrations (light grey) of *N. oleoabundans* cultivated autotrophically in a 20-L PBR in BM medium; **(b)** Time-course variations in the pH in culture media of *N. oleoabundans* cells cultivated autotrophically in a 20-L PBR; **(c)** Growth curve (filled squares) and biomass concentrations (dark grey) of *N. oleoabundans* cultivated in a 20-L PBR in BM medium under mixotrophic conditions (supplemented with 2.5 gL^{-1} of glucose) **(d)** Time-course variations in the pH in culture media of *N. oleoabundans* cells cultivated mixotrophically in a 20-L PBR. In both graphs, curves are constructed on a $\log_2$ scale and for each samples, values are means ± s.d. (n=3)

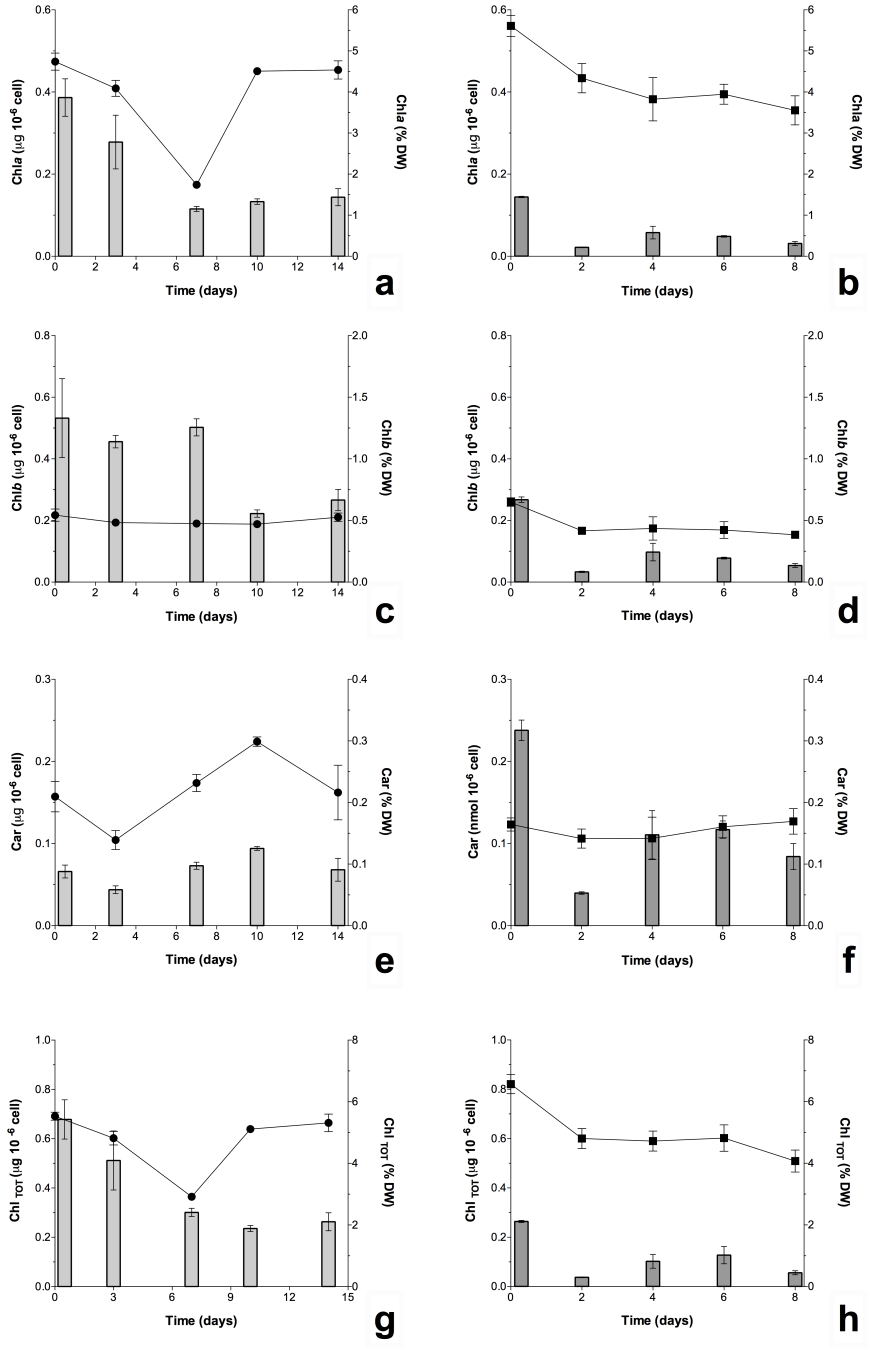

Fig. 2: Time-course variations of pigments content, expressed on cell basis (line) and biomass basis (histograms), of *N. oleoabundans* cells cultivated autotrophycally (filled circles and light grey) and mixotrophycally (filled squares and dark grey) in a 20-L PBR. (**a**) Chl*a* content in *N. oleoabundans* cells cultivated autotrophically and (**b**) mixotrophically; (**c**) Chl*b* content in *N. oleoabundans* cells cultivated autotrophically and (**d**) mixotrophically; (**e**) Car content in *N. oleoabundans* cells cultivated autotrophically and (**f**) mixotrophically; (**g**) total Chl content in *N. oleoabundans* cells cultivated autotrophically and (**h**) mixotrophically. For each samples, values are means ± s.d. (n=3)

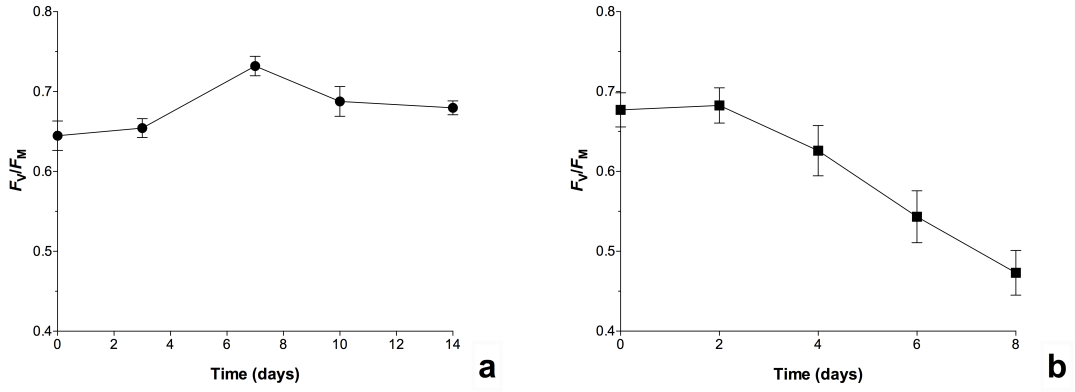

Fig. 3: Time-course variations of PSII maximum quantum yield (F_V/F_M ratio) in *N. oleoabundans* cells cultivated autotrophically **(a)** and mixotrophically **(b)** in a 20-L PBR. Values are means ± s.d. (n=3)

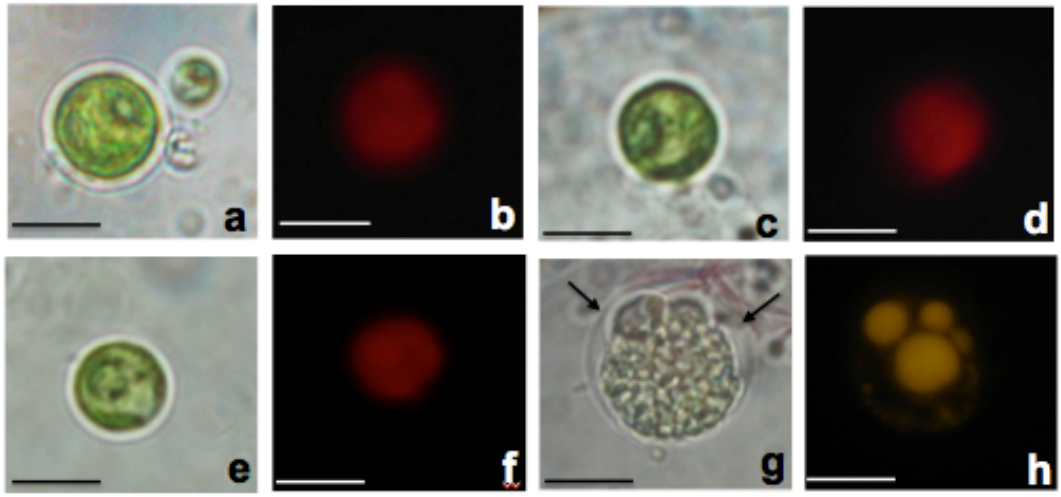

Fig. 4: (a-h) Cell morphology of *N. oleoabundans* cells cultivated in a 20-L PBR. Light **(a, c)** and fluorescence microscope observations **(b, d)** of *N. oleoabundans* cells after 3 **(a, b)** and 14 **(c, d)** days of autotrophic cultivation. Light **(e, g)** and epifluorescence microscope observations **(f, h)** of *N. oleoabundans* cells after 2 **(e, f)** and 8 **(g, h)** days of mixotrophic cultivation. Translucent globules are indicated with arrows. In all micrographs, bars: 2 μm

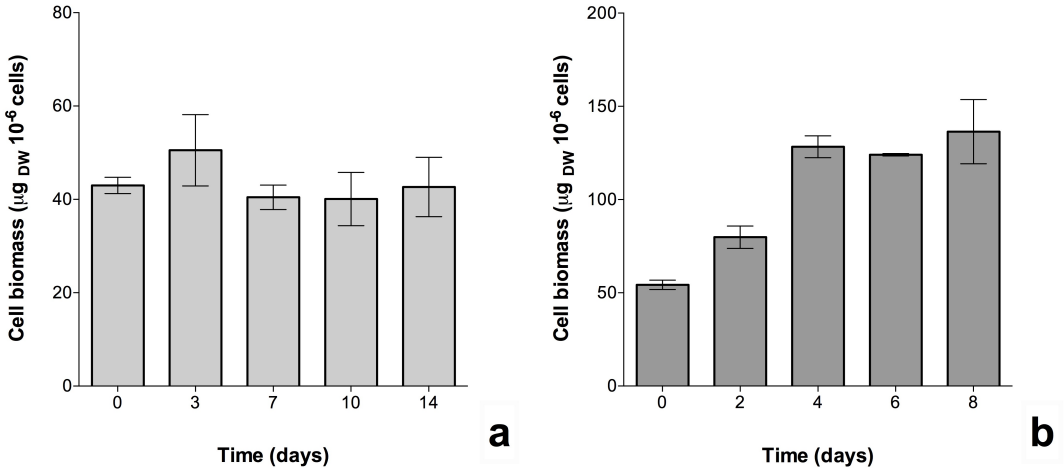

Fig. 5: Time-course variations of the single cell biomass, expressed as micrograms of algal dry weight per one million cells, of *N. oleoabundans* cultivated in a 20-L PBR autotrophically **(a)** and mixotrophically **(b)**. Values are means ± s.d. (n=3)

3. *Re-cultivation of* Neochloris oleoabundans *in exhausted autotrophic and mixotrophic media: the potential role of polyamines and free fatty acids*

3.1 Materials and Methods

3.1.1 Algal strains and culture condition

The strain used in this study was the Chlorophyta *N. oleoabundans* UTEX 1185 (syn. *Ettlia oleoabundans*) (Chlamydomonadales, Chlamydomonadales incertae sedis), obtained from the Culture Collection of Algae of the University of Texas (UTEX, Austin, Texas, USA; www.utex.org). *N. oleoabundans* was cultivated in axenic liquid brackish medium (BM) (Baldisserotto et al. 2012) in a coaxial 20-L capacity PBR (M2M Engineering, Grazzanise, Caserta, Italy). Algae were cultivated autotrophically in BM for 25 days or mixotrophically, by addition of 2.5 g L^{-1} glucose, for 8 days, according to previously described protocols (Baldisserotto et al. 2014; Giovanardi et al. 2014). For autotrophic cultivation, cells were inoculated into the PBR to obtain an initial cell concentration of about 0.6 x 10^6 cells mL^{-1}, while for mixotrophic cultivation initial cell concentration was higher (10 x 10^6 cells mL^{-1}). Culture conditions in the PBR were: 24 ± 1°C; sterile air injection at the bottom of the PBR, with 0.5/3.5 h bubbling/static cycles; irradiance 65 $\mu mol_{photons}$ m^{-2} s^{-1} of PAR (16:8 h light:dark photoperiod). Light was supplied with inner cool–white fluorescent Philips tubes. Algal growth and morphology in the PBR were monitored as described in Section 2.1.1.

3.1.2 Preparation of growth media

Experiments were performed by testing the following culture media:

- C: freshly prepared BM medium (control);
- E: autotrophic exhausted medium;
- EG: mixotrophic exhausted medium after cultivation with glucose;
- E+: autotrophic exhausted medium replenished with nitrate and phosphate concentrations as for BM medium;
- EG+: mixotrophic exhausted medium after cultivation with glucose and replenished with

nutrients as in E+;

- E_2+: 2nd run of autotrophic exhausted medium replenished with nitrate and phosphate concentrations as for BM medium;

- EG_2+: 2nd run of mixotrophic exhausted medium after cultivation with glucose and replenished with nutrients as in E_2+.

In order to obtain the exhausted media for the recycling experiment, *N. oleoabundans* was grown inside the PBR under the culture conditions described in the previous section. For E+ medium preparation, about 500 mL of autotrophic algal culture in the stationary phase of growth (at the 15th day of cultivation in the PBR) were centrifuged at 2000 *g* for 10 min in order to separate the medium from the algae, thus obtaining an autotrophic exhausted medium (E). The medium appeared pale-yellow and was free from algae, bacteria and protozoa; its optical density at a wavelength of 750 nm (OD_{750}) was 0.02. For EG+ medium, at the 8th day of cultivation in the PBR, the same aliquots of algal culture were harvested by centrifugation (2000 *g* for 10 min). In this case, the mixotrophic exhausted medium (EG) was straw-yellow in colour and presented a weak bacterial contamination due to the glucose addition (OD_{750} 0.009). Glucose was proved to be totally consumed during the previous cultivation inside the PBR by 3,5-dinitrosalicylic (DNS; Sigma-Aldrich, Gallarate, Milan, Italy) acid assay, according to Giovanardi et al. (2014). In both exhausted media, pH was not adjusted. After determining the nitrate and phosphate concentrations of the two exhausted media (see "Nitrate and phosphate analyses"), KNO_3 and K_2HPO_4 were axenically added to reach final concentrations of 0.2 and 0.02 g L^{-1}, respectively, i.e., the typical concentration of those components in BM, thus obtaining the replenished exhausted media (E+ and EG+) used for experiments.

3.1.3 Experimental design

Recycling culture media to re-cultivate N. oleoabundans *in unmodified exhausted growth media*

In order to investigate if recycled culture media could be use for the re-cultivation of *N. oleoabundans*, a preliminarily experiment was performed. The growth and morphology were monitored by employing unmodified exhausted growth media (E and EG) to re-cultivate *N. oleoabundans*. When autotrophic cultures of *N. oleoabundans*, grown in BM in the PBR, reached a cell concentration of 10 x 10^6 cells mL^{-1} (after about 9 days of cultivation), aliquots of cells were inoculated into 300 mL Erlenmeyer flasks (150 mL total volume) containing C, E, EG media, to obtain an initial cell concentration of about 0.4 x 10^6 cells

mL^{-1}. The cultures were placed in a growth chamber (24 ± 1 °C, 80 $\mu mol_{photons}$ m^{-2} s^{-1} of PAR with a 16:8 h light-darkness photoperiod), and cultivated with continuous shaking at 80 rpm, without external CO_2 supply. Experiments were performed in triplicate. Aliquots of cultures were collected at different times of cultivation, depending on the analysis.

Exhausted growth media recycling times

In order to find out how many times the exhausted culture media can be recycled, at the end of the 1st run of cultivation, the 100% of E+ and EG+ media were collected to prepare the replenished exhausted media (E_2+ and EG_2+) used for the 2nd run of cultivation as described in the previous section (see "Preparation of growth media"). After determining the nitrate and phosphate concentrations of the two exhausted media (E+ and EG+), KNO_3 and K_2HPO_4 were axenically added to reach final concentrations of 0.2 and 0.02 g L^{-1}, respectively, i.e., the typical concentration of those components in BM, thus obtaining the replenished exhausted media (E_2+ and EG_2+) used for the 2nd run of cultivation. When autotrophic cultures of *N. oleoabundans,* grown in BM in the PBR, reached a cell concentration of 10 x 10^6 cells mL^{-1} (after about 9 days of cultivation), aliquots of cells were inoculated into 300-mL Erlenmeyer flasks (150 mL total volume) containing C, E+, EG+ and in the following experiment E_2+, EG_2+ media, to obtain an initial cell concentration of about 0.5 x 10^6 cells mL^{-1}. The cultures were placed in a growth chamber under the same conditions described above (see "*Recycling culture media to re-cultivate* N. oleoabundans *in unmodified exhausted growth media").* Experiments were performed in triplicate. Aliquots of cultures (cells and media) were collected at different times of cultivation, depending on the analysis.

3.1.4 Growth evaluation

Aliquots of cell samples cultivated in control and exhausted media were counted at different times of cultivation using a Thoma haemocytometer (HBG, Giessen, Germany) under a light microscope (Zeiss, model Axiophot) and growth curves were obtained.

Specific growth rates (μ, number of divisions per day) during the exponential phase were calculated according to Giovanardi et al. (2013).

In parallel to growth, pH was also periodically monitored on small aliquots of samples.

3.1.5 Nitrate and phosphate analyses

After 12 and 25 days of cultivation, samples of C, E+ and EG+ media were harvested by centrifugation to analyze nitrate (N) and phosphate (P) concentrations. These nutrients were quantified colorimetrically using a flow-injection autoanalyzer (FlowSys, Systea, Roma, Italy).

3.1.6 Photosynthetic pigment extraction and quantification

For photosynthetic pigment analysis, cell samples were collected by centrifugation at different times of cultivation. Extraction of photosynthetic pigments was performed according to Baldisserotto et al. (2014). The extracts were measured with the same spectrophotometer described above at 666 (Chl*a*), 653 (Chl*b*) and 470 nm (Car). Quantification was performed according to equations reported in Wellburn (1994).

3.1.7 PAM fluorimetry

The maximum quantum yield of PSII algae was determined using a pulse amplitude modulated fluorometer (ADC Bioscientific Ltd, Hoddesdon, Hertfordshire, UK) at the same cultivation times considered for growth curve measurements. Samples were prepared as reported in Ferroni et al. (2011). After 15 min of dark incubation, initial fluorescence (F_0) and maximum fluorescence (F_M) values were measured flashing the samples with a saturating light impulse. These values were used for the calculation of the maximum quantum yield of PSII: Y(PSII) = F_V/F_M, where variable fluorescence is F_V = (F_M–F_0), F_M (Lichtenthaler et al. 2005).

3.1.8 Light and fluorescence microscopy

For microscopic observations, cell samples were routinely collected throughout the cultivation period inside the PBR and during the experiment with exhausted media. Aliquots of samples were observed using a microscope (Zeiss, model Axiophot) with conventional and fluorescent attachments. The light source for chlorophyll fluorescence observation was a HBO 100 W pressure mercury vapour lamp (filter set, BP436/10, LP470). Pictures of cells were taken with a Canon IXUS 110 IS digital camera (12.1 megapixels), mounted on the ocular lens through a Leica DC150 system (Leica Camera AG, Solms, Germany).

3.1.9 Lipid staining

During experiments, for lipid identification inside cells, the fluorochrome Nile Red (9-diethylamina-5Hbenzo[α]phenoxazine-5-one, 0.5 mg dissolved in 100 mL acetone; Sigma-Aldrich, Gallarate, Milan, Italy), was employed according to Giovanardi et al. (2014). After incubation at 37°C in darkness for 15 min, cells were observed with the same microscope described above at the excitation of 485 nm (filter set, BP485, LP520) to highlight the presence of intracellular neutral lipids as gold–yellowish spots. Pictures were taken with the camera described above.

3.1.10 Transmission electron microscopy (TEM)

TEM observations were made on cells samples of C, E+ and EG+ harvested by centrifugation (600 *g*, 10 min) at the 12th day of cultivation. Cells were fixed, post-fixed and dehydrated as reported in Baldisserotto et al. (2012). Embedding in resin and staining procedures were performed as previously described (Pancaldi et al. 2002). Sections were observed with a Hitachi H800 electron microscope (Hitachi, Tokyo, Japan) at the Electron Microscopy Centre, University of Ferrara.

3.1.11 Extracellular free fatty acids analysis

To analyze the FFA composition of the exhausted E and EG media used to prepare E+ and EG+ replenished media, aliquots of *N. oleoabundans* cultures grown autotrophically and mixotrophically in the PBR were collected at the 15th and at the 8th day of cultivation, respectively. Samples were centrifuged at 2000 *g* for 10 min in order to separate the medium from algae. Fifty mL of media obtained from the autotrophic (E) and mixotrophic (EG) cultures were freeze-dried for analysis. The extracellular FFA composition was determined in duplicate by gas chromatography-mass spectrometry (GC-MS). For extraction, samples were dissolved in 3 mL of hexane, sonicated for 15 min and extracted overnight. Fatty acid methyl esters were prepared by transesterification with 1.5 mL of 5% of sodium hydroxide in methanol solution. Sample volumes of 1 µl were injected into the GC-MS apparatus, which consisted of a Varian Saturn 2100 MS/MS ion trap mass spectrometer. Separations were performed using a Zebron ZB-WAX Phenomenex capillary column (60 cm in length, 0.25 mm i.d) supplied with helium carrier gas at 1 mL min^{-1} constant flow. The injector temperature was 250 °C and the oven temperature program was the following: start 100°C for 2 min, ramp to 200°C at 10 °C/min, and hold for 108 min. The MS acquisitions were

performed by full scan mode.

3.1.12 Determination of polyamines

To analyze the free PA composition of *N. oleoabundans* cells cultivated autotrophically and mixotrophically in PBR and in the corresponding E and EG media, samples were collected as described for FFA analyses. Freeze-dried E or EG media and algae (50 and 20 mg, respectively) were extracted in cold 4% perchloric acid, kept for 1 h on ice, and then centrifuged at 15,000 *g* for 15 min. Aliquots (200 mL) of the supernatants and standard solutions of putrescine (Put), spermidine (Spd) and spermine (Spm) were derivatised with dansyl chloride (Scaramagli et al. 1999). Dansylated derivatives were extracted with toluene, taken to dryness and resuspended in acetonitrile. PAs were separated and quantified by HPLC using a reverse phase C_{18} column (Spherisorb ODS2, 5-µm particle diameter, 4.6 x 250 mm, Waters, Wexford, Ireland) and a programmed acetonitrile:water step gradient (flow rate 1 mL min^{-1}) on a Jasco system (Jasco Corp., Tokyo, Japan) consisting of a PU-1580 pump, an LG-1580-02 ternary gradient unit, a DG-1580-53 three-line degasser and a FP-1529 fluorescence detector, linked to an autosampler (AS 2055 Plus).

3.1.13 Data treatment

Data were processed with Graphpad Prism 6 (Graph Pad Software, San Diego CA, USA). In each case, means ± standard deviations for *n* number of samples are given. Statistical analyses for comparison of means were carried out using ANOVA, followed by Student's *t*-test (significance level, 0.05).

3.2 Results

3.2.1 Growth and morphology of *N. oleoabundans* cultivated autotrophically and mixotrophically in a 20-L PBR

Growth curve and cell morphology of *N. oleoabundans* cultivated autotrophically and mixotrophically in a 20-L PBR are shown in Fig. 1. Autotrophic cells rapidly entered the exponential phase starting on the 2^{nd}-3^{rd} day up to the 7^{th}-9^{th} day of cultivation (Fig 1a). After this period, cultures had a short late exponential phase and then entered the stationary phase of growth, reaching a final cell concentration of about 22×10^6 cells mL^{-1} at the end of the cultivation time (25 days). During the entire period, cells showed the typical morphology of *N. oleoabundans* grown in BM medium, i.e., almost spherical, with a cell diameter of 3-5 µm (Fig. 1b, d). One cup-shaped chloroplast, containing a large pyrenoid, was present and emitted an intense red fluorescence due to the presence of chlorophyll (Fig. 1b, c). On the 25^{th} day of cultivation, cells maintained their normal features and dimensions, but some lipid globules accumulated inside the cytoplasm, as revealed by Nile Red staining (Fig. 1d, e).

During the first four days of mixotrophic growth in the PBR in the presence of glucose, cells showed an evident increase in cell concentration, reaching values of ca. 33×10^6 cells mL^{-1} (Fig. 1f). Subsequently, cells entered the stationary phase of growth, and then cell concentration leveled off to values of about 36×10^6 cells mL^{-1} up to the end of the cultivation period, i.e., eight days. At the beginning of cultivation, both cell shape and size, and chloroplast features in mixotrophic cultures (Fig. 1g, h) were similar to those of autotrophic cultures (Fig. 1b, c). Starting on the 3^{rd} day and up to the end of cultivation, however, mixotrophic cells showed peculiar features. In fact, the chloroplast lost its characteristic cup shape and translucent globules, which tended to occupy almost the entire cell volume, accumulated inside the cytoplasm (Fig. 1i). Nile Red staining confirmed the lipidic nature of these droplets (Fig. 1j).

3.2.2 Growth and morphology of *N. oleoabundans* cultivated in unmodified exhausted growth media and control growth media

In order to investigate if recycled culture media could be use for the re-cultivation of *N. oleoabundans*, a preliminarily experiment was performed by employing unmodified exhausted growth media (E and EG). As shown in Fig. 2a all samples rapidly entered the

exponential phase during the first 3 days up to the 6th day of cultivation. Subsequently, from the 7th day onwards, E and EG samples entered the stationary phase and no relevant differences were observed between them, showing similar stable growth curves and cell concentrations until the end of the cultivation. Conversely, C samples had a short late exponential phase and then entered the stationary phase of growth on 14th day. Is was noteworthy that during the entire experiment C cells reached and maintained the highest cell concentration; the major difference as compared to the other samples was observed starting on the 14th day, with a cell concentration of about 29 × 10^6 cells mL^{-1} (50% more than E and EG samples, respectively; $p < 0.01$ in both cases relative to C samples). At the end of the of cultivation time (28 days), C samples reaching a final cell concentration of about 37 *vs* ca. 14 × 10^6 cells mL^{-1} of E and EG samples. Cell concentration in C was ca. 2.5 times higher than those found in cells grown in unmodified exhausted growth media (E and EG samples, respectively; $p<0.001$ in both cases relative to C samples).

To investigate the morphological changes induced by cultivation in unmodified exhausted media, algal samples were monitored by light microscopy during the entire cultivation time. During the entire period, C cells showed the typical morphology and dimensions of *N. oleoabundans* grown in BM medium, i.e., almost spherical, with a cell diameter of 3-5 μm (Fig. 2b, c). One cup-shaped chloroplast, containing a large pyrenoid, was present and emitted an intense red fluorescence due to the presence of chlorophyll (Fig. 2c). Only at the end of the experiment (28 days) C samples showed some translucent granulation at the cytoplasmic level. Regarding E and EG samples at the beginning of cultivation, both cell shape and size, and chloroplast features were similar to those of C cultures. Starting on the 14th day and up to the end of cultivation, however, E and EG cells showed peculiar features. In fact, the chloroplast lost its characteristic cup shape and translucent globules, which tended to occupy almost the entire cell volume, accumulated inside the cytoplasm (Fig. 2d, f). Nile Red staining confirmed the lipid nature of these droplets (Fig. 2e, g).

3.2.3 Growth curves of *N. oleoabundans* in exhausted and control growth media

As shown in Fig. 3, the growth of *N. oleoabundans* in E+ medium was promoted for the entire experiment relative to controls grown on BM medium; on the contrary, growth in EG+ medium was similar to that of the C. All samples entered the exponential phase very soon (during the first 3 days of cultivation), despite some differences in growth rates (Fig 3b). The highest μ values were observed for algae cultivated in the two exhausted media (E+

and EG+, 1.29 and 1.18 div d^{-1}, respectively; control, 0.98 div d^{-1}; $p < 0.01$ in both cases relative to C) (Fig. 3b). Subsequently, from the 3rd day onwards, no relevant differences were observed between C and EG+ samples, as both of them showed similar growth curves and cell concentrations (Fig. 3a). Conversely, E+ samples reached and maintained the highest cell concentration; the major difference as compared to the other samples was observed starting on the 7th day, with a cell concentration of about 16×10^6 cells mL^{-1} (45 and 50% more than EG+ and C samples, respectively; $p < 0.01$ in both cases relative to E+ samples) at the same time point. After the 17th day, all samples entered the stationary phase, reaching, at the end of the experiment, a final cell concentration of 25-30 $\times 10^6$ cells mL^{-1}. During the experiments, aliquots of samples were periodically harvested to measure the pH of the culture media. As observed in Fig 3c, initial pH was very different between control medium (about 7.0) and exhausted media (about 9.5 in both cases). pH gradually increased up to 9.2 in the control medium, whilst in E^+ and EG^+ media the pH varied between 9.5 and 8.0 without any obvious trend.

3.2.4 Consumption of nitrate and phosphate by *N. oleoabundans* cultivated in exhausted and control growth media

The consumption of nitrate and phosphate by *N. oleoabundans* cells in the course of the experiment is depicted in Fig. 4. At time 0 (inoculation), the exhausted replenished growth media contained approximately 2 mM NO_3^- and 0.115 mM PO_4^{2-}, i.e., the typical concentration of those components in BM medium. By the end of the experiment, cells had consumed practically all nitrate and phosphate present in the growth media. However, nutrient consumption by C, E+ and EG+ algal cultures followed different trends, since C samples maintained higher nitrate and phosphate concentrations than samples in exhausted media. In fact, after 12 days of cultivation, cells grown in E+ and EG+ had consumed about 34% and 68% of nitrate, respectively, while C only 15%. Nitrate concentration decreased from 1.43 mM at time 0 for all culture media to 0.94 mM for E+, 0.45 mM for EG+ and 1.21 mM for C media ($p < 0.001$ in both treated samples relative to C; Fig. 4a). Differently, during the first 12 days of growth, phosphate exhibited a dramatic decrease, with a reduction by cells cultivated in EG+ and E+ of 94% and 98%, respectively, while in C medium it was only about 60%. In fact, phosphate concentration decreased from about 0.086 mM at time 0 for all culture media to 0.002 for E+, 0.005 for EG+ and 0.035 mM for C media ($p < 0.001$ in both treated samples relative to C; Fig. 4b).

3.2.5 Photosynthetic pigment content in *N. oleoabundans* cultivated in exhausted and control media

During the experiment a gradual increase in photosynthetic pigment content was observed in all samples (Fig. 5). Chl*a* in algae grown in C medium showed an evident increase up to the end of the experiment, reaching an average concentration of 0.5 nmol 10^{-6} cells (Fig. 5a). A similar increasing trend was also observed in E+ samples, though with values 10-15% lower than those of controls. From the 17th day of cultivation, Chl*a* content of E+ samples was significantly lower than in C (32%; $p<0.01$). Conversely, in EG+ samples Chl*a* content showed a slightly increasing trend as compared with C and E+ samples; this led to a final value of 0.35 nmol 10^{-6} cells at the end of the experiment (32 and 21% lower than C and E+ samples, respectively; Fig. 5a). An increasing trend in Chl*b* content was also observed for all samples, without significant differences between C and E+ samples (Fig. 5b). Similar to Chl*a* content, EG+ cells contained lower quantities of Chl*b* than C and E+ samples (15-25% and 20-35% lower than C and E+ samples, respectively). More evident differences were observed for Car content (Fig. 5c). In fact, C samples showed an evident increase up to the end of the experiment, always containing higher quantities compared to the other samples (20-45% and 15-30% more than E+ and EG+ samples, respectively; $p<0.05$). It is noteworthy that EG+ and E+ cells shared a similar trend of Car concentration during the experiment (Fig. 5c).

3.2.6 Maximum quantum yield of PSII of *N. oleoabundans* cultivated in exhausted and control media

The variations in PSII maximum quantum yield measured during the experiments are shown in Fig. 6. C, E+ and EG+ samples showed a slight increase of the F_V/F_M ratio during the first three days of growth, reaching values of ca. 0.70. Subsequently, C cells maintained stable values around 0.70-0.75, while the F_V/F_M ratio of E+ and EG+ samples decreased drastically down to values below 0.50 at the end of experiment. In EG+ cells the decrease was dramatic already from the 3rd day of cultivation, reaching the lowest value after 17 days (ca. 0.30 for EG+ *vs* 0.75 for C and 0.50 for E+; $p<0.01$ in both cases); thereafter, samples maintained stable values of ca. 0.35 until the end of the experiment. On the contrary, in E+ cells, F_V/F_M started to decrease very strongly only from the 7th day of cultivation until the

17th day (from about 0.70 at day 7 down to 0.50 at day 17). During the subsequent experimental times (21th and 25th day), the F_V/F_M ratio of E+ cells remained stable around 0.45.

3.2.7 Morphological observations of *N. oleoabundans* cultivated in exhausted and control media

Light microscopy and Nile Red staining

Light microscopy of both control and treated samples showed that *N. oleoabundans* maintained similar cell morphology and dimensions throughout the experiment (Fig. 7, 8). In fact, cells were almost spherical with a cell diameter of 3-5 μm. One cup-shaped chloroplast, containing a large pyrenoid, was present inside the cells (Fig. 7a, c, e). Moreover, the chloroplast emitted an intense red fluorescence due to Chl (Fig. 7b, d, f). Interestingly, after 25 days of cultivation, while cell size and shape remained substantially unchanged, all algal samples showed some translucent granulation at the cytoplasmic level (Fig. 8a, c, e). In order to understand the nature of those granulations and to investigate if the recycled growth media could promote the production of lipid globules, all samples were periodically stained with the lipid-specific fluorochrome Nile Red. The reaction was positive only at the end of the experiment and the translucent globules were then unequivocally identified as lipid droplets (Fig. 8b, d, f).

TEM observations

To investigate the morphological and cytological changes induced by cultivation in recycled growth media, the ultrastructure of C, E+ and EG+ samples was observed by TEM. At 12 days of cultivation, most of the C cell volume was occupied by a characteristic cup-shaped chloroplast. Inside the plastid one large pyrenoid, which was crossed by two elongated and appressed thylakoids, was present (Fig. 9a). In particular, the organelle contained starch in the shape of a shell around the pyrenoid, and showed the typical thylakoid organization (Fig. 9b). Typically featured chloroplasts, as described for cells grown in C, were observed in cells grown in E+ medium (Fig. 9c); however, thylakoids were more appressed than in C (Fig. 9d). EG+ samples showed a more strongly altered chloroplast structure (Fig. 9e), as compared with C and E+ samples. Photosynthetic membranes showed different degrees of thylakoid appression: some portions of thylakoid membranes were appressed while others

were loose and sometimes swollen (Fig. 9f, g). In addition, large portions of the stroma were free of thylakoids and some plastoglobules in proximity of the thylakoid membranes were also visible (Fig. 9g, h). Finally, in EG+ samples the pyrenoid lost its round shape and appeared malformed (Fig. 9e).

3.2.8 Extracellular FFA composition in E and EG media

The extracellular FFA composition in the exhausted autotrophic (E) and mixotrophic (EG) media of *N. oleoabundans* is shown in Table 1. While EG medium comprised both saturated and mono-unsaturated FFAs, E medium contained only saturated FFAs. In fact, both samples contained similar percentages of saturated myristic acid (C14:0) and palmitic acid (C16:0), while the percentage of stearic acid (C18:0) was different between the two samples. In E medium, stearic acid was present at about 64%, while in EG medium at about 56%; however, C18:0 represented the main saturated FFA for both samples. It is noteworthy that only EG medium contained about 6% of the monoenoic oleic acid (C18:1ω9).

3.2.9 Polyamine content in E and EG media and in the corresponding *N. oleoabundans* cells cultivated in a 20-L PBR

As shown in Fig. 10a, the PA composition of *N. oleoabundans* cells cultivated mixotrophically in the PBR were characterized by higher putrescine, spermine, and, especially, spermidine levels compared to cells cultivated autotrophically. In fact, Spm, Put and Spd concentrations in mixotrophic cells were *ca.* 2, 30 and 57 times higher, respectively, than those found in autotrophic cells ($p<0.001$). Conversely, the PA composition in E and EG medium was not significantly different. In fact, both media contained similar amounts of Put and Spm. Interestingly, spermidine was present in high concentration in both media (119 ± 16.91 and 101 ± 5.00 pmol mL^{-1} in E and EG, respectively; Fig. 10b).

3.2.10 Growth curves of *N. oleoabundans* in the 2nd run exhausted growth media and control media

In order to find out to what extent the exhausted culture media can be recycled, at the end of the 1st run of cultivation, 100% of E+ and EG+ was recycled to re-grow *N. oleoabundans*. Growth curves of *N. oleoabundans* cultivated in the 2nd run exhausted growth media is shown

in Fig. 11. All samples entered the exponential phase very soon (during the first 4 days of cultivation). Subsequently, from the 4th day up to 7th day of cultivation, E_2+ and EG_2+ had similar short late exponential phase. Afterwards, from the 7th day onwards, E_2+ and EG_2+ entered the stationary phase and no relevant differences were observed between them, showing similar slight growth curves and cell concentrations until the end of the cultivation. Conversely, C cells showed a more long late exponential phase until the 11th day of cultivation and then entered the stationary phase.

It was noteworthy that no relevant differences in cell concentration were observed between all samples until the 7th day of cultivation. Subsequently, C cells reached and maintained the highest cell concentration relative to E_2+ and EG_2+; the major difference as compared to the other samples was observed starting on the 11th day, with a cell concentration of about 15 × 10^6 cells mL^{-1} (1.6 times higher than those found in cells grown in E_2+ and EG_2+, respectively; $p < 0.01$ in both cases relative to C samples). At the end of the of cultivation time (18 days), C samples reaching a final cell concentration of about 27 *vs* ca. 12 × 10^6 cells mL^{-1} of E_2+ and EG_2+ samples. Cell concentration in C was ca. 2.3 times higher than those found in cells grown in the E_2+ and EG_2+ 2nd run exhausted growth media (E_2+ and EG_2+ samples, respectively; $p<0.001$ in both cases relative to C samples).

3.2.11 Photosynthetic pigment content in *N. oleoabundans* cultivated in the 2nd run exhausted growth media and control media

During the experiment a gradual increase in photosynthetic pigment content was observed in all samples (Fig. 12). Chl*a* in algae grown in C medium showed an evident increase up to the end of the experiment, reaching an average concentration of 0.6 nmol 10^{-6} cells (Fig. 12a). E_2+ samples showed a gradual increase of Chl*a* during the entire experiment, reaching values of ca. 0.30 nmol 10^{-6} cells until the end of the experiment. Conversely, EG_2+ samples, during the first 4 days of cultivation, had stable values of Chl*a* (0.19 nmol 10^{-6} cells). Subsequently, cells from the 4th day up to 11th day of cultivation showed a slightly increasing trend, reaching the same values of E_2+ samples; thereafter, samples maintained stable values of ca. 0.30 nmol 10^{-6} cells up to the end of the experiment. At the end of the experiment, algae grown in C medium reached a final value of 0.6 nmol 10^{-6} cells (50% more than E_2+and EG_2+ samples, respectively; $p < 0.05$ in both case).

An increasing trend in Chl*b* content was also observed for all samples (Fig. 12b). During the 4 days of cultivation, C and E_2+ samples showed the same slightly increasing trend, reaching

the same quantities of Chl*b* (0.09 nmol 10^{-6} cell). Thereafter, C samples had an evident increase until the end of the experiment while in E_2+ cells the increase of Chl*b* was evident until the 7th day of cultivation. Subsequently, cells in E_2+ maintained stable values of ca. 0.15 nmol 10^{-6} cells up to the end of the experiment. Conversely, EG_2+ samples, during the 4 days of cultivation, showed a less decrease of Chl*b* content. Afterwards, EG_2+ showed a similar increasing trend already observed in E_2+samples. At the end of the experiment, E_2+ and EG_2+ cells reaching the value of ca. 0.17 nmol 10^{-6} cells *vs* 0.23 for C, respectively.

Car content in algae grown in C medium showed an evident increase up to the end of the experiment, cells contained higher quantities than E_2+and EG_2+ samples (Fig. 12c). During the 4 days of cultivation, E_2+and EG_2+ cells shared a similar slight increase of Car concentration, reaching the same values of 0.06 nmol 10^{-6} cells. Subsequently, E_2+ showed an evident increase until the 7th day; thereafter, samples maintained stable values of ca. 0.10 nmol 10^{-6} cells up to the end of the experiment. From the 7th day of cultivation, a similar trend was also observed in EG_2+samples, though with lower values than those of E_2+. At the of the experiment, C cells reached the Car content value of 0.15 nmol 10^{-6} cells *vs* 0.11 and 0.10 for E_2+ and EG_2+ samples, respectively.

3.2.12 Maximum quantum yield of PSII of *N. oleoabundans* cultivated in the 2nd run exhausted growth media and control media

The variations in PSII maximum quantum yield measured during the experiments are shown in Fig. 13. C cells showed stable values of the F_V/F_M ratio during the first four days of growth, reaching values of ca. 0.70. During the same experimental time (0-4 days), a similar stable values were also observed in E_2+ and EG_2+ samples, though with lower values than those of C. Subsequently, C cells from the 4th day up to 7th day of cultivation, showed a slight increase of the F_V/F_M ratio, reaching values of ca. 0.75; thereafter, samples maintained stable values of ca. 0.75 until the end of the experiment. On the contrary, the F_V/F_M ratio of E_2+ and EG_2+ samples decreased drastically down to values below 0.20 at the end of experiment. In cells grown in the E_2+ and EG_2+ 2nd run exhausted growth media the decrease was dramatic already from the 7st day of cultivation, reaching value of ca. 0.50 for E_2+ and EG_2+ *vs* 0.75 for C, respectively; $p < 0.0001$ in both cases. During the subsequent experimental times (11th and 18th day), the F_V/F_M ratio of E_2+ and EG_2+ samples started to decrease very strongly (from about 0.50 at day 7 down to 0.30 at day 11). At the of the experiment, E_2+ and EG_2+ reaching the lowest value of ca. 0.20 for E_2+ and EG_2+ *vs* 0.75

for C, respectively; $p < 0.0001$ in both cases.

3.3 Discussion

The recycling of culture medium has been proposed as a possible solution in order to reduce water consumption for algal cultivation, thereby making the process more economically feasible and environmentally sustainable (Yang et al. 2011; Hadj-Romdhane et al. 2013; Farooq et al. 2014; Sabia et al. 2015). Yang et al. (2011) estimated that the large-scale cultivation of the microalga *Chlorella vulgaris* in recycled culture medium could reduce water use by about 84%. Present results clearly suggest that *N. oleoabundans* can also efficiently grow in exhausted growth media, especially in the autotrophic medium replenished with nitrate and phosphate (E+). Observing the consumption trend of these main nutrients (Fig. 4), it is clear that in the exhausted media their concentration decreased faster with respect to controls in parallel to the enhanced cell growth. Moreover, phosphate was consumed faster than nitrate by all samples, probably because of its 100 times lower concentration with respect to the latter nutrient (0.2 gL^{-1} of nitrate *vs* 0.002 gL^{-1} of phosphate; Baldisserotto et al. 2012). Thus, in order to make the use of recycled culture medium feasible, exhausted media should be replenished with the correct ratio of nitrate and phosphate, as they are the main nutrients that guarantee cell growth (Stephens et al. 2010). This aspect was preliminarily verified by employing unmodified exhausted growth media (E and EG) to re-cultivate *N. oleoabundans*. In fact, cells showed a slight growth, reaching, at the end of the cultivation period, low cell concentrations (ca. 14 *vs* 37 × 10^6 cells mL^{-1} of C cultures, Fig. 2a). These cells accumulated intracellular lipids throughout the experiment as a consequence of the limitation of nitrate and phosphate (Mata et al. 2010; Popovich et al. 2012a). These results are consistent with those of Zhu et al. (2013), who found that *C. zofingensis* cultivated in recycled medium, without nitrogen and phosphorus, displayed enhanced lipid production compared to cultures with full nutrients. In order to understand if the growth promotion of *N. oleoabundans* cultivated in E+ and EG+, relative to C, was influenced by some molecules released from the algae, a characterization of autotrophic (E) and mixotrophic (EG) media was performed. First of all, pH of the control and exhausted media was monitored. In particular, an increasing trend was observed in control, which reached the pH of the exhausted media at the end of the experiment. This was not surprisingly, considering that during the microalgal growth pH usually increases

because of the consumption of CO_2 during the photosynthetic process (Zhang et al. 2014). However, considering that in E^+ and EG^+ pH fluctuated around their initial values, or at least gradually decreased, it is not possible to attribute to pH any evident role in the cell growth promotion. Moheimani and Borowitzka (2006) and Stephen et al. (2010) reported that molecules released from cells can positively/negatively alter cell metabolism and biomass production. In the present work, it was showed that both exhausted media contained the main PAs (putrescine, spermidine, and spermine). It is known that these plant growth regulators are involved in a great variety of developmental processes in plant cells, e.g., cell division and protein synthesis (Kaur-Sawhney et al. 2003; Kuznetsov et al. 2006). PA biosynthetic pathways have also been studied in green algae (Cohen et al. 1984; Theiss et al. 2002; Fuell et al. 2010). Although algae produce "unusual" PAs, such as homospermidine and thermospermine (Hamana et al. 2013), the more common PAs (Put, Spd and Spm), when added to the media, promoted growth and metabolism in *C. vulgaris* (Czerpak et al. 2003). PAs are also known to alleviate the effect of biotic and abiotic stress in plants as well as algae (Tate et al. 2013). In the present work, a very strong increase in Put and, especially, Spd concentrations was observed in *N. oleoabundans* mixotrophic cells as compared to the autotrophic ones, both cultivated in a 20-L PBR. The growth-promoting role of PAs could explain the faster (0-4 days) and stronger cell concentration increase (ca. 33 x 10^6 cell mL^{-1}) in mixotrophic cultures relative to the autotrophic ones (0-9 days; ca. 22 x 10^6 cell mL^{-1}). The higher PA concentrations in mixotrophically grown cells also suggest that, under these culture conditions, algal cells may be better protected from stress-inducing factors, e.g., bacterial contamination. It was noteworthy to know that the presence of PAs released into algal growth medium in PBRs has not been documented before. The release of PAs (predominantly Spd) from autotrophic and mixotrophic cells into their respective (E and EG) exhausted media was not significantly different. However, the presence of these plant growth regulators seems to contribute to the promotion of *N. oleoabundans* growth in both media replenished with nitrate and phosphate. In fact, during the entire experiment, a higher cell concentration was observed in E+ relative to C medium. Moreover, the EG+ samples showed a promotion of cell concentration, albeit similar to that of C. Both autotrophic and mixotrophic exhausted media also contained FFAs, which could have induced a change in the normal metabolism of *N. oleoabundans*. This assumption is demonstrated by the more rapid consumption of nitrate and phosphate, the decreased photosynthetic pigments content and the strong decline in the F_V/F_M ratio observed in cells grown in E+ and EG+ media. These results corroborate the assumption that microalgae

release metabolites in high cell density cultures (Richmond 2004) or as a consequence of stressful conditions (Ikawa 2004; Wu et al. 2006). Even if the role of FFAs is currently under debate (Ikawa 2004; Stephen et al. 2010), recent studies showed that they can strongly inhibit growth or exert cytotoxic effects on microalgae (Kogteva and Bezuglov 1998; Wu et al. 2006; Bosma et al. 2008; Stephen et al. 2010). In fact, the presence of these metabolites, and their oxidative products, negatively affects biomass productivity, especially when the microalgae are cultivated in recycled medium (Lívanský et al. 1996; Rodolfi et al. 2003). Granum et al. (2002) reported that the accumulation of intracellular lipids in microalgae enhanced the release of FFAs into the culture medium, and Harun et al. (2010) observed that this release was caused by cell lysis. In this work, FFA composition in autotrophic (E) and mixotrophic (EG) media, after *N. oleoabundans* cultivation, was similar. The only main difference was represented by the presence of the monoenoic oleic acid (C18:1ω9) in EG medium. Oleic acid is one of the major lipid components in lipid-enriched *N. oleoabundans* grown under N-stress conditions (Popovich et al. 2012a). It is reported that mixotrophic cultivation alters N/C ratio, inducing similar lipid production as N-depleted autotrophic cultures (Giovanardi et al. 2014). Probably for this reason, the release of oleic acid occurred only in the mixotrophic growth medium. Moreover, Wu et al. (2006) observed altered plasma membrane permeability due to the toxic effects of FFAs in two Chlorophyta (*C. vulgaris* and *Monoraphidium contortum*) and in a cyanobacterium (*Anabaena* P-9). Then, in addition to the lower concentration with respect to nitrate in BM medium, the rapid consumption of phosphate in both exhausted media could be linked also to an alteration in membrane permeability, due to the activity of FFAs (Ikawa 2004; Wu et al. 2006). In addition, FFAs can cause inhibition of the PSII and PSI electron transport chains (Siegenthaler 1973) and disorganization of thylakoids (Wu et al. 2006). Indeed, *N. oleoabundans* cells in E+ and EG+ were characterized by alterations of the photosynthetic apparatus. These cells, in fact, contained less photosynthetic pigments than C cells, and exhibited a drastic decrease of the PSII maximum quantum yield (F_V/F_M). These variations are linked to an alteration of photosynthetic efficiency, especially as regards PSII (White et al. 2011). The decreased photosynthetic pigment contents, observed in cells grown in E+ and EG+, also reflected the changes in thylakoid membrane arrangement (Baldisserotto et al. 2012). Therefore, correct thylakoid organization is necessary to maintain optimal photosynthetic activity and this is often influenced by culture conditions (Nevo et al. 2012). In addition, the presence of some plastoglobules in chloroplasts of algal cells, grown on exhausted media, could be another indicator of an alteration of

photosynthetic membranes (Besagni and Kessler 2013). On the contrary, *N. oleoabundans* cells grown in C medium showed stable and normal F_V/F_M values throughout the experiment (Giovanardi et al. 2014), and the typical assembly of thylakoid membranes (Nevo et al. 2012; Baldisserotto et al. 2012; Giovanardi et al. 2014). The presence of lipid droplets, observed only at the end of the experiment in C, E+ and EG+ cells, is probably related to aging of the microalgal cultures (Hu et al. 2008; Baldisserotto et al. 2012) rather than to the use of recycled culture media. When exhausted media were recycled for the 2nd run of cultivation an evident decrease of growth ability and a strong impact on photosynthetic performance was observed. Thus, the cell concentration achieved at the end of the 2nd run in E_2+ and EG_2+ media was not satisfactory. One of the reasons might be that algae could withstand metabolites and possible micronutrients limitation in the culture only during the first time of cultivation in recycled media. These results are consistent with those of Zhu et al. (2013), who found that the cultivation of *C. zofingensis* in exhausted medium recycled 3 times began to affect the growth of algae compared to cultures in the 1 and 2 times. On the basis of the above observation, autotrophic and mixotrophic exhausted media could be 100% recycled only one time to prepare a replenished nutrient medium to re-grow *N. oleoabundans* and to obtain high cell concentration cultures of the microalga.

Tables and Figures

Fatty Acids	Abbreviation	E medium	EG medium
Myristic Acid	C14:0	2.16 ± 0.09	2.09 ± 0.04
Palmitic Acid	C16:0	33.54 ± 0.01	34.43 ± 2.80
Stearic Acid	C18:0	64.31 ± 0.10	56.56 ± 2.71
Oleic Acid	C18:1ω9	nd	5.93 ± 1.47

Tab. 1: Extracellular free fatty acids composition in the exhausted E and EG media of *N. oleoabundans* grown in 20-L PBR, after 15 and 8 days of cultivation, respectively. Values are expressed as percentage of total fatty acids and are means ± s.d. (n=2)

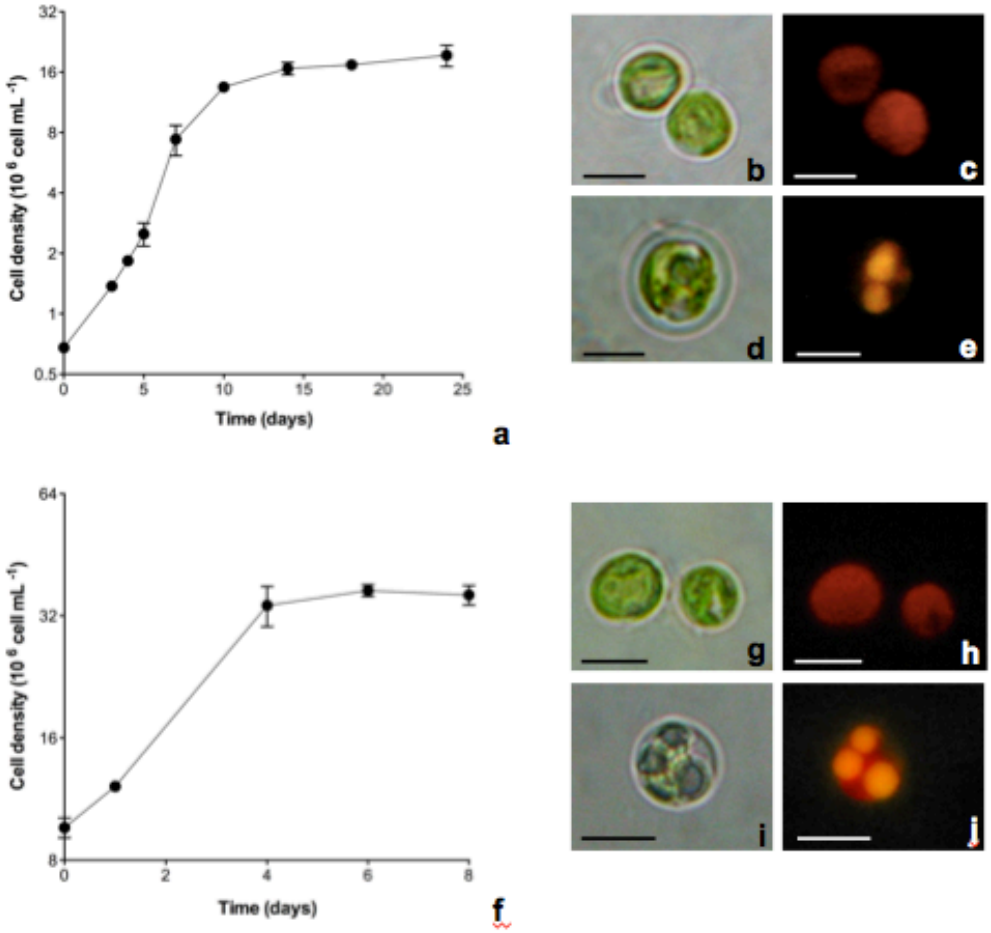

Fig. 1 (a-e): Growth curves **(a)** and morphology **(b-e)** of *N. oleoabundans* cells cultivated autotrophically in a 20-L PBR in BM medium. Light **(b, d)** and fluorescence microscope observations **(c, e)** of *N. oleoabundans* cells after 3 **(b, c)** and 25 **(d, e)** days of autotrophic cultivation. **(f-j)** Growth curves **(f)** and cell morphology **(g-j)** of *N. oleoabundans* cultivated in a 20-L PBR in BM medium under mixotrophic conditions (supplemented with 2.5 gL^{-1} of glucose). Light **(g, i)** and fluorescence microscope observations **(h, j)** of *N. oleoabundans* cells after 3 **(g, h)** and 8 **(d, e)** days of mixotrophic cultivation. In both graphs, curves are constructed on a $\log_2$ scale and data are means ± s.d. (n=3). In all micrographs, bars: 2 μm

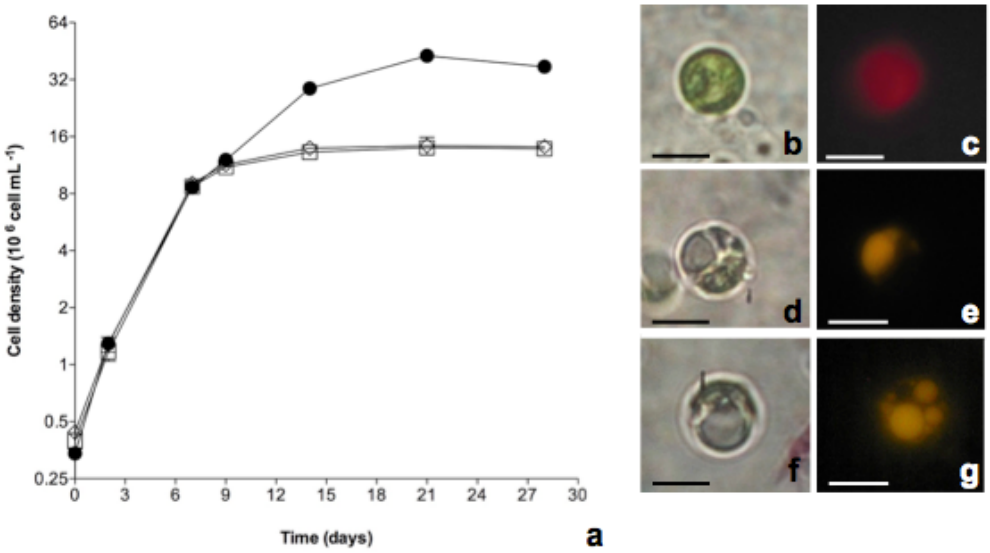

Fig. 2: Growth curves **(a)** and morphology **(b-g)** of *N. oleoabundans* cells in BM medium (filled circles), E medium (empty squares) and EG medium (empty diamonds). Light and epifluorescence pictures of *N. oleoabundans* cells after 21 days of growth. **(b)** Control cells and **(c)** the relative Nile Red-staining observation; **(d)** cell grown in E medium and **(e)** the relative Nile Red-staining observation; **(f)** cell grown in EG medium and **(g)** the relative Nile Red-staining observation. The growth curve is constructed on a $\log_2$ scale and data are means ± s.d. (n=3). In all micrographs, bars: 2 μm

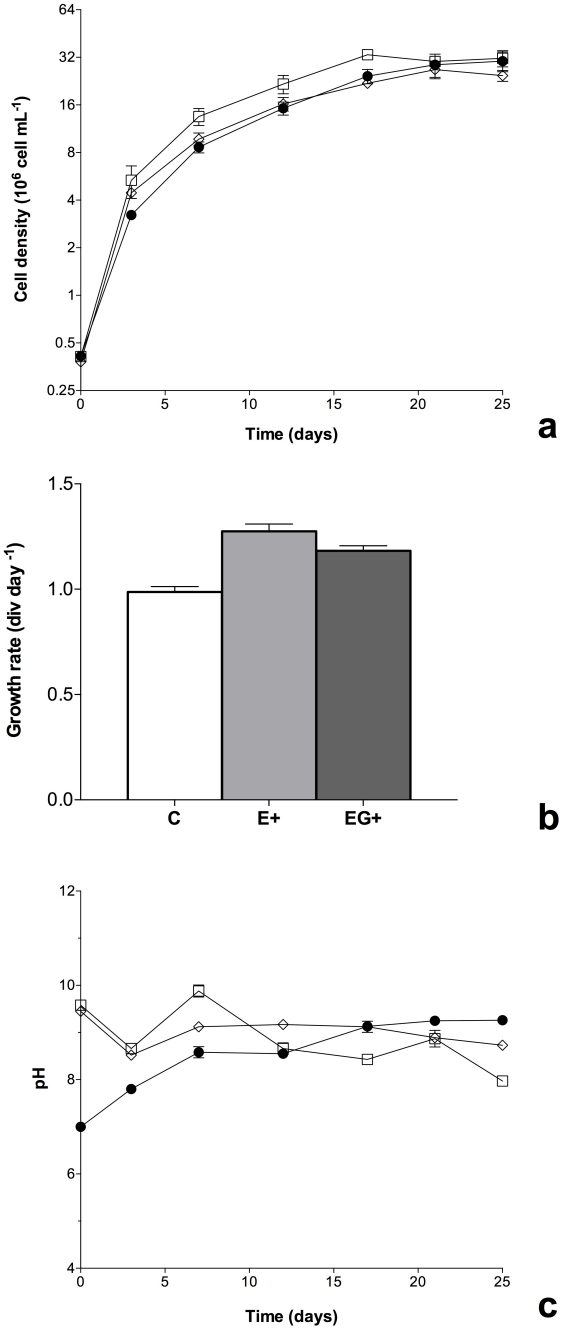

Fig. 3: **(a)** Growth curves of *N. oleoabundans* in BM medium (filled circles), E+ medium (empty squares) and EG+ medium (empty diamonds); **(b)** Growth rates, calculated during the exponential phase (0–3 days time interval of cultivation), of cells grown in BM (white), E+ (light grey) and EG+ (dark grey) media. The growth curve is constructed on a $\log_2$ scale and data are means ± s.d. (n=3); **(c)** Time-course variations in the pH in culture media of *N. oleoabundans* during 25 days of cultivation. Values are means ± s.d. (n=3)

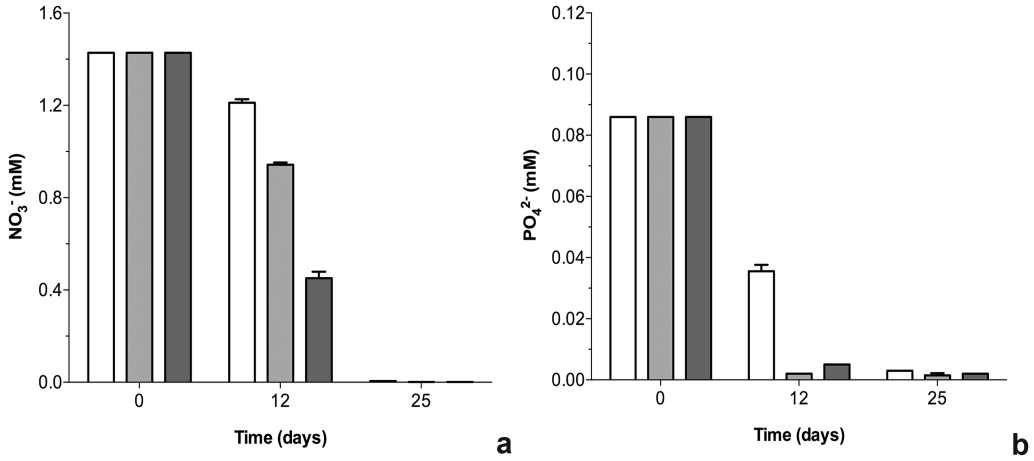

Fig. 4: Time-course variations in the concentrations of nitrate **(a)** and of phosphate **(b)** in culture media of *N. oleoabundans* during 25 days of cultivation on BM medium (white), E+ medium (light grey) and EG+ medium (dark grey). Values are means ± s.d. (n=3)

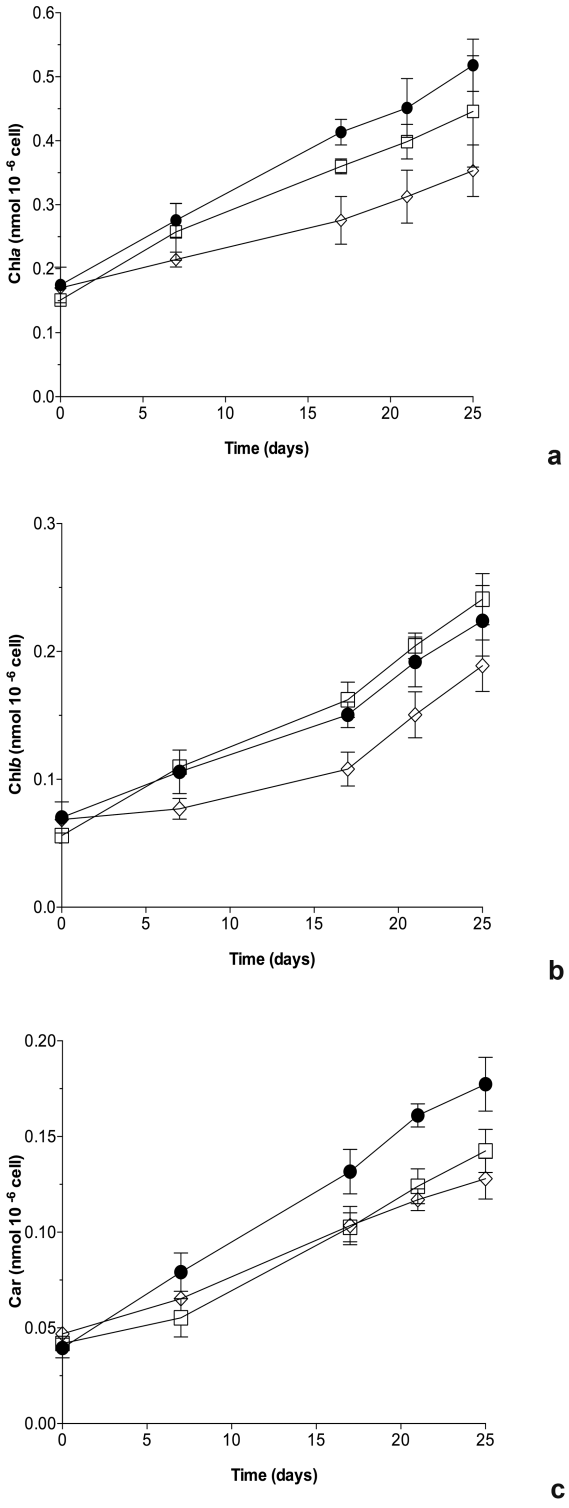

Fig. 5: Time-course variations of Chl*a* (**a**), Chl*b* (**b**) and Car content (**c**) in *N. oleoabundans* cells grown in BM (filled circles), E+ (empty squares) and EG+ (empty diamonds) media during the 25 days of cultivation. Values are means ± s.d. (n=3)

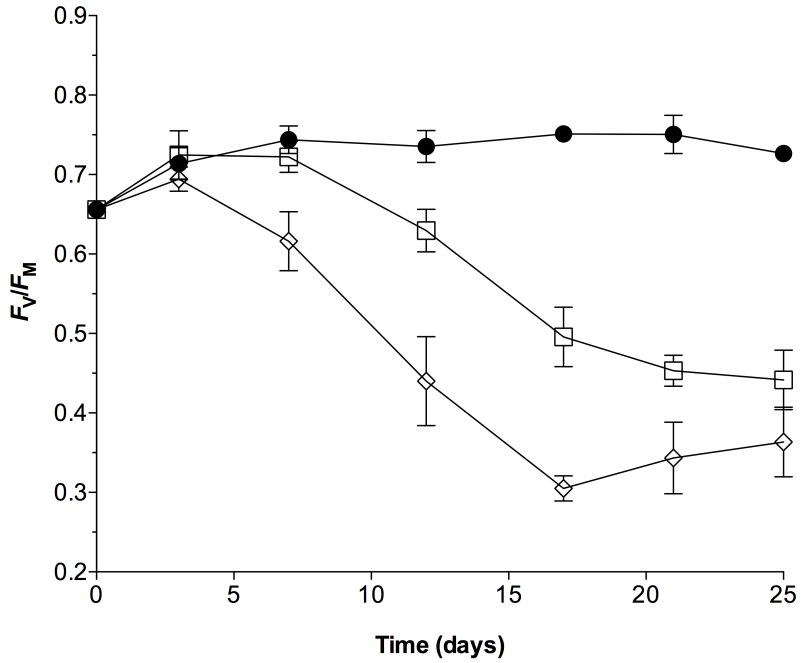

Fig. 6: Time-course variations of PSII maximum quantum yield (F_V/F_M ratio) in *N. oleoabundans* cells grown in BM (filled circles), E+ (empty squares) and EG+ (empty diamonds) media during the 25 days of cultivation. Values are means ± s.d. (n=3)

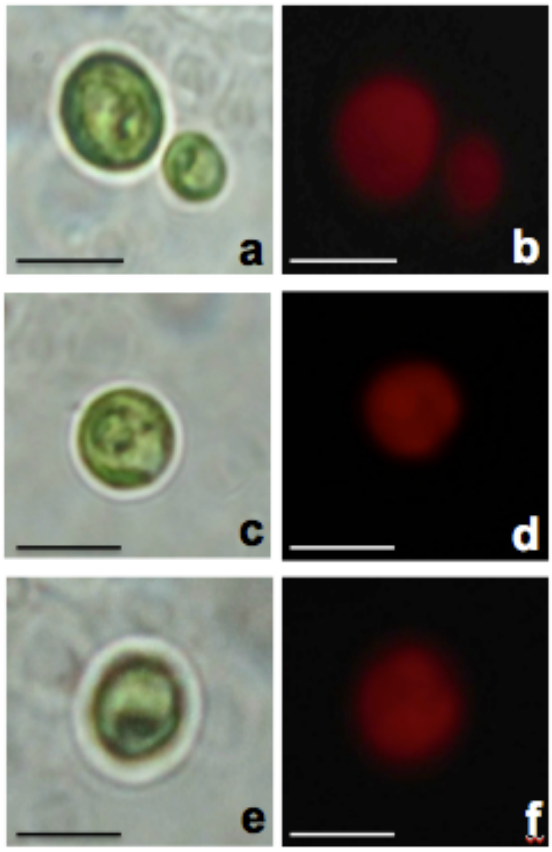

Fig. 7: Light and fluorescence microscopy observations of *N. oleoabundans* cells at 12 days of cultivation. **(a)** Control cells and **(b)** the relative fluorescence of the chloroplast; **(c)** cell grown in E+ medium and **(d)** the relative fluorescence of the chloroplast; **(e)** cell grown in EG+ medium and **(f)** the relative fluorescence of the chloroplast. In all micrographs, bars: 2 μm

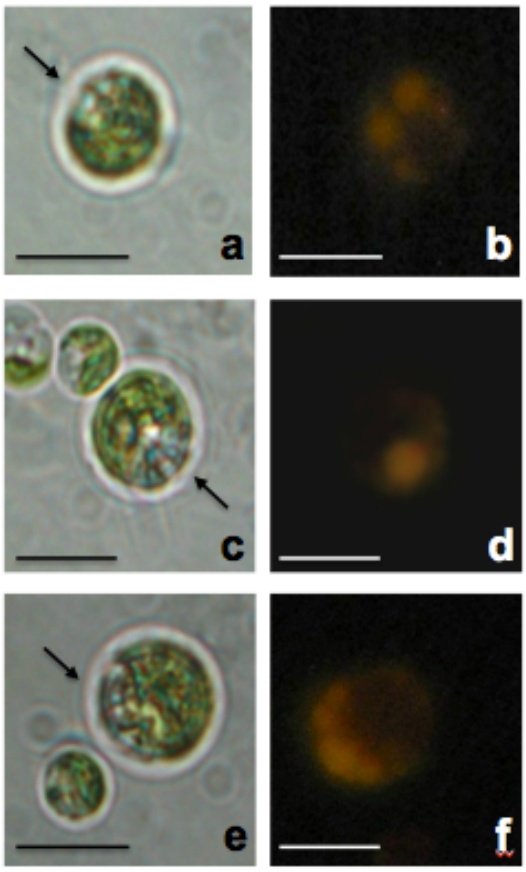

Fig. 8: Light and epifluorescence pictures of *N. oleoabundans* cells after 25 days of growth. **(a)** Control cells and **(b)** relative Nile Red-staining observation, **(c)** cells grown in E+ medium and **(d)** relative Nile Red-staining observation, and **(e)** cells grown in E+ medium and **(f)** relative Nile Red-staining observation in EG+ medium. Translucent globules are indicated with arrows. In all micrographs, bars: 2 μm

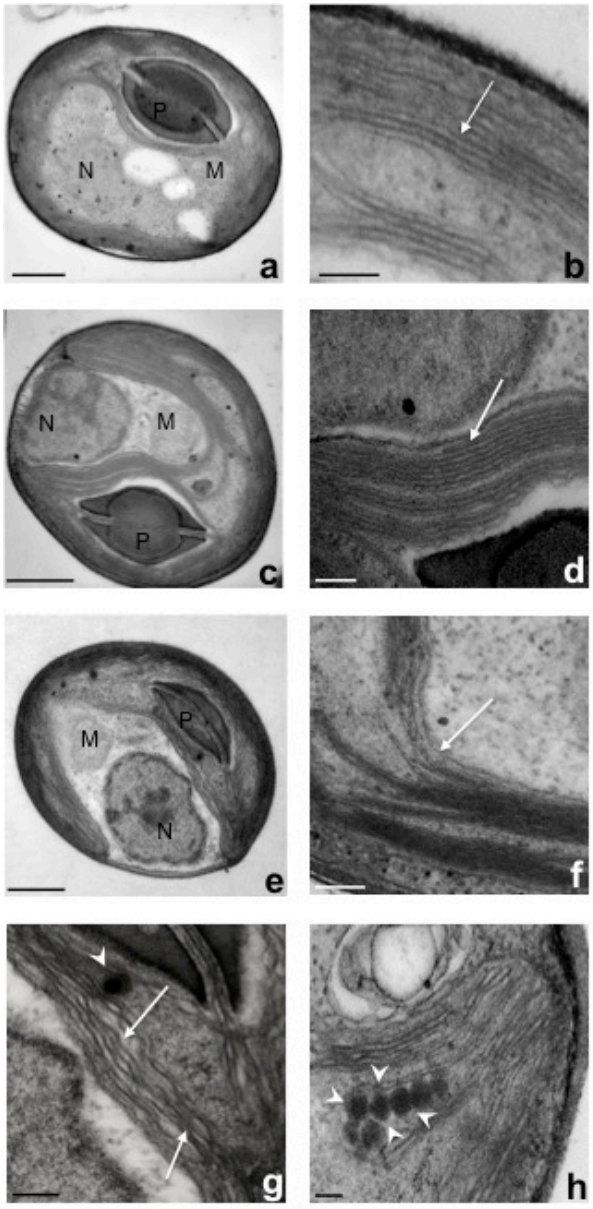

Fig. **9:** Transmission electron micrographs of *N. oleoabundans* cells at 12 days of cultivation. **(a)** Cell grown in BM medium and **(b)** detail of its chloroplast showing the typical thylakoid membranes organization (white arrow). **(c)** Cell grown in E+ medium and **(d)** detail of its chloroplast with quite compact and appressed (white arrow) thylakoids. **(e)** Cell grown in EG+ medium and **(f-h)** details of its chloroplast. The presence of intermediate stages of thylakoid membranes is evident in EG+ cells, showing a general disorganisation of thylakoid membranes **(f-h)**, which appeared wavy, loose and sometimes swollen **(f, g)**. **(h)** Some plastoglobules in proximity of thylakoid membranes are also observed (arrowheads). P, pyrenoid; N, nucleus, M, mithochondrion. Bars: 0.5 μm (a, c, e); 0.05 μm (b, d, f, g, h)

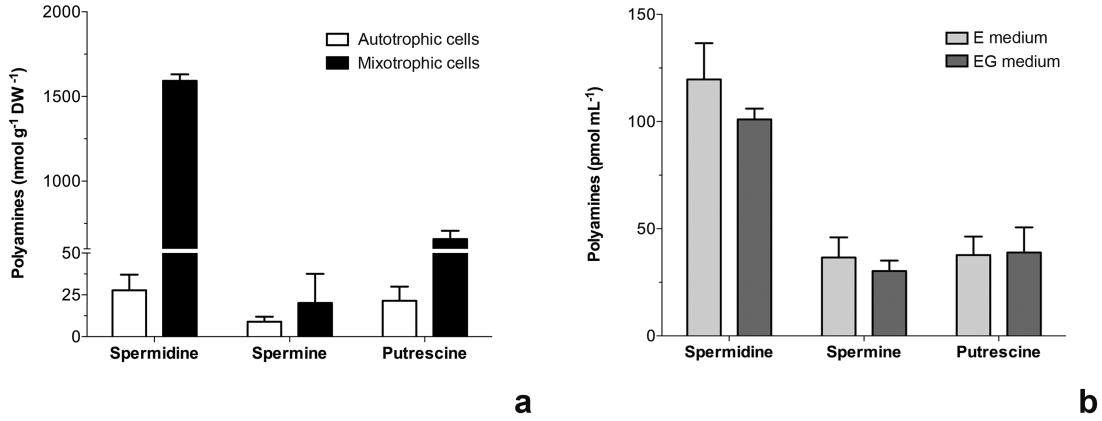

Fig. **10:** Polyamine concentrations **(a)** in *N. oleoabundans* cells cultivated in a 20-L PBR autotrophically and mixotrophically at 15 and 8 days of cultivation, respectively, and **(b)** in the corresponding exhausted E and EG growth media. Autotrophic cells (white), mixotrophic cells (dark), E+ (light grey) and EG+ medium (dark grey). Values are means ± s.d. (n=3)

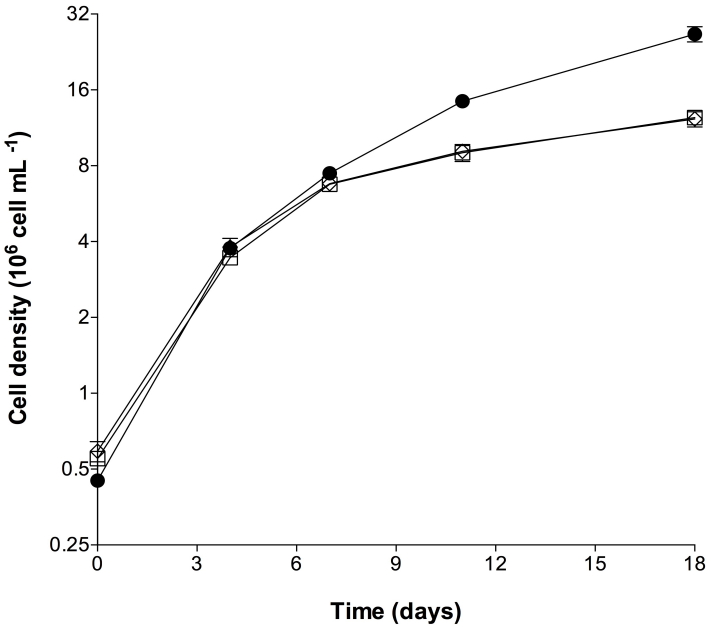

Fig. 11: Growth curves of *N. oleoabundans* in BM medium (filled circles), E_2+ medium (empty squares) and EG_2+ medium (empty diamonds). The growth curve is constructed on a $\log_2$ scale and data are means ± s.d. (n=3)

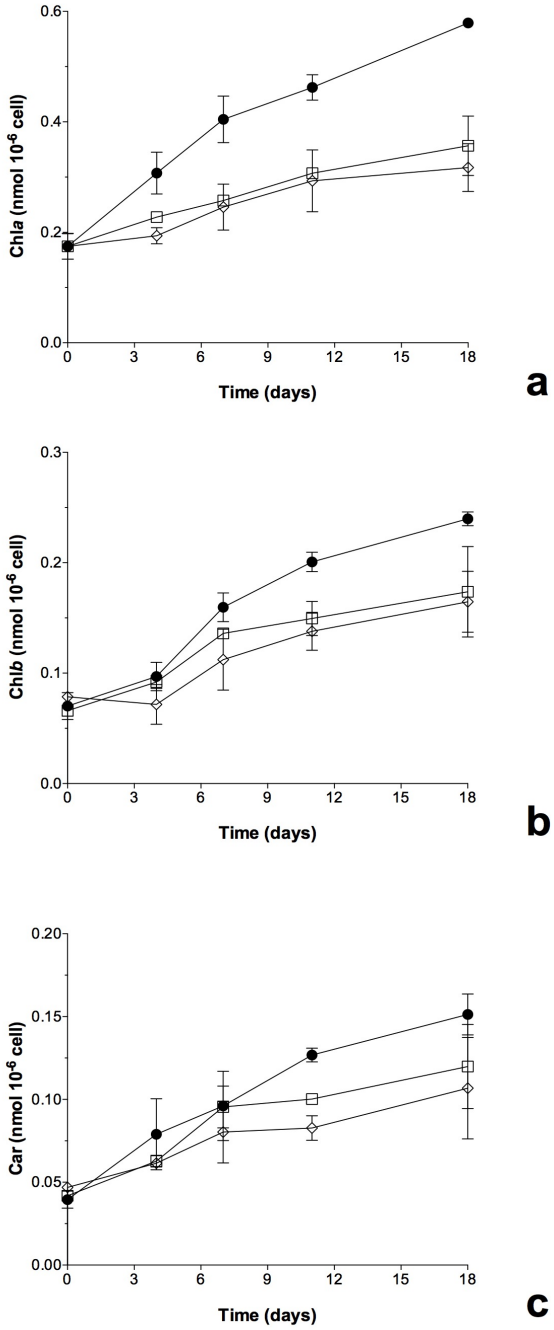

Fig. 12: Time-course variations of Chl*a* **(a)**, Chl*b* **(b)** and Car content **(c)** in *N. oleoabundans* cells grown in BM (filled circles), E_2+ (empty squares) and EG_2+ (empty diamonds) media during the 18 days of cultivation. Values are means ± s.d. (n=3)

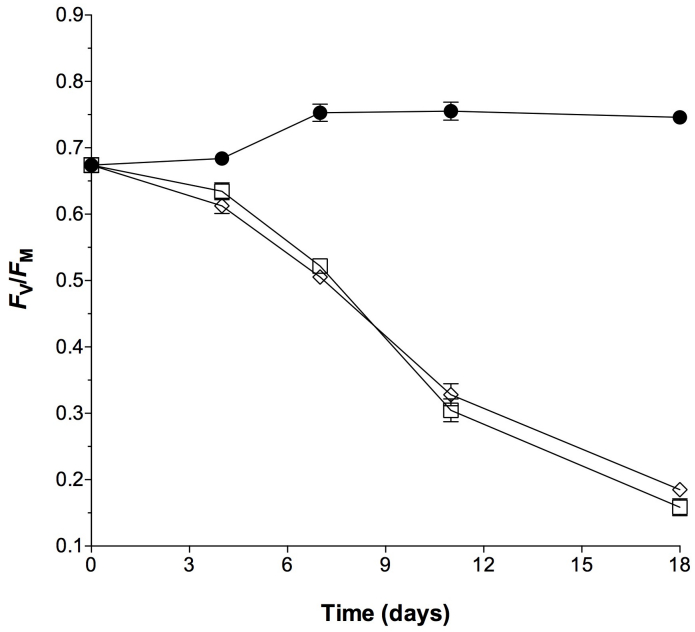

Fig. 13: Time-course variations of PSII maximum quantum yield (F_V/F_M ratio) in *N. oleoabundans* cells grown in BM (filled circles), E_2+ (empty squares) and EG_2+ (empty diamonds) media during the 18 days of cultivation. Values are means ± s.d. (n=3)

4. Conclusion

During the last years, the increasing of global climate change and prices of petroleum and other fossil fuels has led to an urgent need to develop renewable, carbon-neutral, cost-effective alternative feedstock to displace petroleum. Among renewable energy sources, lipid-rich microalgal biomass is being widely studied and proposed as biofuel feedstock (Chisti 2007; Li et al. 2008; Mata et al. 2010; Borowitzka and Moheimani 2013). Nevertheless, one of the main obstacles in microalgal cultivation is the relatively high production and operation cost (Suali and Sarbatly 2012). In order to lower the process costs and, at the same time, to make microalgal production more environmentally sustainable, recycling culture medium has been proposed as a possible solution (Yang et al. 2011; Hadj-Romdhane et al. 2012; Farooq et al. 2014; Sabia et al. 2015).

Neochloris oleoabundans is considered one of the most promising oil-rich microalgae due to its high ability to accumulate lipids (TAGs) under stress culture conditions (Chisti 2007; Li et al. 2008; Gouveia et al. 2009; Pruvost et al. 2009) or mixotrophically (Giovanardi et al. 2014; Silva et al. 2016; Baldisserotto et al. *In Press*).

In this first chapter of the Thesis, the scaling-up of the cultivation and the recycling of growth media for the re-cultivation of the alga were tested with the aim of gaining further knowledge on the potential behaviour of this algal strain in a large-scale cultivation system. With this purpose, the experiments were organized in two different sections.

In the first section, *N. oleoabundans* was cultivated autotrophically and mixotrophically in a 20-L PBR. Results showed that the alga can efficiently achieved higher biomass productivity and lipid content when it was cultivated mixotrophically as compared to the autotrophic conditions. According to Giovanardi et al. (2014), the effect of cultivation of the alga with 2.5 gL^{-1} of glucose on biomass productivity, photosynthetic efficiency and lipid content was very significant respect to the autotrophic one. It is worth noting that, in the present work, the value of biomass productivity (0.28 $gL^{-1}d^{-1}$) achieved by the mixotrophic cultivation of the alga was the second higher value of biomass productivity reported in the literature. In addition, the mixotrophic cultivation had a strong impact on photosynthetic activity and lipid content of *N. oleoabundans*. Accordingly, when the PRB-mixotrophic cells entered in the stationary phase of growth showed a decrease in pigment content and in photosynthetic activity, and, concomitantly, lipid production (Li et al. 2008; Gouveia et al. 2009; Giovanardi et al. 2014; Baldisserotto et al. *In Press*). This study confirmed that the mixotrophic cultivation of the alga in closed photobioreactors is a valid and very efficient

strategy to obtain higher biomass and lipid productivities.

In the second section, *N. oleoabundans* was cultivated in recycling autotrophic and mixotrophic growth media obtained from the previous cultivations in a 20-L PBR with the aim of making the cultivation more economically feasible and environmentally sustainable. Results showed that *N. oleoabundans* can efficiently grow in both exhausted media, if appropriately replenished with the main nutrients (E+ and EG+), especially in E+ and to the same extent as in control medium. Growth promotion of the alga was attributed to PAs and alteration of the photosynthetic apparatus to FFAs. Recycling autotrophic and mixotrophic growth media result to be a suitable solution to obtain high cell concentration cultures of the microalga *N. oleoabundans*.

Collectively, present results represent a valid contribution for improving the scaling-up of microalgal cultivation, providing a more sustainable ecological impact on water resources. On the whole, increasing knowledge of the microalgal physiology is necessary to obtain new useful information for the improvement of culture performance in large-scale cultivation system. Further studies are also needed to deepen knowledge on the nature and the specific role of some molecules that are released into the growth media, in order to obtain useful information for the advancement of the biotechnological use of this strain.

Chapter 3

Comparative analyses of growth, photosynthetic parameters, protein content and pattern in four Chlorophyta species

1. Introduction

The potential of microalgae as a source of renewable energy and high-value bio-products have been widely explored (Mata et al. 2010; Posten and Walter 2012; Borowitzka 2013a; Markou and Nerantzis 2013). One of the main microalgal compounds that might be of particular interest are proteins, which represent up to 50% of total microalgal biomass dry weight (Lourenço et al. 2004; Becker 2007; López et al. 2010; Williams and Laurens 2010). Not only for their high protein content but also for their nutritional quality, that is comparable to other referenced food proteins, various microalgal species are studied as innovative sources of protein in food sectors (Spolaore et al. 2006; Becker 2007; Williams and Laurens 2010). In addition, proteins have been recently considered an attractive feedstock for the production of bio-based chemicals in bio-refinery (Lammens et al. 2012).

In order to enhance the accumulation of protein in microalgal biomass, the choice of the best microalgal strains and their culture condition represent a fundamental and critical step (Borowitzka 2013b; Juneja et al. 2013). It is well known that protein content in microalgae is mainly influenced by culture conditions, growth phases and morphological and structural characteristics of microalgae species (Morris et al. 1974; Piorreck and Pohl 1984; Meijer and Wijffels 1998; Lourenço et al. 2004; Laurens et al. 2014). Moreover, it was reported that the protein pattern also changed in response to culture conditions (Tran et al. 2009). In spite of these aspects, a direct comparison of protein content in different microalgae is also very difficult, due to the several methods employed for protein extraction and quantification (Meijer and Wijffels 1998; Barbarino and Lourenço 2005; López et al. 2010, Schwenzfeier et al. 2011; Servaites et al. 2012; Safi et al. 2013; Ursu et al. 2014).

To allow a better comparison on protein results obtained by different authors, in this study analyses on protein content and pattern were performed on four microalgae species belonging to the same phylum (Chlorophyta) and cultivated under identical culture conditions. Chlorophyta include the majority of the described species of green algae, which are characterized by a great morphological diversity and ability to grow in a wide range of environmental conditions (Proschold and Leliaert 2007; Friedl and Rybalka 2012; Leliaert et al. 2012). Due to these remarkable peculiarities, four Chlorophyta species were selected on the basis of their phylogenetic position in two different classes: Trebouxiophyceae (*Chlorella vulgaris* and *Chlorella protothecoides*) and Chlorophyceae (*Neochloris oleoabundans* and *Scenedesmus acutus*) (Fig. 1). Among the Trebouxiophyceae, *Chlorella vulgaris* is one of the best-studied green microalgae and is considered a model organism in plant science (Krienitz

et al. 2015). Due to its high adaptability to a variety of environmental conditions and high productivity in large-scale cultivation system, this strain has been widely exploited for several biotechnological applications including human and animal nutrition, cosmetics, pharmaceuticals, wastewater treatments or renewable energy source (Spolaore et al. 2006; Görs et al. 2010; Heredia-Arroyo et al. 2011; Safi et al. 2014; Abinandan and Shanthakumar 2015). *Chlorella* (Auxenochlorella*) protothecoides* is chosen due to its capability to produce a large amount of lipids, suitable for biodiesel production, under heterotrophic growth condition (Xu et al. 2006; Heredia-Arroyo et al. 2010; Perez-Garcia et al. 2011). Based on the recent available genome knowledge of this alga, that provides useful information on the regulatory pathway for lipid synthesis in heterotrophic conditions, this species could become a model oleaginous microalga, which could be also used for the application of genetic engineering tools (Gao et al. 2014; Wu et al. 2015). Among the Chlorophyceae, *Neochloris oleoabundans*, recently a taxonomic synonym of *Ettlia oleoabundans*, is an attractive candidate for biodiesel production because of its high capability to accumulate lipids in response to physiological stresses caused by nitrogen starvation (Tornabene et al. 1983; Chisti 2007; Li et al. 2008; Gouveia et al. 2009; Pruvost et al. 2009; Popovich et al. 2012a) or under mixotrophic growth conditions (Giovanardi et al. 2013, 2014; Baldisserotto et al. *In Press*). In attempt to reduce the cultivation costs of this alga and, at the same time, to make its production more environmentally sustainable, this strain was also grown in presence of a wastewater stream (Levine et al. 2011) or in a recycling culture media (Sabia et al. 2015). Finally, the green alga *Scenedesmus acutus* is selected since it is remarkably known for its potential use in wastewater bio-remediation (de Alva et al. 2013; Doria et al. 2012; Giovanardi et al. *In Press*) and its ability to produce large amount of lipids under nitrogen stress condition (Damiani et al. 2014; Giovanardi et al. *In Press*).

In the present work, the four selected Chlorophyta species were cultivated under identical culture condition in order to obtain comparative results about their growth performances, photosynthetic pigment content, PSII maximum quantum yield (F_V/F_M ratio), with special attention to protein content and profile. In addition to these analyses, SDS-PAGE and Blue-native-PAGE in second dimension electrophoresis were performed with the aim to investigate if some differences occurred in the organisation and assembly of the resolved thylakoid complexes of the four green algae.

2. Materials and Methods

2.1 Algal strains and culture condition

The strains used in this study were selected on the basis of their phylogenetic position within the Chlorophyta, in two different classes: Trebouxiophyceae (*Chlorella protothecoides* and *Chlorella vulgaris)* and Chlorophyceae (*Neochloris oleoabundans* and *Scenedesmus acutus)*. The microalgae *Chlorella protothecoides* SAG 211-8d (Chlorophyta, Trebouxiophyceae, Chlorellales, Chlorellaceae, Auxenochlorella) and *Chlorella vulgaris* SAG 211-12 (Chlorophyta, Trebouxiophyceae, Chlorellales, Chlorellaceae, Chlorella) were obtained from the Culture Collection of Algae at the University of Goettingen (SAG, Germany; www.uni-goettingen.de). The microalga *Neochloris oleoabundans* UTEX 1185 (syn. *Ettlia oleoabundans*) (Chlorophyta, Chlorophyceae, Chlamydomonadales, Chlamydomonadales incertae sedis, Neochloris (*syn. Ettlia*) was obtained from the Culture collection of the University of Texas (UTEX, USA; www.utex.org), while the microalga *Scenedesmus acutus* strain PVUW12 (Chlorophyta, Chlorophyceae, Sphaeropleales, Scenedesmaceae, Scenedesmus) (deposited as *Scenedesmus cf. acutus* Meyen CCALA-935 at the Culture Collection of Autotrophic Organisms from Institute of Botany, Academy of Sciences of the Czech Republic, Centre of Phycology, Dukelská) was kindly provided by Prof. Erik Nielsen (Department of Biology and Biotechnology, University of Pavia, Italy).

The microalgae were inoculated at a optical density at 750 nm (OD_{750}) between 0.03 and 0.04 in 500 mL Erlenmeyer flasks (300 mL of total volume) containing BG 11 medium (www.ccap.ac.uk) and cultivated under the same culture conditions in a growth chamber (24 ± 1 °C temperature, 80 $\mu mol_{photons}$ m^{-2} s^{-1} PAR and 16:8 h of light-darkness photoperiod), with continuous shaking at 80 rpm and without external CO_2 supply.

For morpho-physiological and biochemical analyses, aliquots of algae were collected periodically. For protein analyses, aliquots were analysed when the microalgal cultures reached the stationary phase of growth (18^{th} day of cultivation). Experiments were performed at least in triplicate.

2.2 Growth and biomass evaluation

Growth evaluation was carried out on cell samples at different times of cultivation using a Thoma haemocytometer (HBG, Giessen, Germany) under a light microscope (Zeiss, model Axiophot) in order to estimate the specific growth rates (μ). Cell density (cell mL^{-1}) was also

estimated measuring the optical density (OD_{750}) with a Pharmacia Ultrospec 2000 UV-Vis spectrophotometer (1-nm bandwidth; Amersham Biosciences, Piscataway, NJ, USA). Specific growth rates (μ, number of divisions per day) during the exponential phase were calculated according to Giovanardi et al. (2013).

For dry biomass (DW) determination, aliquots of samples (20 mL) were filtered through pre-dried and pre-weighed glass-fiber filters (Whatman GF/F). Filters with cell pellets were rinsed with 20 mL of distilled water and dried 72 h at 60° C, and weighted until they reached constant weight.

During the growth period, the pH in culture replicates was periodically monitored using a pH meter (mod. 3510, Jenway, Bibby Scientific Limited, Staffordshire, UK).

2.3 Photosynthetic pigment extraction and quantification

Extractions of photosynthetic pigments of *C. protothecoides*, *C. vulgaris* and *N. oleoabundans* were performed according to Baldisserotto et al. (2014) with absolute methanol for 10 min at 80°C. For *S. acutus*, due to its thick and rigid cell wall, photosynthetic pigments were extracted with absolute dimethyl sulphoxide (DMSO) for 7 min at 60°C. Absorption of extracts of *C. protothecoides*, *C. vulgaris* and *N. oleoabundans* were measured at 666 (chlorophyll *a* - Chl*a*), 653 (chlorophyll *b* - Chl*b*) and 470 nm (carotenoids - Car), while for *S. acutus* at 665 (Chl*a*), 649 (Chl*b*) and 480 nm (Car) with the same spectrophotometer already described. Pigment concentrations were evaluated according to equations reported in Wellburn (1994).

2.4 PAM fluorimetry

The PSII maximum quantum yield of algae was determined at the same cultivation times considered for growth kinetics measurements. A pulse amplitude modulated (PAM) fluorometer (ADC Bioscientific Ltd, Hoddesdon, Hertfordshire, UK) was used to determine the *in vivo* chlorophyll fluorescence of PSII. The PSII maximum quantum yield is reported as F_V/F_M ratio, *i.e.* $(F_M - F_0)/ F_M$, where variable fluorescence is $F_V = (F_M - F_0)$, F_M is the maximum fluorescence and F_0 is the initial fluorescence of samples (Lichtenthaler et al. 2005). This measurement is considered a valid method to probe the maximum quantum yield of photochemistry in PSII (Kalaji et al. 2014). Samples were prepared as reported in Ferroni et al. (2011) after 15 min of dark incubation.

2.5 Total protein extraction and quantification

Total protein content of the four Chlorophyta species was evaluated with the extraction of two different fractions: a soluble protein fraction and a less-soluble protein fraction, which includes also transmembrane proteins. In detail, aliquots of algal samples were centrifuged for 10 min at 500 *g* and treated according to Ivleva and Golden (2007), with some modifications. Pellets were resuspended in 2 mL of soluble proteins buffer [2mM Na_2EDTA, 5 mM ε-aminocaproic acid, 5 mM $MgCl_2$, 5 mM dithiothreitol dissolved in PBS buffer 1x; PBS buffer (1L, stock solution 10x): 80 g NaCl, 2 g KCl, 14.4 g $Na_2HPO_4.2H_2O$, 2.4 g KH_2PO_4 dissolved in distilled water], transferred into Eppendorf tubes and then centrifuged (10 min, 2000 *g*). Subsequently, pellets were resuspended in 200 µL of soluble proteins buffer. For three times, samples were frozen in liquid N_2 for 2 min and subsequently heated at 80°C for other 2 min, then rapidly frozen in liquid N_2 and kept at -20°C over night. The following day, the samples were added with glass beads (0.40-0.60 µm diameter; Sartorius, Germany) and vigorously vortexed for 10 min (mixing cycles of 30 s followed by cooling on ice for 30 s). After the addition of 100 µL of soluble proteins buffer, samples were centrifugated (10 min, 1500 *g*) and the supernatants (soluble proteins extract) were harvested and rapidly frozen in liquid N_2 and kept at -20°C until quantification. Subsequently, pellets were re-extracted by resuspending with 1 mL of less-soluble proteins buffer (0.1 M NaOH, 1% sodium dodecyl sulphate, 0.5% β-mercaptoethanol dissolved in distilled water), vortexing tubes for 2 min and subsequently boiled at 60°C for other 15 min. After centrifugation (10 min, 1500 *g*) supernatants were harvested (I extract), while pellets were re-extracted by resuspending it in 0.5 mL of less-soluble proteins buffer, then vortexing tubes for 2 min and finally keeping tubes at 60°C for 15 min. Samples were then centrifuged (10 min, 1500 *g*) and the supernatants (II extract) was added to the first one. Finally, the less-soluble proteins extract was rapidly frozen in liquid N_2 and kept at -20°C until quantification. The quantitative estimation of proteins, expressed in terms of percentage of dry weight basis (%DW), was determined with a modified Lowry's method, suitable for a membrane protein determination (Markwell et al. 1981), using bovine serum albumin (BSA) as a standard (Sigma, USA).

2.6 Isolation of thylakoid membranes

Thylakoid membranes of microalgal samples were isolated according to Pantaleoni et al. (2009), with modifications. For extraction, after 1 h of dark incubation, 300 mL of algal

culture in the stationary phase of growth (18th day of cultivation) were harvested by centrifugation at 600 *g* for 10 min. Pellets were transferred to an ice-cold mortar containing sand quartz. The extraction was performed grinding cells with liquid N_2, and then the lysate was resuspended in the grinding buffer (330 mM sorbitol, 50 mM Tricine-NaOH pH 7.5, 2 mM Na_2EDTA pH 8.0, 1 mM $MgCl_2$, 5 mM ascorbate, 0.05% bovine serum albumin, 10 mM NaF) into 15 mL tubes. Samples were centrifuged at 300 *g* for 5 min at 4°C and then at 700 *g* for 5 min at 4°C, in order to remove sand quartz and cell debris. Pellets were discarded and the thylakoids present in the supernatant were collected by centrifugation at 7000 *g* for 10 min at 4°C. The supernatant was discarded and thylakoids were resuspended in 1 mL of shock buffer (5 mM sorbitol, 50 mM Tricine-NaOH pH 7.5, 2mM Na_2EDTA, 5 mM $MgCl_2$, 10 mM NaF) and centrifuged at 7000 *g* for 10 min at 4°C. After that, the supernatant was removed and around 100 µL of storage buffer (100 mM sorbitol, 50 mM Tricine-NaOH pH 7.5, 2 mM Na_2EDTA pH 8.0, 5 mM $MgCl_2$, 10 mM NaF) were added to the pellet. The samples were rapidly frozen in liquid nitrogen and stored at -80°C until further analyses. Manipulation of samples was always performed on ice and in very dim safe light. Quantification of Chls in thylakoid samples was performed according to Porra (1989).

2.7 Denaturing electrophoresis

The different protein fractions and thylakoid membrane proteins were characterized by using SDS-PAGE (15% acrylamide resolving gel containing 6 M urea), according to Laemmli (1970) with minor modifications. For soluble or less-soluble proteins SDS-PAGE gels, 10 or 15 µg of proteins were loaded in each lane respectively, whereas, for thylakoid membrane proteins, samples were loaded on equal Chls basis (2 µg in each lane). After electrophoresis, proteins were visualised by Coomassie staining (0.1% Coomassie Brilliant Blue R250, 7% acetic acid, 40% MeOH in distilled water) following routing protocols. ImageJ software (Version 1.50e, National Institutes of Health, Bethesda, MD) was used for gel bands quantitative densitometric analysis. Selected bands were quantified based on their relative intensities.

2.8 Blue-native PAGE and second dimension (2D) electrophoresis

Blue-native-PAGE was performed as described by Rokka et al. (2005), with minor modifications. Thylakoids containing 8 µg Chl were resuspended in medium A (25 mM Bis-Tris-HCl, pH 7.0, and 20% w/v glycerol and 0.25 mg mL^{-1} Pefabloc), and after that, an

equal volume of 1.5% (w/v) dodecyl β-D-maltoside (Sigma), freshly prepared in medium A, was added. Thylakoids were then solubilised and centrifuged at 18000 *g* at 4°C for 15 min. The supernatant was supplemented with 1/10 volume of SB buffer (100 mM BisTris-HCl, pH 7.0, 0.5 M ε-amino-n-caproic acid, 30% w/v sucrose and 50 mg mL^{-1} Serva Blue G). Samples were loaded on 5-12.5% gradient of acrylamide gel and first-dimension (1D) electrophoresis was performed at 0°C for 6 h by gradually increasing the voltage from 50 to 200 V, using an Owl Separation Systems (mod. P8DS Portsmouth, NH). Quantification of band volume was performed with Image J software. After BN-PAGE, the lanes were cut out and incubated for 1 h in 10%-sodium dodecyl sulphate (SDS) Laemmli buffer containing 5% (v/v) β-mercaptoethanol, followed by separation of the protein subunits of the complexes with SDS-PAGE (15% acrylamide resolving gel containing 6 M urea). After electrophoresis, proteins were visualised by silver staining.

2.9 Data treatment

Data were processed with Graphpad Prism 6 (Graph Pad Software, San Diego CA, USA). In each case, means ± standard deviations for n number of samples are given. The statistical significance of differences was determined by one-way ANOVA followed by a multiple comparison test (Tukey's test). A significance level of 95% ($p < 0.05$) was accepted.

3. Results and discussion

3.1 Comparison of growth parameters

The monitoring of microalgal growth is a fundamental step to evaluate the physiological state of a microalgae strain and to improve its culture performance in biotechnological fields (Havlik et al. 2013). The kinetic parameters are routinely quantified by measuring the optical density (OD), dry weight (DW) biomass concentration, or cell counting (Richmond 2004; Moheimani et al. 2013). OD is frequently used as a rapid and non-destructive indirect measurement of biomass. Another convenient but time-consuming method is the cell DW determination. However, both methods generate mistakes in the biomass estimation because are closely dependent on culture conditions and morpho-physiological state of the cells, namely cell size and shape (Rocha et al. 2003; Andersen 2005; Griffiths et al. 2011; Chekanov and Solovchenko 2015). In this study, in order to accurately estimate

the cell density, cell direct counting was also used. As reported in Fig. 2a, the four selected green algae under identical culture conditions showed similar growth kinetics. All samples promptly entered the exponential phase and grew with a very similar growth rates, ranging between 0.6 and 0.7 div day^{-1} (Fig. 2c). These growth rates values are close to the results reported for some other Chlorophyta species cultivated at irradiance comparable to that used in this study (Singh and Singh 2015). After this period, all cultures had a short late exponential phase and then entered the stationary phase of growth, reaching different final cell densities (Fig. 2a). For a better comparison between samples, biomass yields were also evaluated at the end of the cultivation time (Fig. 2d). Interestingly, despite *C. protothecoides*, *C. vulgaris* and *S. acutus* reached lower cell densities than those observed for *N. oleoabundans*, they yielded higher biomass (61, 32 and 50% higher than *N. oleoabundans*, respectively) (Fig. 2d). Another variable that highly affected microalgal growth is the pH value (Khalil et al. 2010; Moheimani 2013). In this study, pH values displayed a diverse pattern of variance between samples, which it could be attributed to the species-specific assimilation and release of CO_2 during photosynthesis and respiration, respectively. According to previous studies (Moheimani and Borowitzka 2011; Zhang et al. 2014; Difusa et al. 2015; Sabia et al. 2015), under unregulated pH condition, microalgae species showed an overall increase in the pH of the medium with time, which it could be attributed to the consumption of CO_2 during the photosynthetic process (Fig. 2b).

3.2 Comparison of photosynthetic pigment content

Pigment composition of photosynthetic microorganism is linked to its phylogenetic and environmental constraints (Deblois et al. 2013; Esteban et al. 2015). Microalgal cells are known to actively modify their photosynthetic response to the available light in order to maximize their potential growth (Richardson et al. 1983; Falkowski and LaRoche 1991; Rubio et al. 2003; Lichtenthaler and Babani 2004). In addition to light intensity, the cellular pigment composition is modulated by other culture factors such as temperature, nutrient availability and microalgal growth phase (Esteban et al. 2015; Ferreira et al. 2015; Singh and Singh 2015). By considering the analyses on photosynthetic pigment content, in this study the four Chlorophyta species showed a species-specific pattern (Fig. 3). Overall, despite some differences during the exponential phase, concentration of all single pigments inside cell of all samples increased with cultivation time, reaching different final pigments content (Fig. 3). For a better comparison between samples, pigments yields were also

evaluated at the end of the cultivation time (Table 1). It was observed that, independently of the unit of measure employed (on cell or biomass basis), *S. acutus* showed the highest Chl*a* and total Chl content as compared to the other species (ca. 1.5 to 2 times higher than *C. protothecoides*, *C. vulgaris* and *N. oleoabundans*, respectively) (Fig. 3a,d and Table 1). About Chl*b* content, when considering the pigment content per cell, *C. protothecoides* and *S. acutus* contained higher quantities than *C. vulgaris* and *N. oleoabundans* while, on a biomass basis, *C. protothecoides* showed the lowest values as compared to the other species (88, 25 and 33% lower than *C. vulgaris, N. oleoabundans* and *S. acutus*, respectively) (Fig. 3b and Table 1). Similar to Chl content, *S. acutus* contained the highest Car concentrations, on cell basis, as compared to the other strains (ca. 1.5 times higher than *C. protothecoides*, *C. vulgaris* and ca. 3 times higher than *N. oleoabundans*) (Fig. 3c). More evident differences between samples were observed for the Car content expressed as %DW. In detail, *C. vulgaris* showed the lowest Car content as compared to the other species (ca. 3, 4 and 7 and 3 times lower than *C. protothecoides*, *N. oleoabundans* and *S. acutus* respectively) (Table 1). As a consequence, the variability in pigment content between samples resulted in different photosynthetic pigment molar ratios (Fig. 3e,f). Chl *a/b* ratio is an indicator of the light harvesting antennae (LHCII) size and can be affected by the stoichiometry of PSII to PSI (MacIntyre et al. 2002; Deblois et al. 2013; Esteban et al. 2015). As shown in Fig. 3e, this ratio was higher in *S. acutus* (ca. 3) as compared to the other species (ca. 2.5); however, these values are generally found in microalgae (Esteban et al. 2015). According to results obtained by Esteban and co-workers (2015), the higher variability between strains clearly emerged comparing the trend of total Chl/Car molar ratios (Fig. 3f). Alteration of Chl/Car ratios is commonly observed in photosynthetic organisms as a strategy of photoadaptation (MacIntyre et al. 2002; Lichtenthaler and Babani 2004; Mendoza et al. 2008; Solovchenko et al. 2009; Esteban et al. 2015). During the cultivation time, *S. acutus* showed almost a constant ratio, while in the other species, the total Chl/Car molar ratio was subjected to an evident increase until the 5th day. Subsequently, the ratios tended to decrease, reaching, at the end of the experiment, higher values than *S. acutus* (*C. protothecoides, C. vulgaris* and *N. oleoabundans*, 5.8, 4.5 and 5.5, respectively; *S. acutus*, 4) (Fig. 3f). In this study, under identical culture conditions, the species-specific changes in pigment composition mainly depended on the morpho-physiological state of microalgal cells (Richardson et al. 1983; Falkowski and Raven 1997; Deblois et al. 2013).

3.3 Comparison of maximum quantum yield of PSII

PAM fluorometry has become one of the most common, rapid, non-invasive techniques to measure the variability of photosynthetic performance in microalgae (Kruskop and Flynn 2006; Baker 2008; White et al. 2011; Kalaji et. al 2014; Malapascua et al. 2014; Schuurmans et al. 2015). Among PAM parameters, the PSII maximum quantum yield (indicated by F_V/F_M) is widely used to estimate the physiological state of microorganisms under stressful conditions (Parkhill et al. 2001; Kruskop and Flynn 2006; White et al. 2011; Schuurmans et al. 2015). Despite of this, the parameter is also employed to follow and optimize microalgae growth performance (Kruskop and Flynn 2006; Malapascua et al. 2014; Schuurmans et al. 2015). In this study, in order to compare the photosynthetic efficiency of each species, the F_V/F_M ratio was monitored during the experiment (Fig. 4). During the cultivation time, *C. protothecoides* tended to have higher values of F_V/F_M as compared to the other algae, but the values differed significantly only at time 0, 14 and 18th day. In particular at the end of the experiment, *C. protothecoides* showed the highest values of F_V/F_M (0.78) as compared to all other samples (+10, 8% with respect to *C. vulgaris, N. oleoabundans* and *S. acutus*, respectively). No significant differences were reported between the other samples until the 14 days of cultivation, when *C. vulgaris* and *S. acutus* showed similar F_V/F_M ratio, while in *N. oleoabundans* was found to be the lowest as compared to the other species. Overall, present results showed that all selected species had F_V/F_M values that varied in a short range from 0.65 to 0.75 during the cultivation time (Fig. 4). It was reported that, these values in green algae are indicative of non-stressed cultures (White et al. 2011; Masojídek et al. 2013).

3.4 Comparison of protein content and pattern

Several methods have been employed to extract and quantify protein content in microalgal biomass (Meijer and Wijffels 1998; Barbarino and Lourenço 2005; López et al. 2010, Schwenzfeier et al. 2011; Servaites et al. 2012; Safi et al. 2013; Ursu et al. 2014). One of the main obstacles during the protein extraction is the cell wall composition of microalgae; since differences in cell wall composition could affect the efficiency of proteins solubilisation and consequently, the final total protein determination (Lourenço et al. 2004; Servaites et al. 2012; Safi et al. 2013; Ursu et al. 2014). To overcome this drawback, microalgal cells lysis must be achieved to fully release of protein content. In this work, different procedures were tested to extract proteins of the four selected green algae (Meijer

and Wijffels 1998; Ivleva and Golden 2007; Pruvost et al. 2011; Popovich et al. 2012a; Serive et al. 2012). Based on the results obtained from the different extraction methods tested (data not show), the modified procedure (Ivleva and Golden 2007) proposed here allowed to obtain a better extraction of total proteins from all samples, independent of their morphological and biochemical characteristic. According to Chisti and Moo-Young (1986) and Meijer and Wijffels (1998), disruption of the cell by vortexing with glass beads in presence of SDS resulted to be an essential and effective extraction step to lyse the cell walls. In this study, the total protein content of the four Chlorophyta species was evaluated with the extraction of two different fractions: a soluble protein fraction and a less-soluble protein fraction, which includes also transmembrane proteins. After the extraction, the protein content was measured by the modified Lowry's method (Lowry et al. 1951), suitable also for a membrane protein determination (Markwell et al. 1981). It is well known that this is one of the most accurate methods to directly measure the protein content in microalgae biomass (Barbarino and Lourenço 2005; López et al. 2010; Safi et al. 2013). As shown in Table 2, the four selected green microalgae showed some differences in the final total protein content. In detail, the highest value of 53.2 %DW was found in *S. acutus* as compared to the other species (47, 9 and 8% higher than *C. protothecoides*, *C. vulgaris* and *N. oleoabundans*, respectively). *S. acutus* showed also the highest total protein accumulation in the final cell culture (13.3 gL^{-1}). These values are coherent with the results reported by Becker (2007). As shown in Fig. 5a, the most soluble proteins fraction was low in all species. This result might depend on the mild extraction protocol used for soluble proteins.

Qualitative comparison of protein extracts of the four Chlorophyta species was determined by SDS-PAGE. Coomassie-stained gels revealed that the number and intensity of protein bands were different between the four species. With respect to the soluble proteins fraction, as shown in Fig. 5b, the extraction of the less-soluble protein fraction gave the best results in terms of quality and resolution of protein bands. In fact, the protein bands of the soluble fraction appeared poorly defined, even though the same protein amount was loaded in gels (Fig. 5a). In all samples, the proteins extracted were resolved into distinct bands mainly from 76 kDa to 12.3 kDa. The clearly distinct band with a molecular mass of about 55-56 kDa in all selected green algae was assignable as the RuBisCO large subunit (Spreitzer 1993; Park et al. 1999). The protein bands below 30 kDa mainly correspond to subunits of the light-harvesting complex of photosystem II (LHCII) (Bennett 1991). Interestingly, in *C. protothecoides* and *C. vulgaris* LHCII was principally represented by one evident band, while two bands with similar intensity were resolved in the other two species.

3.5 Comparison of thylakoid protein complexes

Photosynthetic organisms regulate the stoichiometry of their photosynthetic complexes in response to variations in light intensity and spectral quality. To avoid light excess and make a profitable use of limiting light, plants and green algae have evolved different acclimation mechanisms that include both short-term and long-term responses (Anderson et al. 1995). Long-term acclimation responses by plants and green algae involve generally changes in the stoichiometry of PSI *versus* PSII and the modulation of the light-harvesting complex of PSII (LHCII) (Humby and Durnford 2006; Wientjes et al. 2013; Büchel 2015; Wobbe et al. 2016). Among green algae, studies on acclimation mechanisms to light intensity are mainly on *Chlamydomonas reinhardtii*, which is widely explored as a photosynthetic model organism (Durnford et al. 2003; Bonente et al. 2012, Mettler et al. 2014). However, contrasting results on acclimation behavior have been reported in other green algae (Falkowski and Owens 1980; Smith et al. 1990), suggesting that acclimation mechanisms in green microalgae are species-specific. In this study, basic information on thylakoid protein complexes in the four selected Chlorophyta was obtained by SDS-PAGE of thylakoid membrane proteins. Some key proteins, belonging to abundant and fundamental thylakoid complexes are easily detectable in SDS-PAGE profiles after staining with Coomassie blue (Fig. 6a) (Minagawa and Takahashi 2004; Dekker and Boekema 2005). In Fig. 6b the densytometric profiles is shown for each species. In each lane in Fig. 6b, the first conspicuous band from the left corresponded to the major PSI polyptides PsaA/PsaB (around 62 kDa). As regards PSII, the band around 43 kDa was assignable to its light-harvesting protein CP43 and the two homologous proteins of nearly 30 kDa corresponded to D1 and D2 subunits of the reaction center of PSII core. Finally, the LHCII were resolved in four or five separated bands below 30 kDa. In general, it was evident that the two Trebouxiophyceae shared a very similar thylakoids membrane protein pattern as compared to the other two species, which instead showed differences mainly in the bands corresponding to the LHCII proteins (Fig. 6a, b). For semi-quantitative comparison between the four green algae, the relative amount (band volume ratio) of different photosynthetic proteins in the thylakoids complexes was quantified. The band volumes were used to calculate band volume ratios that could provide information on the relative variations in fundamental thylakoid complexes stoichiometry. The LHCII/D2 ratio reflects the abundance of LHCII as compared to PSII cores. It was comparable within the same classes of Chlorophyta, but lower in the two Trebouxiophyceae than in the two Chlorophyceae

(Fig. 7a). The PSI/D2 ratio provides information about stoichiometric variations involving the two photosystems. *S. acutus* showed a markedly lower PSI/D2 ratio as compared to all other species (Fig. 7b). Interestingly, this observation matched the results on biomass yields, since this ratio reflected the acclimation of the available light quality (Büchel 2015; Wobbe et al. 2016). In fact the photosystems stoichiometry is modulated to balance the excitation between PSI and PSII; in a more dense culture, such as that of *S. acutus,* the reciprocal shading of cells is expected to increase, leading to not only a less available light intensity, but also to a relative enrichment in far red. Since far red is mainly absorbed by PSI, cells are expected to downregulate PSI in favour of PSII, as observed in *S. acutus.* Since LHCII can serve also PSI as an antenna, the LHCII/PSI band volume ratio was also calculated. However, for this ratio the main aspect observed was its species-specific variability (Fig. 7c). In general, the two Chlorophyceae revealed a higher availability of LHCII antennae for both photosystems. For this reason, it was interesting to analyse if this specificity of Chlorophyceae had an impact on native interactions.

In order to obtain information on native interactions of photosynthetic protein complexes, thylakoid membrane were mildly solubilized using dodecyl β-D-maltoside and separated by blue native PAGE (BN-PAGE) (Rokka et al. 2005). This technique represents a very reliable method for analysis of protein interaction and assembly, mainly used for analysis of higher plants thylakoids (Kügler et al. 1997; Hippler et al. 2001; Eubel et al. 2005). Applications of this technique in green microalgae has been almost limited to *C. reinhardtii* and only seldom used for other microalgae, with uncertain results (Kantzilakis et al. 2007; Katz et al. 2007; Tran et al. 2009; Szyszka-Mroz et al. 2015). In Giovanardi, Doctoral dissertation, (2013), native complexes in thylakoids from the model organism *Arabidopsis thaliana* and autotrophic *N. oleoabundans* have been separated by BN-PAGE (Fig. 8a), in order to identify the resolved bands of the alga with those of *A. thaliana.* Accordingly to Giovanardi (2013, Doctoral dissertation), in this study all the four green microalgae exhibited the presence of five distinct bands, which were ordered from I to V in Fig. 8b. Interestingly, some evident differences emerged comparing the samples (Fig. 8b). The first band with higher molecular mass in the uppermost part of the gel was more visible in *S. acutus* as compared to the other species, while in *N. oleoabundans* the band IV was clearly less intense. However, by comparison with many published profiles of thylakoid complexes in higher plants (Aro et al. 2005; Kügler et al. 2007; Järvi et al. 2011; Ferroni et al. 2014; Albanese et al. 2016), those of microalgae were singularly scanty. This may depend on the harsh extraction protocol used in this study, and in general with microalgae covered by a cellulose cell wall. This

extraction method cannot give with certainty the possibility to preserve the native interactions of photosynthetic protein complexes, especially the more labile interactions. Independent of the methodological limitation, it is widely accepted that the thylakoid protein patterns of microalgae were less rich as compared to higher plants (Dekker and Boekema 2005; Kouřil et. al 2012). In addition, green algae lack Lhcb6 (CP24), which is necessary for binding some of the LHCII trimers (M-trimer) to PSII core dimer, resulting in an intrinsically more labile LHCII-PSII interaction (Kouřil et. al 2012; Büchel 2015). Nevertheless, it was interesting to investigate if some differences occurred in the organisation and assembly of the resolved thylakoid complexes of the four green algae. Each stripe from the BN-PAGE was further analysed by SDS-PAGE in 2D, enabling the separation of different protein complexes into constituting subunits (Kügler et al. 1997). By considering each 2D image, each species showed some peculiarities in their protein thylakoid patterns. Since in the BN-PAGE the pattern of protein complexes of *S. acutus* resulted to be more abundant as compared to the other species, it was chosen for the identification of the components of the major thylakoid membrane complexes. The heaviest complexes on the left in Fig. 9 in *S. acutus* corresponded to various assemblages of PSI, LHCI and PSII-LHCII, and therefore were attributed to the PSI-(PSII)-LHCII megacomplexes. These results were in agreement with SDS-PAGE analysis and further showed that the higher LHCII/PSI ratio favours a more stable interaction of LHCII with PSI. Concerning the PSII monomers (Lane III), it was observed that in *S. acutus* it was present in low amounts. Lanes IV and V were resolved into the LHCII components, which formed large spots on the right side of the 2D gels. *S. acutus* exhibited the presence of similar amounts of LHCII trimers and monomers, accompanied by a various spectrum of single subunits migrating as free proteins of different molecular weights. The 2D profile also characterized a high molecular mass protein (> 130 kDa) that in *Chlamydomonas* sp. UWO 241 was recently assigned to components of a PSI-associated pigment-protein complex (Szyszka-Mroz et al. 2015). Regarding the other Chlorophyceae *N. oleoabundans* in Fig. 10, the first conspicuous band from the left corresponded to PSI-LHC complexes. The observation of abundant PSI-LHCII complexes but not PSII-LHCII supercomplexes again suggested a weak capability of this alga to preserve the PSII-LHCII interaction. The following lane (III) contained PSII in the monomeric form with subunits migrating with different molecular weight. The resolution of bands corresponding to LHCII trimers and monomers showed that in *N. oleoabundans* LHCII was mostly present in the monomeric form, indicating that in this alga also the interaction between LHC subunits in trimers is

very weak. The Trebouxiophyceae *C. vulgaris* showed a very similar protein thylakoid patterns to that of *N. oleoabundans* (Fig. 11). The main differences emerged comparing the LHCII complexes since in *C. vulgaris* it was found that LHCII was mostly present as trimers. Finally, regarding *C. protothecoides*, the extraction method did not allow to preserve the native interactions of photosynthetic protein complexes, because the majority of proteins were separated at the right end of the gel (Fig. 12). The 130-kDa proteins were resolved and abundant in all algae.

In conclusion, by comparing the 2D/BN-PAGE of all samples some general considerations emerged: i) LHCII was mainly present in the form of free complexes, more abundant trimers than monomers, suggesting that LHCII did not take part to associations sufficiently stable with the two photosystems. Apparently, the more stable interactions were those with PSI rather than PSII; ii) the high molecular mass protein (>130 kDa) assigned to components of a PSI-associated pigment-protein complex was most stable component especially in high molecular mass complexes (Szyszka-Mroz et al. 2015). Interestingly, the molecular mass of this complex corresponds to the heterodimer PsaA-PsaB, which forms the core of PSI (Nelson and Ben-Shem 2004). This would mean that in microalgae PsaA/PsaB could be covalently bound.

4. Conclusion

Recently, modern molecular studies revealed that the green algal classes Chlorophyceae and Trebouxiophyceae, which are characterized by similar morphological shape but by a remarkable diversity of physiological and biochemical constraints, evolved in independent phylogenetic lineages as a result of convergent evolution within the Chlorophyta phylum (Leliaert et al. 2012; Krienitz et al. 2015). By comparing the results on growth performance, under identical culture conditions, all the selected species grew with a very similar rate on a cell basis, but reaching different final cell densities. Interestingly, despite *N. oleoabundans* had reached the highest cell density, the alga showed significant lower values of biomass as compared to the other species at the end of the experiment. The specific variability of each strain clearly emerges comparing the time-course analysis of the photosynthetic pigment content on cell basis, suggesting that each species developed kind of a unique metabolic framework, certainly determined by their phylogenetic and environmental constraints

(Deblois et al. 2013; Esteban et al. 2015). In order to obtain useful information for the biotechnological exploitation of these algae, the pigment yields, expressed on biomass basis, were compared between samples. Interestingly, *S. acutus* showed significant higher values of total Chl and Car (%DW) as compared to all other species. Data on F_V/F_M ratio, which it is a useful parameter to monitor the photosynthetic performance of microorganisms, were shown similar in all the Chlorophyta species during the entire experiment. These higher values, ranging between 0.65 and 0.78, are indicative of non-stressed green microalgal cultures (White et al. 2011; Masojídek et al. 2013). The protein content and profile of these four Chlorophyta species was evaluated in order to identify if some differences occurred between samples. It is well known that a comparison of protein content in different microalgae is very difficult due to the morphological and biochemical characteristics of each microalgae species but also to the several methods employed for protein extraction and quantification (Meijer and Wijffels 1998; Barbarino and Lourenço 2005; Becker 2007; Safi et al. 2013; Ursu et al. 2014). As a first step, in this study, several protein extraction methods were tested. Based on the results obtained, the modified protein extraction procedure (Ivleva and Golden 2007) proposed here allowed to obtain a better comparison of protein content of different green microalgae, independently of their morphological and biochemical characteristics. Interestingly, *S. acutus* showed not only the highest value of protein content (53.2 %DW) but also the highest total protein accumulation in the final cell culture (13.3 gL^{-1}) as compared to the other samples. In order to evaluate if the higher capability to accumulate protein of this alga reflected also in a more abundant profile of thylakoids membrane protein, SDS-PAGE of thylakoid membrane was performed. By considering the results obtained, the two Trebouxiophyceae shared a very similar thylakoids membrane protein pattern as compared to the other two species, which instead showed differences mainly in the bands corresponding to the LHCII proteins. To evaluate if the selected species showed significant variations in fundamental thylakoid complexes stoichiometry, the relative amount (band volume ratio) of some key proteins in the thylakoids were compared. The LHCII/D2 ratio, which reflects the abundance of LHCII as compared to PSII cores, was found to be comparable within the same classes of Chlorophyta, but lower in the two Trebouxiophyceae than in the two Chlorophyceae. Interestingly, the results obtained on PSI/D2 ratio, which provides information about stoichiometric variations involving the two photosystems, showed that *S. acutus* had a markedly lower value as compared to all other species. Concerning the LHCII/PSI ratio, the main aspect observed was its species-specific variability. In general, the two

Chlorophyceae revealed a higher availability of LHCII antennae for both photosystems. Finally, with the aim to analyse if this specificity of Chlorophyceae had an impact on native interactions, BN/SDS-PAGE in second dimension was performed. By comparing the 2D/BN-PAGE, each species showed some peculiarities in their protein thylakoid pattern. In particular, the pattern of protein complexes of *S. acutus* resulted to be more abundant as compared to the other species, which also showed the presence of PSI-(PSII)-LHCII megacomplexes. These results were in agreement with SDS-PAGE analysis and further showed that the higher LHCII/PSI ratio favours a more stable interaction of LHCII with PSI in the alga. Regarding the Chlorophyceae *N. oleoabundans*, BN-PAGE revealed the presence of more abundant PSI-LHCII complexes with respect to the PSII-LHCII supercomplexes, suggesting a weak capability of this alga to preserve the PSII-LHCII interaction. In addition, in *N. oleoabundans* LHCII was mostly present in the monomeric form, indicating that in this alga also the interaction between LHC subunits in trimers is very weak. *C. vulgaris* showed a very similar protein thylakoid patterns to that of *N. oleoabundans,* however in this alga the LHCII were mostly present as trimers. Finally, regarding *C. protothecoides*, the extraction method did not allow to preserve the native interactions of photosynthetic proteins. In general, each species were characterized to have a high molecular mass protein (>130 kDa), which was recently assigned to components of a PSI-associated pigment-protein complex (Szyszka-Mroz et al. 2015).

Collectively, the results obtained in this work provide useful information for the exploitation of these microalgae species in biotechnological applications. Nevertheless, these results have also highlighted the metabolic uniqueness of each strain, resulting in a non-obvious comparison with other species, even the most related ones. Finally, it can be concluded that *S. acutus* resulted to be the most promising species to use for several biotechnological applications, since it had showed high capability to accumulate a large amount of proteins and photosynthetic pigment content, which is also accompanied by a more abundant thylakoid protein complexes profile as compared to all the other species.

Tables and Figures

Strains	Photosynthetic pigment content (%DW)		
	Chl*a*	Chl*b*	Car
Chlorella protothecoides	$2.23^{a} \pm 0.05$	$0.92^{a} \pm 0.03$	$0.35^{a} \pm 0.05$
Chlorella vulgaris	$2.88^{b} \pm 0.13$	$1.80^{b} \pm 0.10$	$0.11^{b} \pm 0.01$
Neochloris oleoabundans	$2.88^{b} \pm 0.33$	$1.17^{ac} \pm 0.17$	$0.46^{c} \pm 0.02$
Scenedesmus acutus	$3.89^{c} \pm 0.26$	$1.25^{c} \pm 0.08$	$0.79^{d} \pm 0.04$

Tab. 1: Photosynthetic pigment content expressed in percentage of dry weight biomass (%DW) of *C. protothecoides*, *C. vulgaris*, *N. oleoabundans* and *S. acutus*. Values are means ± s.d. (n=3), ANOVA $P<0.05$, means in columns for each photosynthetic pigment content followed by the same superscripts are not significantly different at the 5% level according to Tukey's multiple comparison test

Strains	Soluble proteins (%DW)	Less-soluble proteins (%DW)	Total proteins (%DW)	Total proteins (gL^{-1})
Chlorella protothecoides	$3.4^{a} \pm 0.4$	$32.7^{ab} \pm 4.9$	$36.1^{ab} \pm 5.2$	$9.6^{ab} \pm 0.5$
Chlorella vulgaris	$1.8^{b} \pm 0.5$	$46.8^{a} \pm 5.9$	$48.6^{a} \pm 6.5$	$10.9^{ab} \pm 3.7$
Neochloris oleoabundans	$3.8^{a} \pm 0.6$	$45.7^{a} \pm 7.8$	$49.5^{a} \pm 8.1$	$8.2^{b} \pm 0.6$
Scenedesmus acutus	$1.1^{b} \pm 0.1$	$52.1^{ac} \pm 4.2$	$53.2^{ac} \pm 4.3$	$13.3^{a} \pm 0.1$

Tab. 2: Protein content expressed in percentage of dry weight biomass (%DW) and grams liter (gL^{-1}) of *C. protothecoides*, *C. vulgaris*, *N. oleoabundans* and *S. acutus*. Values are represented as means ± s.d. (n=3), ANOVA $P<0.05$. Different superscripts denote significant ($P<0.05$) differences between the means in columns of each protein fraction

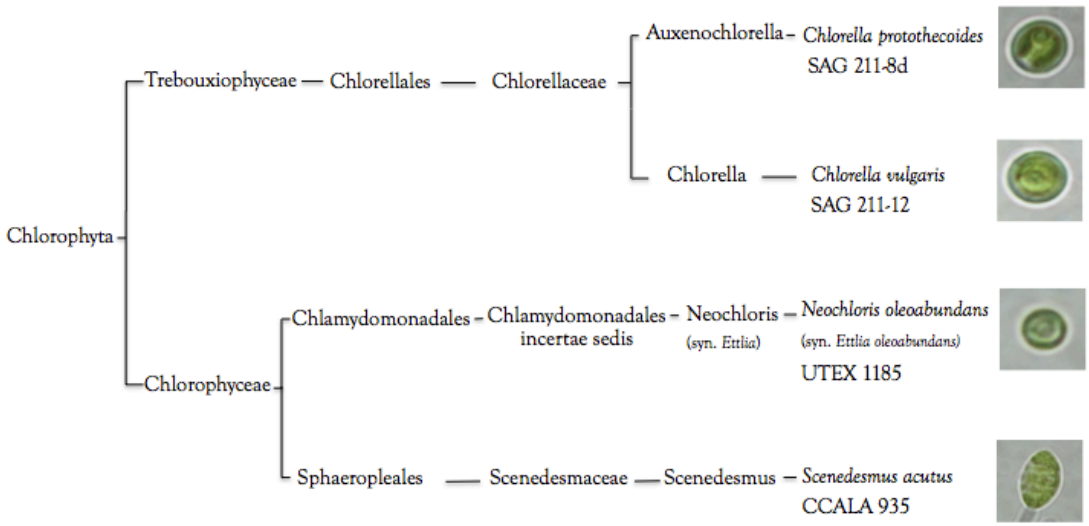

Fig. 1: The phylogenetic position of the four selected Chlorophyta species: two Trebouxiophycea (*Chlorella prototheocoides* and *Chlorella vulgaris)* and two Chlorophyceae (*Neochloris oleoabundans* and *Scenedesmus acutus)*

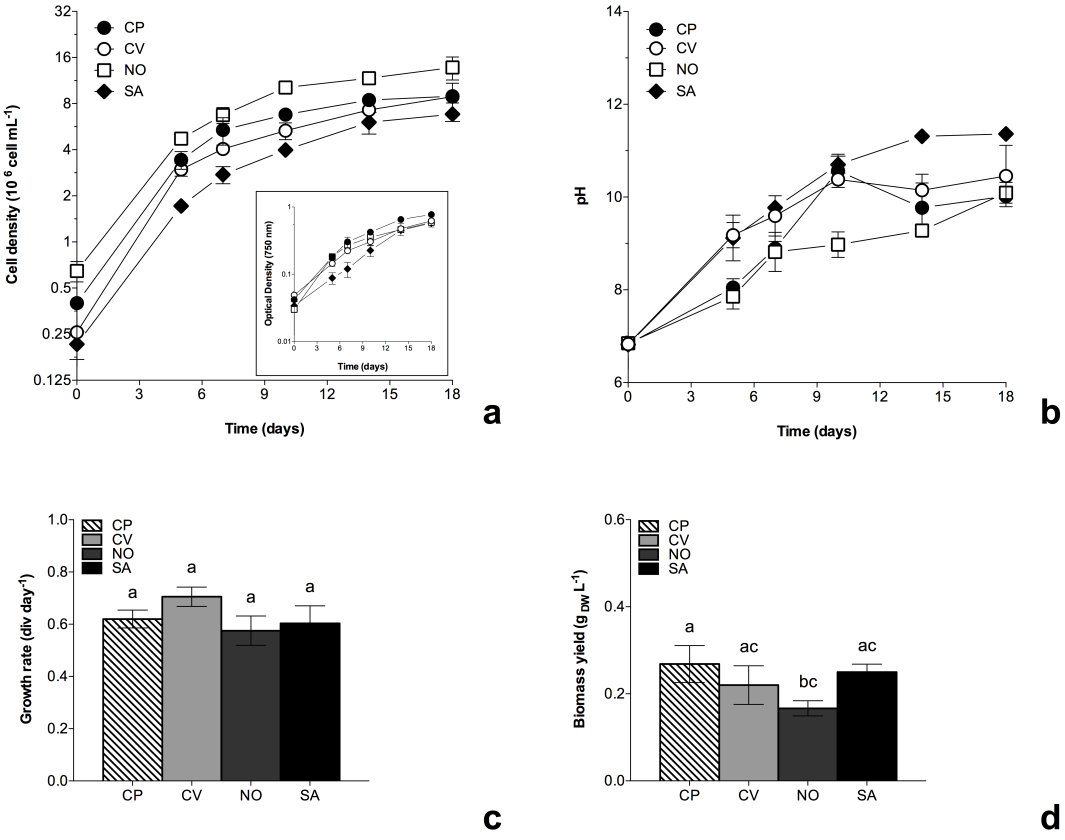

Fig. 2: Growth parameters of *C. protothecoides*, *C. vulgaris*, *N. oleoabundans* and *S. acutus*. **(a)** Growth curves and **(b)** time-course variations in the pH in culture media of *C. protothecoides* (filled circles), *C. vulgaris* (empty circles), *N. oleoabundans* (empty squares) and *S. acutus* (filled diamonds). **(c)** Growth rates (div day^{-1}), calculated during the exponential phase (0–5 days time interval of cultivation) and **(d)** Biomass yields at 18^{th} day of cultivation of *C. protothecoides* (striped), *C. vulgaris* (light grey), *N. oleoabundans* (dark grey) and *S. acutus* (dark). Values are represented as means ± s.d. (n=3), ANOVA $P<0.05$. Different superscripts denote significant ($P<0.05$) differences between the samples.

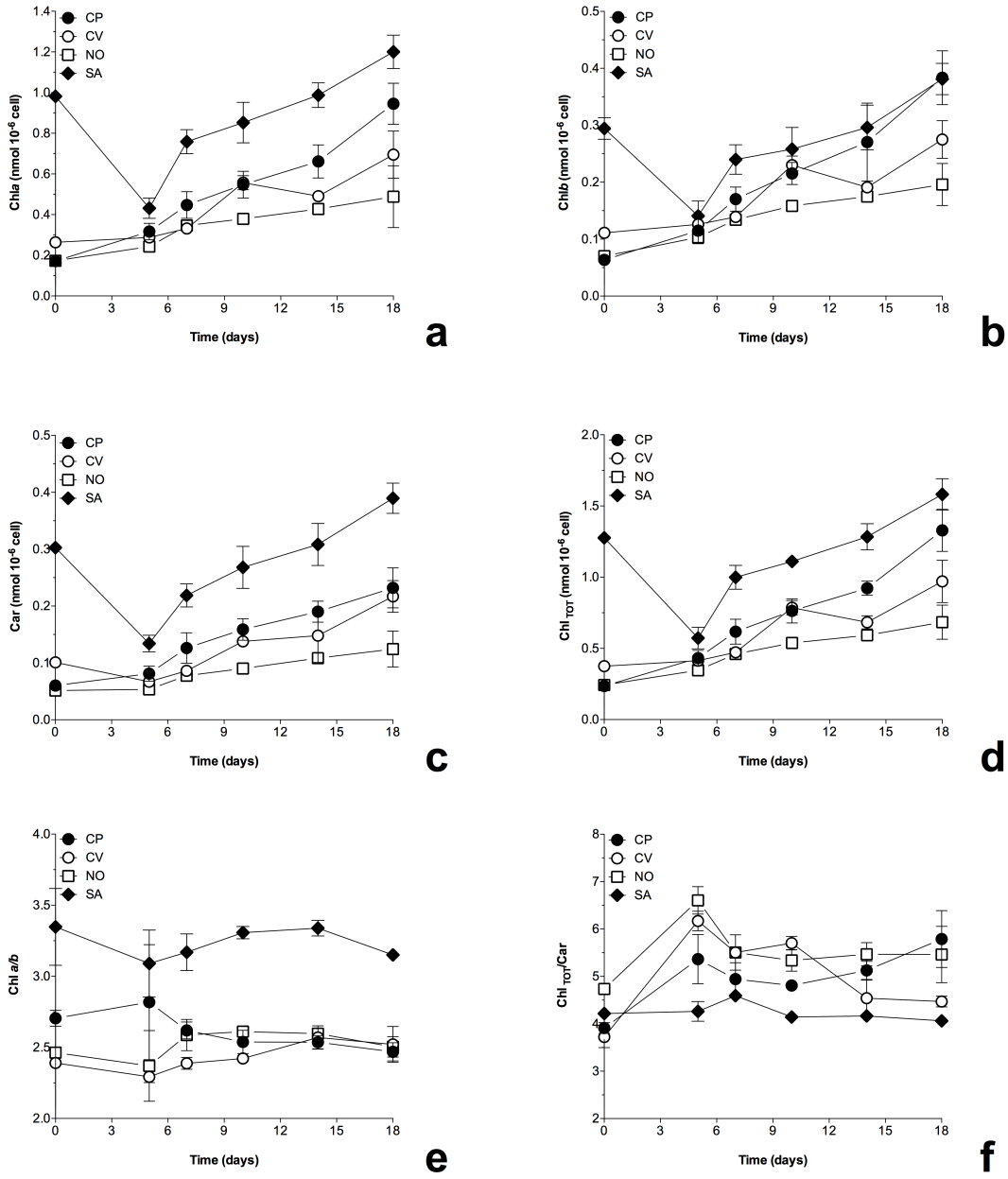

Fig. 3: Time-course variations of photosynthetic pigment content and their ratios in *C. protothecoides* (filled circles), *C. vulgaris* (empty circles), *N. oleoabundans* (empty squares) and *S. acutus* (filled diamonds). **(a)** Chl*a*, **(b)** Chl*b*, **(c)** Car, **(d)** total Chl content; **(e)** Chl *a/b* ratio and **(f)** total Chl/Car ratio**.** Values are means ± s.d. (n=3)

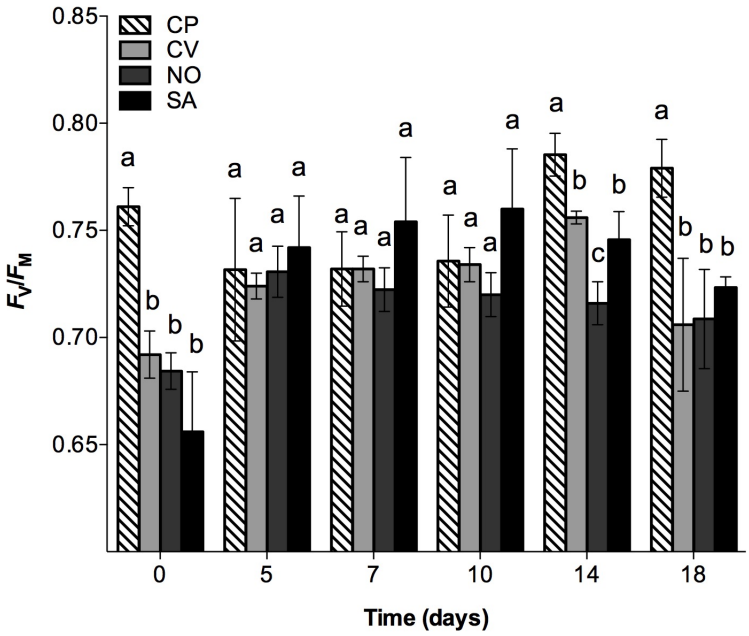

Fig. 4: Time-course variations of PSII maximum quantum yield (F_V/F_M ratio) in *C. protothecoides* (striped), *C. vulgaris* (light grey), *N. oleoabundans* (dark grey) and *S. acutus* (dark). Values are represented as means ± s.d. (n=3), ANOVA P<0.05. Different superscripts denote significant (P<0.05) differences between the samples within each day of cultivation

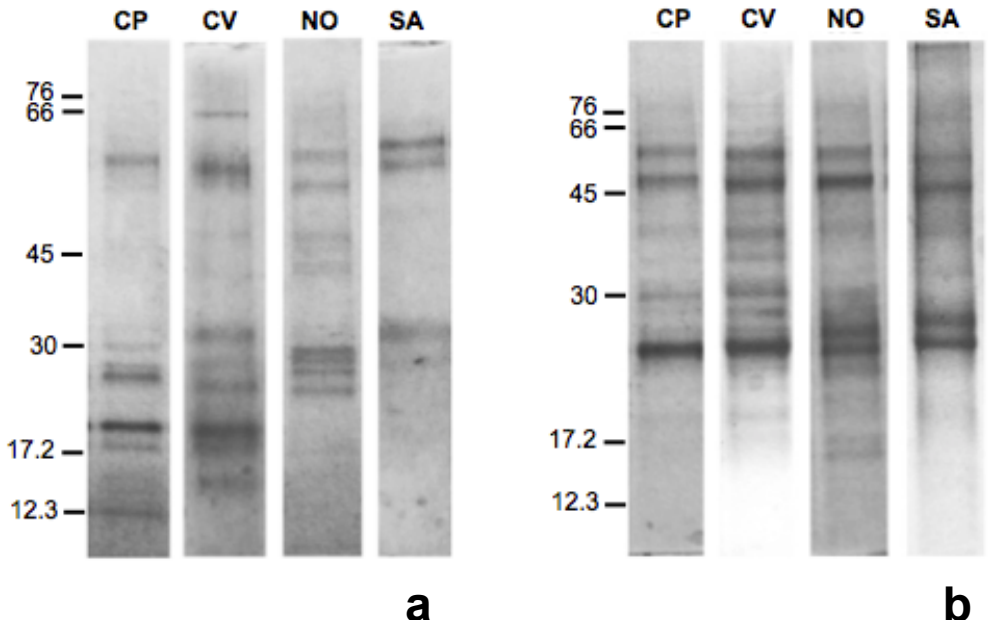

Fig. 5: (a) Coomassie-stained SDS-PAGE of the soluble and (b) less soluble protein fraction of *C. protothecoides* (CP), *C. vulgaris* (CV), *N. oleoabundans* (NO) and *S. acutus* (SA). On each lane, 10 μg (a) and 15 μg of proteins (b) were loaded. Molecular weight (kDa) marker is reported on the left in each gel

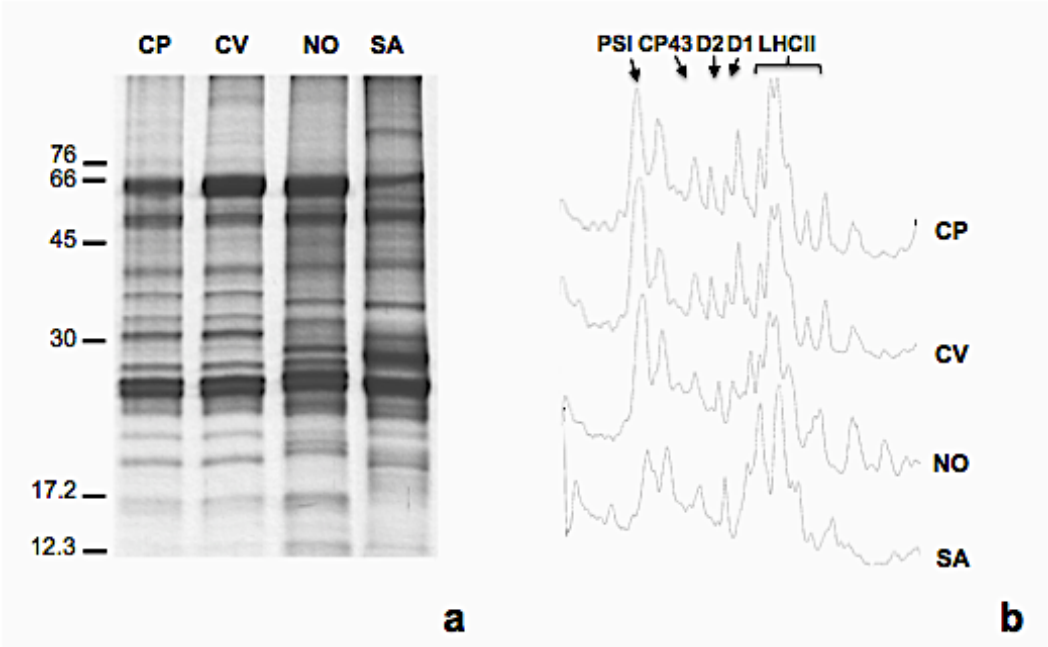

Fig. 6: (a) Coomassie-stained SDS-PAGE of thylakoids membrane proteins and (b) densitometric profiles of *C. protothecoides* (CP), *C. vulgaris* (CV), *N. oleoabundans* (NO) and *S. acutus* (SA). On each lane, 2 μg of Chl were loaded. Molecular weight (kDa) marker is reported on the left in gel

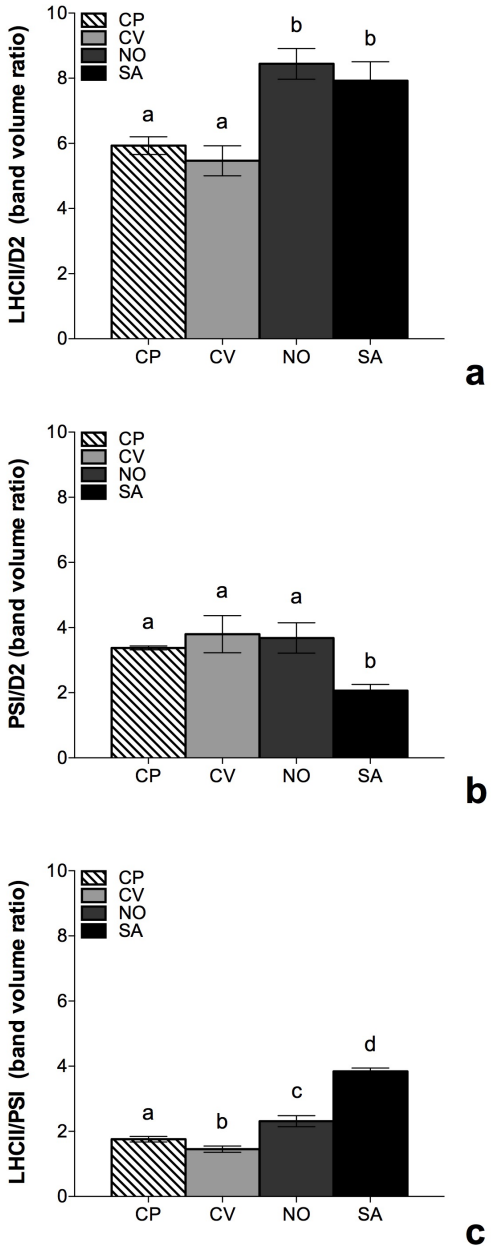

Fig. 7: Semi-quantitative analyses of the band intensities (band volume ratio) of different photosynthetic proteins in thylakoids membrane of *C. prototheco*ides (striped), *C. vulgaris* (light grey), *N. oleoabundans* (dark grey) and *S. acutus* (dark). Values are represented as means ± s.d. (n=3), ANOVA $P<0.05$. Different superscripts denote significant ($P<0.05$) differences between the samples

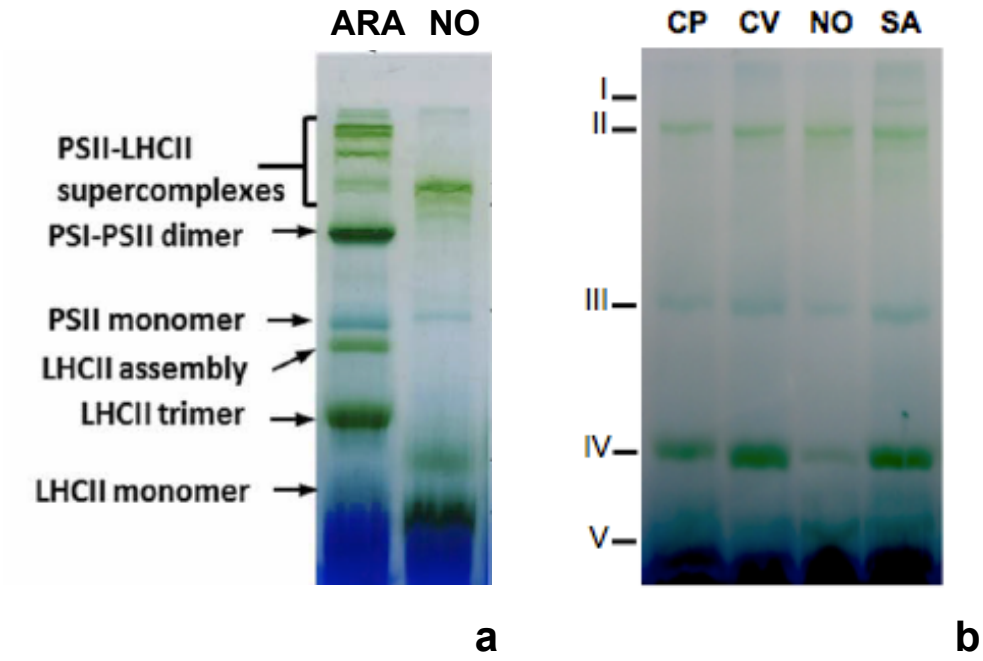

Fig. 8: Distribution of different protein complexes in BN-PAGE profile of thylakoids membranes. **(a)** Reference BN-PAGE profile of thylakoids from *Arabidopsis thaliana* (Ara) and autotrophic *N. oleoabundans* (NO). Figure is taken and modified from Giovanardi, (2013, Doctoral dissertation). Purified thylakoids were solubilized with 1% (v/v) dodecyl β-D-maltoside, loaded on an equal chlorophyll (5 μg) basis and separated on 5-12.5% gradient native gel. **(b)** BN-PAGE of thylakoid membrane complexes of *C. protothecoides* (CP), *C. vulgaris* (CV), *N. oleoabundans* (NO) and *S. acutus* (SA). Purified thylakoids were solubilized with 1.5% (v/v) dodecyl β-D-maltoside, loaded on an equal chlorophyll (8 μg) basis and separated on 5-12.5% gradient native gel

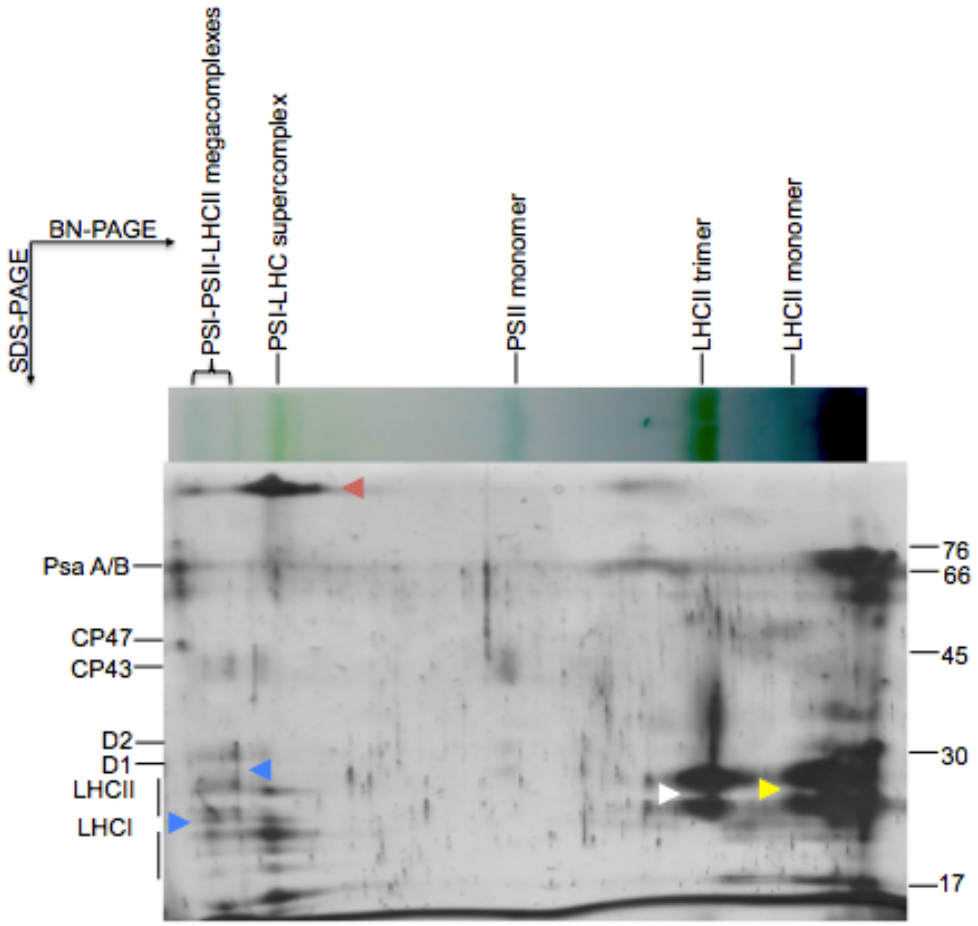

Fig. 9: BN-PAGE gel strip of *S. acutus* was treated with 10% (v/v) SDS and 5% (v/v) β-mercaptoethanol and denatured subunits were separated using SDS-PAGE. Second dimension gels were silver stained. Molecular masses are indicated on the right (kDa). The highlighted silver-stained spots corresponded to PSI-associated pigment-protein complex (red arrowhead), PSI-PSII-LHCII megacomplexes and supercomplex (blue arrowheads), LHCII trimers (white arrowhead) and LHCII monomers (yellow arrowhead)

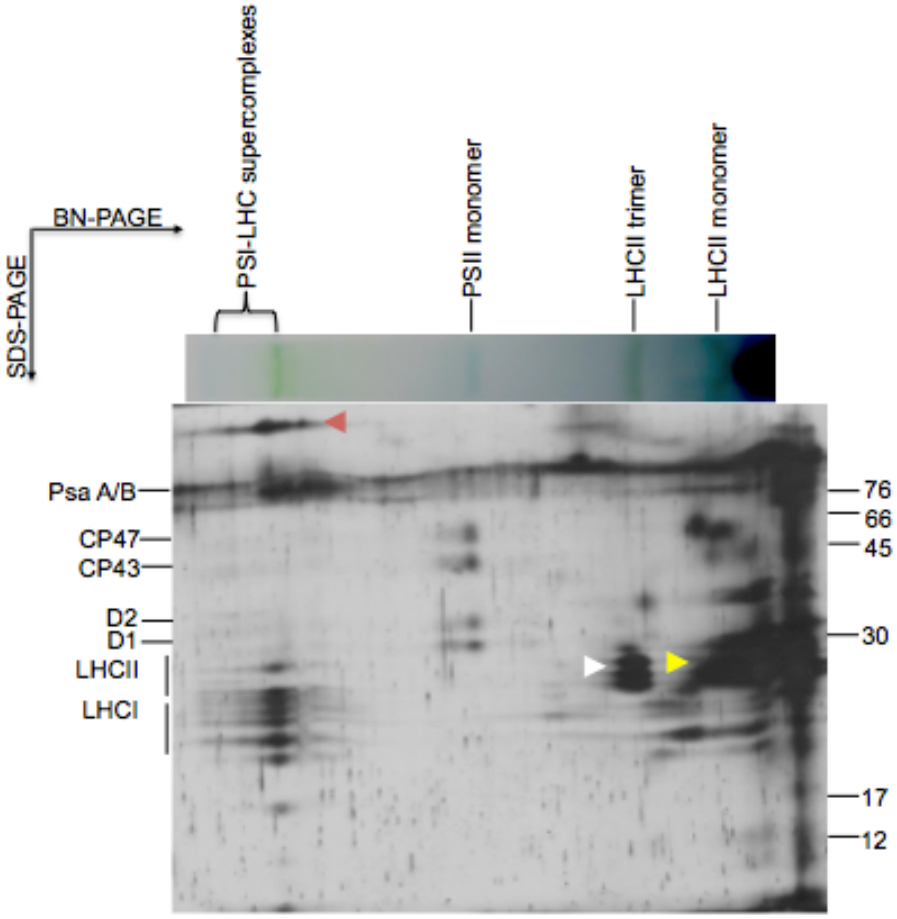

Fig. 10: BN-PAGE gel strip of *N. oleoabundans* was treated with 10% (v/v) SDS and 5% (v/v) β-mercaptoethanol and denatured subunits were separated using SDS-PAGE. Second dimension gels were silver stained. Molecular masses are indicated on the right (kDa). The highlighted silver-stained spots corresponded to PSI-associated pigment-protein complex (red arrowhead), LHCII trimers (white arrowhead) and LHCII monomers (yellow arrowhead)

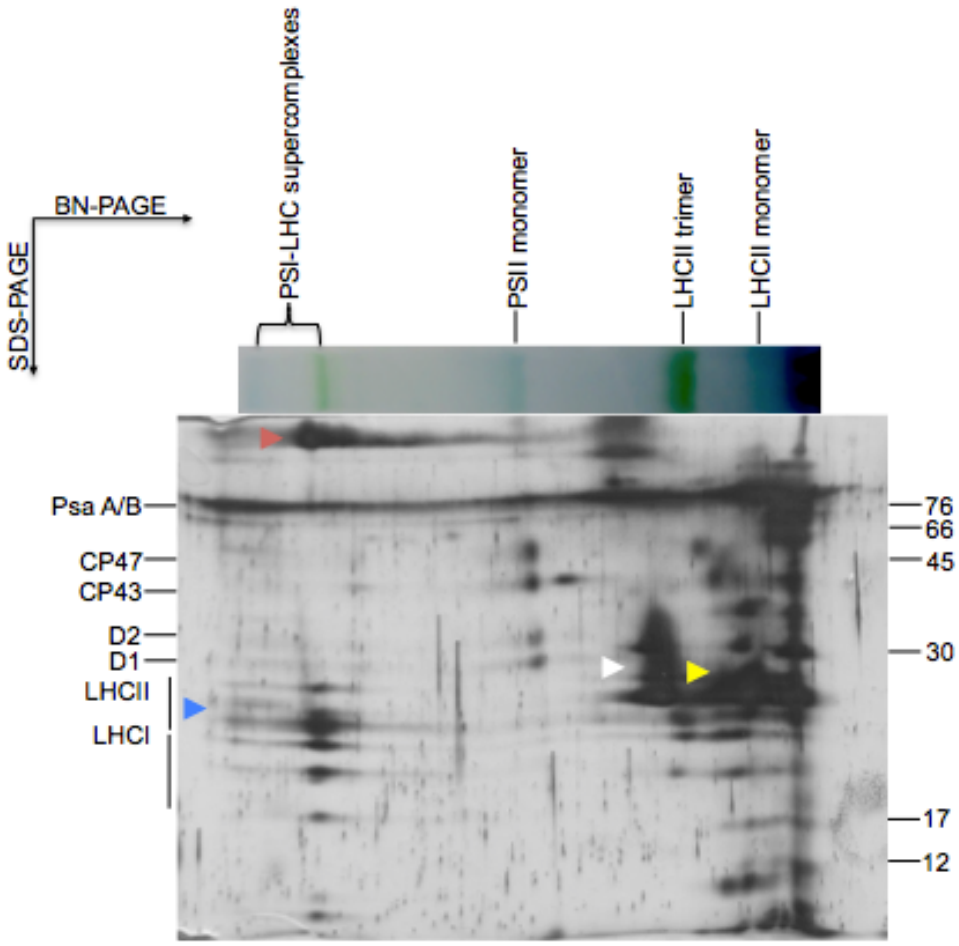

Fig. 11: BN-PAGE gel strip of *C. vulgaris* was treated with 10% (v/v) SDS and 5% (v/v) β-mercaptoethanol and denatured subunits were separated using SDS-PAGE. Second dimension gels were silver stained. Molecular masses are indicated on the right (kDa). The highlighted silver-stained spots corresponded to PSI-associated pigment-protein complex (red arrowhead), PSI- LHC supercomplexes (blue arrowheads) LHCII trimers (white arrowhead) and LHCII monomers (yellow arrowhead)

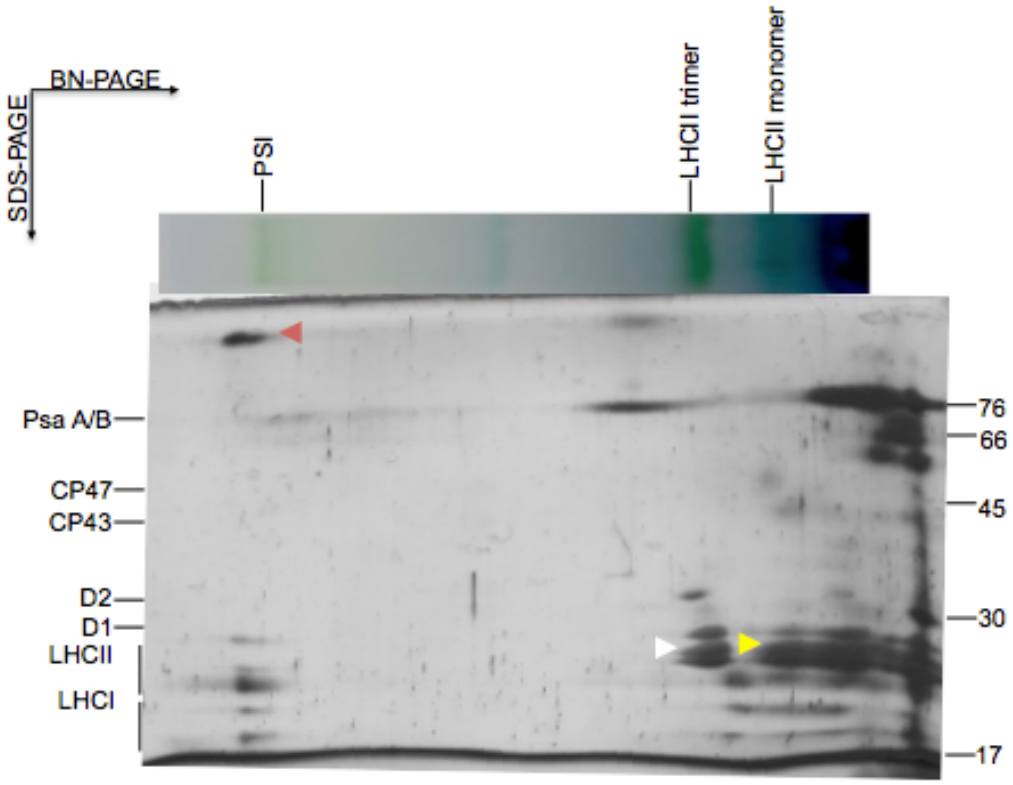

Fig. 12: BN-PAGE gel strip of C. *protothecoides* was treated with 10% (v/v) SDS and 5% (v/v) β-mercaptoethanol and denatured subunits were separated using SDS-PAGE. Second dimension gels were silver stained. Molecular masses are indicated on the right (kDa). The highlighted silver-stained spots corresponded to PSI-associated pigment-protein complex (red arrowhead), LHCII trimers (white arrowhead) and LHCII monomers (yellow arrowhead).

Chapter 4

Thalassiosira pseudonana:

a promising marine diatom for lipid production

1. General introduction

The potential role of diatoms for biotechnological applications

Diatoms are unicellular photosynthetic eukaryotes, which are considered the most productive microalgae on the planet. These microorganisms, which are the dominant component of phytoplankton, are responsible for up to 25% of the global CO_2 fixation and contribute up to 40% of marine primary production (Dugdale and Wilkerson 1998; Falkowski et al. 1998; Field et al. 1998; Granum et al. 2005; Hildebrand et al. 2012). Characterized by complex evolutionary history and recurrent genetic rearrangements, diatoms developed a unique metabolism and subcellular organization, allowing them to greatly adapt to changes in environmental stress conditions (Armbrust 2009). The overall faster growth rates, higher rates of carbon dioxide fixation, higher photosynthetic efficiency and higher biomass and lipid productivities, collocate diatoms among the best candidates for several applications in different biotechnological fields. Food, pharmaceutical, bio-remediation, bio-energy and nanotechnology are the most common biotechnological applications of diatoms (Lebeau and Robert 2003a,b; Lopez et al. 2005; Kroth 2007; Bozarth et al. 2009; Hildebrand et al. 2012; Levitan et al. 2014; Fu et al. 2015). These microorganisms are excellent accumulators of polyunsaturated fatty acids (PUFAs), in particular eicosapentaenoic (EPA) and dodecahexaenoic acids (DHA), which were used as a nutritionally food in aquaculture (Dunstan et al. 1994; Tonon et al. 2002; Lebeau and Robert 2003b; Lopez et al. 2005). Diatoms have been also explored as sources of carotenoids. In particular, fucoxanthin has received much attention for its pharmaceutical role as an antioxidant, anti-inflammatory and anticancer molecule (Peng et al. 2011; Chung et al. 2013; Fu et al. 2015). Due to their high bioabsorption ability, diatoms have been frequently used for the phytoremediation of heavy metals contamination (Lebeau and Robert 2003b; Mehta and Gaur 2005; Monteiro et al. 2012). Currently extensive researches have been explored the potential applications of diatom frustules in nanotechnology (Lebeau and Robert 2003b; Kroth 2007; Gordon et al. 2009). Among which engineered biosensors (Bismuto et al. 2008), drug delivery systems (Zhang et al. 2013), molecular filtration (Kroth 2007), solar cells, conductive electronic devices (Jeffryes et al. 2011) and enzyme immobilizers (Poulsen et al. 2007) has been examined.

In spite of these attractive attributes, only recently diatoms have been considered as a potential algal taxon for the biodiesel production (Hu et al. 2008; Griffiths and Harrison 2009; Hildebrand et al. 2012; Levitan et al. 2014; d'Ippolito et al. 2015). A recent review

highlighted the higher diatoms productivity performances as compared to those of the most common green algal strain used in the biofuel field (Hildebrand et al. 2012). Diatoms produce a large amount (i.e. 40-60% dry cell weight) of triacylglycerides (TAGs) as a storage lipid, which represent the best substrate for the production of biodiesel (Hu et al. 2008; Bozarth et al. 2009; Hildebrand et al. 2012; Levitan et al. 2014; d'Ippolito et al. 2015). It is widely accepted that diatoms increased TAGs synthesis and accumulation in response to environmental stress conditions, such as low temperature and nitrate or silicate starvation (Hu et al. 2008; Yu et al. 2009; Hildebrand et al. 2012; Yang et al. 2013; Levitan et al. 2014; d'Ippolito et al. 2015). Lately, some studies reported that genetic modification could boost the neutral lipid accumulation in diatoms (Armbrust et al. 2004; Poulsen et al. 2006; Radakovits et al. 2011; Trentacoste et al. 2013). These promising results suggest that targeted metabolic manipulation can be applied greatly to improve the economic feasibility of biodiesel production.

A model species, *Thalassiosira pseudonana*

Thalassiosira pseudonana (Husted) Hasle and Heimdal (Heterokontophyta, Thalassiosirales, Thalassiosiraceae) is a marine centric diatom, which represents one of the main components of phytoplankton. Due to its faster growth rate and natural features this strain has long been used in experimental studies. Data in literature about this diatom have been concerned several topics ranging from studies about the ecology and evolution of marine diatoms to studies about their determinant role in the global primary productivity and marine geochemical cycles (Nelson et al. 1995; Armbrust et al. 2004; Armbrust 2009; Bowler et al. 2010; Gao et al. 2012b). Other studies have been concerned the influence of irradiance, nutrients, temperature, CO_2 concentrations on growth and metabolism (Berges et al. 2002; Claquin et al. 2002; Stramski et al. 2002; Sobrino et al. 2008; Bender et al. 2012; McCarthy et al. 2012; Mejia et al. 2013; Hennon et al. 2014). This strain was also used in studies about silica biomineralization (Frigeri et al. 2005; Mock et al. 2008; Sumper and Brunner 2008). Recently, its available genome sequence established this strain as a first model organism for genomic, transcriptomic and metabolic studies in diatom research (Armbrust et al 2004; Poulsen et al. 2007; Trentacoste et al. 2013). Due to its genomic data and physiological characteristics, *T. pseudonana* represents an optimal model organism for studies on lipid accumulation for biofuels. Nutrient deficiency (nitrate or silicate starvation) has been well documented to increase TAG accumulation in this diatom (Yu et al. 2009; Zendejas et al. 2011; Hockin et al. 2012; Roleda et al. 2013; Trentacoste et al. 2013; d'Ippolito et al. 2015), although it is well known that a variety of other environmental stresses induce the accumulation of lipids in microalgae (Hu et al. 2008; Yu et al. 2009; Popovich et al. 2012b). In conclusion, *T. pseudonana* was used as object of this chapter in order to improve knowledge about the biotechnological potential use of this diatom in the bioenergetics field.

Aim of the work

The third chapter of this Thesis is focusing on different cultivation strategies for enhancing the biomass productivity and lipid production of the marine diatom *Thalassiosira pseudonana*.

The aim is to gain further knowledge on the morpho-physiological and biochemical aspects of this algal strain in order to improve its culture performance and development strategies for its biotechnological exploitation towards biofuel production.

The first section is a morpho-physiological and biochemical study about the effects of mixotrophic growth on lipid production in the diatom *T. pseudonana*. The cultivation of the diatom was tested in presence of different organic carbon source and with the supply of a low-cost carbon source, crude glycerol, which is one of the major by-products of the biodiesel industry. Growth was estimated in parallel to biomass concentration; photosynthetic pigment content; PSII maximum quantum yield; cell morphology, with special attention to intracellular lipid accumulation.

In the second section, *T. pseudonana* was cultivated under increasing CO_2 concentrations in order to evaluate its potential for biodiesel production coupled with the reduction of CO_2 emissions. The influence of the different CO_2 concentrations on diatom growth, cell morphology, photosynthetic pigment content, total nitrogen (TN), and dissolved inorganic (TIC) and organic carbon (TOC) content were investigated. The lipid content was also estimated in order to evaluate the effects of elevated CO_2 concentrations on lipid profile.

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2. Effect of mixotrophy on cell growth, lipid accumulation and photosynthetic performance of the marine diatom **Thalassiosira pseudonana**

2.1 Introduction

During the last years, concerns about global climate change and rises in prices of fossil fuels have prompted intense interest in the ability of microalgae to produce lipids that can be easily converted to biodiesel, which is considered a sustainable and environmentally friendly alternative to petroleum (Hu et al. 2008; Griffiths and Harrison 2009; Mata et al. 2010; Demirbas and Demirbas 2011; Borowitzka and Moheimani 2013; Singh et al. 2014). Among microalgae, diatoms are the most productive and environmentally flexible eukaryotic microalgae on the planet (Field et al. 1998; Hildebrand et al. 2012). The ability of these microorganisms to synthesize and accumulate large amounts of TAGs, collocate diatoms among the best candidates for biodiesel production (Hu et al. 2008; Griffiths and Harrison 2009; Hildebrand et al. 2012; Levitan et al. 2014; d'Ippolito et al. 2015). Therefore, the biodiesel production from microalgae is technically feasible, but, for an effective use of this renewable resource, it is necessary to reduce the algal production costs (Sheehan 2009; Solomon 2010; Wijffels and Barbosa 2010; Suali and Sarbatly 2012). In this perspective, a considerable effort has been made for the development of more efficient culture conditions (Scott et al. 2010; Chen et al. 2011; Suali and Sarbatly 2012; Sabia et al. 2015). Recently, mixotrophy has been considered an alternative method for achieving higher biomass densities and lipid production as compared to autotrophic or heterotrophic cultivation (Brennan and Owende 2010; Heredia-Arroyo et al. 2011; Cheirsilp and Torpee 2012; Chandra et al. 2014). Mixotrophy is the condition by which some microalgae can use organic carbon source together with light for their growth (Ogbonna et al. 2002; Chandra et al. 2014). Many studies reported that green algae are able to grow mixotrophically, achieving higher biomass and lipid content in presence of an appropriate organic carbon source (Fang et al. 2004; Liang et al. 2009; Heredia-Arroyo et al. 2011; Giovanardi et al. 2014; Li et al. 2014; Silva et al. 2016). Also some diatoms are known to grow mixotrophically (Cerón García et al. 2006; Liu et al. 2009a; Wang et al. 2012;

Shishlyannikov et al. 2014). However, the cost of organic carbon source represents the 50-80% of the cost of microalgal growth medium (Cheng et al. 2009; Heredia-Arroyo et al. 2010; Bhatnagar et al. 2011). For this reason, alternative organic carbon sources, derived as by-products from industrial processes, are desirable to supply during microalgal cultivation (Brennan and Owende 2010; Heredia-Arroyo et al. 2010; Giovanardi et al. 2013; Baldisserotto et al. *In Press*; Giovanardi et al. *In Press*).

Among diatoms, *Thalassiosira pseudonana*, is one of the most studied species for the production of biofuels up to now (Yu et al. 2009; Zendejas et al. 2011; Roleda et al. 2013; d'Ippolito et al. 2015). Furthermore, it is a model diatom species, which genome has been recently sequenced (Armbrust et al. 2004; Trentacoste et al. 2013). To the best of our knowledge this is the first study on the potential of mixotrophic growth of *T. pseudonana* for biodiesel production. For this reason, the aim of this research was: a) to test the effect of different organic carbon sources on growth rates; b) to analyse the effect of different concentrations of the best organic carbon source on growth and lipid production and c) to test if such organic compound can be supplied as a low-cost derivative of an industrial activity.

2.2 Materials and Methods

2.2.1 Algal strain and culture condition

The strain used in this study was the marine centric diatom *Thalassiosira pseudonana* (Husted) Hasle and Heimdal CCAP 1085/12 (Heterokontophyta, Thalassiosirales, Thalassiosiraceae), obtained from the Culture Collection of Algae and Protozoa (CCAP; Scotland, United Kingdom; (www.ccap.ac.uk). Cells were grown in artificial seawater medium (ESAW) (Berges et al. 2001) enriched with f/2 nutrients (Guillard and Ryther 1962). The *T. pseudonana* cultures were placed in a growth chamber (24 ± 1 °C, 80 μmolphotons m^{-2} s^{-1} of PAR with a 16:8 h light-darkness photoperiod), and cultivated with continuous shaking at 80 rpm. To test the microalgal growth under different organic carbon sources concentrations, cells were inoculated at a cell density between 1.4 and 1.6 x 10^5 cells mL^{-1} in 100 mL Erlenmeyer flasks (50 mL of total volume) in ESAW medium enriched with f/2 nutrients containing increasing concentrations from 0 to 5 gL^{-1} of glucose, sodium acetate and glycerol, in the same growth chamber described above. For the subsequent experiments, cells were inoculated at a cell densities between 4 and 4.5 x 10^5 cells mL^{-1} in

300 mL Erlenmeyer flasks (150 mL of total volume) in ESAW medium enriched with f/2 nutrients containing 0 gL^{-1} (control), 1, 2.5 or 5 gL^{-1} of pure glycerol and cultivated under the same conditions describe above. Finally, to test the mixotrophic cultivation of *T. pseudonana* in presence of a low-cost organic carbon source, cells were inoculated at a cell density between 4 and 4.5 x 10^6 cells mL^{-1} in 300 mL Erlenmeyer flasks (150 mL of total volume) in ESAW medium enriched with f/2 nutrients containing 0 gL^{-1} (control) and 2.5 gL^{-1} of pure glycerol or crude glycerol derived from biodiesel production, and cultivated in the same growth chamber described above. Crude glycerol and its characterization were obtained from Masol Continental Biofuel (MCB crude glycerol) (Livorno, Italy). The characteristics of MCB crude glycerol are shown in Table 1.

Aliquots of samples were collected at different times of cultivation, depending on the analysis. Experiments were performed in duplicates for small culture volumes and in triplicates for larger culture volumes.

2.2.2 Growth and biomass evaluation

Growth evaluation was carried out on cell samples at different times of cultivation using a Thoma haemocytometer (HBG, Giessen, Germany) under a light microscope (Zeiss, model Axiophot) and growth curves were obtained. Specific growth rates during the exponential phases were calculated according to Giovanardi et al. (2013), whereas biomass productivities according to equations reported in Hempel et al. (2012).

For dry biomass determination, aliquots of samples were filtered through pre-dried and pre-weighed glass-fiber filters (Whatman GF/F). Filters with cell pellets were rinsed with 20 mL of distilled water and dried 72 h at 60° C, and weighted until they reached constant weight.

2.2.3 Photosynthetic pigment extraction and quantification

For photosynthetic pigment analysis, cell samples were collected by centrifugation and the extraction was performed according to Baldisserotto et al. (2014) with absolute methanol for 10 min at 80°C under a dim green light to avoid photodegradation. The extracts were measured with Pharmacia Ultrospec 2000 UV-Vis spectrophotometer (1-nm bandwidth; Amersham Biosciences, Piscataway, NJ, USA) at 665 (Chl*a*), 632 (Chl*c*) and 480 nm (Car). Quantification of Chls was performed according to equations reported in Ritchie (2006), whereas Car quantification according to equations reported in Wellburn (1994).

2.2.4 PAM fluorimetry

The maximum quantum yield of PSII algae was determined using a pulse amplitude modulated fluorometer (ADC Bioscientific Ltd, Hoddesdon, Hertfordshire, UK) at the same cultivation times considered for growth curve measurements. Samples were prepared as reported in Ferroni et al. (2011). After 15 min of dark incubation, initial fluorescence (F_0) and maximum fluorescence (F_M) values were measured flashing the samples with a saturating light impulse. These values were used for the calculation of the maximum quantum yield of PSII: Y(PSII) = F_V/F_M, where variable fluorescence is $F_V = (F_M - F_0)$, F_M (Lichtenthaler et al. 2005). This measurement is considered a valid method to estimate the physiological state of plants and microorganism (White et al. 2011).

2.2.5 Microscopy

Light and fluorescence microscopy

Microscopic observations were made on cell samples using a Zeiss model Axiophot microscope with conventional and fluorescent attachments. The light source for chlorophyll fluorescence observation was a HBO 100 W pressure mercury vapour lamp (filter set, BP436/10, LP470). Pictures of cells were taken with a Canon IXUS 110 IS digital camera (12.1 megapixels), mounted on the ocular lens through a Leica DC150 system (Leica Camera AG, Solms, Germany).

2.2.6 Lipid analyses

Nile Red staining

In order to evaluate the intracellular lipids accumulation, cells were stained with the fluorochrome Nile Red (9-diethylamina-5Hbenzo[α]phenoxazine-5-one) 0.5 mg dissolved in 100 mL acetone (Sigma-Aldrich, Gallarate, Milan, Italy), as described in Giovanardi et al. (2014). The fluorescence emitted by stained samples containing 1.5 x 10^6 cells mL^{-1} was measured with a spectrofluorimeter (Spex Fluoromax-2 equipped with Hamatsu R928 photomultiplier), using excitation wavelength of 488 nm and emission wavelength between 550 and 660 nm. The relative fluorescence was obtained by subtraction of the autofluorescence of non-stained cells and self-fluorescence of the fluorochrome. After staining, cells were also observed with the microscope described above at an excitation of 485 nm (filter set, BP485, LP520). Photographs were taken with the camera already

described.

2.2.7 Data treatment

Data were processed with Graphpad Prism 6 (Graph Pad Software, San Diego CA, USA). In each case, means ± standard deviations for n number of samples are given. Statistical analyses for comparison of means were carried out using ANOVA, followed by Student's *t*-test (significance level, 0.05).

2.3 Results

2.3.1 Effect of different organic carbon sources on *T. pseudonana* growth in small culture volumes (50 mL)

In a comparative experiment, the growth ability, intended as cell divisions per day, was tested in *T. pseudonana* cultivated in the presence of three alternative organic carbon sources (glucose, sodium acetate and glycerol) supplied at increasing concentration. These comparative tests were performed in small culture volumes of 50 mL.

As shown in Fig. 1, only in cultures supplemented with glycerol the growth was clearly stimulate at all tested concentrations as compared to the controls. In particular, glycerol significantly promoted growth at the concentration of 1 and 5 gL^{-1} ($p < 0.05$ in both cases). Conversely, the addition of glucose in the medium did not influence the specific growth rate, but inhibited it, significantly at the concentrations of 0.5 and 5 gL^{-1}. Finally, the cultivation in presence of sodium acetate resulted in lower specific growth rates at all tested concentrations.

From these experiments, the most promising carbon sources for the diatom growth appeared to be glycerol, whose effects were further characterised.

2.3.2 Effect of pure glycerol on *T. pseudonana* growth and lipid induction in larger culture volume (150 mL)

In order to obtain more information about the capability of this diatom to grow mixotrophically in presence of 1, 2.5 or 5 gL^{-1} of pure glycerol, a first scaling-up of the cultivation volume to 150 mL was performed. As shown in Fig. 2, which reports data on growth rates and cell densities of control and glycerol-treated cells, no statistically

differences were observed between samples. The cultivation of the diatom in autotrophic and mixotrophic condition allowing to obtain similar growth rates, ranging between 0.72 and 0.75 div day^{-1}, and similar cell densities, ranging between 28 and 38 x 10^5 cells mL^{-1} at the end of stationary phase (7^{th} day) (Fig. 2a-b). Interestingly, for all samples, biomass yields apparently did not reflect the cell density achieved at the end of the experiment (Fig. 2c); in particular, biomass of cells grown in presence of 2.5 gL^{-1} of glycerol achieved the highest values (0.5 $g_{DW}L^{-1}$) as compared to other samples ($p<0.05$ with respect to autotrophic cells). This is justified by the characteristics of the single cell biomass and size. In fact, the 2.5 gL^{-1} glycerol-treated samples achieved higher values of single cell biomass than autotrophic and other tested glycerol concentrations ($p<0.05$ with respect to cells cultivated with 1 gL^{-1}) (Fig. 2d). This result also explained the increase in biomass concentration but not in cell density of cells grown in the presence of 2.5 gL^{-1} of glycerol; in fact cells were less numerous but heavier.

To find a possible relation between glycerol supply and lipid synthesis, measurements of neutral lipid accumulation were performed by spectrofluorimetric quantification of the relative fluorescence intensity emitted by Nile Red-stained cells. The relative fluorescence intensity was measured at the 7^{th} day of cultivation, when cells were in stationary phase. In all tested glycerol concentrations, the relative emission intensity was found to be 6 times higher than controls ($p<0.05$; Fig. 3). In addition, the presence of oil globules in both control and glycerol-treated samples were observed by light microscopy. In fact, at the 7^{th} day of cultivation, both control and glycerol-treated cells showed translucent granulation at the cytoplasmic level. In detail, lipid droplets, giving an intense positive reaction with the fluorochrome, occupied most of the cell volume of the diatom grown in the presence of 2.5 gL^{-1}. On the contrary, autotrophic cells showed fewer lipid globules, which had a slight positive reaction (Fig. 4).

Overall, these results clearly suggest that 2.5 gL^{-1} can be selected as the optimal glycerol concentration, allowing to obtain the best compromise between biomass production and lipid accumulation. For this reason, this concentration was used in the subsequent mixotrophic experiments.

2.3.3 Effect of crude glycerol on growth, photosynthetic apparatus and lipid production of *T. pseudonana* in a larger scale volume

In order to test if the mixotrophic cultivation with pure glycerol can be substitute with the supply of a low-cost carbon source, *T. pseudonana* was cultivated in presence of crude glycerol, one of the major by-products of the biodiesel industry.

As shown in Fig. 5b, the diatom *T. pseudonana* cultivated autotrophically and in presence of 2.5 gL^{-1} of pure glycerol or crude glycerol grew at similar division rates (0.67, 0.65 and 0.69 div d^{-1}, respectively) and reached the stationary phase of growth during 7 days of cultivation (Fig. 5a, b). However, cell grown in presence of glycerol or crude glycerol achieved higher biomass productivities than control cultures (42 and 40% higher than control cells, respectively; $p=0.14$ in pure glycerol cells and $p=0.18$ in crude glycerol cells) (Fig. 5c). In order to evaluate if the cultivation in presence of crude glycerol could also affected the photosynthetic activity of the diatom, the photosynthetic pigment content and the variations in PSII maximum quantum yield were also analysed. As reported in Fig. 6, samples shared a similar trend of Chl*a*, Car and total Chls concentrations during the cultivation period. Chl*a* and Car showed a strong decrease during the exponential phase; thereafter an evident increase in Chl*a* and Car content was observed in all samples. In particular, at the end of the experiment mixotrophic cells cultivated with pure glycerol achieved higher quantities of Chl*a* as compared to autotrophic and crude glycerol samples (39 and 52% higher than control and crude glycerol; respectively; $p<0.05$ in crude glycerol-treated cells) (Fig. 6a). About the Car content, cell grown in presence of pure glycerol tended to higher final values than autotrophic and crude glycerol-treated cells (50 and 38% higher than control and crude glycerol; respectively) (Fig. 6c). Interestingly, a different trend for Chl*c* content was observed between samples. During the exponential phase, Chl*c* in cell cultivated with pure glycerol showed similar quantities to those measured at day 0. Conversely, in autotrophic and crude glycerol-treated cells Chl*c* content strongly decreased during this cultivation period. Chl*c* quantities in control and crude glycerol cells were 37 and 46% lower as compared to pure glycerol cells ($p<0.05$ in both cases). Subsequently, a slightly decrease in Chl*c* was observed in pure glycerol-treated cells. At the end of the experiment, Chl*c* content tended to be higher in pure glycerol-treated cells than control and crude glycerol cultures (50 and 45% higher than pure glycerol, respectively) (Fig. 6b). Interestingly, at the end of the cultivation period, control and crude glycerol cultures

showed similar quantities to those measured at day 4. Similar to Chl*a* and Chl*c*, pure glycerol cultures contained higher quantities of total Chls at the end of the cultivation period (40 and 51% higher than control and crude glycerol, respectively) (Fig. 6d). The effects of crude glycerol on photosynthetic activity were also evaluated by PAM fluorimetry as F_V/F_M ratio. The variations in PSII maximum quantum yield measured during the cultivation period are shown in Table 2. Control, pure or crude glycerol cells showed an evident increase of the F_V/F_M ratio during the first 4 days of growth, reaching values of ca. 0.70-0.72 (+21, 28 and 23% with respect to time 0, respectively; $p<0.05$ in control cells; $p<0.01$ in pure or crude glycerol cells). The F_V/F_M values in pure glycerol cells tended to be higher than control and crude glycerol cells ($p=0.17$ and $p=0.33$, respectively). Subsequently, the F_V/F_M ratio in all samples decreased down to values of 0.52-0.56. Interestingly, at the end of the experiment all samples showed similar values of F_V/F_M to those measured at day 0 (inoculum).

2.4 Discussion

The choice of the most adequate microalgal growth conditions is a fundamental requisite in order to achieve the productivities required for biodiesel production under a profitable cost (Borowitzka 1999; Scott et al. 2010; Chen et al. 2011; Suali and Sarbatly 2012). Mixotrophic cultivation has been repeatedly proposed as a feasible alternative, allowing obtaining higher biomass and lipid productivity as compared to the most common autotrophic or heterotrophic cultivation (Heredia-Arroyo et al. 2011; Cheirsilp and Torpee 2012; Giovanardi et al. 2014; Chandra et al. 2014; Silva et al. 2016; Baldisserotto et al. *In Press*). Unfortunately, most microalgae are obligate photoautotrophs and are unable to make use of external organic carbon sources (Heredia-Arroyo et al. 2011). Among microalgae, diatoms often have higher trophic flexibility than other algal classes (Hildebrand et al. 2012). Most diatom species are photoautotrophic but, under certain conditions, are able to grow mixotrophically exploiting a variety of organic molecules (Kitano et al. 1997; Cerón García et al. 2006; Wang et al. 2012; Shishlyannikov et al. 2014). Among diatoms, *T. pseudonana* is widely studied for its ability to accumulate large amount of lipids suitable for biodiesel production (Yu et al. 2009; Zendejas et al. 2011; Roleda et al. 2013; d'Ippolito et al. 2015). Such studies take also advantage from its recently sequenced genome (Armbrust et al. 2004; Trentacoste et al. 2013). In spite of the current extensive studies performed on

this species, there is still no information on its possible mixotrophic growth, which, if demonstrated, could allow a significant advancement for the biotechnological use of this alga. Present results clearly suggest that *T. pseudonana* is able to grow mixotrophically. In a comparative experiment, *T. pseudonana* was cultivated in presence of increasing concentrations of three alternative carbon organic sources (glucose, sodium acetate and glycerol). It is known that glucose, sodium acetate and glycerol were the most commonly used organic carbon sources in the mixotrophic microalgal cultivation (Cerón García et al. 2006; Heredia-Arroyo et al. 2011; Wang et al. 2012; Giovanardi et al. 2013; Silva et al. 2016). Conflicting results on the effect of different carbon sources on microalgal growth, promoting or inhibiting were reported in many studies (Cerón García et al. 2006; Heredia-Arroyo et al. 2011; Wang et al. 2012). Among the different organic substrates tested, present results clearly suggest that 2.5 gL^{-1} can be selected as the optimal glycerol concentration, allowing to obtain the best compromise between biomass production and lipid accumulation. These results are coherent with previous studies, which found that glycerol was the most effective carbon source for achieving higher biomass and lipid production as compared to autotrophic and other carbon sources supplied (Cerón García et al. 2006; 2013; Liu et al. 2009a). Conversely to those observed by Liu et al. (2009a) and Wang et al. (2012), in this study no stimulatory effect on growth was observed during the cultivation in presence of glucose and sodium acetate. However, glycerol is one of the most costly carbon source employed as substrate for microalgal growth (Cheng et al. 2009; Heredia-Arroyo et al. 2010). In order to promote the biodiesel production on large scale and reduce the cost of algal growth medium, low-cost carbon sources are desirable to supply during microalgal cultivation (Brennan and Owende 2010; Heredia-Arroyo et al. 2010; Giovanardi et al. 2013; Giovanardi et al. *In Press*; Baldisserotto et al. *In Press*). During the biodiesel production, one of the major by-products is crude glycerol (Thompson and He 2006; Cheng and Walker 2011; Abad and Turon 2012; Yang et al. 2012). The increasing production of crude glycerol, as a consequence of the rise of biodiesel processes, would also impact the refined glycerol market (Cheng and Walker 2011; Abad and Turon 2012, Yang et al. 2012). For this reason, it is necessary to find a sustainable application for utilizing this excess of raw glycerol (Thompson and He 2006; Da Silva et al. 2009; Yang et al. 2012). Anyway, before considering crude glycerol as a feasible substrate, its chemical characterization is necessary. In fact, it is known that the composition of raw glycerol depends on the different feedstocks and biodiesel production operations employed (Thompson and He 2006; Cheng and Walker 2011; Abad and Turon 2012; Yang et al.

2012). Pharmaceutical, food and cosmetic industries are the most common applications of crude glycerol. However, for such applications a costly purification process of crude glycerol is required to remove impurities (Abad and Turon 2012; Yang et al. 2012). To overcome this aspect, crude glycerol has recently been proposed as a substrate for microalgal cultivation (Pyle et al. 2008; Ethier et al. 2011; Cheng and Walker 2011; Kong et al. 2013). Present results clearly showed that *T. pseudonana* could use crude glycerol as a carbon substrate. Comparing the cultivation of the diatom in presence of 2.5 gL^{-1} of pure glycerol or crude glycerol, the results on biomass productivities are similar. It was noteworthy that the biomass productivities in mixotrophic cultures are found 40 and 42%, respectively, higher than autotrophic cells. Recently, some studies reported that mixotrophic cultivation could affect the photosynthetic activity of microalgae (Heifetz et al. 2000; Radchenko et al. 2004; Liu et al. 2009a; Liu et al. 2009b; Giovanardi et al. 2014; Baldisserotto et al. *In Press*). However, data in literature about the effect of mixotrophic cultivation on photosynthetic apparatus reported conflicting results depending on the organic carbon source and microalgal strains employed (Cerón García et al. 2006; Radchenko et al. 2004; Liu et al. 2009a; Baldisserotto et al. 2014; Giovanardi et al. 2014). In agreement with such literature data, in this study mixotrophic samples cultivated with pure glycerol contained higher quantities of photosynthetic pigments than autotrophic cultures (Cerón García et al. 2006; 2013; Kong et al. 2013). Differently, crude glycerol-treated cells showed similar quantities to those measured for the controls. This result could be attributed to some impurities, which were remained in the raw substrate tested. Despite of this, Liu et al. (2009a, b) have reported that after a long period of adaptation to mixotrophic conditions, the photosynthetic pigment content was reduced in *Phaeodactilum tricornutum*. In this study, both autotrophic and mixotrophic cultures exhibited an initial decrease of Chl*a*, Car and total Chls content during the exponential phase. This aspect in all samples could be attributed to the initial pigment content in the high-density starter cultures that were used for the cell inoculum. Similarly, the higher photosynthetic pigments quantities at the end of the cultivation period could be linked to the increase in cell density. In fact, as reported by Schenk et al. (2008) in diluted cultures the light penetration is usually higher than in dense cultures, then a decrease in pigments after day 0 could be due to the effect of dilution.

It has been widely accepted that F_V/F_M is a valid method to probe the maximum quantum yield of photochemistry in PSII (Kalaji et al. 2014). In this study, cells grown in presence of pure or crude glycerol had similar higher F_V/F_M values to those observed in autotrophic cells. The mixotrophic cultivation with glycerol not influenced negatively the photosynthetic

efficiency of the diatom. These results were different from previous studies, which have observed that the cultivation in presence of glycerol depressed the photosynthetic activity of *P. tricornutum* (Liu et al. 2009a,b). According to previous studies (White et al. 2011; Giovanardi et al. 2014; Baldisserotto et al. *In Press)*, when cells were in stationary phase and lipid production was induced, cell showed a decrease in photosynthetic efficiency, confirming a reverse correlation between high photosynthetic efficiency and lipid synthesis, which occurred in response to the physiological stress caused by nutrient consumption.

Tables and Figures

Composition	% (m/m)
Glycerol	≥ 80
Water	7.7
Methanol	0.18
Other impurities	0.16

Tab.1: Masol Continental Biofuel crude glycerol composition

PSII maximum quantum yield (F_V/F_M)	Growth conditions		
	Photoautotrophy	Mixotrophy with pure glycerol	Mixotrophy with crude glycerol
0 day (inoculum)	0.570 ± 0.009	0.570 ± 0.009	0.570 ± 0.009
4th day	0.691 ± 0.046	0.731 ± 0.017	0.704 ± 0.028
7th day	0.561 ± 0.014	0.578 ± 0.027	0.527 ± 0.033

Tab. 2: Time-course variations of PSII maximum quantum yield (F_V/F_M ratio) of *T. pseudonana* cells grow autotrophically and mixotrophically with 2.5 gL^{-1} of pure glycerol or crude glycerol. Values are means ± s.d. (n=3)

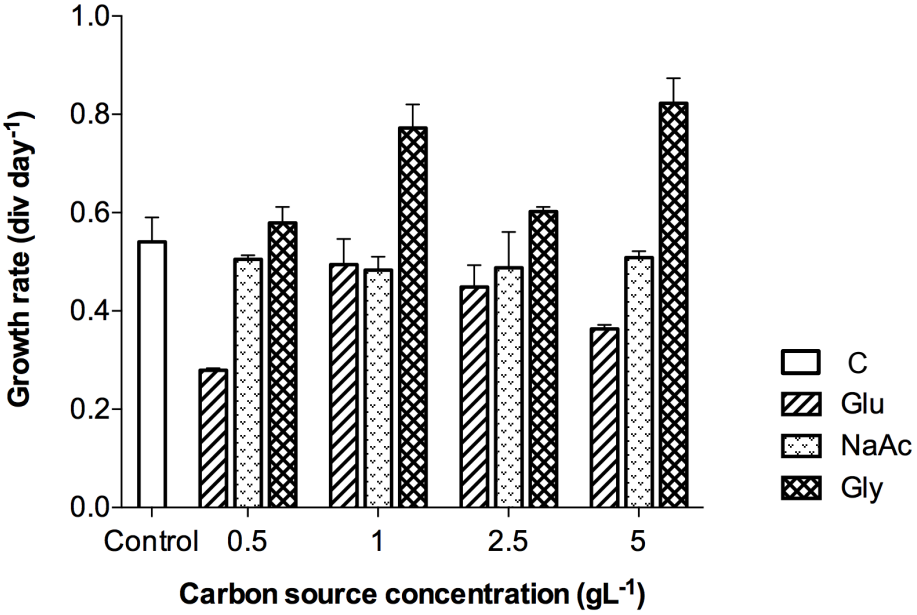

Fig. 1: Variation of growth rates (div day^{-1}) of *T. pseudonana* cultivated autotrophically (white) and in presence of increasing concentrations of glucose (striped), sodium acetate (dotted) and glycerol (diamond-shape) in small culture volumes (50 mL). Values are means ± s.d. (n=2)

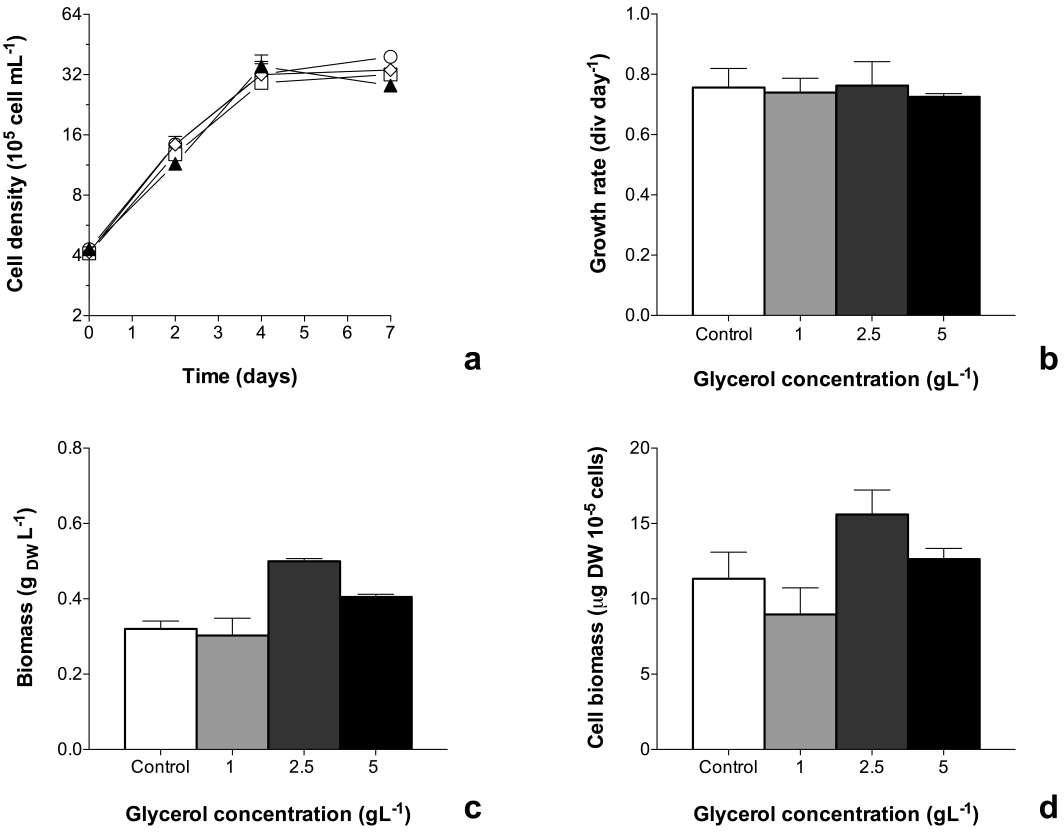

Fig. 2: Growth parameters of *T. pseudonana* cultivated with increasing glycerol concentrations in larger culture volumes (150 mL). **(a)** Growth curves of *T. pseudonana* grown autotrophically (filled triangles), in presence of 1 gL^{-1} (empty diamonds), 2.5 gL^{-1} (empty squares) and 5 gL^{-1} (empty circles) of glycerol. **(b)** Growth rates (div day^{-1}), calculated during the exponential phase (0–4 days time interval of cultivation). **(c)** Biomass yields and **(d)** single cell biomass at 7th day of cultivation of autotrophic cells (white), 1 gL^{-1} (light grey), 2.5 gL^{-1} (dark grey) and 5 gL^{-1} (dark) of glycerol-treated cells. Values are means ± s.d. (n=3)

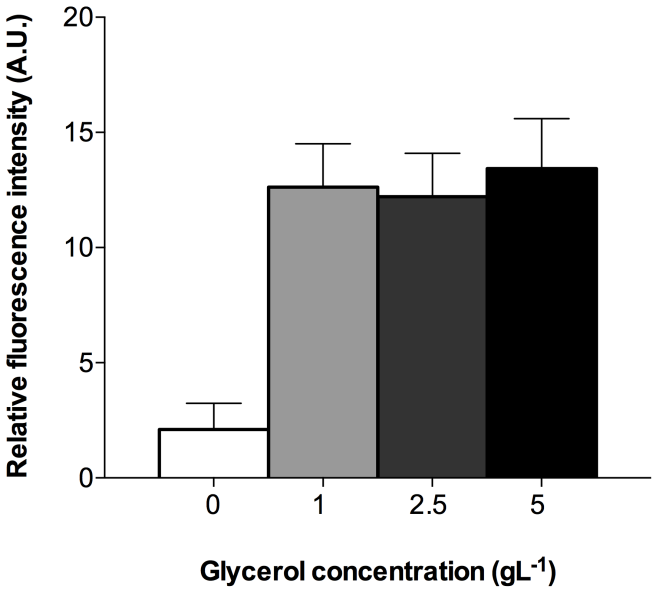

Fig. 3: Relative fluorescence intensity emitted by *T. pseudonana* cells cultivated with 0 gL^{-1} (white), 1 gL^{-1} (light grey), 2.5 gL^{-1} (dark grey) and 5 gL^{-1} (dark) of glycerol and stained with Nile Red after 7 days of growth. For each sample, values are means ± s.d. (n=3)

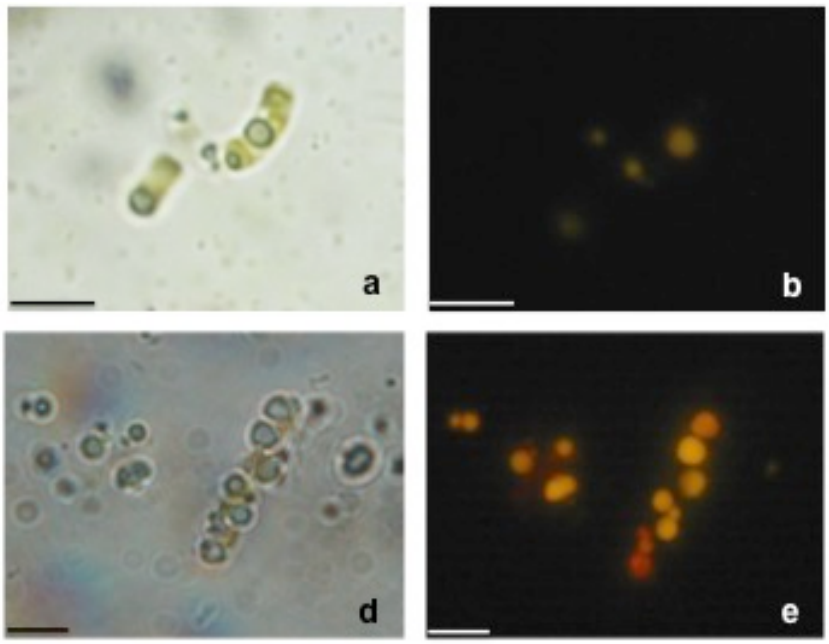

Fig. 4: Light and epifluorescence pictures of *T. pseudonana* cells after 7 days of growth. **(a)** Autotrophic cells and **(b)** relative Nile Red-staining observation, **(c)** cells grown in presence of 2.5 gL^{-1} of glycerol and **(d)** relative Nile Red-staining observation.

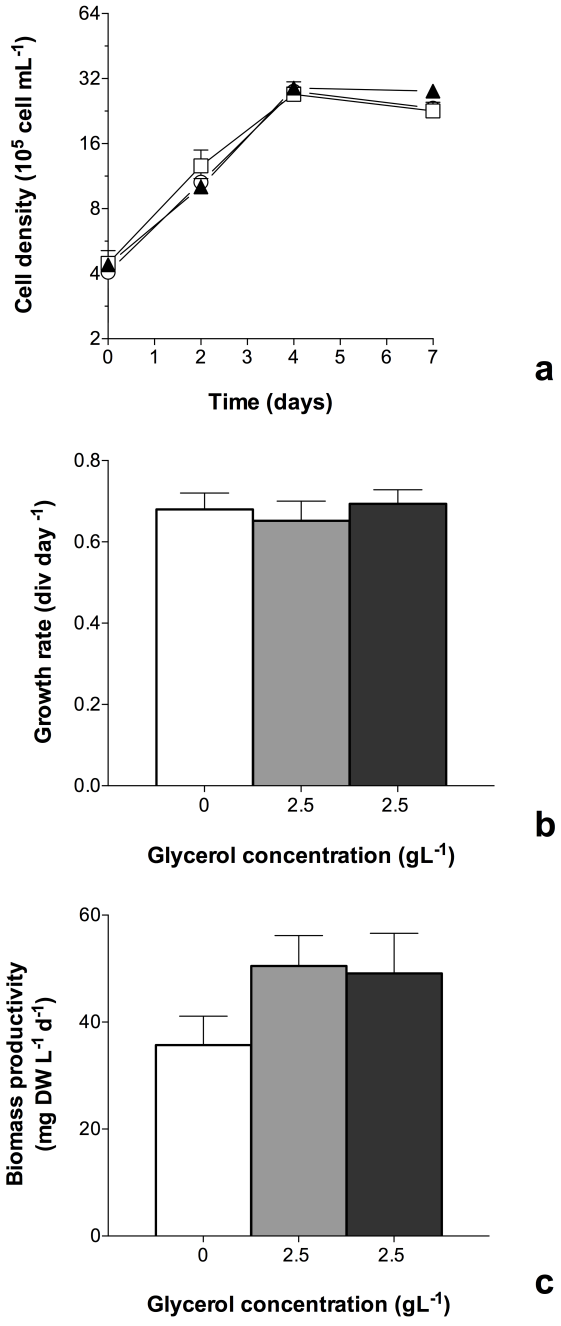

Fig. 5: Growth parameters of *T. pseudonana* cultivated with 2.5 gL^{-1} of pure glycerol or crude glycerol. **(a)** Growth curves of *T. pseudonana* grown autotrophically (filled triangles), in presence of 2.5 gL^{-1} of pure glycerol (empty squares) and 2.5 gL^{-1} of crude glycerol (empty circles). **(b)** Growth rates (div day^{-1}), calculated during the exponential phase (0–4 days time interval of cultivation) and **(c)** Biomass productivity at 7^{th} day of cultivation of autotrophic cells (white), 2.5 gL^{-1} of pure glycerol-treated cells (light grey) and 2.5 gL^{-1} of crude glycerol-treated cells (dark grey). Values are means ± s.d. (n=3)

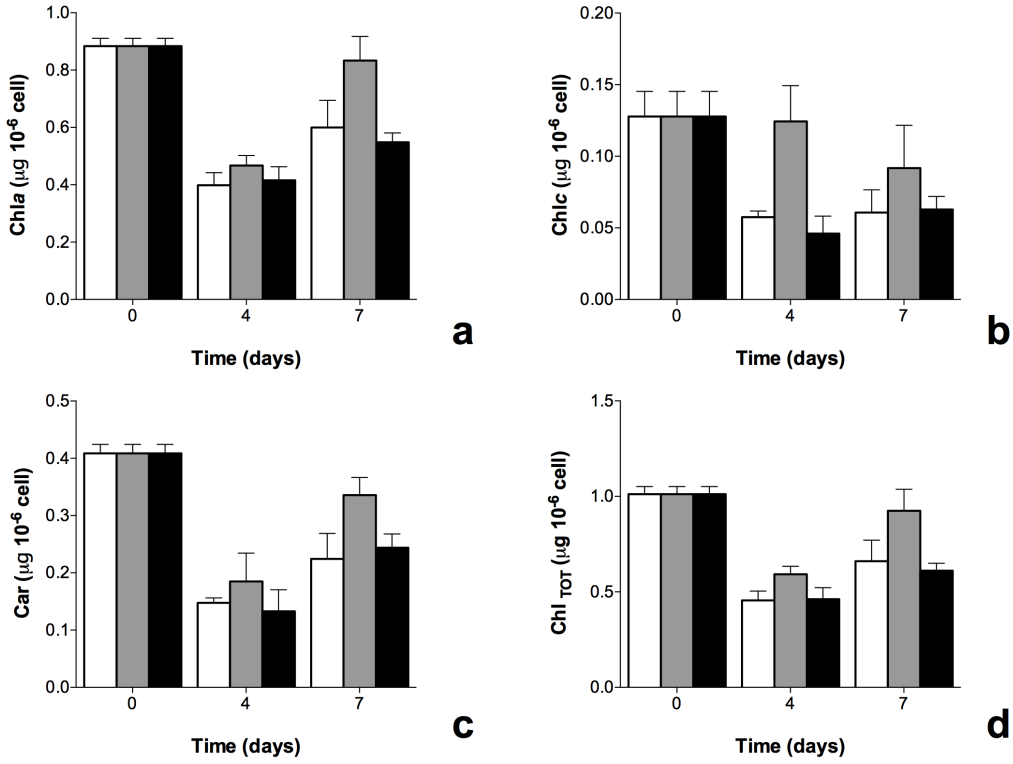

Fig. 6: Time-course variations of Chl*a* (**a**), Chlc (**b**) Car content (**c**) and total Chl content (**d**) of *T. pseudonana* cells grow autotrophically (white), with 2.5 gL^{-1} of pure glycerol (light grey) and 2.5 gL^{-1} of crude glycerol (dark grey) during the cultivation period. Values are means ± s.d. (n=3)

3. *Effect of different CO_2 concentrations on biomass, pigment content and lipid production of the marine diatom* Thalassiosira pseudonana

3.1 Introduction

The increasing concentration of greenhouse gases in the atmosphere has received great concern. Carbon dioxide (CO_2), the principal greenhouse gas, has contributed to a 40% increase in the atmospheric CO_2 and its concentration has increased rapidly since the onset of industrialization (Blasing et. al 2009; Shen 2014; Cheah et al. 2015). The anthropogenic emissions from power plants contain approximately 10–15% v/v CO_2 and are responsible for up to 7% of the global release of CO_2 (de Morais and Costa 2007). As a consequence of this global phenomenon, extensive studies have been focus on the development of an eco-friendly approach to reduce the CO_2 emissions. Among the various physical, chemical, and biological methods that can be used to capture CO_2, the CO_2 biological fixation by microalgae is thought to be one of the most important and effective approaches and is considered a renewable, energy-saving, sustainable technology for the reduction of CO_2 emissions (Wang et al. 2008; Kumar et al. 2010; Ho et al. 2011; Lam et al. 2012; Razzak et al. 2013; Maity et al. 2014; Shen 2014). Moreover, microalgae can be used to produce biofuels (Hu et al. 2008; Mata et al. 2010). These microorganisms have faster growth rates, higher photosynthetic efficiencies, higher rates of carbon dioxide fixation, and higher biomass and lipid productivities as compared to conventional oil crop plants (Chisti 2007; Hu et al. 2008; Harun et al. 2010; Mata et al. 2010; Scott et al. 2010). Currently extensive researches have been concerned the choice of the most adequate microalgal strain that could tolerate elevated CO_2 concentrations and simultaneously, produce lipids suitable for biodiesel production (Yoo et al. 2010; Zeng et al. 2011; Lam et al. 2012; Singh and Singh 2014). However, most of these microalgal strains studied for this purpose are freshwater species (Yoo et al. 2010; Tang et al. 2011; Lam et al. 2012). With the aim of making the microalgal large-scale production increasingly sustainable, it is necessary to identify a species that can grow in saltwater to avoid the competition with freshwater resources (Hu et al. 2008; Doan et al. 2011; Popovich et al. 2012a; Singh et al. 2014). Marine diatoms may be

considered as an attractive feedstock for combined CO_2 fixation and lipid production (Hildebrand et al. 2012; Wang et al. 2014; Vinayak et al. 2015). Diatoms, due to their higher trophic flexibility, are considered the most productive microalgae on the planet (Field et al. 1998; Hildebrand et al. 2012). These microorganisms, which are the main component of phytoplankton, significantly contribute to the global primary productivity and marine geochemical cycles (Falkowski et al. 1998; Field et al. 1998; Granum et al. 2005). Moreover, they are excellent lipid accumulators, especially triacylglycerides (TAGs), which were synthetized and accumulated in the stationary phase of growth (Hu et al. 2008; Hildebrand et al. 2012; Levitan et al. 2014; d'Ippolito et al. 2015; Vinayak et al. 2015). It is well known that TAGs represent the best substrate for the biodiesel production (Chisti et al. 2007; Hu et al. 2008). Nevertheless, only few studies have valorised the diatom lipid profile for biodiesel (Francisco et al. 2010; Popovich et al. 2012b; Zendejas et al. 2011). *Thalassiosira pseudonana*, a model diatom species, which genome has been recently sequenced (Armbrust et al. 2004; Trentacoste et al. 2013), is one of the most studied species for the production of biofuels up to now (Yu et al. 2009; Zendejas et al. 2011; Roleda et al. 2013; d'Ippolito et al. 2015). In addition, recent studies have been concerned the effect of elevated CO_2 concentrations on growth and photophysiology of this diatom species (Trimborn et al. 2009; Crawfurd et al. 2011; Yang and Gao 2012; McCarthy et al. 2012; Li and Campbell 2013).

The present work is focused on the potential of the marine diatom *Thalassiosira pseudonana* as a promising candidate strain for the cultivation in presence of higher CO_2 levels coupled with biodiesel production. The algal growth, photosynthetic pigment content, lipid content and profile were monitored under increasing CO_2 concentrations (0.04 (ambient air), 1 and 5%).

3.2 Materials and Methods

3.2.1 Algal strain and culture condition

The strain used in this study was the marine centric diatom *Thalassiosira pseudonana* (Husted) Hasle and Heimdal CCAP 1085/12 (Heterokontophyta, Thalassiosirales, Thalassiosiraceae), obtained from the Culture Collection of Algae and Protozoa (CCAP; Scotland, United Kingdom; www.ccap.ac.uk). *T. pseudonana* was inoculated in 6-L Erlenmeyer flasks with a 4-L working volume of filtered (0.45 μm) autoclaved seawater

enriched with f/2 nutrients (Guillard and Ryther 1962) at 25±2°C and under an exposure to an irradiance of 250 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ provided by cool white fluorescent lamps (OSRAM L30W/865 Lumilux) under a cycle period of 16:8 h light-darkness. The *T. pseudonana* cultures were aerated at a rate of 250 mL min^{-1} (i.e., 0.0625 vvm, volume gas per volume media per min) of air enriched with CO_2 to obtain final concentration of 0.04, 1 and 5% CO_2. The air-CO_2 mixtures were obtained by combining rotameter flows of air and pure CO_2. The percentage of CO_2 in the air-CO_2 mixture was verified with an infrared analyzer (Servomex 4900C1). Experiments were performed in triplicate. Aliquots of cultures were collected at different times of cultivation, depending on the analysis.

3.2.2 Growth, cell size and biomass evaluation

Algal cells were counted daily using an Improved Neubauer hemocytometer under a light microscope (Zeiss, model Axioscope A1) equipped with DIC (Differential Interference Contrast) optics in order to estimate the division rates (k, number of divisions per day). Cell density (cell mL^{-1}) was also measured by the absorbance at 750 nm (A_{750}) in 96 well plates with the microplate reader (Infinite Tecan M200Pro). Absorbance measures were converted to optical density according to the manufacturing instructions. Cell size measurements were done with the microscope described above and measured with the aid of the software (ProgRes Capture Pro v. 2.8) and a digital camera (Jenoptik ProgRes Speed XT Core5).

The division rates (k, number of divisions per day) during the exponential phase was calculated with the following equation:

$$k\ (div\ d^{-1}) = (\log_2 N_t - \log_2 N_0) / (t_t - t_0),$$

where k is the division rate, N_t the cell number at time t_t, N_0 the cell number at time 0 and $t_t - t_0$ the time interval (days) (Andersen 2005).

The biomass productivity ($mg_{AFDW}\ L^{-1}\ d^{-1}$) was calculated from the variation in the biomass concentration ($mg_{AFDW}\ L^{-1}$) within a specific cultivation time (d) according to the following equation:

$$BP\ (mg_{AFDW}\ L^{-1}\ d^{-1}) = (X_1 - X_0) / (t_1 - t_0),$$

where BP is the biomass productivity, X_1 the biomass concentration at time t_1, X_0 the biomass concentration at time 0 and $t_1 - t_0$ the time interval (days) (Hempel et al. 2012).

Algal biomass concentration, expressed as ash free dry weight (AFDW) was measured according to the methods as described by Zhu and Lee (1997), with some modifications. In detail, after the filtration of culture replicates (30-50 mL) (onto tare GF/F Whatman filters

which had been previously precombusted), the filtered samples were washed with sodium chloride to remove adhering salts and dried in the oven for 72 h at 60°C. Subsequently, the filters were placed in a muffle furnace for 4 h at 550°C and re-weighed. AFDW corresponds to the difference of the filter weight after the oven treatment minus the filter weight after the muffle.

3.2.3 Photosynthetic pigment extraction and quantification

For photosynthetic pigment analysis, cell samples were collected by centrifugation and the extraction was performed according to Baldisserotto et al. (2014). The extracts were measured with the same microplate reader, already described, at 665 (Chl*a*), 632 (Chl*c*) and 480 nm (Car). Quantification of Chls was performed according to equations reported in Ritchie (2006), whereas Car quantification according to equations reported in Wellburn (1994).

3.2.4 Lipid staining

Cell lipid content was estimated by neutral lipid staining with the fluorochrome Nile Red at the 4th day of cultivation, when the microalgal cultures reached the stationary phase of growth, according to Chen et al. (2009). First, algal samples were diluted to attain a final cell concentration of 0.06 (A_{750}). After the algal suspension were stained with Nile Red (9-diethylamina-5Hbenzo[α]phenoxazine-5-one, 0.5 μg dissolved in 100 mL acetone) and 25% of absolute dimethyl sulphoxide (DMSO). Fluorescence was measured with the microplate reader, already described, at excitation and emission wavelengths of 530 nm and 580 nm, respectively. The relative fluorescence was obtained by subtraction of the autofluorescence of non-stained cells and self-fluorescence of the fluorochrome. After staining, cells were also observed with the microscope already described.

3.2.5 Chemical analyses

During the growth period, the pH in culture replicates was monitored daily using a pH meter. The total nitrogen (TN), the dissolved inorganic (TIC) and dissolved organic carbon (TOC) concentration in the culture medium were measured by a Multi N/C 3100 Analytic Jena analyser. 20 mL of the algal cultures were centrifuged at 4000 rpm for 15 min and the supernatant was used for TIC, TOC and TN measurements.

3.2.6 Data treatment

Data were processed with Graphpad Prism 6 (Graph Pad Software, San Diego CA, USA). In each case, means ± standard deviations for *n* number of samples are given. Statistical analyses for comparison of means were carried out using ANOVA, followed by Student's *t*-test (significance level, 0.05).

3.3 Results

3.3.1 Effect of different CO_2 concentrations on *T. pseudonana* growth

As shown in Fig. 1a, the diatom *T. pseudonana* cultivated with 0.04 (ambient air), 1 and 5% CO_2 promptly entered the exponential phase and reached the stationary phase of growth during 4 days of cultivation. However, it was apparent from Table 1 that the highest CO_2 concentration increased the division rates of the diatom as compared to cells cultivated with 0.04 and 1% CO_2. Interestingly, at the end of the experiment the biomass concentrations of cells cultivated with 1 and 5% CO_2 were 190 and 210 mg L^{-1} respectively, which were 1.58 and 1.75 times higher than that of air grown cultures ($p < 0.01$ in both cultures enriched with CO_2) (Table 1). In this study, the addition of different CO_2 concentration not only had a positive effect on growth but also influenced the cell volume of the diatom. In detail, a gradual but evident increasing trend was observed in cells cultivated with 1 and 5% CO_2 until the end of the experiment (Fig. 1b). Conversely, the cell volume of air grown cultures remained almost stable during the cultivation period. At 4^{th} day of cultivation, the cell volume achieved by cultures enriched with CO_2 was found 2 times higher than that of cell cultivated with 0.04% CO_2 ($p < 0.001$ in both cultures enriched with CO_2) (Fig. 2b). Therefore, these results reflected the higher biomass productivities achieved by cultures grown at 1 and 5% CO_2 (69 and 84% higher than 0.04% CO_2-treated cells, respectively; $p < 0.001$ in both cultures enriched with CO_2) (Table 1). As shown in Table 2, with the increase of CO_2 concentrations from 0.04% to 5%, the TIC concentration in the culture medium increased from 16.83 mg L^{-1} to 43.30 mg L^{-1} (+117 and 157% higher than air grown cultures in cells cultivated with 1% and 5% CO_2, respectively; $p < 0.001$ in both cultures enriched with CO_2). Despite, no great differences were reported between 0.04% and 1% of CO_2 treatments regarding TOC concentrations, the maximum TOC values in the culture medium were found under 5% CO_2 (14.63 mg L^{-1}, $p < 0.05$ in cells cultivated

with 0.04% and 1% CO_2). However, higher CO_2 concentrations resulted in lower pH. As shown in Table 2, the pH value of the culture medium decreased from pH 9.4 to pH 6.5 with the increase of CO_2 concentrations from 0.04% to 5% ($p < 0.001$ in both cultures enriched with CO_2). Further, the TN concentration in the culture medium decreased by 13% and 43% in cells cultivated with 1% and 5% CO_2, respectively; ($p < 0.001$ in cells cultivated with 5% CO_2).

3.3.2 Effect of different CO_2 concentrations on *T. pseudonana* photosynthetic pigment content

In order to evaluate if the cultivation in presence of elevated CO_2 concentrations could also affected the photosynthetic activity of the diatom, the photosynthetic pigment content were also analysed at the end of the cultivation period. The increase of CO_2 concentrations resulted in significant increases in Chl*a* and Car content as compared to those of air grown cultures (Table 3). In detail, Chl*a* quantities in cells cultivated with 1% and 5% CO_2 were 27 and 77% higher than 0.04% air grown cultures ($p < 0.05$ in 1% CO_2-treated cells and $p < 0.001$ in 5% CO_2-treated cells). Despite, no great differences were reported between 0.04% and 1% of CO_2 treatments about Chl*c* content, the highest Chl*c* values were found in cells cultivated with 5% CO_2 (57 and 37% higher than air and 1% CO_2 grown cultures, respectively; $p < 0.05$ in both cases). About Car content, cells cultivated with 0.04% CO_2 contained 21 and 64% lower quantities as compared to cultures enriched with elevated CO_2 ($p < 0.05$ in 1% CO_2-treated cells and $p < 0.001$ in 5% CO_2-treated cells). The increases in total Chl content were greater than differences in the Car content, resulting in higher total Chl to Car ratios in cultures enriched with elevated CO_2 as compared to air grown cultures, but the ratios did not differ significantly at the 95% level. However, no significant effects on Chl*c* content were observed between cells cultivated with 1% and 5% CO_2 compared with that under air conditions, resulting in a significant lower Chl*a* to *c* ratios (44 and 48% lower than 0.04% CO_2-treated cells cultivated, respectively; $p < 0.05$ in both cases) (Table 3).

3.3.3 Effect of different CO_2 concentrations on *T. pseudonana* lipid production

To find a possible relation between CO_2 treatments and lipid synthesis, measurements of neutral lipid accumulation were performed by spectrofluorimetric quantification of the relative fluorescence intensity emitted by Nile Red-stained cells. The relative fluorescence

intensity was measured at the 4th day of cultivation, when cells were in stationary phase. Lipid content, as revealed by Nile Red staining, was 3-3.5 times higher in cultures enriched with elevated CO_2 than in air grown cultures ($p< 0.05$ in 1% CO_2-treated cells and $p< 0.001$ in 5% CO_2-treated cells) (Fig. 2). After staining, cells were also observed by light microscopy. In detail, while cells cultivated with 0.04% CO_2 showed slightly positive reaction, the cultures enriched with elevated CO_2 accumulated abundant lipid droplets, giving an intense positive reaction with the fluorochrome (data not shown).

3.4 Discussion

This study clearly indicates that the marine diatom *T. pseudonana* could tolerate elevated CO_2 concentrations and simultaneously, produce lipids suitable for biodiesel production. In spite of extensive studies performed on this species, there is still no information on the possible effects of increasing CO_2 concentrations on lipid accumulation, which, if demonstrated, could allow a significant advancement for the biotechnological use of this alga. A recent review summarized how higher CO_2 conditions could affected the diatom growth and physiology (Gao and Campbell 2014). Some authors reported that elevated CO_2 concentrations could have positive effects on diatom growth (Wu et al. 2010; McCarthy et al. 2012; Wang et al. 2014), while other observed neutral (Crawfurd et al. 2011; Gao et al. 2012b) or negative effects (Sobrino et al. 2008; Torstensson et al. 2012; Mejia et al. 2013). These conflicting results reported in literature, even in the same species studied, could be attributed to the different experimental conditions such as light intensity (Li and Campbell 2013), pH (Wu et al. 2010), temperature (Torstensson et al. 2012), but also to the physiological complexity of algal responses to elevated CO_2 concentrations (Trimborn et al. 2009; Wu et al. 2010; Yang and Gao 2012; Hennon et al. 2014). In agreement with such literature data, which reported a positive effect of elevated CO_2 concentrations on *T. pseudonana* growth, in this study the addition of 5% CO_2 increased the diatom growth rates by 15% and biomass concentration by 75% (Yang and Gao 2012; McCarthy et al. 2012; Li and Campbell 2013). Interestingly, observations on diatom cell size during the experiment showed remarkable changes in response to the CO_2 treatments. A 2-fold increase in cell volume of the diatom was observed as CO_2 increase from 0.04 to 5%. These results are coherent with previous studies, which found an evident increase in cell size in centric diatom cultivated under high CO_2 levels (Hoogstraten et al. 2012; Reinfelder 2012; Li and

Campbell 2013). The dissolved inorganic carbon (TIC) in culture medium represents the carbon sources for the microalgal growth. As expected, the cultivation of *T. pseudonana* under increasing CO_2 concentrations resulted in higher TIC values (Tang et al. 2011). It has long been known that the inorganic carbon system is closely related to the pH, as it is the main buffering system in the culture medium. In this study, the higher CO_2 concentrations have lead to higher diatom biomass productivities, but also caused a decrease in the pH. Lower pH values could be attributed to the enhanced respiration of the cells and to the buffering properties of the carbonate system (Wu et al. 2010; Kumar et al. 2010; Yang and Gao 2012; Moheimani 2013; Gao and Campbell 2014). The cultivation with the highest CO_2 resulted also in higher TOC concentrations in culture medium, which could be attributed to the frequently extracellular release of algal exudates during microalgal growth (Engel 2002; Hulatt and Thomas 2010). In addition, in this study the increase of CO_2 resulted in higher pigment content per cell of *T. pseudonana*. However, no significant effects on pigment content of this diatom species were observed after a long period of adaptation to elevated CO_2 (Crawfurd et al. 2011; McCarthy et al. 2012; Li and Campbell 2013). These responses were more related to photo-acclimation of cultures rather than variation in CO_2 availability (Crawfurd et al. 2011; Li and Campbell 2013). The effects of elevated CO_2 concentrations on fatty acids composition and content of microalgae have been already reported (Muradyan et al. 2004; Ota et al. 2009; Tang et al. 2011). Present results clearly indicated that the increase of CO_2 concentrations resulted in higher lipid content of *T. pseudonana*, as revealed by Nile Red staining. Nile Red fluorescence has been widely accepted as a valid method for analyzing TAGs in algal cultures (Yu et al. 2009; Chen et al. 2009). Several studies have been showed that diatoms accumulated lipids as a result of N limitation and increased the proportion of TAGs in the stationary phase of growth (Yu et al. 2009; Hildebrand et al. 2012; Yang et al 2013; Levitan et al. 2014; d'Ippolito et al. 2015). Accordingly, in this study when 1 and 5% CO_2-treated cells were in stationary phase showed a 3-fold increase in lipid content and coincidently, decreased clearly the TN concentrations in culture medium.

Tables and Figures

CO_2 concentration (%)	k (div d^{-1})	X (mg_{AFDW} L^{-1})	BP (mg_{AFDW} L^{-1} d^{-1})
0.04	1.13 ± 0.08	120 ± 0.005	26 ± 0.001
1	1.14 ± 0.09	190 ± 0.010	44 ± 0.002
5	1.29 ± 0.08	210 ± 0.015	48 ± 0.003

Tab. 1: The division rates (k), biomass concentrations (X) and biomass productivities (BP) of *T. pseudonana* cultivated under different CO_2 concentrations. Values are means ± s.d. (n=3)

CO_2 concentration (%)	pH	TIC (mg L^{-1})	TOC (mg L^{-1})	TN (mg L^{-1})
0.04	9.36 ± 0.03	16.83 ± 0.52	12.25 ± 0.12	18.84 ± 0.32
1	7.34 ± 0.03	36.64 ± 0.38	12.52 ± 0.39	16.59 ± 0.38
5	6.49 ± 0.03	43.30 ± 0.19	14.63 ± 0.64	13.16 ± 0.38

Tab. 2: The pH, dissolved inorganic carbon (TIC), dissolved organic carbon (TOC) and total nitrogen (TN) concentration in the culture medium of *T. pseudonana* cultivated under different CO_2 concentrations at 4^{th} of cultivation. Values are means ± s.d. (n=3)

CO_2 concentration (%)	Pigment content (μg 10^{-6} cells)			Chl*a* /Chl*c*	Chl tot/Car
	Chl*a*	Chl*c*	Car		
0.04	0.86 ± 0.09	0.14 ± 0.02	0.42 ± 0.05	6.83 ± 0.43	1.58 ± 0.02
1	1.10 ± 0.04	0.16 ± 0.01	0.51 ± 0.02	4.75 ± 0.25	1.64 ± 0.05
5	1.52 ± 0.05	0.22 ± 0.02	0.69 ± 0.02	4.62 ± 0.37	1.68 ± 0.02

Tab. 3: Photosynthetic pigments content of *T. pseudonana* cultivated under different CO_2 concentrations after 4 days of growth. Values are means ± s.d. (n=3)

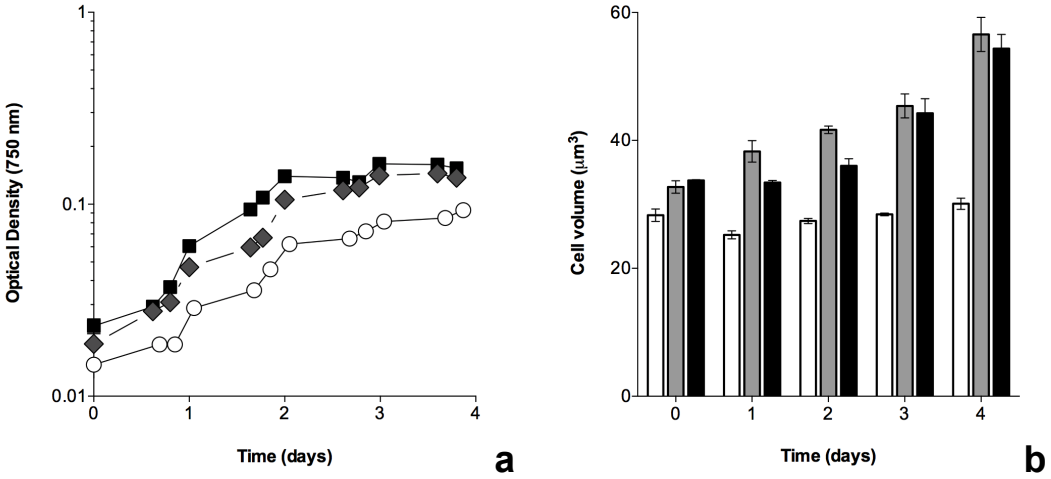

Fig. 1: (**a**) Growth curves and (**b**) cell volumes of *T. pseudonana* cultivated under 0.04% (white circles and bars), 1% (grey diamonds and bars) and 5% CO_2 (dark squares and bars). Values are means ± s.d. (n=3).

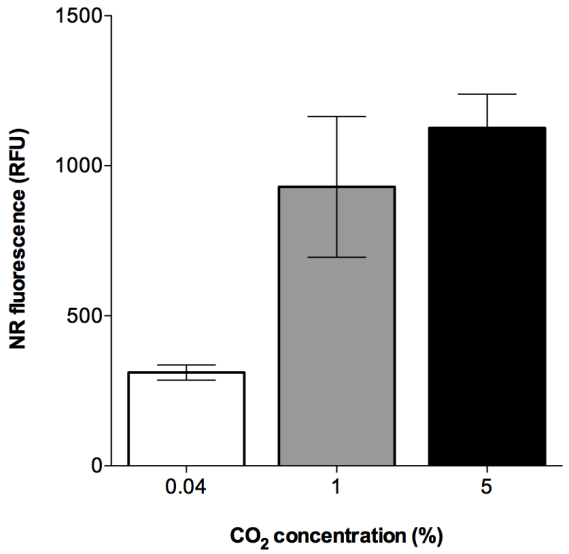

Fig. 2: Relative fluorescence intensity emitted by *T. pseudonana* cells cultivated under 0.04% (white), 1% (grey) and 5% CO_2 (dark) and stained with Nile Red after 4 days of growth. For each sample, values are means ± s.d. (n=3)

4. Conclusion

During the last years, extensive studies have been focus on the development of renewable, carbon-neutral feedstock to displace petroleum and to mitigate anthropogenic emissions of CO_2 (Kumar et al. 2010; Razzak et al. 2013; Maity et al. 2014; Shen 2014). Due to its genomic data and physiological characteristics, the marine diatom *T. pseudonana* represents an optimal model organism for studies concerning the lipid accumulation for biofuels coupled with the CO_2 fixation (Hildebrand et al. 2012).

In this chapter of the Thesis, two different cultivation strategies were tested for enhancing the biomass productivity and lipid content in the marine diatom with the aim of gaining further knowledge on its biotechnological potential use in the bioenergetics field. With this purpose, the experiments were organized in two different sections.

In the first section, *T. pseudonana* was cultivated autotrophically and mixotrophically under different organic carbon source and with the supply of a low-cost carbon source. In the preliminary experiment, results showed that the diatom grown mixotrophically in the presence of 2.5 gL^{-1} of pure glycerol achieved higher biomass concentration (0.5 $g_{DW}L^{-1}$) and lipid content (6 times higher) as compared to the autotrophic condition (0.3 $g_{DW}L^{-1}$). It is worth noting that, in the following experiment, the cultivation of the diatom in the presence of 2.5 gL^{-1} of crude glycerol, one of the major by-products of the biodiesel production, allow to achieved similar and high value of biomass productivity of that obtained with the supply of pure glycerol. In order to evaluate how these culture conditions have influenced the lipid profile of the diatom, the fatty acids characterization will be estimated by Raman spectroscopy, which it is a new method for direct, quantitative, in vivo lipid profiling of oil-producing microalgae (Wu et al. 2011). This analysis is still in progress in collaboration with Dr. Carlo Liberale (Dep. of Bioscience, Univ. of KAUST, Saudi Arabia). Despite of this, the present results suggested that *T. pseudonana* is a very promising organism to be exploited in the green-energy fields because of its capability to accumulate lipids under mixotrophic cultivation. Moreover, the use of biodiesel-derived products such as crude glycerol could provide the organic carbon source, which is required for the mixotrophic growth. This could make mixotrophy economically advantageous, especially in the context of industries that need to dispose this kind of waste products within a biorefinery approach.

In the second section, *T. pseudonana* was cultivated in the presence of increasing CO_2 concentration with special attention to lipid accumulation. Present results claearly indicated

that the diatom cultures grown at 1 and 5% CO_2 presented higher growth rate (1.14 and 1.29 div d^{-1}, respectively) and biomass productivity (44 and 48 $mg_{AFDW}L^{-1}d^{-1}$) than air grown cultures (with 1.13 div d^{-1} and 26 $mg_{AFDW}L^{-1}d^{-1}$). Moreover, lipid content, as revealed by the lipid-specific fluorochrome Nile Red, was 2 times higher in cultures enriched with CO_2 than in air grown cultures. In order to obtain information on lipid profile, the fatty acids characterization is still in progress in collaboration with Prof. Joan Salvadò (Dept. of Chemical Engineering, Universitat Rovira I Virgili, Tarragona, Spain).

Despite of this, the present results suggest that the diatom *T. pseudonana* has a great potential for mitigating CO_2 and biodiesel production.

Chapter 5

Biofertilizer properties of the microalga Chlorella vulgaris

1. Introduction

During the last decades, the global food demand is drastically rising due to the continuous increase of world population (Tilman et al. 2002; Godfray et al. 2010; Foley et al. 2011). As a result, the global demand for agricultural products is expected to continue to rise (Tilman et al. 2002). Increase crop productivity to meet the growing food request has been made possible with the use of agrochemicals. However, the indiscriminate and excessive use of chemical fertilizers has significantly contributed to the global environmental pollution, leading to soil infertility, biodiversity loss, land degradation, eutrophication and global warming (Kirchmann and Thorvaldsson 2000; Tilman et al. 2002; Chagnon et al. 2014; Van der Sluijs et al. 2015). As a consequence of these environmental stresses, extensive studies have been focused on the development of renewable, carbon-neutral, and sustainable alternative to synthetic fertilizers. Biofertilizers have emerged as a potential eco-friendly approaches to be exploited in sustainable agriculture (Kawalekar 2013; Raja et al. 2013; Bhardwaj et al. 2014). Biofertilizers are products that contain living microorganisms or natural metabolites derived from organisms such as bacteria, fungi, and algae that are capable of improving soil fertility, plant growth and its tolerance to abiotic and biotic stress (Goyal 2002; Vessey 2003; Abdel-Raouf et al. 2012; Bhardwaj et al. 2014). In this perspective, microalgae could represent a potential source in agricultural sector (Metting 1990). Microalgae has been widely proposed as promising renewable source for the production of biodiesel and a wide range of high-value bio-products (Chisti 2007; Mata et al. 2010; Borowitzka 2013a; Markou and Nerantzis 2013). Nevertheless, significant obstacles still need to be solved before microalgal production becomes a cost-effective process (Solomon 2010; Wijffels and Barbosa 2010; Suali and Sarbatly 2012). In this perspective, most of the research has been focused on potential products and by-product applications that can make microalgal production more environmentally sustainable and economically feasible (Singh and Gu 2010; Subhadra et al. 2011). Among the potential by-product applications of microalgae, only recently the use of microalgal biomass as potential biofertilizer has been tested in agriculture (Shaaban 2001a,b; Ördög et al. 2004; Abdel-Raouf et al. 2012; Trentacoste et al. 2015). Several studies have reported a positive effect on seed germination, plant growth and yield after the addition of algal extracts as a soil amendment or foliar spray (Faheed and Abd-El Fattah 2008; Shaaban 2001a,b; Shaaban et al. 2010; Grzesik and Romanowska-duda 2014; Garcia-Gonzalez and Sommerfeld 2015). Among the Chlorophyta, *Chlorella vulgaris* is one of the best-studied and exploited green

microalgae due to its high adaptability to a variety of environmental conditions and high productivity in large-scale cultivation system (Spolaore et al. 2006; Faheed and Abd-El Fattah 2008; Görs et al. 2010; Safi et al. 2014). For this reason, it was chosen for the present study.

The objective of the last Chapter of the Thesis was to investigate the potential application of the green microalga *C. vulgaris* as biofertilizer and assess its effects on plant growth of garden pea plants (*Pisum sativum* L. var. Paladio nano) under greenhouse conditions.

2. Materials and Methods

2.1 Plant material

Garden pea plants (*Pisum sativum* L. var. Paladio nano) (Fabales, Fabaceae) were employed in this study. After germination on wet filter paper, the seedlings were grown and maintained into 10-cm pots (one seedling per pot) containing potting soil in an outdoor greenhouse at the Botanical Garden of Ferrara (Italy). After 10 days of growing, seedlings were transplanted in an outdoor greenhouse.

2.2 Algal material for biofertilizer experiment

The strain used in this study was the Chlorophyta *Chlorella vulgaris* SAG 211-12 (Trebouxiophyceae, Chlorellales, Chlorellaceae), obtained from the Culture Collection of Algae at the University of Goettingen (SAG, Germany; www.uni-goettingen.de).

C. vulgaris was cultivated in 10-L Erlenmeyer flasks (5-L working volume) in BG 11 medium (www.ccap.ac.uk) supplied by continuous air bubbling, at 22±2°C and under an exposure to an irradiance of 60 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ of PAR (16:8 h light:dark photoperiod). *C. vulgaris* extracts for biofertilizer treatments were prepared harvesting the biomass at 21 days of cultivation (optical density (OD_{750}) of ca. 0.9, total fresh weight (FW) of 2.5 gL^{-1} and total dry weight (DW) of 0.4 gL^{-1}) and then stored at 4°C until used.

2.3 Biofertilizer experiment

The experiment was conducted under greenhouse conditions at approximately 12±5°C, in 75% relative humidity, from October to November 2014. The experiment consisted of two treatments at various *C. vulgaris* concentrations (0, 15 and 30%) diluted in water. Each

treatment consisted of four replicates (one seedling per replicate) and was set up in a completely randomized block design. Each plant received two foliar spray applications; the first, at 5 mL, was applied after 10 days of the seedlings transplant in the greenhouse conditions and the second, at 5 mL as foliar spray application, 1 week later. The biofertilizer treatments were conducted in the morning when the stomata were open due to water pressure, thus enabling greater foliar penetration. All plants were watered as needed throughout the experiment.

Treatments were as follows:

1. Control, 0 % of *C. vulgaris*, 100 mL water
2. T1-15% (v/v) 15 mL of *C. vulgaris* in 100 mL water
3. T2- 30% (v/v) 30 mL of *C. vulgaris* in 100 mL water

2.4 Determination of growth parameters of pea plant

Morphometrics and biomass analysis

The stem height (mm) and diameter (mm), leaf size, calculated as leaf area (mm^2), of pea plants were measured during the biofertilizer experiment.

One week later the second foliar spray application, two samples per treatment were chosen at random to measure total fresh and dry plant weight (g). The harvested plants were dried at 60°C for 72h and weighted until they reached constant weight.

Photosynthetic pigment analysis

One week later the second foliar application, pigments were extracted from small leaf pieces with 80% aqueous acetone (v/v) for at least 24h in complete darkness at -20°C. The extracts were clarified by centrifugation and analysed with a Pharmacia Ultrospec 2000 UV-Vis spectrophotometer (1-nm bandwidth; Amersham Biosciences, Piscataway, NJ, USA). For Chl and carotenoid determinations, absorption was measured at 663 (chlorophyll a - Chl*a*), 646 (chlorophyll b - Chl*b*) and 470 nm (carotenoids - Car). Pigment concentrations, expressed on biomass basis (mg g^{-1} FW), were evaluated according to equations reported in Wellburn (1994).

Maximum quantum yield of photosystem II

A portable pulse amplitude modulated (PAM) fluorometer (ADC OS1-FL, ADC BioScientific Ltd., Hoddesdon, UK) was used for in vivo measurements of chlorophyll fluorescence of PSII of pea plants collected one week later the second foliar application. The PSII maximum quantum yield, evaluated after 20 min of dark incubation of samples, is reported as F_V/F_M ratio, *i.e.* $(F_M - F_0)/ F_M$, where variable fluorescence is $F_V = (F_M - F_0)$, F_M is the maximum fluorescence and F_0 is the initial fluorescence of samples (Lichtenthaler et al. 2005). This measurement is considered a valid method to probe the maximum quantum yield of photochemistry in PSII (Kalaji et al. 2014).

2.5 Data treatment

Data were processed with Graphpad Prism 6 (Graph Pad Software, San Diego CA, USA). In each case, means ± standard deviations for n number of samples are given. The statistical significance of differences was determined by one-way ANOVA followed by a multiple comparison test (Dunnett's test). A significance level of 95% ($p < 0.05$) was accepted.

3. Results and Discussion

Most of the research reported in literature about the agricultural applications of algae has been focused on the use of cyanobacteria (Pereira et al. 2008; Sharma et al. 2010; Dhar et al. 2015) or on macroalgae (Khan et al. 2009; Hernández-Herrera et al. 2014; Ramya et al. 2015). Only recently, microalgae are proposed as a potential biofertilizers that can be effectively used in sustainable agriculture (Faheed and Abd-El Fattah 2008; Shaaban et al. 2010; Grzesik and Romanowska-duda 2014; Garcia-Gonzalez and Sommerfeld 2015).

In the present study, the application of *C. vulgaris* as biofertilizer for pea plants clearly led to an increase in plant growth, enhancing many of the studied plant growth parameters (Fig. 4). In detail, the stem height increased significantly when plants were treated with 15 and 30% of *C. vulgaris* (+12 and 13% than controls; respectively) (Fig. 1a). However, not significant effects in stem diameter were observed between all samples (Fig. 1b). Also the leaves of the pea plants were greatly influenced by the addition of *C. vulgaris*, especially after the second foliar application. As shown in Fig. 2, the leaf area of treated plants with 15 (T1) or 30% (T2) of *C. vulgaris* were found to be 36 and 45% bigger than controls; respectively.

Consequently, the total fresh and dry weight of treated pea plants was found to increase significantly after the algal application. At the end of the experiment, the T1 and T2 treatments led to ca. 2 times higher total fresh plant weight compared to the control group. The dry weight also significantly increased when plants were treated with 15% and 30% of *C. vulgaris* (more than 70% with respect to the control). These results were in agreement with those of Shaaban (2001a,b) and Faheed and Abd-El Fattah (2008), which have noted an increase of growth and yield in wheat, maize and lettuce plants after the treatments with *C. vulgaris*. These promoting effects of algal extracts may be associated with some plant growth regulators (gibberellins, auxin, cytokinin, polyamines) present in the algal biomass, which are known to play crucial roles in plant development. These compounds are also involved in mitigating various types of biotic and abiotic stress (Ördög et al. 2004; Tarakhovskaya et al. 2007; Tate et al. 2013; Bhardwaj et al. 2014; Sabia et al. 2015). The microalgal biomass, which is known to contain high quantities of micro and macroelements, was also proposed as an alternative approach to overcome the problems connected with the soil infertility (Tilman et al. 2002; Graham 2008). The micro and macronutrients deficiency derived from the intensive crop cultivation is known to cause several problems in plant metabolism and its development. However, several studies reported that the improvements in plant growth after the algal treatments depended also on the methods of applications and on microalgal species employed. As observed in Chapter 3 of this Thesis, *C. vulgaris* had showed high growth rate and protein content (40% of its dry weight biomass), suggesting that this alga could be a promising natural source of nitrogen fertilizer. Among the various methods of algal treatments, the foliar spray applications resulted to be more effective than soil applications, since they allowed obtaining the best uptake of nutrients requested for plant growth (Shaaban 2001b,c; Kassab et al. 2004; Arif et al. 2006, Shaaban et al. 2010; Ramya et al. 2015). For this reason, in this study, *C. vulgaris* was given as foliar spray. The present results are consistent with previous studies (Shaaban et al. 2010; Garcia-Gonzalez and Sommerfeld 2015; Ramya et al. 2015), suggesting that the increase of growth in treated pea plants may be attributed to the presence of micro and macroelements and growth promoting substances in the extract of *C. vulgaris*.

In order to estimate also the physiological state of control and treated plants, data on photosynthetic pigment content and maximum efficiency of PSII are reported in Tab. 1. After the second foliar spray applications of *C. vulgaris*, treated plants contained higher quantities of total chlorophyll as compared to the controls, with values significantly different in treated-T2 plants (32% more than controls). These results were in agreement

with those obtained by other authors (Faheed and Abd-El Fattah 2008; Grzesik and Romanowska-duda 2014), which have showed an increase of chlorophyll content after the algal application. Indeed, control and treated plants showed similar carotenoid content at the end of the experiment. As observed in Tab. 1, the maximum PSII quantum yields of T1 and T2 treated-plants showed higher and similar values of F_V/F_M (around 0.83) of that of the control, indicating a good photosynthetic activity of all samples.

Collectively, the present results demonstrated that the application of *C. vulgaris* as biofertilizer, especially at the higher concentration tested (T2-30%), had positively affected the cellular metabolism in treated plants leading to enhanced growth and yield of pea plants.

4. Conclusion

With the increasing of global food demand and the environmental stresses derived from the intensive agriculture, microalgae could represent a sustainable and valid alternative to chemical fertilizers. The present results show that enhancement of growth, yield and physiological parameters of treated pea plants might be due to the presence of some plant growth regulators and micro and macroelements in the foliar extracts of *C. vulgaris*. Further studies, however, should be conducted to study if *C. vulgaris* has the same stimulating growth effects under open field conditions.

Collectively, present results represent a valid contribution to improve the metabolism of plants and their productivity; however additional studies should be conducted to discover and exploit the great potential of microalgae in the agricultural sector.

In conclusion, this study also emphasizes that among the several biotechnological applications, the use of microalgae as biofertilizers represents not only an eco-friendly approach but also a possible way to make microalgal production more economically feasible.

Tables and Figures

Treatment (14th day)	PSII maximum quantum yield (F_V/F_M)	Photosynthetic pigment content (mg g^{-1} FW)	
		Total Chl	Car
Control	$0.835^{a} \pm 0.02$	$1.19^{a} \pm 0.09$	$0.20^{a} \pm 0.02$
T1-15%	$0.831^{a} \pm 0.01$	$1.46^{ab} \pm 0.24$	$0.22^{a} \pm 0.07$
T2-30%	$0.839^{a} \pm 0.01$	$1.57^{b} \pm 0.10$	$0.22^{a} \pm 0.07$

Tab. 1: Effects of *C. vulgaris* as foliar treatments on photosynthetic parameters of pea plants. Values are represented as means ± s.d. (n=4). Columns denoted by different superscripts are significantly different at $P<0.05$

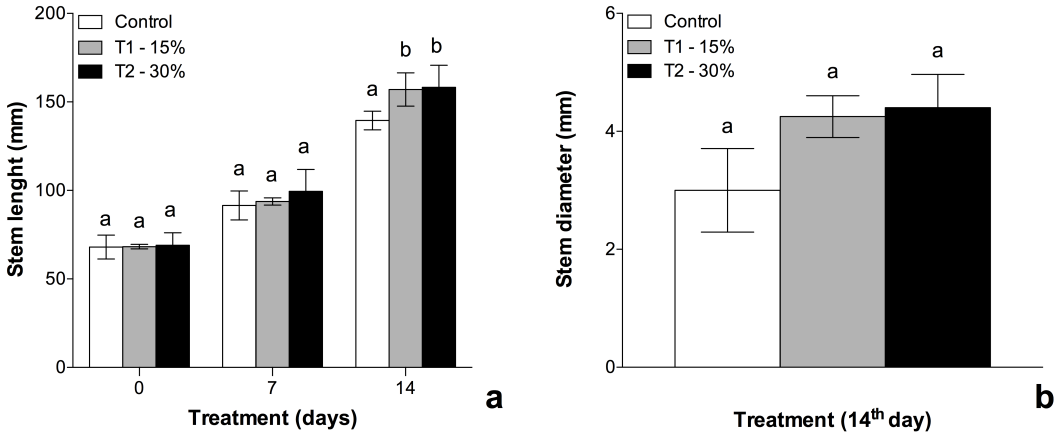

Fig. 1: (a) Effects of *C. vulgaris* as foliar treatments on stem length and **(b)** stem diameter of pea plants. Values are represented as means ± s.d. (n=4), ANOVA $P<0.05$. Different superscripts denote significant ($P<0.05$) differences between the samples

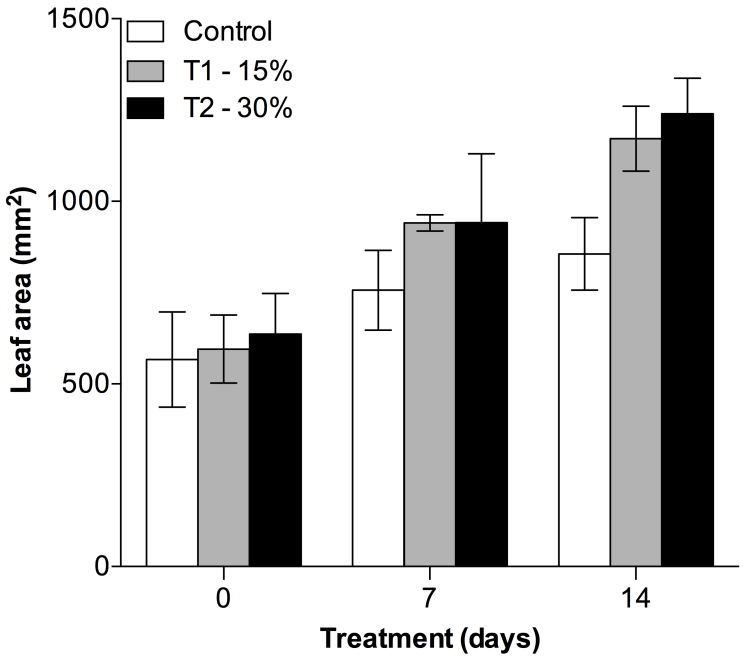

Fig. 2: Effects of C. *vulgaris* as foliar treatments on leaf area of pea plants. Values are represented as means ± s.d. (n=5). No significant (P<0.05) differences were observed between samples within each day of treatments

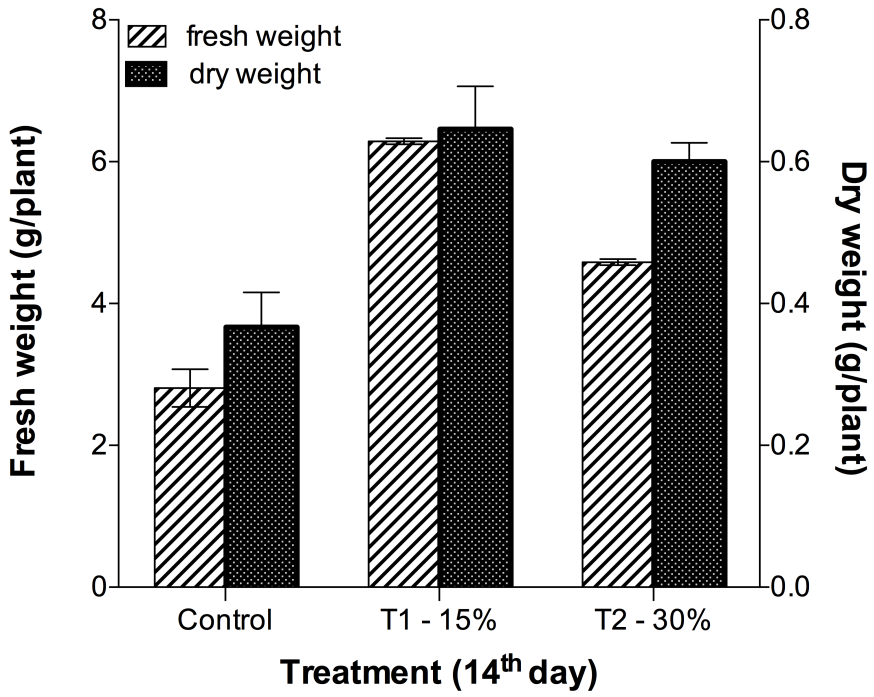

Fig. 3: Effects of *C. vulgaris* as foliar treatments on total fresh plant weight (striped) and total dry plant weight (dotted) of pea plants. Values are represented as means ± s.d. (n=2). Samples are significantly different at $P<0.05$

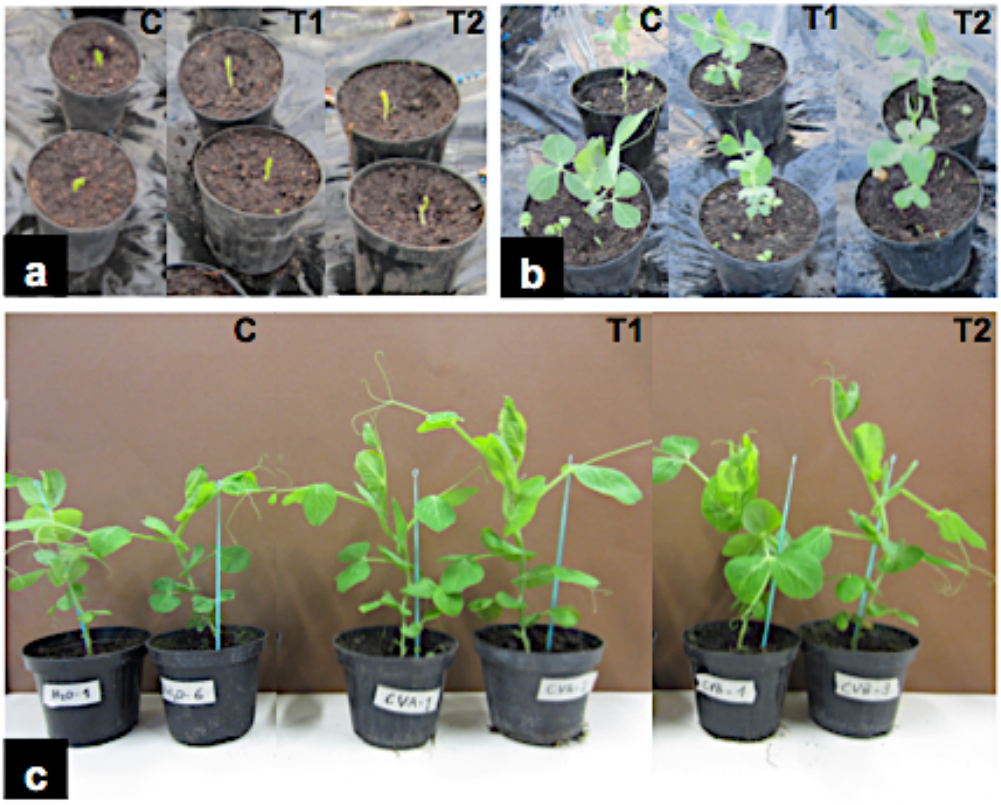

Fig. 4: Effects of C. *vulgaris* as biofertilizer on pea plants: **(a)** after 10 days of seed germination; **(b)** at the beginning of the first foliar treatment and **(c)** one week later the second foliar treatment. Pea seedlings-treated showed bigger and greener leaves as compared to control. In all micrographs, control (C), T1-15% (T1) and T2-30% (T2)

Concluding remarks

In recent decades, extensive researches have been explored the great potential of microalgal production for biofuels and specific high-value compounds. However, significant obstacles still need to be solved before microalgal production becomes a competitive and cost-effective process. In this Thesis, different cultivation strategies were applied aiming to improve the growth performance and biomass composition of some microalgal species in view of their exploitation in different biotechnological fields.

In detail, it has been demonstrated that mixotrophy can be considered a valid and efficient strategy of cultivation, allowing to obtain the best compromise between biomass production and lipid accumulation in the scaling-up of the green microalga *N. oleoabundans*. This Thesis has also showed that the recycling of autotrophic and mixotrophic growth media, can be efficiently considered a suitable solution in order to reduce the cultivation costs, and at the same time providing a more sustainable ecological impact on water resources. Moreover, it has been demonstrated that the use of biodiesel-derived products, such as crude glycerol, could provide the organic carbon source, which is required for the mixotrophic growth of the marine diatom *T. pseudonana*. This could make mixotrophy economically advantageous, especially in the context of a biorefinery approach. In this perspective, the results on the diatom *T. pseudonana* cultivated under increasing CO_2 concentration, have clearly demonstrated that this species has a great potential for mitigating CO_2 emission coupled with the biodiesel production.

This Thesis provides also advanced insights the protein content and profile of different microalgae species belonging to the algal classes Chlorophyceae (*N. oleoabundans* and *S. acutus*) and Trebouxiophyceae (*C. vulgaris* and *C. protothecoides*), which are characterized by similar morphological shape, but a remarkable diversity of physiological and biochemical constraints.

Finally, it has demonstrated that the use of microalgae as biofertilizers represents not only a valid method to improve the metabolism and productivity of plants but also an eco-friendly alternative to chemical fertilizers to be exploited in sustainable agriculture.

Collectively, the results obtained in this Thesis provide useful information for the advancement of the biotechnological application of these microalgae species. Nevertheless, further studies are also needed in order to remove some bottlenecks, which occurred during the microalgal process. To this aim, a multidisciplinary approach is suggested, such as the integration of the engineering with discoveries in algal biology, within a bio-refinery strategy.

Acknowledgments

The sea, once it casts its spell,
holds one in its net of wonder forever
(Jacques Yves Cousteau)

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