Algal Research 16 (2016) 255–265

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Photosynthetic aspects and lipid profiles in the mixotrophic alga *Neochloris oleoabundans* as useful parameters for biodiesel production

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ARTICLE INFO

Article history:
Received 21 July 2015
Received in revised form 4 February 2016
Accepted 14 March 2016
Available online 29 March 2016

Keywords:
Neochloris oleoabundans
Mixotrophy
Photosynthetic apparatus
PSII
Lipid profiles
Ultrastructure

ABSTRACT

Since fossil fuels are expected to run out within few decades, attention has increasingly been focused on renewable energy sources, including microalgae. *Neochloris oleoabundans* (Chlorophyta) has a capability to accumulate lipids, in particular triacylglycerols (TAG), useful for biodiesel production; furthermore, it can grow mixotrophically. The present work deals with two fundamental steps of mixotrophic cultivation with glucose (late exponential – 6 days – and late stationary – 14 days – phases of growth), focusing on the relationship between photosynthesis and lipid production. Results confirmed that the use of glucose induces a high biomass productivity, which is associated to a rapid cell replication until day 6 followed by cell enlargement until day 14. At day 6, mixotrophic cells contained numerous stromatic starch grains, while at day 14 lipids were highly accumulated and starch tended to reduce. Photosynthetic pigment and protein content decreased under mixotrophy. The degree of photoinhibition under high light was not significantly affected by mixotrophic cultivation at both experimental times. The creation of a reducing environment due to the photosynthetic activity, together with alterations of N:C ratio, promoted the lipid synthesis. Neutral lipids increased under mixotrophy and oleic acid was the major component, while linolenic acid decreased; these aspects match requirements for biodiesel production.

1. Introduction

The world energy demand is rapidly increasing because of the continuous rise of human population, urbanization and modernization [1]. At present, energy is mainly supplied by fossil fuels (about 80%), while minor inputs derive from renewable sources (13.5%) and nuclear power (6.5%) [1]. The large use of fossil fuels raises many issues, in terms of both environmental pollution and geopolitical aspects. Moreover, the depletion time for the fossil fuel reserves is calculated in few decades [2]. Research efforts worldwide aim at increasing and improving energy supply by renewable, clean sources, instead of non-renewable. Among renewable energy sources, lipid-rich microalgal biomass is proposed as a useful biofuel feedstock [3–7]. *Neochloris oleoabundans* (syn. *Ettlia oleoabundans*) is a green unicellular alga largely studied for its capability to accumulate lipids, especially triacylglycerols (TAGs), inside the cytoplasm [8–11]. *N. oleoabundans* is characterised by a high lipid content under different growth conditions, such as N-starvation, high-light exposure, pH variations, and mixotrophy [8,10–14]. Moreover, it was shown that, in *N. oleoabundans*, N-starvation has a negligible impact on the qualitative lipid profile [8,10]. However, in general, the proportion of lipid classes that differ with respect to the length of C-chain or degree of unsaturation can considerably change depending on environmental conditions [15]. Among culture conditions useful for both biomass and lipid production, the algal cultivation realised in the presence of organic carbon sources (glucose, acetate, organic acids, etc.) and light exploits a metabolic condition, called mixotrophy, shared by many algal species, *N. oleoabundans* included [11,14,16–20]. It has been recently demonstrated that *N. oleoabundans* can grow mixotrophically in the presence of pure glucose, but also of a glucose-containing apple waste product (AWP). In both cases, biomass production increased and lipids

Abbreviations: ALA, linolenic acid; AWP, apple waste product; Car, carotenoid; Chl, chlorophyll; DW, dry weight; FAME, fatty acid methyl ester; F_0, minimum fluorescence in the dark-adapted state; F_M, maximum fluorescence in the dark-adapted state; F_M', maximum fluorescence in the light-adapted state; F_S, steady state fluorescence; F_V, variable fluorescence ($F_M - F_0$); MUFA, monounsaturated fatty acid; PSII, photosystem II; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TAG, triacylglycerol; TEM, transmission electron microscopy; Y(NO), quantum yield of non-regulatory thermal dissipation and fluorescence; Y(NPQ), quantum yield of regulatory thermal dissipation; Y(PSII), quantum yield of PSII photochemistry.

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http://dx.doi.org/10.1016/j.algal.2016.03.022
2211-9264/

accumulated inside the alga [11,14,18]. In particular, growth rates under mixotrophic cultivation were higher than under autotrophy, leading to 6–7 times higher cell density and biomass productivity [11,14,18]. The biomass increase, observed when either pure glucose or AWP were added, was supported by a good photosynthetic activity of photosystem II (PSII), even if accompanied by different photosynthetic pigment patterns [11,14,18]. Only when the PSII maximum quantum yield decreased, lipids started to accumulate [14,18]. In fact, a decrease in F_V/F_M ratio reflects a damage to PSII, symptomatic of an unbalanced accumulation of reducing power, which in turn promotes the synthesis of lipids [21,22]. It is known that there is a close link between energy carrier accumulation, due to a feedback inhibition of photosynthate buildup, and decrease in the rate and photon efficiency of photosynthesis [22]. This confirms the great complexity of the relationship between photosynthesis and lipid synthesis, especially in the case of mixotrophy, as already highlighted by the complex behaviour of the photosynthetic apparatus of *N. oleoabundans* grown in the presence of glucose or AWP [14,18]. In this perspective, the link between these metabolic pathways (photosynthesis and lipid synthesis) under mixotrophic growth conditions deserves further investigations. Moreover, lipid quality, which is basic information for any bioenergetic applications, still remains poorly characterised under such conditions, especially for microalgae belonging to *Neochloris* genus [20].

In the present work, in order to better exploit the biotechnological potential of *N. oleoabundans*, we provide an in-depth characterisation of morpho-physiological aspects of the alga at two crucial steps (late exponential and late stationary phases of growth) during the lipid synthesis process induced by the mixotrophic cultivation with glucose. It was reported, in fact, that the alga replicates rapidly, but does not produce lipids, when a sufficient combination of nutrients is available in the culture medium (i.e. up to the late exponential phase of growth), while it slows replication, with a concomitant lipid synthesis induction, when nitrogen becomes limiting [14,18,23,24]. Moreover, we included a comparison of lipid production and quality in *N. oleoabundans* cultivated mixotrophically in the presence of AWP, in order to test if the lipid profile remains unchanged upon cultivation with different mixotrophic substrates.

2. Materials and methods

2.1. Culture conditions

Axenic cultures of *N. oleoabundans* UTEX 1185 (Sphaeropleales, Neochloridaceae) (syn. *E. oleoabundans*) were grown and maintained in liquid BM brackish medium in static conditions inside a growth chamber (24 ± 1 °C temperature, 80 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ PAR and 16:8 h of light:darkness photoperiod; light was supplied by cool-white fluorescent Philips tubes) [18,25]. For experiments, algae were inoculated in BM medium containing 2.5 $g\ L^{-1}$ of glucose at a cell density of 0.5–0.7 × 10^6 cells mL^{-1} in 500 mL flasks (300 mL of total culture volume); flasks were maintained under continuous shaking at 80 rpm, as reported in Giovanardi et al. [18]. Controls in BM medium containing 0 $g\ L^{-1}$ of glucose were set up as well and cultivated in the same culture conditions described above (initial cell density: 0.5–0.7 × 10^6 cells mL^{-1}; stirring; 24 ± 1 °C temperature; 80 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ PAR and 16:8 h of light:darkness photoperiod) [18]. Experiments lasted 14 days and were performed at least in triplicate. For analyses (growth measurements excluded), aliquots of cells were collected after 0 (inoculation), 6 (late exponential phase) and 14 days (late stationary phase) of cultivation.

To compare the effect of a different mixotrophic substrate on the lipids produced by the alga, *N. oleoabundans* was also cultivated for 28 days in static conditions in the presence of a diluted apple waste product (AWP; 1:20 dilution in BM medium), as reported in Baldisserotto et al. [14]. For details on AWP preparation and composition see Giovanardi et al. [11]. Aliquots of cells were harvested for lipid analyses at 28 days of cultivation, when lipids were accumulated [14]. Controls in BM medium without AWP were done in parallel [14] and experiments were performed in triplicate.

2.2. Analyses on growth

2.2.1. Growth parameters of microalgae

Control and glucose-treated cells were periodically counted with a Thoma haemocytometer under a light microscope (Zeiss, Mod. Axiophot, Jena, Germany); cell densities were plotted on a logarithmic scale to obtain the growth kinetics. Cell biomass ($\mu g_{DW}\ 10^{-6}$ cells), biomass concentration ($g_{DW}\ L^{-1}$) and biomass productivity ($g_{DW}\ L^{-1}\ d^{-1}$) were calculated on the basis of dry weight of samples collected after 0, 6 and 14 days of cultivation. For dry weight determination, cell samples were treated as reported in Popovich et al. [10].

2.2.2. Nitrate quantification in the culture media

For nitrate concentration analysis, autotrophic and mixotrophic culture media were harvested by centrifugation (2000 *g*, 10 min) after 0, 6 and 14 days of cultivation. Nitrate was quantified colorimetrically using a flow-injection autoanalyser (FlowSys, Systea, Roma, Italy).

2.3. Transmission electron microscopy (TEM)

After 6 and 14 days of cultivation, control and glucose-treated cells were harvested by centrifugation (500 *g*, 10 min) and prepared for transmission electron microscopy as reported in Baldisserotto et al. [25]. Sections were observed with a Hitachi H800 electron microscope (Electron Microscopy Centre, University of Ferrara). Images were employed to calculate the cell volume.

2.4. Analysis of photosynthetic parameters

2.4.1. Photosynthetic pigments extraction and quantification

Aliquots of cell suspensions from both autotrophic controls and glucose-treated cultures were harvested by centrifugation at 8000 *g*, 10 min. Then, pellets were extracted with absolute methanol at 80 °C for 10 min under a dim green light to avoid photo-degradation [14]. The extracts were clarified by centrifugation and analysed with an UV/Vis spectrophotometer (Pharmacia Biotech Ultrospec® 2000) (1 nm resolution). For chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*) and carotenoid (Car) quantification, the extracts were measured at 666 nm (Chl*a*), 653 nm (Chl*b*) and 470 nm (Car) and the equations proposed by Wellburn [26] were applied. Pigments were expressed as percentage of total dry weight (% DW) by dividing pigment content by biomass concentration. Pigment content was also expressed on a cell basis, as $nmol_{pigment}\ 10^{-6}$ cells.

2.4.2. Pulse amplitude modulated fluorimetry (PAM) analyses

For analyses, aliquots of cells from glucose-treated cultures and their controls were collected by centrifugation (8000 *g*, 5 min); then, pellets, drop by drop, were put onto small pieces of wet filter paper (Schleicher & Schuell) [27]. After 15 min of dark adaptation PSII maximum quantum yield [$F_V/F_M = (F_M - F_0) / F_M$] was measured with a pulse amplitude modulated fluorimeter (ADC-OS1-FL, ADC Bioscientific Ltd., Herts, UK). Furthermore, induction/relaxation curves of fluorescence parameters were obtained by applying standard protocols [28]. In detail, the dark-adapted pellets were illuminated with a halogen lamp through a fibre-optic system for 5 min at an irradiance of 1100 $\mu mol_{photons}\ m^{-2}\ s^{-1}$ and a saturation pulse was applied every minute. After the induction phase, pellets were returned to darkness for relaxation and a saturation pulse was applied after 1, 2 and 5 min. In particular, induction/relaxation kinetics were recorded for the actual PSII quantum yield $Y(PSII) = (F_{M'} - F_S) / F_{M'}$ [29], the quantum yield of the regulated energy dissipation $Y(NPQ) = (F_S / F_{M'}) - (F_S / F_M)$ and the combined yield of fluorescence and

constitutive thermal dissipation Y(NO) = (F_S / F_M) [30]. The degree of PSII photo-inhibition was calculated as the non-relaxed fraction of PSII yield after the 5 min of dark relaxation.

2.5. Biochemical analyses

2.5.1. Total protein extraction and quantification

Aliquots of control autotrophic and glucose-treated mixotrophic cells (about 100 mL with an optical density of 0.5 at 750 nm) were centrifuged for 10 min at 500 *g* and treated according to Ivleva and Golden [31], with some modifications. In detail, pellets were resuspended in a small quantity (2 mL) of washing buffer [2 mM Na_2EDTA, 5 mM ε-aminocaproic acid, 5 mM $MgCl_2$, 5 mM dithiothreitol dissolved in PBS buffer 1×; PBS buffer (1 L, stock solution 10×): 80 g NaCl, 2 g KCl, 14.4 g $Na_2HPO_4 \cdot 2H_2O$, 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 the extraction buffer (0.1 M NaOH, 1% sodium dodecyl sulphate, 0.5% β-mercaptoethanol dissolved in distilled water). 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 overnight. 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 centrifugation (1500 *g*, 10 min) supernatants were harvested (I extract). Pellets were re-extracted by resuspending with 0.5 mL of extraction buffer, vortexing tubes for 2 min and finally keeping tubes at 60 °C for 15 min. Samples were then centrifuged (1500 *g*, 10 min) and the supernatant (II extract) was added to the first one. Finally, the total extract was rapidly frozen in liquid N_2 and kept at −20 °C until quantification. Proteins were quantified following the Lowry's method [32].

2.5.2. Total lipid extraction

For both mixotrophic samples (glucose- and AWP-treated algae) at the late stationary phase of growth (14 or 28 days of cultivation for glucose- and AWP-treated samples, respectively), lipid extraction was performed according to a modified Folch's method [33]. Autotrophic samples and algae harvested at the inoculation time were processed as well. In detail, duplicated freeze-dried samples of 200 mg of biomass were vortexed thoroughly for 30 s, ultrasonicated for 30 min at ambient temperature in 25 mL chloroform:methanol (2:1, v:v) and centrifuged (3000 *g*, 5 min) three times. Between each interval, the chloroform:methanol solution (25 mL) in the vial was collected. The mixture of supernatants was placed and shaken in a separatory funnel with 12.5 mL NaCl 0.9% (w/w) to create a biphasic system. The upper phase contains all of the non-lipid substances, while the lower phase contains essentially all the lipids. After a period of time, the lower phase (containing the extracted lipids) was recovered. This procedure was repeated three times to ensure an adequate washing. Then, the lipid extract was evaporated to dryness under nitrogen and kept at −20 °C. All chemicals used were of analytical grade. After a comparison with the modified Weldy and Huesemann's method [34], the modified Folch's method was selected because no significant differences had been found and for its simplicity.

2.5.3. Lipid fractionation and fatty acid profile determination

Lipid fractionation into neutral lipids, glycolipids and phospholipids was performed using a silica Sep-Pack cartridge (SP) of 1000 mg (J. T. Baker Inc., Phillipsburg, N.J., USA), according to Popovich et al. [35]. The efficiency of SP separation was verified by thin layer chromatography (Silica gel G 60 70–230 mesh, Merck, Darmstadt, Germany). New plates were pre-run in a tank containing chloroform:methanol (50:50, v/v) in order to remove contaminants from the silica gel. Concentrated solutions of each fraction in chloroform (10 mg mL^{-1}) were applied to the bottom of the plates and the plates were developed with chloroform:methanol (2:1, v/v). After solvent evaporation, the plates were sprayed with phosphomolybdic acid and heated at 120–130 °C. Fatty acid profile was determined according to Popovich et al. [35], by methyl ester derivation and gas chromatographic (GC) analysis, with a HP Agilent 4890D gas chromatograph (Hewlett Packard Company, USA), equipped with a flame-ionisation detector at a temperature of 260 °C, a split/splitless injector (175 °C) and a capillary column SP–2560 (100 m, 0.25 mm and 0.2 μm; Supelco Inc., Bellefonte, PA).

2.6. Statistical data treatment

Data were processed with Microcal Origin 6.0 software (OriginLab, Northampton, MA, USA). Data of control and glucose-treated samples were compared by using the Student's *t* test (significance level, 0.05). Data are expressed as means ± standard deviations (s.d.) for n number of samples (n ≥ 3, depending on analysis). Asterisks are used to identify the levels of significance: *, $p \le 0.05$; **, $p \le 0.01$; and ***, $p \le 0.001$. For data treatment of results on lipids of control, glucose and AWP samples, ANOVA was applied (significance level, 0.05).

3. Results

3.1. Growth aspects

3.1.1. Growth

Autotrophic and glucose-treated cultures of *N. oleoabundans* showed an evident difference as regards growth kinetics. In fact, mixotrophic cultures were characterised by a very effective exponential phase during the first 6–7 days of cultivation. Then they promptly entered the stationary phase, which lasted approximately a week; the 14th day corresponded indeed to the late stationary/early decline phase of growth (Fig. 1A). In fact, mixotrophic cultures reached a cell density of about 35 and 50 × 10^6 cells mL^{-1} at the 6th and 7th days of cultivation, respectively, and then yielded almost stable cell density values, with a small decrease only at the end of experiment. In spite of being characterised by a similar growth curve in terms of general features, the control samples yielded lower cell densities (about 3 and 8 × 10^6 cell mL^{-1} after 6 and 14 days of cultivation, respectively) (Fig. 1A). On the whole, two growth points were highlighted: the 6th day, corresponding to the late exponential phase, and the 14th day, corresponding to the late stationary phase. Interestingly, the glucose-treated samples, starting from the same initial biomass of about 0.07 g_{DW} L^{-1} as the autotrophic ones, very strongly increased their biomass especially during the 6–14 day time interval, from about 0.4 to nearly 1.8 g_{DW} L^{-1} (Fig. 1B). On the contrary, during the same time interval, control samples increased their biomass from about 0.10 to only 0.25 g_{DW} L^{-1}, which was parallel to the cell density increase (Fig. 1A, B). Moreover, it was interesting to note that, after 6 days of experiment, treated cells were lighter than their controls, but also than the mixotrophic cells after 14 days (about 10 μg_{DW} 10^{-6} cells for 6-day-mixotrophic cells vs more than 30 μg_{DW} 10^{-6} cells for controls and 14-day-mixotrophic cells), while the controls maintained stable values of about 30–35 μg_{DW} 10^{-6} cells throughout the experiment (Fig. 1C). Noteworthy was the biomass productivity, which was greatly enhanced in the treated samples as compared to controls at both examined time points (Fig. 1D).

3.1.2. Nitrate content in culture media

Nitrate in the culture media was about 1.43 mM for both autotrophic and mixotrophic cultures at the beginning of experiment. Media harvested from control cultures underwent a small decrease in nitrate content during experiment (17% from 0 to 14 days of cultivation) reaching values of about 1.36 and 1.18 mM at the 6th and 14th days of experiment, respectively. Differently, media of glucose-treated cultures were characterised by values of nitrate content near zero yet after 6 days of cultivation (0.034 and 0.004 mM at days 6 and 14, respectively).

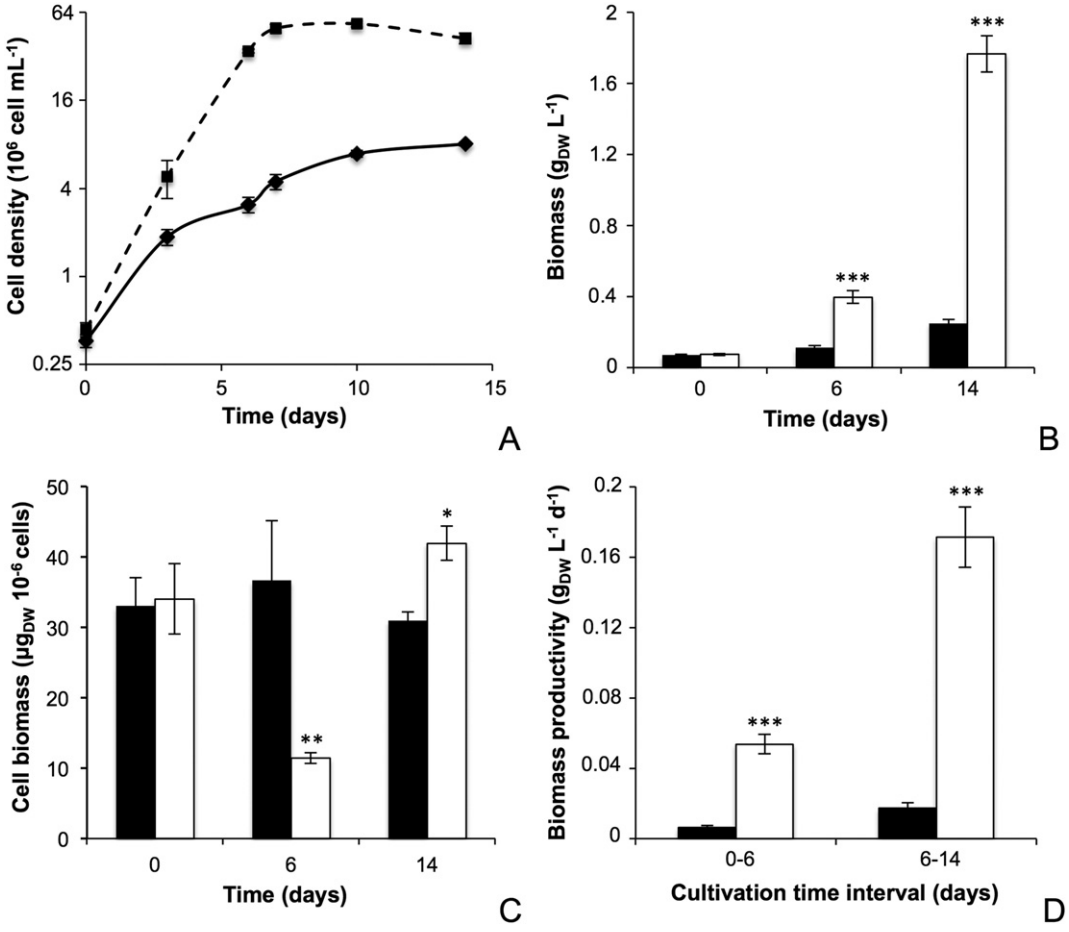

Fig. 1. Growth parameters of *N. oleoabundans* cultivated under autotrophic and mixotrophic (glucose-induced) conditions for 14 days. A) Growth kinetics plotted using a logarithmic scale. B) Biomass yield expressed as grams of algal dry weight per litre. C) Single cell biomass expressed as micrograms of algal dry weight per one million cells. D) Biomass productivity, as grams of algal dry weight per litre per day, during the 0–6 days and 6–14 days of cultivation intervals. In A, solid black line = autotrophic cultures; dash black line = mixotrophic cultures. In B–D, black histograms = autotrophic cultures; white histograms = mixotrophic cultures. Data refer to means ± standard deviations ($n \geq 3$). Asterisks identify significant differences between control and mixotrophic samples: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

3.2. Morphology: cell ultrastructure

Fig. 2 reports images of ultrastructural aspects of *N. oleoabundans* cells after 6 (Fig. 2A–D) and 14 (Fig. 2E–H) days of cultivation under autotrophic (Fig. 2A, E) and mixotrophic conditions (Fig. 2B–D, F–H). In detail, TEM analyses showed that 6-day-grown control cells were typically characterised by a big cup-shaped chloroplast containing a large pyrenoid surrounded by a starch shell and crossed by one–two thylakoids (Fig. 2A). Inside the cytoplasm, the nucleus and mitochondria were also observable (Fig. 2A). After 14 days, controls maintained similar features, but cells were sometimes vacuolated (Fig. 2E). In both cases, thylakoids were elongated and only locally appressed (Fig. 2A, E). Different aspects were observed for treated cells (Fig. 2B–D, F–H). In fact, after 6 days of cultivation, the mixotrophic populations were generally characterised by smaller cells than in the controls (about 28% in volume; $p < 0.05$) and by morphologically different cells according to a kind of gradient (Fig. 2B–D). In fact, some cells showed an overall morphology similar to that of controls, but with an evident increase in stromatic starch and more extensive thylakoid appression (Fig. 2B), while other cells contained plastids very enriched in starch, which occurred both as stromatic and as a shell surrounding the pyrenoid that had assumed an altered feature (Fig. 2C, D). Only sometimes cells with the latter feature contained small cytoplasmic lipid globules (Fig. 2C, D). Also in these cells, thylakoid membranes appeared to be more appressed than in controls (Fig. 2A, C, D). Sporadic sporocysts, which had not yet released the young cells, were still observable in glucose-treated samples (Fig. S1). At the 14th day of cultivation, glucose-treated cells became larger than at day 6 (about 50% in volume; $p < 0.001$) and reached, sometimes slightly exceeding, the dimensions of the control cells ($p > 0.05$) (Fig. 2E–H). As for cells at the previous step of cultivation, a morphological gradient was observable. Some cells, in fact, contained a chloroplast with large stromatic starch grains and a still identifiable pyrenoid surrounded by an evident starch shell; lipid globules increased in number and size (Fig. 2F, G). Other cells contained a few small starch grains, but very large lipid droplets (Fig. 2H). As regards thylakoids, they were reduced in number and extension as compared to those contained in treated samples at the previous time of experiment (Fig. 2B–D) and to those hosted in controls (Fig. 2E); however, these few thylakoids were less appressed than at the previous time of experiment, but similar to those in controls (Fig. 2F, G).

3.3. Photosynthetic aspects

3.3.1. Photosynthetic pigments

Photosynthetic pigment content was expressed both as a fraction of total biomass and in terms of quantity of pigment inside cells (Fig. 3). It was noted that, during the time interval 0–6 days of cultivation, the concentrations of all pigments inside autotrophic control samples tended to decrease (ca. 25 to 60% depending on the pigment), while from 6 to 14 days of cultivation their pigments tended instead to increase independent of the unit of measure employed (ca. 1.5 to 2

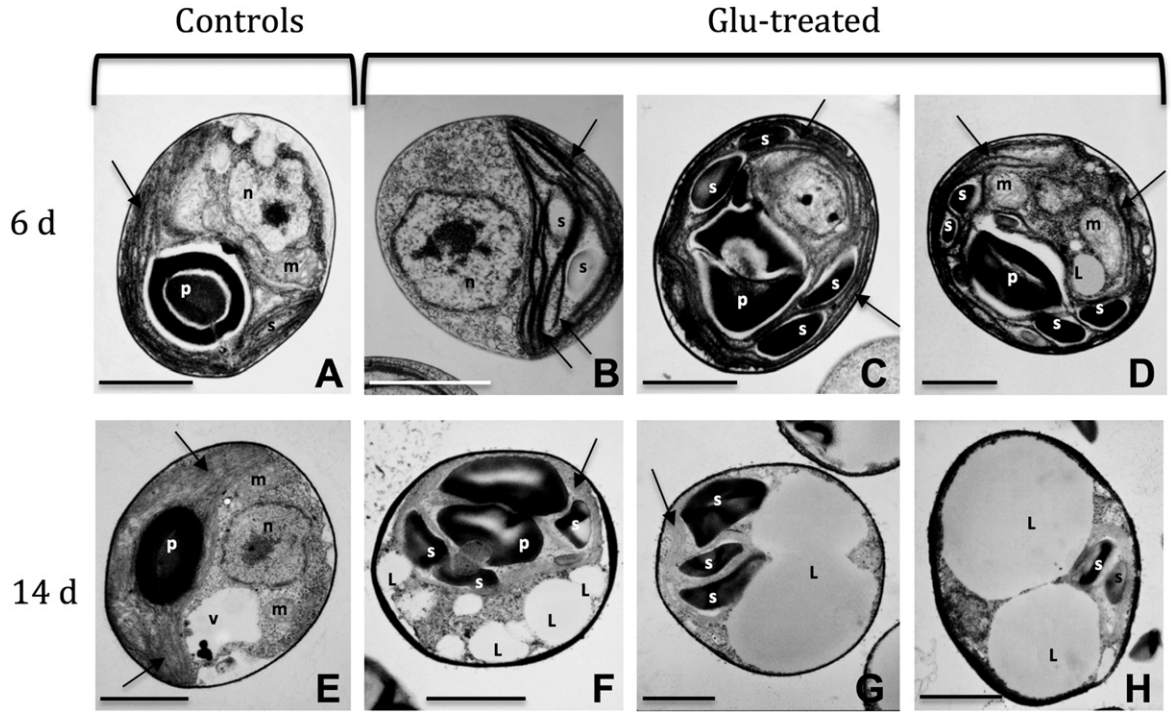

Fig. 2. TEM images of *N. oleoabundans* cells after 6 (Fig. 2A–D) and 14 (Fig. 2E–H) days of cultivation under autotrophic control (Fig. 2A, E) and mixotrophic conditions (Fig. 2B–D, F–H). p, pyrenoid; m, mitochondrion; n, nucleus; v, vacuole; s, stromatic starch; L, lipid droplets; arrow, thylakoids. Bars: 1 μm.

times, Fig. 3A–F). Differently, such trends were not observed for treated samples. In detail, when considering the pigment content per cell (Fig. 3B, D, F), an evident decrease was observed throughout the experiment: 64, 68 and 77% decrease for Chl*a*, Chl*b* and Car, respectively, from 0 to 6 days, and a further significant, though smaller, decrease by 37 ($p < 0.001$) and 27% ($p < 0.01$) for Chl*a* and Chl*b*, respectively, during the time interval 6–14 days of mixotrophic cultivation. In the latter interval, no differences were observed for the Car content ($p = 0.569$). The pigment content expressed as % DW, so on a biomass basis, did not show a similar trend (Fig. 3A, C, E). During the first time interval (0–6 days), Chl*a* content increased significantly by about 30% ($p < 0.001$) and Chl*b* by about 12% ($p < 0.05$), while Car decreased by about 26% ($p < 0.001$); only during the second time interval (6–14 days), all pigments strongly decreased (about 4 times less Car and nearly 6 times less Chl; $p < 0.001$ in all cases). Concomitantly, in comparison with controls all pigments were less abundant in treated cells on a cell basis (40–80% depending on the pigment; $p < 0.001$), while, on a biomass basis, in glucose-treated algae an increase by about 40–45% ($p < 0.001$) occurred at day 6 and was followed by a strong decline (about 80%; $p < 0.001$) at the 14th day.

Photosynthetic pigments, however, maintained a quite stable stoichiometry, the Chl*a*/Chl*b* molar ratio was indeed substantially unchanged between samples (differences <10%; $p = 0.08$ at all three experimental times considered). Total Chls/Car ratio was significantly lower only at the end of the experiment, showing a 41% difference between treated and control algae ($p < 0.01$) and a 48% difference when mixotrophic algae were compared between times 6 and 14 days ($p < 0.01$) (Fig. 3G, H). In fact, at the 6th day, the 20% difference in the ratio between control and treated samples was not significant ($p = 0.098$).

3.3.2. PSII fluorescence analyses

The effects on the use of light energy of *N. oleoabundans* cultivated mixotrophically or autotrophically were evaluated by PAM fluorimetry.

Starting from values of the PSII maximum quantum yield (F_V/F_M) around 0.600 at the beginning of experiment, both autotrophic and mixotrophic samples were characterised by increased F_V/F_M ratios at the 6th day of cultivation. The value was significantly higher in glucose-treated cells than in autotrophic controls (+12%; $p < 0.001$), 0.764 and 0.674 respectively (Fig. 4A). Differently, at the 14th day of experiment, F_V/F_M value in mixotrophic algae decreased to 0.608, i.e. 13% lower than that recorded for controls ($p < 0.05$), which instead maintained stable values during time. In parallel, these differences in PSII efficiency were accompanied by interesting photoinhibition data (Fig. 4B). In fact, exposure to high light (1100 $\mu mol_{photons}\ m^{-2}\ s^{-1}$) did not influence the photosynthetic efficiency of the treated samples, which gave not significantly different responses from those of controls after both 6 days (lower value, but $p = 0.132$) and 14 days of cultivation (higher value, but $p = 0.072$) (Fig. 4B). Furthermore, a study on the use of the light energy absorbed by the algae was performed after 6 and 14 days of cultivation, separating the three fractions in which energy itself is converted: Y(PSII), Y(NO) and Y(NPQ) (Fig. 4C–H). As regards Y (PSII), i.e. the operating PSII efficiency in the light, in mixotrophic algae it was about 50% higher as compared to controls at the 6th day ($p = 0.032$), but strongly decreased at the end of experiment, showing values more than 3 times lower than that of controls ($p = 0.015$) (Fig. 4C). The corresponding induction/relaxation kinetics detailed better this response, showing the strong impact of light on old mixotrophic cells, which had Y(PSII) values around 0.100 during the high light exposure (Fig. 4D). In addition, Y(NO), i.e. the energy constitutively dissipated as heat or fluorescence emission by non-functional and closed PSII, was significantly lower in treated cells after 6 days of cultivation ($p = 0.023$), but increased, even if not significantly ($p = 0.077$), at the 14th day (Fig. 4E). In the induction/relaxation curves, controls at both times of cultivation and glucose-treated cells at the 6th day were characterised by a relative stability during light exposure, while mixotrophic cells at the late stationary phase of growth showed a decreasing trend of Y(NO) values already starting from the first minute of high light exposure (Fig. 4F). Finally, Y(NPQ) values, which represent

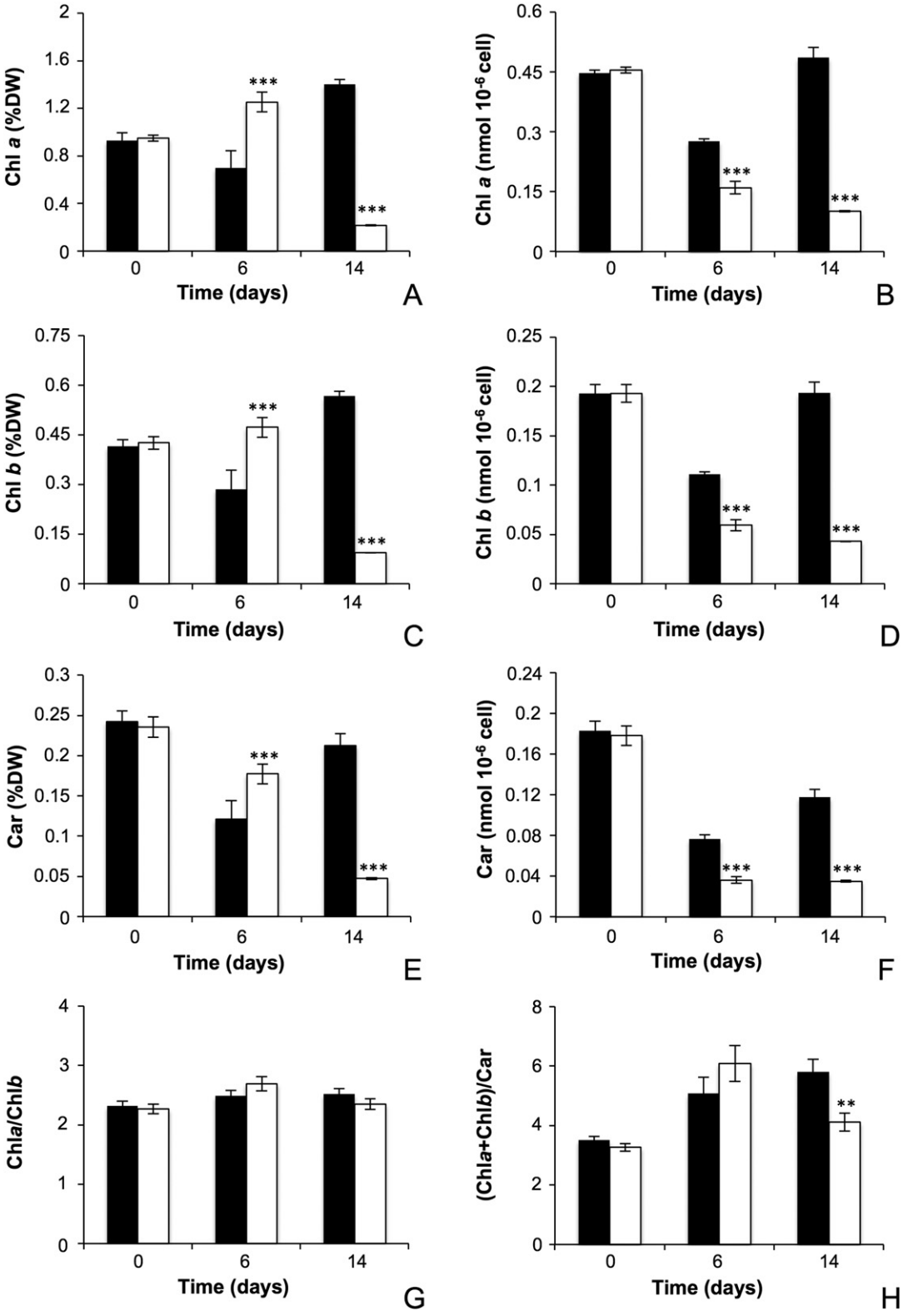

Fig. 3. Photosynthetic pigments content and their molar ratios in control and glucose-cultivated *N. oleoabundans* cells at the inoculum time (0 days), the late exponential (6 days) and late stationary (14 days) phases of growth. Pigment concentrations are reported both as percentage of dry weight (% DW) (A, C, E) and as nanomoles per million of cells ($nmol_{pigment}$ 10^{-6} cells) (B, D, F). Black histograms = autotrophic cultures; white histograms = mixotrophic cultures. Data refer to means ± standard deviations (n ≥ 3). Asterisks identify significant differences between control and mixotrophic samples: **, p ≤ 0.01; ***, p ≤ 0.001.

light-dependent dissipation mechanisms for energy dissipation as heat, were substantially the same in control and mixotrophic cells at the 6th day of cultivation, but were significantly higher ($p = 0.041$) in treated cells vs controls at the 14th day (Fig. 4G). Interestingly, the corresponding kinetics maintained quite similar features in both controls and in 6-day mixotrophic cells, reaching a plateau during the light exposure, which conversely did not characterise the glucose-treated cells after 14 days of cultivation (Fig. 4H).

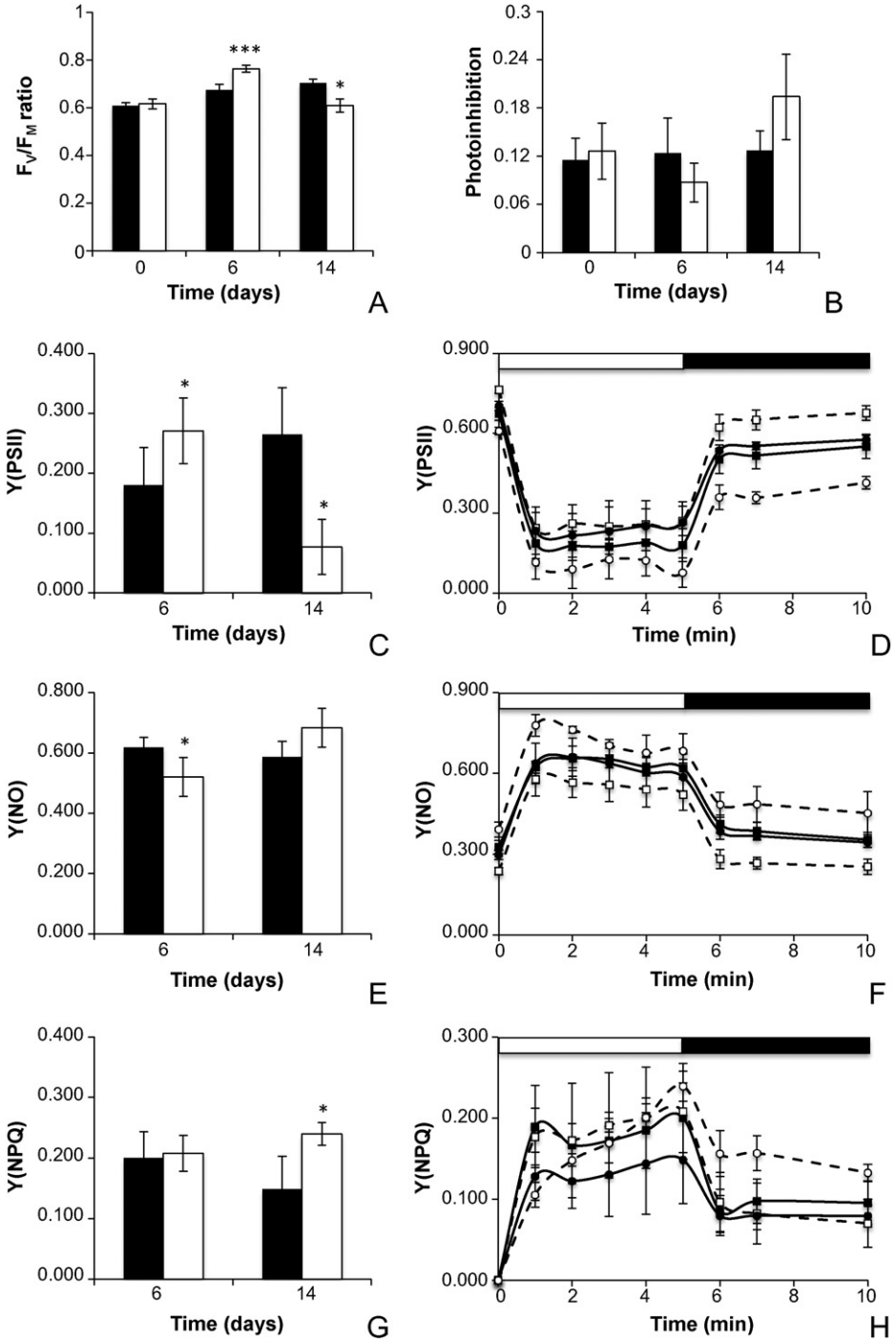

Fig. 4. Chlorophyll fluorescence parameters of control and glucose-cultivated *N. oleoabundans* cells at the inoculum time (0 days), the late exponential (6 days) and late stationary (14 days) phases of growth. A) PSII maximum quantum yield (F_V/F_M). B) Photoinhibition values. C, D) Actual yield of PSII, Y(PSII); E, F) yield of constitutive thermal dissipation and fluorescence emission, Y(NO); and G, H) yield of non-photochemical quenching, Y(NPQ). In C, E and G yields are expressed, while D, F and H report the corresponding induction/relaxation kinetics. In A–C, E and G, black histograms = autotrophic cultures; white histograms = mixotrophic cultures. In D, F and H, solid black line = autotrophic cultures at 6 (black squares) and 14 days (black circles) of cultivation; dash black line = mixotrophic cultures at 6 (open squares) and 14 days (open circles) of cultivation; white rectangle on the top = 5 min high light exposure (induction phase); black rectangle on the top = 5 min dark exposure (relaxation phase). Data refer to means ± standard deviations (n ≥ 5). Data refer to means ± standard deviations (n ≥ 5). Asterisks identify significant differences between control and mixotrophic samples: *, $p \le 0.05$; ***, $p \le 0.001$.

3.4. Biochemical properties of algae useful for biotechnological application

3.4.1. Total proteins

With an initial total protein content of about 13% DW (corresponding to 7.5 μg 10^{-6} cells), at the 6th day of cultivation, in the whole mixotrophic population proteins, expressed as percentage on DW, was about 3 times higher than in controls ($p < 0.001$) (Fig. 5A). However, at the same cultivation time, the amount of proteins accumulated inside cells of control and glucose-treated samples was not significantly different (Fig. 5B) ($p = 0.49$). Conversely, at the late stationary phase of growth, i.e. at the 14th day, total proteins were unequivocally lower in treated samples as compared to controls, both considering the single cells and the biomass (43–60% depending on the considered parameter) ($p < 0.05$) (Fig. 5). An evident decrease in

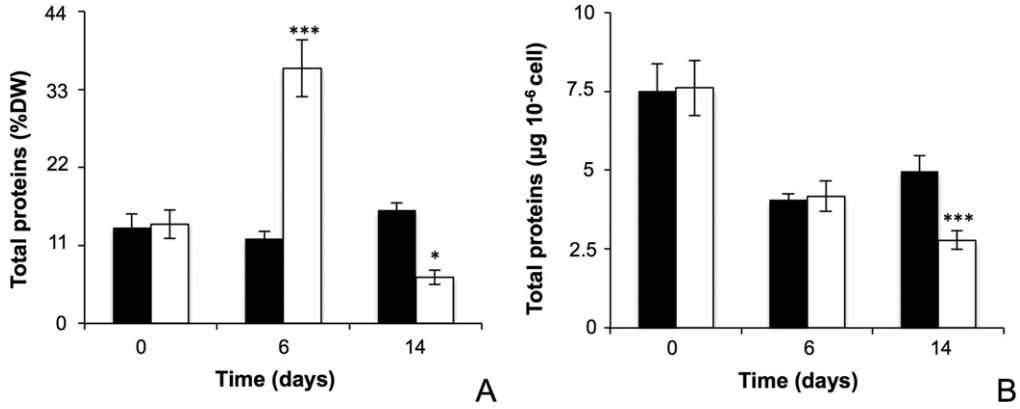

Fig. 5. Total proteins content in control and glucose-cultivated *N. oleoabundans* cells at the late exponential (6 days) and late stationary (14 days) phases of growth. Protein concentrations are reported both as percentage of dry weight (% DW) (A) and as micrograms per million of cells (μg 10^{-6} cells) (B). Black histograms = autotrophic cultures; white histograms = mixotrophic cultures. Data refer to means ± standard deviations (n ≥ 3). Asterisks identify significant differences between control and mixotrophic samples: *, p ≤ 0.05; ***, p ≤ 0.001.

protein content was also observed by comparing treated samples after 6 and 14 days of cultivation, irrespective of parameter through which such amount was expressed ($p < 0.001$).

3.4.2. Lipid quantification and characterisation

Starting from samples characterised by a total lipid content of about 14–16% DW, further analyses were performed at the 14th day of cultivation, when mixotrophic cells were full of lipid globules (Fig. 2F–H). For comparison of lipid production and quality of *N. oleoabundans* under mixotrophic conditions, lipids were extracted and thoroughly analysed at the late stationary phase of growth by using both glucose-treated algae, supported by the morphological observations described in this paper (Fig. 2), and mixotrophic algae cultivated in the presence of an apple waste product (AWP), according to a previous work [14]. Table 1 shows the lipid composition (% DW) of *N. oleoabundans* under control and mixotrophic conditions. The total lipid content under control conditions was 20.3% DW and increased significantly ($p < 0.05$) up to 27.06% DW and 27.59% DW in cells grown with glucose and AWP, respectively (Table 1). Neutral lipids increased significantly in mixotrophy (Table 1), reaching up to ca. 76% and ca. 71% of total lipids under glucose and AWP conditions, respectively, TAGs being the only source of fatty acids. Neither diacylglycerols nor monoacylglycerols were detected.

The fatty acid profiles of *N. oleoabundans* grown under control and mixotrophic conditions are shown in Table S1. The most important fatty acids were the saturated palmitic (C16:0), the monounsaturated oleic (C18:1n-9c) and the polyunsaturated linoleic (C18:2n-6c) and linolenic (C18:3n-3) acids (Table S1; Fig. 6A). However, the lipid classes showed differences in their proportions (Table S1; Fig. 6B), especially in the neutral fraction. The percentages of saturated fatty acids (SFAs) were significantly higher in cultures grown with glucose (ca. 27.6%) and AWP (ca. 33.6%) when compared to control conditions (ca. 22%). The monounsaturated fatty acids (MUFAs) were the major class under mixotrophy. They were significantly higher ($p < 0.05$) than in controls, reaching the maximum average value (ca. 55.5%) in the glucose-treated cells because of a high content of oleic acid (ca. 53%), while for the AWP-cultured algae they remained at slightly lower values (MUFA, 36.7%; oleic acid, 32%). Regarding polyunsaturated fatty acids (PUFAs), there was a significant decrease in the mixotrophic condition as compared with the control one (Table S1; Fig. 6B). In particular, PUFA levels decreased significantly owing to a decline in the proportion of linolenic acid, from ca. 19% in controls to ca. 2% in glucose medium and to 9% in AWP medium (Table S1; Fig. 6B).

Table 1
Total lipid and lipid fractions – neutral lipids, glycolipids and phospholipids – (in percentage of dry weight biomass = % DW) of *N. oleoabundans* growing under different culture conditions (control, glucose, AWP). Values are means ± standard deviations of two or three replicates. Differences were not significant ($p > 0.05$) for groups with the same superscript.

Conditions	Total lipids (% DW)	Neutral lipids (% DW)	Glycolipids (% DW)	Phospholipids (% DW)
Control	$20.30^{a} \pm 0.54$	$10.62^{c} \pm 0.66$	$7.13^{e} \pm 0.33$	$2.55^{g} \pm 0.21$
Glucose	$27.06^{b} \pm 0.63$	$20.55^{d} \pm 1.99$	$4.04^{f} \pm 0.85$	$2.47^{g} \pm 0.55$
AWP	$27.59^{b} \pm 2.47$	$19.51^{d} \pm 1.91$	$6.86^{e} \pm 1.06$	$1.44^{h} \pm 0.43$

4. Discussion

N. oleoabundans is widely considered an important microalga to be potentially used as a green feedstock of lipids for biofuel production [8,10,23,24,36,37]. An interesting opportunity is given by the mixotrophic behaviour of the alga, which both promotes biomass

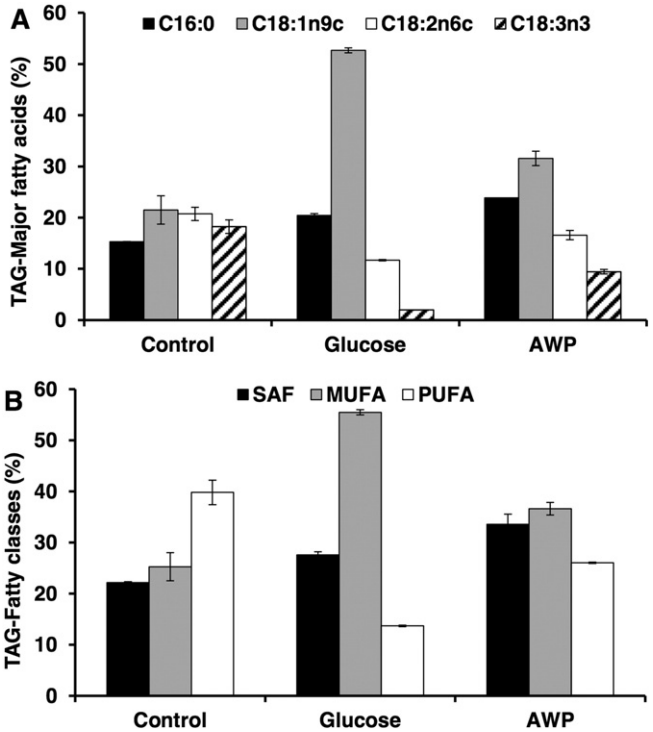

Fig. 6. Data on lipid analyses on control and mixotrophic cultures (glucose- and AWP-cultured cells) of *N. oleoabundans* at the late stationary phase of growth. A) Major fatty acids (in percentage of total fatty acids = %) in the TAG fraction. B) Relative proportions of fatty acid classes (SFA, MUFA and PUFA in %) in the TAG fraction. In A and B, the values presented are means ± standard deviations of 4 replicates.

productivity, combining photosynthesis with sugar uptake, and lipid accumulation [11,14,18]. For the biotechnological exploitation of the alga, we investigated the link between photosynthesis, biomass productivity and lipid synthesis.

An interesting aspect emerging from the mixotrophic growth kinetics seemed to be its apparent discrepancy with biomass productivity, since the higher cell density observed at 6–7 days for mixotrophic algae compared to controls (around 10–12×) was accompanied by relatively low values of biomass (Fig. 1). However, this is justified by some characteristics of the single cell biomass and size. In fact, 6-day mixotrophic cells were very numerous, but lighter and smaller than control cells, while 14-day mixotrophic cells were again very numerous, but heavier and bigger (Fig. 1A, C). Accordingly, the life cycle of *N. oleoabundans* is characterised by the release of young small cells from sporocysts and their subsequent maturation to larger cells [38,39]. Our results support the inference by de Winter et al. [40] that the cell cycle of *N. oleoabundans* plays an important role in biomass production owing to the differences in cell morphology occurring during the life cycle. We extend this observation to the mixotrophic mode of cultivation. We observed that after 6 days of cultivation with glucose, the cells consumed almost all nitrogen in the culture medium for their duplication and, at the same time, they also absorbed and used part of the glucose [18]; subsequently, they increased their size exploiting the uptake of the glucose still available in the medium, the starch consumption and the photosynthetic activity to produce carbohydrates and lipids, i.e. N-free molecules. By comparison, autotrophic algae consumed only a low quantity of nitrate up to 14 days, as also reported in a recent work on *N. oleoabundans* [41]. Moreover, in mixotrophic glucose-treated cells after 6 days of cultivation, the protein content per cell was similar to that of controls, a sign of a sufficient supply of nitrogen in the culture medium and of its efficient employment by cells. Concomitantly, actively photosynthesizing cells showed a very large pyrenoid surrounded by starch shells linked to RuBisCO activity, but accumulated also many non-photosynthetic stromatic starch granules [42]. Therefore, the cells, during the first phase of cultivation, employed exogenous glucose in the culture medium to produce starch as a storage of exceeding reducing power and carbon skeletons [43,44]. Subsequently, they gradually started to use the carbon deriving from starch degradation to produce lipids. The metabolic pathways of starch and lipids share, in fact, common precursors [44]. This behaviour was also found in *N. oleoabundans* cultivated with AWP as a mixotrophic substrate [11,14].

Interestingly, as regards specific photosynthetic aspects highlighted by ultrastructural analyses, the plastids of 6-day mixotrophic cells were characterised by a strong appression of thylakoids, probably due to a new effective set-up of thylakoid membranes, which ensured an ability of photochemical energy conversion even higher than in controls, as attested by F_V/F_M ratio (Fig. 4A). Interestingly, this seemed not to be linked to a quantitative variation in LHCII antennae, in fact, Chl*a*/*b* molar ratio, which reflects LHCII amount [45–47], was substantially unchanged between control and glucose-treated samples (Fig. 3G). The analyses of photosynthetic pigments also gave further information. In fact, a significant decrease in the photosynthetic pigment content per cell, in line with previous observations [18], was detected. Such decrease, which was not associated with important variations in the stoichiometry of pigments, testifies to a switch of the metabolism from autotrophic to mixotrophic. Many microalgae, including those belonging to *Neochloris* genus, reduce their photosynthetic pigment content under mixotrophy, i.e. under a cultivation condition less influenced by light availability than autotrophy [11,19,48,49]. However, by expressing pigment accumulation as % DW, after 6 days of cultivation, the mixotrophic algal biomass contained larger amounts of pigments compared to controls, making mixotrophic *N. oleoabundans* an interesting candidate in applicative activities as a source of coloured molecules, i.e. chlorophylls and carotenoids to be employed as pigments and/or antioxidants [50]. The high quantity of pigments found in the mixotrophic biomass was related to the specific cell characteristics at 6 days: each mixotrophic single cell contained a small quantity of pigment (because cells were indeed small), but the whole algal biomass was rich in pigments (because cells were very numerous). After the duplication phase, the entrance of cells in the stationary phase of growth was accompanied by events repeatedly reported, such as decrease in pigment content, decrease in photosynthetic activity, reduction of thylakoid system and, concomitantly, lipid production [11,14,16,18–21,39].

From analyses, it was clear that mixotrophy had a very strong impact on the organisation of the photosynthetic apparatus in *N. oleoabundans*. Important modulations of the photosynthetic activity appeared to follow both the switch of metabolism to mixotrophy and the transition to the lipid production phase. This presupposes a different availability of reducing equivalents in different phases and a very modulable use of light energy depending on the growth phase. This hypothesis was tested through an energy partitioning approach, i.e. calculating the quantum yields of PSII photochemistry (Y(PSII)) and competing regulatory (Y(NPQ)) and non-regulatory (Y(NO)) dissipative processes [51]. Values of Y(PSII), Y(NO) and Y(NPQ) after 6 days of cultivation indicate that mixotrophic cells "work" even better than controls during a high light exposure. In particular, they are able to maintain a high Y(PSII) by keeping Y(NO) at a low value. Y(NO) can be used as a simple index of the reduction state of plastoquinones in the photosynthetic membranes [52]. To ensure a good preservation of photosystems under high light, plants, algae included, aim at minimizing the reduction state of plastoquinone [53]. From this point of view, mixotrophic cells succeeded in keeping the photosynthetic electron transport chain under control better than autotrophic cells. The biochemical reason for this is unknown, but it evidently results in the increased thylakoid appression observed with TEM. The very active photosynthesis allowed cells to sustain all cell syntheses and possibly also the uptake of glucose from the medium and its temporary storage as stromatic starch. However, it should be noted that in static mixotrophic *N. oleoabundans*, cultivated with a glucose-rich waste (AWP), the Y(PSII) was also higher than in autotrophic cultures, but this was linked to a different modulation of energy use [more Y(NPQ)], showing that different culture conditions play important roles in the photosynthetic metabolism under mixotrophy [14]. As expected, a drastic drop in Y(PSII) occurred during the stationary phase of growth. However, the decay in Y(PSII) was not mainly the consequence of more reduced plastoquinones; this was testified by a non-significant increase in Y(NO) at the steady state. Conversely, mixotrophic cells emphasised their ability to safely dissipate the excess of energy as attested by increased Y(NPQ) [51]. While for autotrophic samples and for 6-day mixotrophic cells the generation of Y(NPQ) was dominated by a rapid induction of the ΔpH-dependent quenching (qE) [54], in 14-day mixotrophic algae a different situation occurred. In fact, in the mixotrophic cells at the stationary phase of growth, the fast induction of qE was followed by a second, evident induction phase. Such induction was slower but progressive up to the end of the light exposure and can be attributed to a qZ quenching dependent on the production of the dissipative carotenoid zeaxanthin [54]. qZ corresponds to a component of thermal dissipation, characterised by a slower induction in the light as compared to qE, i.e. in the order of some minutes [54]. It is attributed to the conversion of the carotenoid violaxanthin to the dissipative carotenoid zeaxanthin, a process triggered by ΔpH that enhances the potential of photoprotection of the thylakoid membrane [54]. Our finding was consistent with the lower Chl/Car molar ratio recorded at the end of experiment. In other words, after the consumption of nitrogen, but with glucose still available [18], *N. oleoabundans* cells down-regulated PSII activity, but were still capable of preserving the photosystem from photoinhibition. In this way, photosynthesis could continue to cooperate in providing energy to the growing cells. The availability of reducing power and ATP, generated by photosynthesis and respiration, not only allows cell enlargement, but also triggers the shift from metabolism to the lipid synthesis. It is interesting to observe that, in a previous work,

AWP was also able to induce lipid synthesis, but this was associated with a higher reduction of the membrane carriers and with a down-regulation of Y(NPQ) [14]. So, the hallmark of lipid synthesis induction is presumably the decay in Y(PSII). It is not known if a different partitioning of energy has an impact on lipid profile.

In the present study, the total lipids in mixotrophic cultures of *N. oleoabundans* increased as compared to controls as a result of TAG accumulation. However, a minor lipid content in glucose-treated cells was observed by comparison with the content reported by Giovanardi and co-workers [18], probably because of the variability of the experiments. No previous data are available for AWP. Yang et al. [55] found for *Chlorella pyrenoidosa* C-212 that the supplied energy (light and glucose) was not utilised efficiently in the mixotrophic cultivation due to a decrease in pigment content of the cells. Despite these differences, our results indicated that, under mixotrophy, the cells channelise the excess of carbon and energy into TAGs predominantly made of saturated and monounsaturated fatty acids, which represent up to 83% and 70% of the total fatty acids in glucose and AWP treatments, respectively.

For biodiesel purposes, fatty acid profiles rich in SFAs and MUFAs, which can be transesterified to produce biodiesel [56], are the most interesting profiles. Structural lipids typically have high PUFA contents, which are employed for pharmaceutical or food applications. In general, SFA production is favoured under heterotrophic conditions, while high PUFA (C16:3 and C18:3) contents are mainly produced under autotrophy [57]. According to their behaviour patterns, mixotrophy could be interpreted as an intermediate alternative, since both light and glucose are sources for ATP production in mixotrophic cultures [55]. In this study, the concentration of TAGs, enriched in SFAs and MUFAs, increased significantly under mixotrophy, while PUFAs decreased. As reported by Knothe [58], the fatty acid profiles, enriched in MUFAs and, particularly, in oleic acid improve the quality of biodiesel helping to balance its oxidative stability and cold flow properties. In the present study, at the stationary phase of growth the cultures supplemented with glucose showed the highest MUFA percentage owing to levels of oleic acid up to 53% of total fatty acids. This value is higher than others reported in *N. oleoabundans* under N-stress conditions (e.g., 36% [8] and 46.5% [10] of oleic acid in neutral lipid fraction). On the other hand, oleic acid reached up to ca. 32% in AWP-cultured cells at the late stationary phase. The differences observed in SFA and MUFA proportions between the mixotrophic treatments may be due to the carbon source that was used. On the whole, the total amount of organic carbon made available by the AWP, was estimated to be 3.35% (w/v), glucose, fructose and sucrose being the carbohydrates present in AWP [11]. Morales-Sanchez et al. [37] reported that *N. oleoabundans* did not use sucrose or fructose for metabolism under strict heterotrophic conditions. Thus, our species growing in AWP under mixotrophy may present a limited carbon source to synthesise MUFAs, which require more energy for their synthesis. Glucose possesses more energy content per mole compared with other substrates [57]. A limited ability to synthetize MUFAs could also have an impact on the photosynthetic membranes. The pattern of glycolipids, which characterise thylakoids, remained similar in AWP and controls, while it changed in favour of MUFAs in glucose-grown cells. Only the latter proved to be permissive for a sufficient photo-protection, which was not achievable in AWP-grown algae to the same extent and resulted in a higher reduction state of the membranes [14]. Therefore, the oils accumulated in *N. oleoabundans*, grown in the presence of glucose as the only carbon source, are enriched in oleic acid and exhibit a combination of improved fuel properties with emphasis in cold flow issue.

Regarding PUFAs, the European EN 14214 standard limits linolenic acid's methyl ester (ALA) for vehicle use to 12% (w/w) and the methyl esters with four and more double bonds to a maximum of 1% (w/w). These limits are essential to avoid autoxidation deriving from the presence of double bonds in the chains of many unsaturated fatty acid methyl esters (FAMEs), which cause problems during fuel storage [59]. In this study, the levels of PUFAs in *N. oleoabundans* were lower under mixotrophic rather than autotrophic conditions. For example, the SFA + MUFA/PUFA ratios were 1.19, 6.07 and 2.69 under control, glucose and AWP treatments, respectively, indicating that the minimum PUFA levels and particularly the ALA ones (ca. 2%) were obtained in cells grown with glucose. Although the oils extracted from mixotrophic cultures of *N. oleoabundans* presented ALA contents within specifications, the oils that come from cultures growing in glucose obtained the best performance in terms of oxidative stability. The reduction of both thylakoid membranes and photosynthetic pigments (Chl*a*, Chl*b* and carotenoids) during mixotrophy observed in this study may partially explain the PUFA decrease. In addition, the sampling times may also have had an influence on the proportions of lipid classes, since, as an example, Shishlyannikov et al. [60] reported a different lipid profile in different growth phases in the diatom *Synedra acus*.

We found that mixotrophic cultivation of *N. oleoabundans* with glucose promotes biomass yield, which can be used in different fields since the chemical composition of the biomass differs after different times of cultivation. The creation of a reducing environment due to the photosynthetic activity, together with alterations of N:C ratio, promoted lipid synthesis. Interestingly, lipid profile and photosynthetic properties are substrate-dependent, glucose being linked to the best oil profile for biodiesel production.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2016.03.022.

Author contributions

Conception and design: CB, SP
Analysis and interpretation of the data: CB, CP, SP, PL, LF, MG
Drafting of the article: CB, CP, SP
Critical revision of the article for important intellectual content: SP, PL
Technical support: MG, LF, AS, DC
Collection and assembly of data: CB, CP, AS, DC, MG.

Aknowledgements

This work was financially supported by grants from the Consorzio Universitario Italiano per l'Argentina (CUIA); the University of Ferrara, Italy; the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) de la República Argentina, PIP 112-200801-00234 and the Secretaría de Ciencia y Tecnología de la Universidad Nacional del Sur, PGI TIR. We are grateful to Dr. Roberta Marchesini of the University of Ferrara for nitrate quantification.

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