



***In vivo* measurements to estimate culture status and neutral lipid accumulation in *Nannochloropsis oculata* CCALA 978: implications for biodiesel oil studies**

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With 5 figures

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Abstract: The development of efficient, rapid and species-specific techniques is indispensable for assessing growth and neutral lipid yield in microalga cultures for biodiesel oil production. *Nannochloropsis oculata* is a small microalgae with a thick cell wall. *In vivo* techniques to estimate cell density, chlorophyll a and neutral lipids are reported. A calibration curve of cell density versus optical density was obtained and validated at 540 nm, under different growth phases. Intracellular neutral lipid storage was evaluated with fluorometry and epifluorescent microscopy employing fluorochrome Nile Red. The addition of 5 % dimethyl sulfoxide enhanced 12.5 times the fluorescence signal efficiency. *In situ* fluorescence measurements allowed estimating the neutral lipid content (NR-FI). Besides, no significant differences were found in the lipid neutral content between gravimetric and triolein methods. The relationship between NR-FI and chlorophyll fluorescence signals was used as a neutral lipid accumulation index, which is useful in order to establish the optimum harvesting time. Thus, these procedures may be applied for a better monitoring mode of growth and neutral lipid accumulation in *N. oculata*'s cultures at commercial scale.

Keywords: *Nannochloropsis oculata*, optical density, chlorophyll a, neutral lipids, Nile Red

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1. Introduction

There is an energy crisis worldwide due to depletion of finite fossil fuel resources. Besides, greenhouse gases accumulate increasingly in the atmosphere (Demirbas & Demirbas 2011). Therefore, biodiesel is currently receiving widespread attention owing to its non-toxicity, biodegradability and potential as sustainable and environmentally friendly alternative to petroleum fuels (Ma & Hanna 1999, Leonardi et al. 2011). Microalgae are an especially attractive raw material because they have high biomass yields and solar energy conversion rates. Furthermore, many species can grow under marine and brackish waters, which is an interesting and economic alternative for large scale cultures (Mata et al. 2010). The genus *Nannochloropsis* has been suggested as a renewable feedstock of oil for biodiesel production due to their capacity of accumulating neutral lipids under stress conditions (Rodolfi et al. 2009, Bondioli et al. 2012). In addition, transesterification of *N. oculata*'s oils supported a biodiesel yield of 97.5 wt % (w/w) (Umdu et al. 2009).

Some criteria used for microalgal species selection for biofuel production include: 1) determination of growth rate, 2) knowledge of culture physiological status, and 3) determination of lipid productivity and quality, mainly of triacylglycerides -TAGs- (Hu et al. 2008). After selecting the right candidate strain, it is crucial to optimize the effective cost of cultures and bioprocesses. Therefore, a practical and operational monitoring of all these parameters is necessary in order to evaluate the culture status as well as the bioproduct accumulation. For example, the determination of neutral lipid accumulation time is vital to establish the harvesting time of microalgal cultures for biodiesel production. The decisions must often be taken within short time periods and mistakes may lead to a significant reduction in productivity or in total culture loss (Gitelson et al. 2000). In the present study, *in vivo* measurements are considered in order to determine the culture status and to predict the neutral lipid accumulation time in a strain of *Nannochloropsis oculata* CICALA 978 isolated from South Atlantic coast. In this context, the development of efficient, rapid and mainly species-specific techniques is of considerable importance in order to monitor cultures at a larger scale. In addition, an ultrastructural analysis was done to evidence the intracellular localization of accumulation lipid bodies and the cell wall structure.

2. Materials and methods

2.1. Algal strain and culture conditions

Nannochloropsis oculata from Atlantic South was gently provided by CRIAR, Instituto de Biología Marina y Pesquera Almirante Storni, Argentina. This strain was deposited in the Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences of the Czech Republic as strain CICALA 978. The cells of *N. oculata* were cultured in f/2 medium, prepared with aged natural seawater at 30 PSU of salinity. Different experiments were carried out by triplicate in 250 mL Erlenmeyer

flasks with 100 mL of f/2 medium at $24^{\circ} \pm 1^{\circ}\text{C}$ with continuous bubbling of air ($500\text{--}700\text{ cm}^3\text{ min}^{-1}$). Light was supplied by cool-white fluorescent lamps in a 16:08 h light: dark photoperiod in order to get an average light irradiance of $100\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$. Under these conditions, different cell densities were obtained during exponential and stationary growth phases from complete medium-cultures. In addition, two experiments were carried out to evaluate the neutral lipid accumulation, as follows: (a) an inoculum was resuspended in 3 L of complete medium for 14 days (control) and harvested by centrifugation (10 min at 3600g) for quantitative lipid analysis and (b) an inoculum was resuspended in 3L of complete medium, harvested by centrifugation (10 min at 3600 g) at the end of log-phase culture and transferred to 3 L of nitrogen-free medium for 11 days (N-deficient culture), and finally harvested for quantitative lipid analysis. *In situ* cell density and both chlorophyll *a* and Nile Red fluorescence intensities were measured approximately every two days throughout experiences.

2.2. Model as estimator of cell density

Cell density was evaluated by both direct counting and optical density measurements from several dilutions. Genuine triplicates were prepared by dilution of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mL of homogenized cultures in exponential phase to a final volume of 4.0 mL with f/2 medium. Optical density (OD) was spectrophotometrically determined at 540 nm in a UV-visible spectrophotometer (Shimadzu 1603; double beam; slit size: 2 nm; photometric mode: T %) using 10 mm light path cells and f/2 in the reference cell. The remaining samples were preserved with Lugol's iodine solution and cells were counted in a Neubauer haemocytometer under optical microscope Leica DM 200 at 400X. Percentage transmittance (T %) was converted to optical density as follows: $\text{OD} = \log(100/\text{T}\%)$. A calibration curve of cell density versus OD was established and validated. For analytical validation, calculated and estimated cell densities under exponential and stationary phases from complete medium-cultures were obtained by means of both methods. The validation set was adjusted by using the orthogonal distance regression method and a statistical comparison was performed through the F-test.

2.3. *In vivo* chlorophyll *a* measurements

Genuine triplicates from complete medium-cultures (exponential and stationary phases) and N-deficient cultures were diluted 1:10 with f/2 medium for fluorometric chlorophyll *a* (Chl *a*) detection of living microalgal cells. Excitation wavelength was set at 430 nm and emission wavelength was scanned from 600 to 750 nm (spectrum mode with excitation and emission slits set at 5 nm) using a spectrofluorometer (Schimadzu RF-5301PC, spectrum mode with excitation and emission slits set at 5 nm). Emission wavelength peak was selected at 670 nm.

2.4. Neutral lipid analysis

2.4.1. Optimization of Nile Red staining methods

Fluorescence intensity measurements

A previous scan of excitation and emission wavelengths of both neutral lipid standard and NR stained cell samples was performed by spectrofluorometry. According to this choice, excitation and emission wavelengths were selected at 480 nm and 570 nm, respectively. Fluorescence intensity (FI) was measured by using a spectrofluorometer (Schimadzu RF-5301PC, spectrum mode with excitation and emission slits set at 5 nm). Then, relative fluorescence intensity (RFI) was calculated as the difference between FI of stained cell samples and their corresponding blanks. In order to optimize NR staining protocols, different staining times and pre-treatments were tested.

Optimization of NR staining time

Four staining times were tested: 7 min, 20 min, 30 min and 40 min. A solution of 5 μL of Nile Red (NR) (9-diethylamino-5H-benzo[a] phenoxazine-5-one, Sigma CAS number: 7385-67) in acetone (1 mg mL^{-1}) (Priscu et al. 1990) was introduced into individual glass tubes containing 250 μL of algal suspension, which were agitated on a vortex mixer for 1 minute at 120 rpm. After the incubation time, 5 mL of ultrapure water were added to stained algae cells and the suspensions were analysed by spectrofluorometry. For these tests, the corresponding blanks were: (a) cell-blank: 250 μL of algal sample plus 5 mL of ultrapure water, and (b) NR-blank: 250 μL of f/2 medium plus 5 μL of NR and 5 mL of ultrapure water. Three replicates of each experience were made.

Pre-treatments

Two pre-treatments were performed. First, 250 μL of cell suspension with 5 μL of NR solution were heated up at 70 °C during 10 min. Secondly, 250 μL or 1000 μL of five organic solvents – acetone (Ac), methanol (Met), ethanol (Et), dimethyl sulfoxide (DMSO), or isopropanol (Iso) – were added to 250 μL of cell suspension with 5 μL of NR solution in order to get two final concentrations of 5 % (v/v) and 20 % (v/v), respectively. In all the tested pre-treatments, the suspensions were agitated on a vortex mixer for 1 minute at 120 rpm. After 30 minutes at room temperature (22 °C) under darkness, 5 mL of ultrapure water were added and the suspensions were analysed by spectrofluorometry. The following blanks were used: (a) cell-blank: 250 μL of algal sample plus 5 mL of ultrapure water, and (b) NR-solvent blank: 250 μL of f/2 medium plus 5 μL of NR solution plus 250 μL or 1000 μL of solvents – according to the assessed final concentration – and 5 mL of ultrapure water. Three replicates of each pre-treatment were made.

2.4.2. Quantitative determination of neutral lipids

For neutral lipid content analysis, the biomass was harvested at the end of control and N-deficient cultures, washed three times, freeze dried and reserved for gravimetric and fluorometric quantification, respectively. For the gravimetric determination, duplicated samples of 200 mg were extracted with chloroform/methanol (2:1, v:v) according to modified Folch et al. (1957). Then, a lipid fractionation into neutral lipids, glycolipids and phospholipids was performed using a silica Sep pack cartridge of 1000 mg (J. T. Baker Inc., Phillipsburg, N. J.) according to Berge et al. (1995). Each fraction was collected into a conical vial and evaporated to dryness under nitrogen and weighed. For the fluorometric determination, RFI of stained cells from control and N-deficient cultures was quantified with triolein (TO) (1, 2, 3-trioleoyl-sn-glycerol, C₅₇H₁₀₄O₆, Sigma). Then, the neutral lipid contents obtained by both methods were compared.

2.5. Lipid detection

2.5.1 Transmission electron microscopy

Cells of *N. oculata* in exponential and stationary phases were fixed in 3 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) containing 0.25 M sucrose. Fixation was followed by a series of rinses in 0.1 M Na-cacodylate buffer with gradually decreasing sucrose concentrations. Then, samples were post-fixed in 1 % O₃O₄ in 0.1 M Na-cacodylate buffer, dehydrated in acetone and infiltrated in Spurr's resin over 4 days. Ultrathin sections were stained with aqueous uranyl acetate followed by lead citrate and observed in a JEOL 100CX-II TEM operated at 80 Kv.

2.5.2. Confocal fluorescence microscopy

Nannochloropsis oculata's samples from N-deficient cultures were stained with NR (according to the most efficient method tested) and analysed by means of a Leica DMIRE2 Confocal TCS SP2 SE microscope with a 475 nm band-excitation filter and a 580 nm band-emission filter.

2.6. Statistical analysis

Differences in mean values from several treatments were assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's test in order to identify the sources of detected significance. In all cases, comparisons that showed a p value < 0.05 were considered significant.

3. Results and discussion

3.1. *In situ* cell density measurements

Nannochloropsis oculata is a marine microalga, whose size varies from 2 to 4 μm (Hibberd 1981). Therefore, a rapid monitoring to assess growth rate results difficult due to its small size. Figure 1A shows the model proposed to estimate cell density from optical density values in *N. oculata*. A linear relationship between OD at 540 nm and cell density was found in a range of 3×10^6 cells mL^{-1} to 21×10^6 cells mL^{-1} , corresponding to values of OD from 0.05 to 0.35. The model was obtained by using 21 points in order to cover 7 levels of concentration that correspond to exponential and stationary phases from complete medium-cultures. Griffiths et al. (2011) indicated strategies to minimize errors in microalgal biomass quantification from OD including: (1) selection of a wavelength that minimizes absorbance by the pigment (e.g. 750 nm or 550 nm for cells where chlorophyll is the major pigment), and (2) generation of a

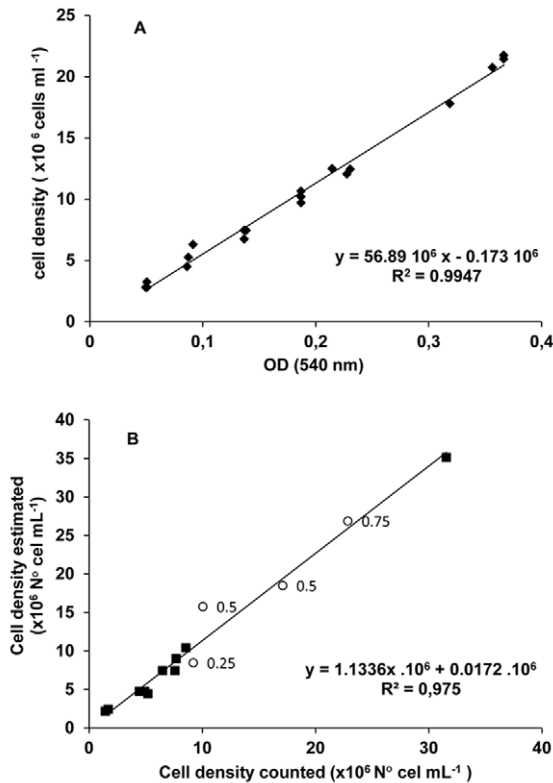


Fig. 1. *Nannochloropsis oculata*. **A.** Calibration curve of cell concentration versus OD (540 nm) corresponding to exponential and stationary phases from complete medium-cultures. **B.** Validation curve. Full squares indicate samples at early and late exponential growth phase and empty circles indicate samples at stationary phase diluted at 25 %, 50 % and 75 %.

standard curve across the entire growth cycle. Several wavelength values have been used to estimate biomass in different strains of *N. oculata*, e.g., 682 nm (Chiu et al. 2009), 625 nm (Converti et al. 2009) and 680 nm (Gu et al. 2012) and no mention was made with respect to growth phases. Spolaore et al. (2006) used OD at 540 nm to estimate cell density in *N. oculata*. However, no calibration curve was presented. Rocha et al. (2003) showed in *N. gaditana* an adequate calibration curve (cell density vs OD at 540 nm), but only for the exponential phase. In the present study, a calibration curve of cell density versus OD in *N. oculata* was estimated by: (1) using an adequate wavelength (540 nm) and (2) covering several growth phases, in order to minimize errors in cell-density quantification according to Griffiths et al. (2011). The model suitability was confirmed by using a validation data set. The Fig. 1B shows the graphic validation with a set of 12 points from exponential and stationary growth phases in complete medium-cultures. Cell densities from N-deficient cultures were similar to those found under stationary growth phase from complete medium-cultures (data not shown).

3.2. Fluorescence staining optimization of neutral lipids

In situ Nile Red fluorescence staining procedure is a rapid sensitive screening tool for neutral lipid detection in some microalgae (Doan & Obbard 2011). However, it has been unsuccessful for many other microalgae, particularly those with thick and rigid cell walls that prevent the penetration of the fluorescent dye into the cell (Chen et al. 2011). Therefore, in these cases special pre-treatments are necessary to increase the staining method efficiency. In addition, the peak signal of NR fluorescence depends on the incubation time of NR-stained cells among other factors (Pick & Rachutin-Zlogin 2012). In our study, RFI increased from 7 to 30 min after NR addition ($p < 0.05$), and stabilized after 30 min ($p > 0.05$) (Fig. 2A). A similar time was indicated by Eley et al. (2007) for *Nannochloropsis* sp. They obtained the maximum emission intensity over a period ranging between 30–40 minutes after NR addition without applying any pre-treatment, indicating a variation in NR diffusion rates through intracellular regions. Doan & Obbard (2011) found the best staining times between 5 and 10 min for *Nannochloropsis* sp., but they worked with glycerol and 15 % DMSO. So, in the present study different pre-treatments were tested (Fig. 2B). With the exception of 20 % Ac, the pre-treatments increased fluorescence signals with respect to the control (non-treated cells), particularly 20 % Et and 5 % DMSO. For the last one, the RFI was 12.5 times higher than the control; while for 20 % Et it was 10 times higher. Then, we compared only the most efficient pre-treatments (5 % DMSO and 20 % Et) with a NR stained cell suspension heated up at 70 °C, being 5 % DMSO the most efficient (Fig. 2C). Although some reports indicate that high DMSO concentrations affect survivor cells (Poncet & Veron 2003), in the present study the DMSO pre-treatment is only proposed in order to monitoring the lipid accumulation in cultures. Figure 2D shows a characteristic spectrum of stressed cells (N-deficient culture) and treated with 5 % DMSO. The fluorescence emission spectra revealed two peaks: one at 570 nm indicative for neutral lipid signal and another one at 670 nm corresponding to autoflu-

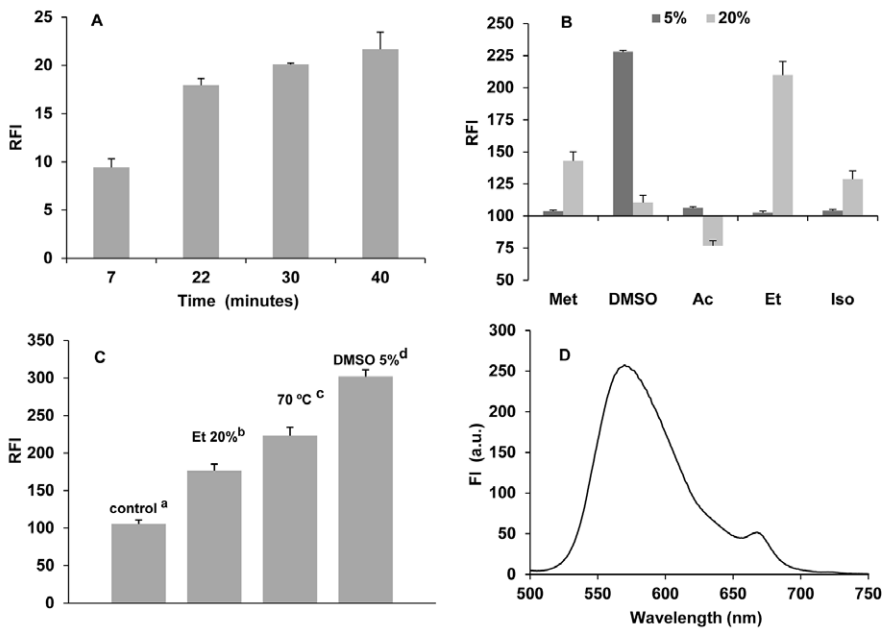


Fig. 2. Optimization of Nile Red staining methods. **A.** Relative fluorescence intensity (RFI) of NR stained cells using different staining time (control: non-treated cells). **B.** RFI deviations from control (non-treated cells) of different solvents at 5 % and 20 %. Met: methanol; DMSO: dimethyl sulphoxide; Ac: acetone; Et: ethanol; Iso: isopropanol. **C.** RFI of 5 % DMSO v/v, 20 % Et v/v and heating up at 70 °C compared with control. Data are means \pm standard deviation for three genuine replicates. Mean values with different superscript letters were significantly different ($p < 0.05$, Bonferroni). **D.** Fluorescence emission spectrum of *N. oculata* under N-deficient conditions.

orescence emission peak of Chl a. The first peak was similar to the ones reported for others species of *Nannochloropsis* (Doan & Obbard 2011, Elsey et al. 2007).

3.3. Quantitative determination of neutral lipids

Lipid standard triolein (TO) have been successfully applied in spectrofluorometric analyses in order to quantify neutral lipids in microalgae (Chen et al. 2009). This method allows the quantification and the monitoring of neutral lipids' accumulation kinetics in microalgal cultures without needing to involve a gravimetric method of quantification. In the present study, the standard TO calibration was made after the optimization of staining time and the pre-treatment selection (indicated in the section 3.2). Therefore, the TO calibration curve was obtained by using blanks and a series of standard solutions prepared in f/2: ultrapure water (1:20) and 5 % DMSO. A linear correlation between RFI (y) and the concentration of TO (x) was established in the range 5 to 30 $\mu\text{g ml}^{-1}$ according to equation: $y = (9.582 \pm 0.310 x) + (10.123 \pm 5.487)$;

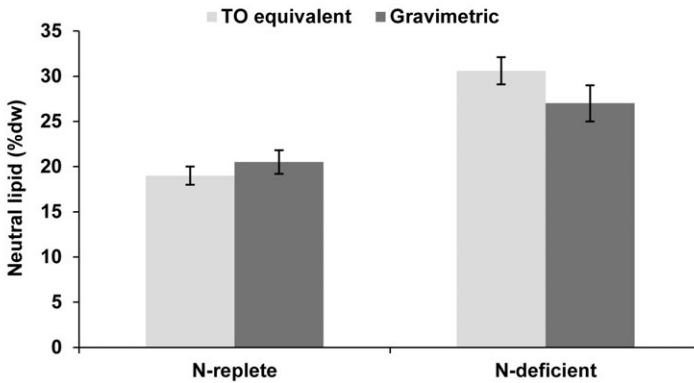


Fig. 3. Neutral lipid contents obtained through the lipid standard TO and the conventional gravimetric method. The values are expressed as a percentage of dry weight and they are means \pm standard deviation of two replicates.

$R^2 = 0.995$. Figure 3 shows the neutral lipid content determined by both gravimetric and standard TO methods. Neutral lipid contents obtained by the gravimetric method were 20.5 (± 1.3) % dry weight (dw) and 27.0 (± 2.0) % dw at N- replete (control) and N-deficient cultures, respectively; while neutral lipid concentrations obtained by standard TO method were 19.0 (± 1.0) % dw and 30.6 (± 1.5) % dw, under the same culture conditions, respectively. No significant differences were detected between the means from both methods ($p > 0.05$).

3.4. Estimation of the culture status and neutral lipid accumulation

In situ measurements were performed to estimate the culture status and neutral lipid accumulation. Figure 4A shows the time course of cell density, Chl a fluorescence intensity (Chl a-FI, in arbitrary units, au) and Nile Red fluorescence intensity (NR-FI, in au) in *N. oculata* under control and N-deficient conditions. The cell density was estimated with the model obtained in this study. Under control conditions, the cell number increased exponentially reaching stationary growth phase on day 9, with a maximum value of 19×10^7 cell mL⁻¹. The Chl a-FI showed the same trend as the cell density, increasing from around 800 to 2000 au, while the NR-IF increased from around 208 to 1500 au. Under N-deficient conditions, the cell number decreased slightly until a value of 17×10^7 cell mL⁻¹, while the Chl a-FI showed a sharp fall at the beginning of the stress, reaching a lower signal than the one reported at baseline control. The NR-IF increased up to a maximum signal of 3500 au. These observations are consistent with reports about the relationship between the decline of Chl a and the increase of total fatty acids under stressful condition in *Nannochloropsis* sp. (Solovchenko et al. 2011) as well as in other microalgal species (e.g. *Neochloris oleoabundans*, Popovich et al. 2012). Two days later, the harvest was done considering that NR-IF remained constant. It was observed that neutral lipids over-accumulated as soon as the chloro-

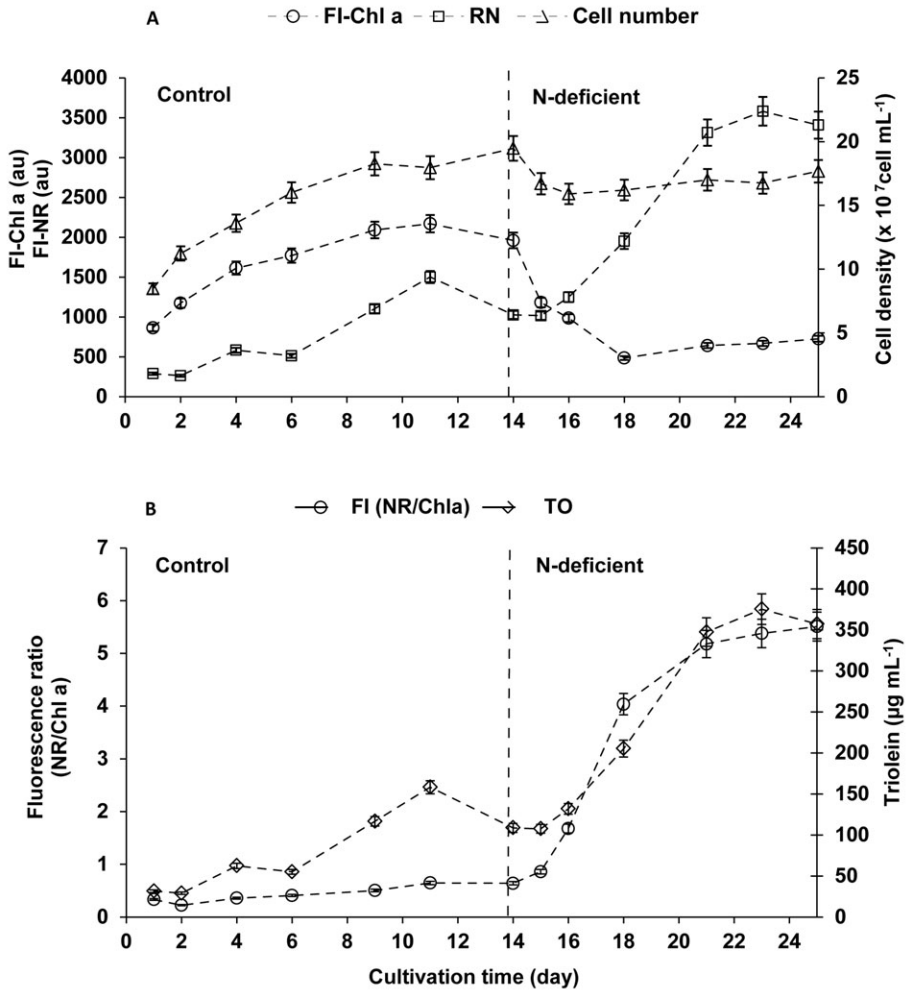


Fig. 4. *Nannochloropsis oculata*. **A.** Time courses of cell density (obtained from OD 540 nm), chlorophyll fluorescence intensity-Ex/Em: 430/670 nm-(Chl a-FI), Nile Red fluorescence intensity-Ex/Em: 480/570 nm-(NR-FI) under control and N-stress conditions. **B.** Time courses of NR-FI/Chl a-FI ratio and triolein equivalent concentrations under control and stress conditions. Data are means \pm standard deviation of three genuine replicates.

phyll reached a minimum value, while the cell density remained relatively constant. According to these results, both Chl a-FI and NR-FI showed a higher sensitivity to the lack of N than the cell density. Lipid accumulation has been mainly related to the nitrate concentration in the medium, while biomass growth could be supported at least partly by an intracellular storage of nitrogen (Pruvost et al. 2009). Therefore, the relationship between NR-FI and Chl a-FI (RN-FI/Chl a-FI) was used in *N. oculata* as a neutral lipid accumulation index to set the harvesting time (Fig. 4B). This ratio

did not present significant differences during the growth control conditions, being the values less than 1, while it increased significantly from 0.86 up to 5.5 under stress conditions, indicating a neutral lipid over-accumulation. Although in this study the harvest was performed on day 11 of N-deficient conditions, the proposed index indicates that it should be done at day 7.

3.5. Ultrastructural cell analysis and neutral lipid detection

In exponential phase, *N. oculata* cells showed a parietal chloroplast that occupied most of the cytoplasm and a thick wall surrounding the cell (Fig. 5A). In stationary phase, the cells presented a median-electron-dense body, which occupied most of the

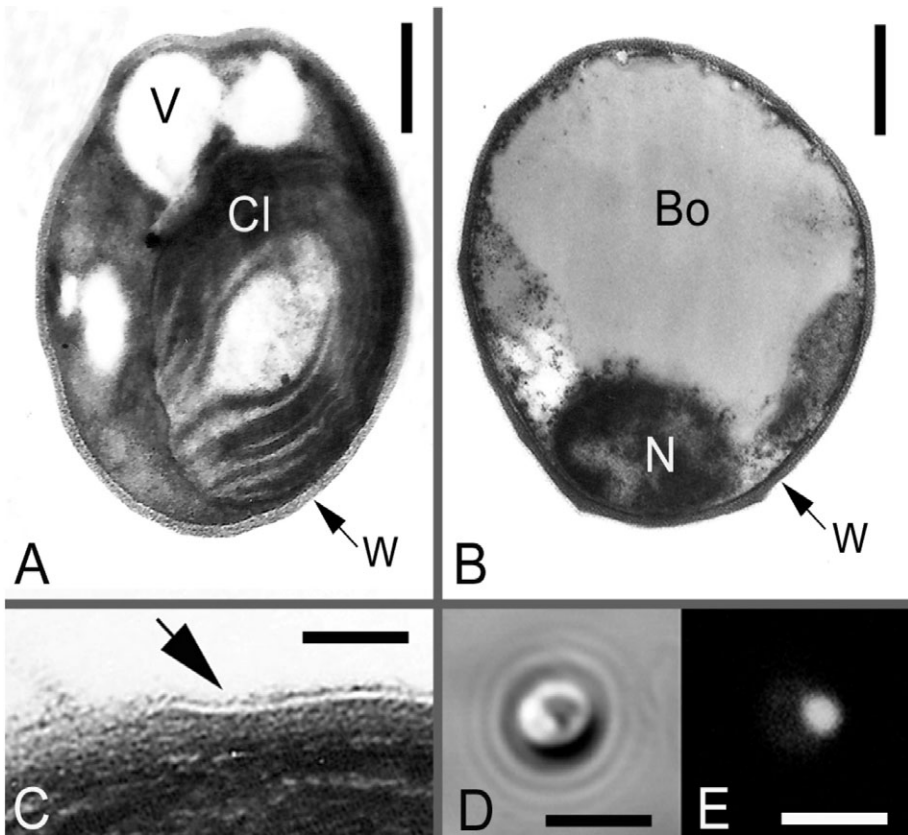


Fig. 5. *Nannochloropsis oculata*. A–C. Transmission electron micrographs. A. Cell in exponential phase with parietal chloroplast (Cl) and thick cell wall (W). B. Cell in stationary phase. Note the median-electron-dense body (Bo) occupying most of the cytoplasm and a thick cell wall. N: nucleous; V: vacuole. Scale bars: A, B = 0.6 μm, C = 0.2 μm. C. Detail of cell wall to show the trilaminar layer (arrow). D–E. Light micrographs of a cell in stationary phase. D. Phase contrast microscopy. E. Epifluorescent microscopy. A neutral lipid droplet is shown. Scale bars = 2 μm.

cytoplasm, and a thick cell wall was also observed (Fig. 5B). It is remarkable that in *N. oculata* the cell wall thickness remained constant during all stages of growth and it presented a trilaminar layer (Fig. 5C). Cell walls with similar appearance were observed in the marine species *N. salina* and *Nannochloropsis* sp. CCAP 849/7 by Gelin et al. (1997). The authors indicated the presence of an insoluble and non-hydrolysable biopolymer named algaenan responsible for its hardness.

Regarding the median-electron-dense bodies observed in *N. oculata*, similar structures were reported in *Nannochloropsis* sp. as storage globuli or accumulation bodies (Fisher et al. 1998, Rodolfi et al. 2003, Sukenik & Carmeli 1989) and in *N. limnetica*, as lipid droplets (Fietz et al. 2005). In *N. oculata* the presence of neutral lipids in the median-electron-dense-bodies was ascertained by the observation of a yellow-gold droplet in cells in stationary phase after Nile Red staining (Figs 5D, E).

4. Conclusions

Defining the neutral lipid accumulation period accurately is crucial to establish the harvesting time in microalgal cultures for biodiesel production. This period varies according to species, environmental conditions, availability of nutrients and growth phases. In this work, we have presented a model to estimate cell density and a DMSO-Nile Red staining method for *in situ* detection of neutral lipids in *Nannochloropsis oculata*. Besides, *in situ* Chl *a* and NR fluorescence measurements allowed to have a simultaneous rapid estimation of the maximum neutral lipid accumulation period and the lowest harvesting time. In addition, these analytical techniques require small quantities of culture samples. It is expected that these results provide baseline information in order to establish predictive models from an automated on-line monitoring in *N. oculata* large-scale cultures for biodiesel oil production.

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