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Haematococcus pluvialis as a source of fatty acids and phytosterols: potential nutritional and biological implications

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Abstract The main aim of this work was to describe the effect of light-induced stress on bioactive lipid accumulation in *Haematococcus pluvialis* CCALA 1081. To this end, the lipid profile was analyzed in order to determine triacylglyceride (TAG) and phytosterol content, the fatty acid profile of TAGs and the composition of the sterol fraction. After 3 days of light-induced stress, the content of both bioactive lipids significantly increased compared to controls. Palmitic, linoleic, and α linolenic fatty acid content was higher whereas caproic acid content diminished in *H. pluvialis* under stress. High irradiance also reduced β-sitosterol content whereas chlerosterol, brassicasterol, and Δ7-campesterol content was higher in the phytosterol fraction. No significant levels of lipid peroxidation were detected after 3 days of light-induced stress. In addition, *H. pluvialis* phytosterols displayed radical scavenging capacity and also a cytotoxic effect associated with the induction of apoptosis in human IMR-32 neuroblastoma cells. Our results show that high light stress induced the accumulation of commercially valuable compounds in *H. pluvialis* that are of potential benefit to human health.

Keywords *Haematococcus pluvialis* · Triacylglycerides · Phytosterols · Biological activity

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Introduction

In the last few years, interest has surged in microalgae as natural sources of commercially produced high-value bioactive compounds such as proteins, vitamins, pigments, among others (de Jesus Raposo et al. 2013). *Haematococcus pluvialis* is a green unicellular biflagellate oleaginous microalga considered to be the best natural source of the red pigment and potent antioxidant astaxanthin (Boussiba et al. 1999; Grünewald et al. 2001; Boussiba 2000). Accumulation of this carotenoid is correlated with triacylglyceride (TAG) storage in cytoplasmic globules under various stress conditions (nutrient limitation, high irradiance, osmotic stress) (Boussiba et al. 1999; Grünewald et al. 2001; Boussiba 2000; Zhekisheva et al. 2002; Damiani et al. 2010). In this way, *H. pluvialis* has been postulated as a potential feedstock for biodiesel production (Damiani et al. 2010; Razon and Tan 2011; Prommuak et al. 2012). However, since the same conditions that induce TAG synthesis and accumulation lead to growth cessation and the development of rigid cell walls, a number of issues must first be resolved in this context (Damiani et al. 2006, 2010; Peled et al. 2011).

Whereas microalgae-derived fatty acids have been studied to assess their role as biodiesel feedstock other lipids from this source, such as sterols, have been poorly explored. Phytosterols are cholesterol-like molecules mainly found in the cellular membranes of plants and algae (Luo et al. 2015) and are characterized by a tetracyclic cyclopenta(α)phenanthrene

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structure and an aliphatic side chain. They differ from cholesterol in the carbon side chain and/or in the presence of a double bond (Lopes et al. 2013). Scientific interest in these compounds emerged because of their ability to reduce blood cholesterol concentration, thus preventing cardiovascular disorders (Gylling et al. 2014). In addition, immunological and antiproliferative activities of plant sterols were reported in various human cancer cells (Hartmann 1998; Khalos et al. 1989; Awad and Fink 2000; Awad et al. 2000a, b). Furthermore, phytosterols have been shown to accumulate in the brain (Jansen et al. 2006; Vanmierlo et al. 2011). Thus, in the present work we used the human neuroblastoma cell line IMR-32, a cell model that undergoes a transformation and displays high rate of division and growth (Neill et al. 1994; Salvador and Oteiza 2011).

To date, higher plants have been the main industrial sources of phytosterols (Fernandes and Cabral 2007). Recent studies suggest the presence of phytosterols in some microalgae species such as *Dunaliella salina*, *Dunaliella tertiolecta*, *Phaeodactylum tricornutum*, *Pavlova lutheri*, *Nannochloropsis gaditana*, *Isochrysis galbana, Tetraselmis* sp., among others (Francavilla et al. 2010; Ahmed 2015; Ryckebosch et al. 2014; Véron et al. 1996). However, the potential of microalgae as sources of phytosterols remains to be fully explored (Borowitzka 2013).

In this work, we studied the content and composition of bioactive lipids such as TAGs and phytosterols in *H. pluvialis* CCALA 1081 after high light exposure. We further assessed the biological activity of phytosterols isolated from *H. pluvialis* CCALA 1081 in human IMR-32 neuroblastoma cells.

Materials and methods

All the chemicals used in the present study were of the highest purity available.

Microalgae culture and light-induced stress experiences

Unialgal cultures of *Haematococcus pluvialis* CCALA 1081 were maintained in Bold's Basal Medium (BBM) (Damiani et al. 2006). Cell concentration was determined by counting three replicate samples using a Neubauer chamber. An inoculum of 2.56×10^4 flagellate cells mL^{-1} was incubated in a final volume of 500 mL of BBM. Cells were kept at 24 °C with continuous bubbling of air (500–700 cm^3 min^{-1}). For control conditions, cells were grown under 90 μmol photons m^{-2} s^{-1} of light intensity with a 16:8-h light/dark photoperiod, as *H. pluvialis* cells grow optimally under low irradiation (Boussiba 2000). For stress (also called high-light) conditions, cultures were exposed to continuous levels of high irradiance at 300 μmol photons m^{-2} s^{-1}. This light intensity has been shown to induce the synthesis of astaxanthin in *H. pluvialis* (Zhekisheva et al. 2002; Wang et al. 2003). In addition, in a previous work by our group, we observed that this light intensity induced the accumulation of neutral lipids in *H. pluvialis* CCALA 1081 (Damiani et al. 2010).

Finally, cells were harvested by centrifugation at 800×*g* during 10 min at room temperature after 3, 6, and 12 days of culture. Light-stress experiments were performed at least in triplicate and two replicates of each condition were made.

Dry biomass determination

For dry biomass determination, samples were filtered through pre-dried and pre-weighted Whatman GF/C filters. The filters were then dried for 12 h at 100 °C, cooled in a dessiccator and weighed (Bongiovani et al. 2014). Three replicates of each sample were made.

Protein content

After high light treatment *H. pluvialis* samples were harvested by centrifugation (see "Microalgae culture and light-induced stress experiences" section) and washed three times with phosphate buffer saline (PBS). Total proteins were extracted using a lysis buffer containing 100 mM Tris HCl (pH 7.5), 300 mM NaCl, 4 mM EDTA, 4 mM EGTA, 0.2 % Triton X-100, 2 % Nonidet P-40, 5 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupeptin, 1 μg mL^{-1} pepstatin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, 2 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), and 1 mM Na_3VO_4. The lysates were immediately frozen in liquid nitrogen and subjected to sonication until the samples were thawed. These steps were repeated several times after which samples were centrifuged for 15 min at 14,000×*g* to remove insoluble material and unbroken cells. Proteins were quantified in the supernatant spectrophotometrically by the Bradford method (Bradford 1976) using bovine serum albumine (BSA) (2 μg μL^{-1}) as standard.

Chlorophyll content

For chlorophyll determination, after high light treatment and cell harvesting by centrifugation (see "Microalgae culture and light-induced stress experiences" section), the pellet was resuspended in absolute methanol, frozen in liquid nitrogen, and subjected to sonication until the samples were thawed. These steps were repeated several times until the resulting pellet became white/light gray. Chlorophyll content was determined spectrophotometrically by measuring the absorbance of the resulting extracts at different wavelengths according to Jeffrey and Humphrey (1975).

Triacylglycerol and phytosterol extraction, separation, and measurement

Lipids were extracted from *H. pluvialis* according to the method of Folch et al. (1957). An additional step of sonication was added to improve lipid extraction. The resulting extract was subsequently washed with 0.2 volumes of 0.05 % $CaCl_2$ and the lowest phase was obtained after centrifugation at 900×*g* for 5 min. During all procedures, lipids were kept under N_2 atmosphere. For TAG and sterol separation, lipid extracts were subjected to one-dimensional thin-layer chromatography (TLC) using silica gel G plates (Merck) in a mobile phase consisting of n-hexane/diethyl ether (80:20 *v*/*v*), along with commercial standards (β-Sitosterol certified reference material (100 μg mL^{-1}) from Sigma-Aldrich, USA, Triolein standard (2 g L^{-1}) from Wienner Lab, Rosario, Argentina. Lipids were then visualized by exposure of the plate to UV light after spraying with 2′,7′-dichlorofluorescein. The spots corresponding to TAGs and phytosterols were scraped off the silica and eluted. This was performed by three successive extractions with chloroform/methanol/water (5:5:1, *v*/*v*/*v*), thoroughly mixing, centrifuging, collecting the solvents, and partitioning with 4 volumes of water to recover the lipids in the chloroform phase. After elution, the resulting extracts were dried under N_2 gas and resuspended in 100 μL of isopropyl alcohol. TAGs and phytosterol content were determined spectrophotometrically using commercially available kits TG color GPO/PAP AA and Colestat enzimático (Wienner Lab), respectively, following the manufacturer's instructions (Sánchez Campos et al. 2015).

Lipid peroxidation assay

In order to evaluate the effect of high light on lipid peroxidation, we performed the thiobarbituric acid reactive substances (TBARS) assay. This determination involves derivatization of malondialdehyde with thiobarbituric acid (TBA) to produce a pink product that is quantified in a UV–VIS spectrophotometer. Briefly, after high light treatment, microalgae were mixed with 300 μL of ice-cold water and 0.5 mL of 30 % trichloroacetic acid after which 50 μL of 5 N HCl and 0.5 mL of 0.75 % TBA (Sigma-Aldrich, USA) were added. Tubes were capped, the mixtures were heated at 100 °C for 30 min in a boiling water bath and the samples were centrifuged at 1,000×*g* for 10 min. TBARS were measured spectrophotometrically in the supernatant at 532 nm (Mateos et al. 2008). Results are expressed as a percentage of the control.

Fatty acid (FA) analysis by gas chromatography

TAGs were extracted from *H. pluvialis*, then separated, visualized, and eluted as described previously (see "Triacylglycerol and phytosterol extraction, separation, and measurement" section). The resulting TAG extracts were dried under N_2 gas and fatty acid methyl esters (FAMEs) were obtained by trans-esterification with a cold methanolic solution of potassium hydroxide (International Olive 2001a). An HP 4890D gas chromatograph equipped with a split/splitless injector and a flame-ionization detector, both at a temperature of 260 °C, a capillary SP2560 column (100 m, 0.25 mm, and 0.2 μm) (Supelco Inc., USA) and a HP3398a GC Chemstation Software (Hewlett Packard, USA) for data processing were used. Operating conditions were: column temperature = 140 °C (15 min) –4 °C min^{-1} to 240 °C (15 min), carrier gas = hydrogen (18 cm min^{-1}), split injection ratio = 1:100. No internal standard was used. Instead, FAMEs identification was performed by comparison with standard certificate material, Supelco FAME 10 mix 37 (CRM47885), according to AOCS Official Method Ce 1b-89.

Phytosterol analysis by gas chromatography

Phytosterols from *H. pluvialis* were extracted, separated, visualized, and eluted as previously described (see "Triacylglycerol and phytosterol extraction, separation, and measurement" section). The resulting sterol extracts containing 5-α-cholestan-3-β-ol (from Fluka Switzerland, purity 95 %, Sigma-Aldrich, Switzerland) as internal standard were dried under N_2 gas. Separation and identification of the silanized compounds was performed according to AOCS (International Olive 2001b) and carried out in an Agilent 7820A gas chromatograph equipped with a split/splitless injector and a flame-ionization detector and a 30 m SE 54 capillary column of 0.25 mm i.d. and 0.2 μm film thickness (Supelco Inc.). The operating conditions were as follows: oven temperature, 260 °C (2 min)–1 °C min^{-1}–265 °C (20 min); injector temperature, 280 °C; FID temperature, 300 °C; injection volume, 1 μL; and carrier gas, hydrogen at 37 cm s^{-1}. Two replicates of each sterol analysis were made.

Determination of antioxidant activity

The antioxidant activity of the sterol fraction isolated from *H. pluvialis* was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical 2,2-diphenyl-1-picrylhydrazyl stable radical ($DPPH^{\cdot}$, from Sigma-Aldrich, USA). Briefly, an aliquot of the sterol fraction of *H. pluvialis* was placed in a cuvette together with 0.050 mL of a methanolic solution of DPPH (0.5 mg mL^{-1}). Then, 1 mL of methanol was added and the decrease in absorbance at 520 nm was determined. Methanol was used to zero the spectrophotometer. Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution. All determinations were performed in triplicate. Results are expressed as a percentage of the control.

Cell culture

IMR-32 human-derived neuroblastoma cells (from the American Type Culture Collection, USA) were grown in DMEM high-glucose medium supplemented with 10 % (*v*/*v*) fetal bovine serum (FBS, Natocor, Villa Carlos Paz, Argentina), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0.25 μg mL^{-1} amphotericin B at 37 °C under 5 % CO_2. Cultures (between passages 10 and 25) were trypsinized every 3 days and fresh medium with 10 % FBS was added.

Neuroblastoma cell treatments

IMR-32 cells were plated in 35 × 10-mm cell culture dishes and grown to 80–90 % confluence in serum-containing medium. Then, cells were serum-deprived 30 min before sterol treatments. The phytosterol fraction isolated from *H. pluvialis* and resuspended in DMSO (0.05 % *v*/*v*) was subsequently added to the cell culture and incubated for 24 h at 37 °C under 5 % CO_2. Phytosterol concentrations used ranged from 25 to 200 μM, and are detailed in each case. In humans, the daily intake of plant sterols and stanols together with their excretion through the bile by hepatocytes will determine their plasma concentration and tissue availability. As an example, a daily intake of 300 and 20 mg of plant sterols and stanols, respectively, leads to 7–24 μmol L^{-1} of plasma phytosterols and to 0.05–0.3 μmol L^{-1} of stanols (AbuMweis et al. 2009; Gylling et al. 2014). Considering this, the phytosterol concentrations assayed in the present work could be physiologically relevant. Control conditions received vehicle alone (DMSO 0.05 %).

Assessment of cell viability

To assess the biological activity of *H. pluvialis* phytosterols, we determined whether these compounds had any effect on IMR-32 cell viability. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay. MTT is a water-soluble tetrazolium salt that is reduced by metabolically viable cells to a colored, water-insoluble formazan salt. After IMR-32 cell treatments, MTT (5 mg mL^{-1} from Sigma-Aldrich, USA) was added to the cell culture medium at a final concentration of 0.5 mg mL^{-1}. After incubating the plates for 2 h at 37 °C in a 5 % CO_2 atmosphere, the assay was stopped and the MTT-containing medium was replaced with solubilization buffer (20 % SDS, pH 4.7). The extent of MTT reduction was measured spectrophotometrically at 570 nm (Uranga et al. 2009). The results obtained were analyzed for normal distribution and were finally expressed as a percentage of the control.

Nuclear staining

To determine if *H. pluvialis* phytosterols affected the nuclear morphology of IMR-32 cells, cell nuclei were stained with Sytox Green and Hoechst. Briefly, IMR-32 cells were grown in serum-containing medium onto glass coverslips until 50–60 % confluence. After cell treatment with phytosterols isolated from *H. pluvialis* (50 μM) or vehicle (DMSO 0.05 %), cells were washed three times with M1 buffer (150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM KCl in 20 mM HEPES buffer, pH 7.4) and stained for 15 min with SYTOX Green nucleic acid dye (Thermo Fisher Scientific, USA), 5 μM in M1 buffer at room temperature. This nucleic acid dye is a green-fluorescent nuclear and chromosome counterstain that is impermeant to live cells, making it a useful indicator of dead cells within a population. Cells were then washed three times with M1 buffer and fixed with 4 % paraformaldehyde. After rinsing three more times with M1 buffer, cells were incubated with Hoescht nuclear dye (Thermo Fisher Scientific) to visualize total nuclei, 1:12,000 in M1 buffer, for 10 min at room temperature. Finally, cells were washed three times with M1 buffer and were observed with a Nikon Eclipse E-600 microscope, using a K2E Apogee CCD camera driven by CCDOPS software (Santa Barbara Instrument Group, Goleta, CA, USA) to visualize stained nuclei (Sánchez Campos et al. 2015). A positive control of apoptosis was performed by exposing IMR-32 cells to 200 μM of H_2O_2.

Measurement of lactate dehydrogenase leakage

To asses if *H. pluvialis* phytosterols induced changes in IMR-32 cell membrane permeability, we determined the activity of the lactate dehydrogenase (LDH) leakage in the incubation medium. Briefly, after cell treatment with phytosterols isolated from *H. pluvialis*, incubation medium was centrifuged at 1,000×*g* for 10 min at 4 °C. The resulting supernatant was used to determine LDH activity, which was measured spectrophotometrically using LDH-P UV AA kit (Wienner Lab), following the manufacturer's instructions. Briefly, the conversion rate of reduced nicotinamide adenine dinucleotide to oxidized nicotinamide adenine dinucleotide was followed at 340 nm (Sánchez Campos et al. 2015). A positive control of apoptosis was performed by exposing IMR-32 cells to 200 μM of H_2O_2. Results are expressed as a percentage of the control.

Caspase-3 activity assay

To assess whether the cytotoxic effect of *H. pluvialis* phytosterols on IMR-32 cells was associated with the induction of apoptosis, we determined the activity of the pro-apoptotic enzyme caspase-3 in IMR-32 cell lysates through the spectrophotometric detection of the chromophore p-nitroaniline (p-

NA), generated by cleavage from the labeled substrate N-acetyl-Asp-Glu-Val-Asp-pNA. Briefly, after incubation with phytosterols isolated from *H. pluvialis*, IMR-32 cells were washed twice with PBS, scraped and centrifuged (10,000×*g* for 5 min at 4 °C). They were then lysed by the addition of a lysis buffer (50 mM HEPES, 1 mM DTT, 0.1 mM EDTA, 150 mM NaCl, Igepal 0.1 % (*v*/*v*)) (Hanzel and Verstraeten 2009). Next, the lysates were incubated on ice for 5 min, vortexed for 45 s and maintained on ice for another 5 min. After centrifugation at 12,000×*g* and 4 °C for 15 min the supernatant was collected and proteins were quantified by the Bradford method (Bradford 1976). The obtained lysates were incubated overnight at 37 °C with the labeled substrate N-acetyl-Asp-Glu-Val-Asp-pNA (from Sigma-Aldrich, USA). The p-NA light emission was finally quantified using a spectrophotometer at 405 nm. A positive control of apoptosis was performed by exposing IMR-32 cells to 200 μM of H_2O_2. Caspase-3 activity is expressed as a percentage of the control.

Statistical analysis

Data are shown as means ± standard deviation (S.D.). Statistical differences between groups were calculated by a two-tailed *t* test. $P < 0.01$ (**) and <0.05 (*) were considered highly statistically significant and statistically significant, respectively. Data from cell viability were first analyzed with a one-way ANOVA test; this analysis was followed by application of the multiple comparison Bonferroni test to compare means.

Results

A one-stage cultivation strategy was applied by directly exposing green vegetative biflagellate cells to optimal light intensity (control light conditions) (Fig. 1a–c) or to high light intensity (light-stress conditions) (Fig. 1d–f) for 3, 6, or 12 days. Control cells change their morphology from ellipsoidal green vegetative flagellate cells (3 days) (Fig. 1a) to non-motile spherical palmelloid cells (12 days) (Fig. 1c). In addition, cells exposed to high-light intensity were transformed from spherical palmelloid green cells with a central red circle of secondary carotenoids (3 days) (Fig. 1d) into reddish aplanospores with a thick and rigid cell wall (12 days) (Fig. 1f).

Figure 2a reveals that *H. pluvialis* CCALA 1081 cultures grown under high light intensity during 3, 6, and 12 days showed a marked decrease in their protein content when compared to control light conditions. In addition, chlorophyll *a* and *b* significantly decreased after 3, 6, and 12 days of high-light-induced stress (Fig. 2b).

After light-induced stress experiences, the amount of TAGs was assessed in *H. pluvialis* CCALA 1081. As Fig. 3a shows, TAG content significantly increased by approximately 250 % with respect to the control light condition after 3 days of high-light cell exposure. Furthermore, no marked differences in the amount of TAGs were observed after 6 days of light-induced stress whereas a significant decrease (~150 % fewer than the respective control) was detected after 12 days of high-light exposure.

In determining the presence of phytosterols in *H. pluvialis* CCALA 1081 we detected the highest amount of sterols within 3 days of high-light intensity exposure (Fig. 3b). The light-stress condition accumulated ~1,200 % more sterols than the respective control. This percentage is equivalent to ~2.0 % dry weight. In addition, a similar amount of phytosterols (1.8 % dry weight) was found after 6 days of high-light-induced stress.

In order to evaluate the light effect on *H. pluvialis* lipids, their peroxidation level was analyzed by the TBARS assay. The level of TBARS was significantly higher after 6 and 12 days of *H. pluvialis* high-light exposure (approximately 250 and 450 %, respectively, higher than each control; Fig. 4). Nevertheless, no increase in *H. pluvialis* TBARS

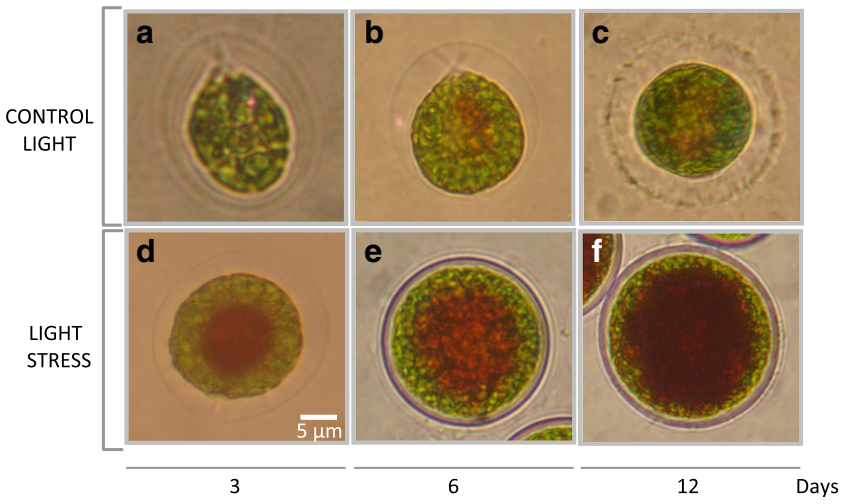

Fig. 1 Effect of high-light exposure on *H. pluvialis* CCALA1081 cells. Cells grown under optimal light intensity (control light) (**a**–**c**) and high-light intensity (light stress) (**d**–**f**) for 3, 6, and 12 days

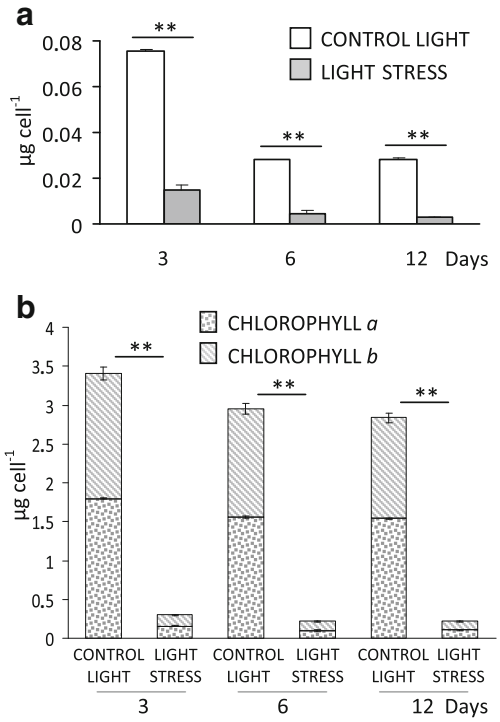

Fig. 2 Effect of high-light exposure on the amounts of proteins and chlorophylls *a* and *b* in *H. pluvialis* CCALA1081 cells. **a** Protein content (in microgram per cell) and **b** determination of the amount of chlorophylls *a* and *b* (in microgram per cell) in cells grown under optimal (control light) and high-light intensity (light stress). Both results shown are representative of, at least, five independent experiments. Means ± standard deviation are given; $^{**}p < 0.01$ where *p* value refers to significant differences in the total amount of proteins (**a**) or chlorophylls (**b**)

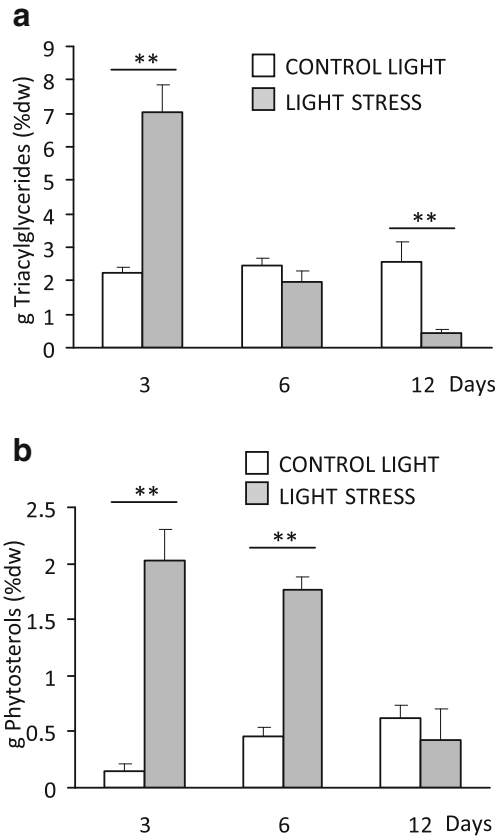

Fig. 3 Effect of high-light stress on triacylglyceride and phytosterol contents in *H. pluvialis* CCALA 1081. Spectrophotometric detection of TAGs (**a**) and phytosterols (**b**) in cells exposed for 3, 6, and 12 days to optimal light (control light) or high-light intensity (light stress). Results are expressed as percentage of dry weight biomass (%dw) and are, at least, from three independent experiments. Means ± standard deviation are given. $^{**}p < 0.01$, where *p* value refers to differences in TAG (**a**) or phytosterol (**b**) content

levels were observed after 3 days of light-induced stress with respect to the control light condition.

Based on the maximal levels of TAGs and phytosterols found in *H. pluvialis* after 3 days of high-light exposure, and with no sign of lipid peroxidation under these experimental conditions, all remaining experiments were performed only at this incubation time. Fatty acid composition of TAGs from *H. pluvialis* revealed that under control light conditions the major fatty acids were caproic (18 %), oleic (16 %), and α linolenic acids (21 %); whereas palmitic (22 %), oleic (16 %), linoleic (23 %), and α linolenic (12 %) were the main fatty acids after light-induced stress (Table 1). The percentage of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) of *H. pluvialis* exposed to high-light intensity showed no significant differences compared to control light conditions. However, PUFA were the main fatty acids found in the TAG of *H. pluvialis* in both control light and light-stress conditions, representing 50 % of total fatty acids (Table 1).

Phytosterol composition revealed that light-induced stress reduced β-sitosterol and 24-methylene cholesterol contents by 25 and 10 %, respectively; whereas chlerosterol, brassicasterol, Δ7-campesterol, and sitostanol were 20, 10, 8, and 5 % higher than in the respective control light condition (Fig. 5).

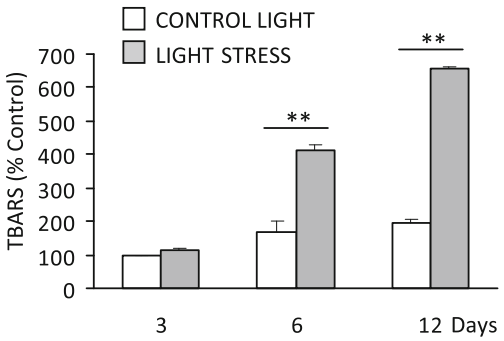

Fig. 4 Effect of high-light intensity in lipid peroxidation in *H. pluvialis* CCALA 1081. Spectrophotometric quantification of TBARS after 3, 6, and 12 days of cell exposure to optimal light (control light) or high-light intensity (light stress). Results are expressed as percentage of the control light condition (3 days) (% control) and were obtained from, at least, three independent experiments. Means ± standard deviation are given. $^{**}p < 0.01$, where *p* value refers to significant differences in TBARS with respect to each control condition

Table 1 Fatty acid composition of TAGs from *H. pluvialis* CCALA1081 after 3 days of optimal and high-light-intensity exposure

Fatty acids	Control light	Light stress
C6:0 (caproic)	18.23 ± 0.010	1.99 ± 0.048
C8:0 (caprylic)	3.20 ± 0.490	0.87 ± 0.050
C14:0 (myristic)	3.10 ± 0.150	0.61 ± 0.170
C16:0 (palmitic)	4.38 ± 0.640	22.94 ± 2.160
C16:1 (palmitoleic)	0.33 ± 0.021	0.35 ± 0.077
C17:1 (heptadecenoic)	0.97 ± 0.051	nd
C18:0 (stearic)	2.16 ± 0.162	1.15 ± 0.323
C18:1n9t (elaidic)	nd	5.46 ± 0.511
C18:1n9c (oleic)	16.11 ± 1.132	16.28 ± 1.196
C18:2n6t (linolelaidic)	3.67 ± 0.372	6.64 ± 0.330
C18:2n6c (linoleic)	7.04 ± 0.706	23.88 ± 1.813
C18:3n6 (γ linolenic)	4.30 ± 0.284	2.19 ± 0.156
C18:3n3 (α linolenic)	21.20 ± 0.917	12.52 ± 1.115
C20:2	6.98 ± 0.418	2.21 ± 0.191
C20:4n6 (arachidonic)	4.79 ± 0.065	1.92 ± 0.137
C24:0 (lignoceric)	nd	0.33 ± 0.074
C20:5n3	3.54 ± 0.257	0.66 ± 0.040
% SFA	31.06 ± 3.96a	27.89 ± 3.56a
% MUFA	17.43 ± 1.40b	22.09 ± 1.70b
% PUFA	51.51 ± 3.03c	50.02 ± 3.79c

TAGs are expressed as percentage of total fatty acids. Values are shown as means ± standard deviations of three independent light-stress experiments. In addition, two replicates of each fatty acid analysis were done. An ANOVA test was performed to establish differences between conditions [p(ANOVA) < 0.01]. The letters indicate the results of Bonferroni's test for mean comparison. Same letters for experimental groups indicate $p > 0.05$, whereas different letters indicate $p < 0.01$

To assess the biological activity of *H. pluvialis* phytosterols, we determined whether these compounds had any effect on cell viability. For this purpose, human IMR-32 neuroblastoma cells were exposed to increasing concentrations of phytosterols isolated from both control light and high light stress conditions. As shown in Fig. 6a, *H. pluvialis* phytosterols (50 μM) isolated after high-light-intensity exposure significantly decreased IMR-32 cell viability. Also, concentrations 100 and 200 μM of phytosterols diminished neuronal viability by 60 %. In this case, no marked differences in cell viability were observed between phytosterols isolated from control light conditions and those obtained by exposing *H. pluvialis* cells to high light intensity. Therefore, the lowest phytosterol concentration showing a significant lethal effect (50 μM) was used in subsequent experiments.

Beyond the cytotoxic effect, high-light *H. pluvialis* phytosterols also showed increased radical scavenging activity (488 %) with respect to phytosterols isolated from *H. pluvialis* grown under control light conditions (Fig. 6b).

To determine whether the cytotoxic effect of *H. pluvialis* phytosterols on IMR-32 cells was associated with the induction of apoptosis, cell nuclei were stained with both Sytox Green and Hoechst. Figure 7a shows that control nuclei (Control (DMSO 0.05 %)) were only stained with Hoechst and remained intact, whereas IMR-32 cells exposed to *H. pluvialis* phytosterols showed significant nuclear condensation and were also stained with Sytox dye. A positive control of apoptosis performed with 200 μM H_2O_2 (positive control (H_2O_2)) showed nuclear condensation and was stained with both dyes, whereas cells exposed to vehicle (control (H_2O)) showed typical IMR-32 cell nuclei morphology and were only stained with Hoechst (Fig. 7b). In line with these results, phytosterols from *H. pluvialis* increased IMR-32 cell membrane permeability by 460 % over the control, as revealed by the release of LDH to the incubation medium (Fig. 8a).

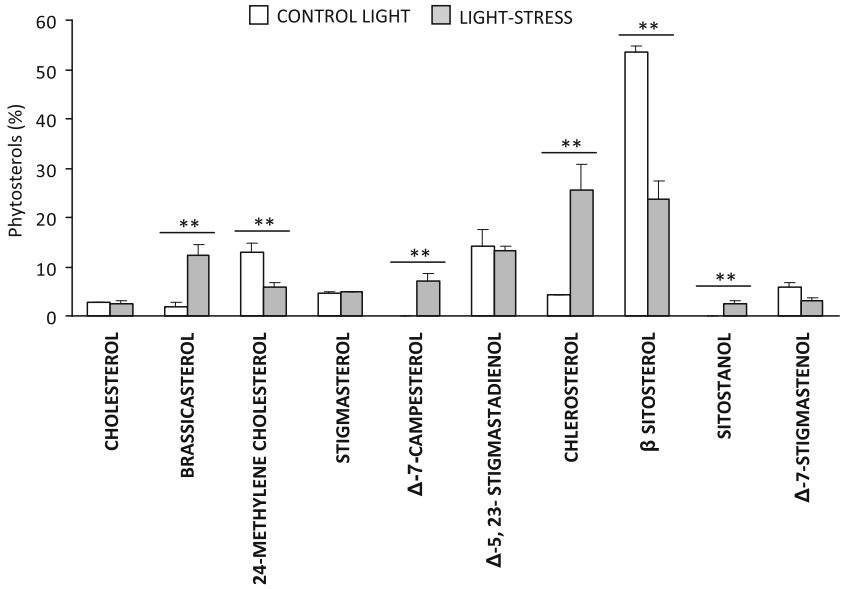

Fig. 5 Composition of the phytosterol fraction of *H. pluvialis* CCALA1081 after 3 days of optimal and high-light intensity exposure. Results are expressed as the percentage of total phytosterols. Values are means ± standard deviations of three independent light-stress experiments. In addition, two replicates of each sterol analysis were done. $^{**}p < 0.01$, where p values refer to differences in *H. pluvialis* phytosterols obtained from control light and high-light exposure conditions

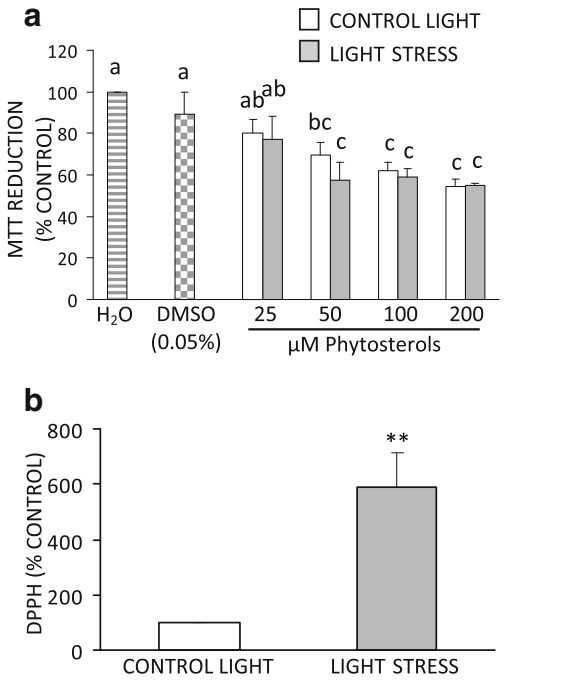

Fig. 6 **a** Cytotoxic effect of phytosterols isolated from *H. pluvialis* CCALA1081 in human neuroblastoma IMR-32 cells. Cell viability was assessed after treatment of IMR-32 cells during 24 h with the indicated concentrations of *H. pluvialis* phytosterols (25, 50, 100, and 200 μM) isolated after optimal (control light) and high-light exposure (light stress). Results are expressed as percentage of a control (H_2O) performed using water as vehicle. Data represent means ± SD of three independent experiments. An ANOVA test was performed to establish differences between conditions [p(ANOVA) < 0.01]. The *letters* indicate the results of Bonferroni test for mean comparison. *Same letters* for experimental groups indicate $p > 0.05$, whereas different letters indicate $p < 0.01$. **b** Radical scavenging activity of *H. pluvialis* CCALA1081 sterols. DPPH· assay results are expressed as percentage over the control light condition (% control), and are representative of, at least, six fractions isolated from different *H. pluvialis* light-stress experiments. Means ± SD are given. **$p < 0.01$, where p values refer to differences in antioxidant activity of *H. pluvialis* phytosterols obtained from control light and high-light irradiance conditions

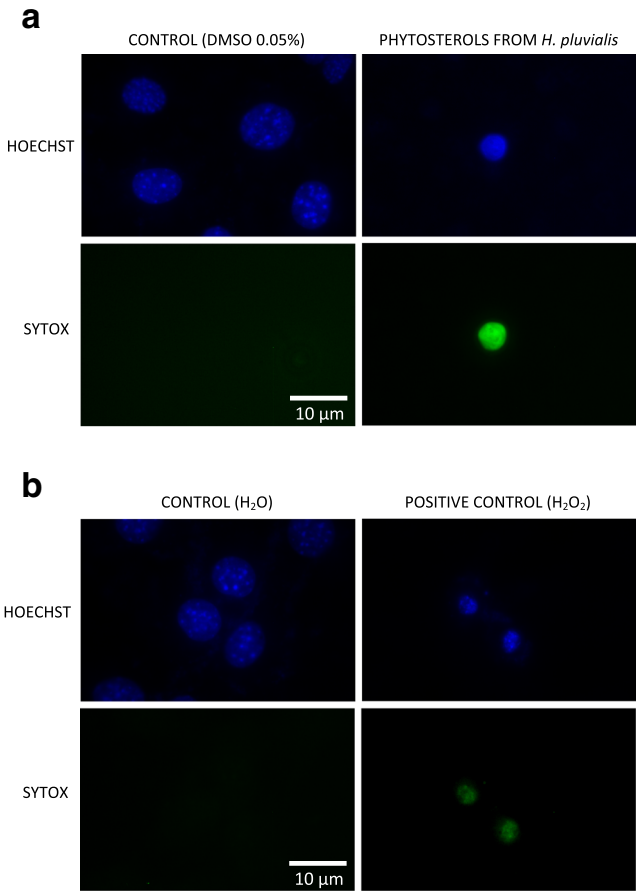

Fig. 7 Effect of *H. pluvialis* CCALA1081 phytosterols on nuclear morphology of IMR-32 cells. **a** Nuclear morphology was assessed after treating IMR-32 cells for 24 h with either vehicle (DMSO 0.05 % *v*/*v*; control) or phytosterols isolated from *H. pluvialis* (50 μM) after high-light exposure (phytosterols from *H. pluvialis*). **b** Nuclear morphology was also assessed after exposing IMR-32 cells to vehicle (control H_2O) or to 200 μM of H_2O_2 for 8 hours (positive control). In both cases, the results shown are representative of, at least, three independent experiments

Finally, in order to better characterize the effect of *H. pluvialis* phytosterols on neuronal cell death, we assayed a typical marker of apoptosis: caspase-3 activity. To assess the involvement of this enzyme in phytosterol-induced apoptosis, we determined its activity in IMR-32 cell lysates. As can be seen in Fig. 8b, phytosterols obtained from *H. pluvialis* stimulated caspase-3 activity significantly above the control level (DMSO 0.05 %), and as much as the hydrogen peroxide (positive control (H_2O_2)).

Discussion

During the life cycle of *H. pluvialis*, four types of cells can be distinguished: microzooids, flagellated macrozooids, non-motiled palmelloid forms, and aplanospores or cysts (Elliot 1934). It is well-known that when *H. pluvialis* flagellate green cells are confronted by stress conditions, they differentiate from the vegetative stage into a resting one, characterized by the formation of non-motile spherical reddish cells (aplanospores) (Boussiba et al. 1999). These changes are also accompanied by cessation of cell division, increase of the cell volume and also development of a resistant cell wall (Grünewald et al. 2001; Damiani et al. 2006). As a consequence, the use of valuable compounds synthesized by *H. pluvialis* after being exposed to stress conditions is restricted to both: the cell number and the rupture of its hard cell wall (Han et al. 2013). In this work, the high-light-stress experiments performed showed that *H. pluvialis* accumulates TAGs and phytosterols. Both lipids were easily extracted by using the Folch method (Folch et al. 1957). This method is widely used for isolation of total lipid from animal tissues, and in our case the lipid extraction was possible due to the non-motile

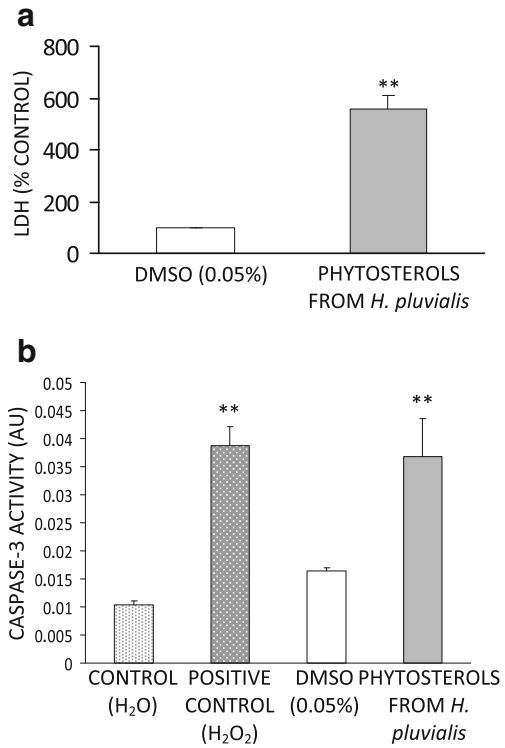

Fig. 8 Phytosterols from *H. pluvialis* CCALA1081 induce apoptosis in IMR-32 cells. **a** *H. pluvialis* phytosterols affect cell membrane permeability. The activity of LDH leakage is expressed as percentage over the control (DMSO 0.05 %) (% control), and is representative of, at least, four different experiments. Means ± SD are given. $^{**}p < 0.01$, where p value refers to differences in LDH leakage. **b** *H. pluvialis* phytosterols induce caspase-3 activity in IMR-32 cells. The caspase-3 activity assay results are expressed as arbitrary units (AU), and are representative of, at least, four different experiments. Means ± SD are given. $^{**}p < 0.01$, where p values refer to significant differences in caspase-3 activity between conditions

palmelloid stage achieved by *H. pluvialis* after a short period of time (3 days) of direct high-light exposure of motile cells. In addition, *H. pluvialis* CCALA 1081 grown under control conditions accumulated a small detectable amount of TAGs, as previously reported in *H. pluvialis* NIES144 (Wang et al. 2014), *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica* (Liu et al. 2013).

Han and collaborators (2012) reported that *H. pluvialis* CCAP 34/12 motile cells became highly vacuolated after high-light exposure. In contrast, micrographs of our experiences with *H. pluvialis* CCALA 1081 showed the absence of vacuoles after high-light exposure. The reddish aplanospores, which were the predominant form after 12 days of high-light exposure, developed a thick and rigid cell wall. This dense layer can be disrupted only by applying vigorous and expensive extraction methods. Thus, we attribute the apparent decrease in both TAG and phytosterol content observed after 12 days of high-light exposure to this phenomenon. In addition, *H. pluvialis* micrographs displayed low chlorophyll *a* and *b* levels in coincidence with the quantitative values obtained by the spectrophotometric measurements. In addition, a marked decrease in the protein content was observed. In agreement with our results, these physiological changes were also observed in other strains of *H. pluvialis* subjected to different stress conditions (Zhekisheva et al. 2002; Han et al. 2012, 2013).

High-light intensity can generate an excess of reactive oxygen species (ROS), harmful agents that can damage diverse cell components as DNA or lipids, and resulting in oxidative stress (Apel and Hirt 2004). Thus, lipid peroxidation was assessed in *H. pluvialis* CCALA 1081 cultures exposed to high-light. The TBARS assay suggested a significant increase in lipid peroxidation after 6 and 12 days of high-light exposure. However, no lipid peroxidation changes were detected after 3 days of light-induced stress. Opposite to our results, Han and collaborators and also Gwak and collaborators (Han et al. 2012; Gwak et al. 2014) both reported the generation of excessive ROS after 24 h of high-light exposure in *H. pluvialis* CCAP34/12 and UTEX #2505, respectively. Nevertheless, these differences could be due to application of different cell growth conditions.

A great variety of secondary biologically active metabolites can be produced by microalgae in the effort to adapt to changing environmental conditions (Borowitzka 2013). Recently, it has been shown the presence of phytosterols in some microalgal strains as in the case of *Isochrysis galbana*, *Nannochloropsis gaditana*, *Nannochloropsis* sp., and *Phaeodactylum tricornutum*. There, phytosterol content ranged from 0.7 to 3.4 % (of dry weight) (Véron et al. 1996; Francavilla et al. 2010; Ryckebosch et al. 2014; Ahmed 2015). Our study provides evidence, to our knowledge for the first time, on the phytosterol content and composition of *H. pluvialis* CCALA 1081 cells. The amount of phytosterols found in this strain is comparable to that reported for the strains aforementioned. Microalgae produce a very wide range of phytosterols including brassicasterol, sitosterol, and stigmasterol. However, phytosterol content and composition can vary according to the growth conditions (growth stage, light intensity, temperature) (Véron et al. 1996; Fábregas et al. 1997). In the case of *H. pluvialis* CCALA 1081, high-light exposure reduced the amount of β-sitosterol but increased chlerosterol, brassicasterol, and Δ7-campesterol content compared to cells grown under optimal light intensity conditions. Thus, *H. pluvialis* phytosterols might be used as supplements in functional foods.

Phytosterols derived from other sources different to microalgae have shown antioxidant activity (Lee et al. 2003; Panda et al. 2009). Consistent with these findings, we observed increased radical scavenging activity in sterols isolated from *H. pluvialis* CCALA 1081 after high-light exposure.

Also, some microalgal sterols have shown in vitro (Kim et al. 2013, 2014) and in vivo bioactivity (Francavilla et al. 2012). Phytosterols from *H. pluvialis* CCALA 1081 showed

potent cytotoxicity against IMR-32 human neuroblastoma cells. Supporting this result, previous studies have shown the anti-proliferative effect of sterols in various human cancer cells (von Holtz et al. 1998; Awad et al. 1998, 2000a, b; Kim et al. 2013, 2014). Also, in concordance with this cytotoxic effect, lyophilized extracts of another *H. pluvialis* strain showed significant toxic activity on diverse tumoral cells in vitro (Rosa et al. 2005). Nevertheless, additional studies should be performed in order to determine if the lethal effect is specific or not for tumoral cells.

Recently, it has been proved out that plant sterols can be accumulated in the brain (Jansen et al. 2006; Vanmierlo et al. 2011). In addition, studies reported effects of plant-derived sterols on the central nervous system (Khabazian et al. 2002; Wilson et al. 2002. In line with these findings, sterols isolated from the microalga *Dunaliella tertiolecta* showed a neuromodulatory action in vivo in selective brain areas of rats (Francavilla et al. 2012). Together, these findings suggest the possibility that *H. pluvialis* phytosterols might be considered anti-proliferative agents for neuroblastoma cells in vivo, although an exhaustive investigation on the effects of chronic sterol exposure is needed.

In addition, the cytotoxic effect exerted by *H. pluvialis* sterols in IMR-32 neuroblastoma cells occurred through the induction of apoptosis, as revealed by the microscopy studies and the caspase-3 activity assay. Similar findings were reported for sterols isolated from the algae *Navicula incerta* and *Codium fragile*. Specifically, stigmasterol isolated from *N. incerta* showed apoptosis inductive effect in human hepatocarcinoma (HepG2) cells; whereas chlerosterol from *C. fragile* showed the same effect on human melanoma A2058 cells (Kim et al. 2013, 2014). These findings give significance to ours as chlerosterol is one of the main components of the sterol fraction obtained from *H. pluvialis* after high-light exposure.

Another characteristic of *H. pluvialis* is its ability to accumulate large amounts of neutral lipids (Damiani et al. 2010). Considering this, we determined the composition of the fatty acids from TAGs in *H. pluvialis* CCALA 1081 motile cells directly exposed to high light for 3 days. The results showed similar fatty acid profile than that reported previously for aplanospores of this strain during 15 days of cultivation under high-light stress (Damiani et al. 2010). As other species of Chlorophyceae, C16:0 and C18:1 fatty acids are the predominant saturated and mono-unsaturated fatty acids (Hu et al. 2008). On the other hand, the fatty acid composition of our control condition differed considerably from the one obtained in previous work (Damiani et al. 2010), mainly by the presence of an important amount of caproic acid. This may be due to the fact that light experiments were performed during different periods of time. In general, fatty acid biosynthesis occurs in the plastids, with consecutive attachment of two carbon units to a growing fatty acid chain resulting in the production of C16, C18, and/or C18:1. If the chain-elongation process is finished earlier, the resulting products will be short- or medium-chain fatty acids (Dyer et al. 2008). In line with this, it is known that some algae can synthesize medium-chain fatty acids (e.g., C8, C10, C12, C14) (Hu et al. 2008). In addition, significant amounts of both caproic and caprylic fatty acids were found in the green microalga *Picochlorum* sp. subjected to different nutrient stresses (El-Kassas 2013).

PUFA represented 50 % of the fatty acids found in the TAG of *H. pluvialis* in both control and stress conditions. The essential linoleic (C18:2n6) and alpha linolenic (C18:3n3) fatty acids were the main components of that fraction. Thus, *H. pluvialis* should be considered as human dietary fatty acid supplement as is the case of other microalgal strains (Lu et al. 2015).

Our results point out the potential of *H. pluvialis* as a source of commercially valuable compounds, particularly regarding phytosterols and PUFA. However, this is the standpoint for optimizing both culture conditions and extraction methods in order to maximize the yield of this natural biorefinery.

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