Artículo original de investigación

Effect of irradiance on the cell density, size and lipid accumulation of *Neochloris oleoabundans*

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Abstract

Neochloris oleoabundans (Chlorophyceae) has shown potential as source of lipids for biodiesel production. Nevertheless, scant information is available about the effect of light intensity on the growth and biochemical composition of this microalga. Furthermore, no reports are available related to the effect of irradiance on its cell size. The aim of this work was to evaluate the effect of three different light intensities (50, 94 and 136 µmol $m^{-2}s^{-1}$), on the cell density and cell size of *N. oleoabundans* cultivated in a modified BBM, as well as to evaluate the presence of intracellular lipids using Sudan III. It was found that cell density was highest at 136 µmol $m^{-2}s^{-1}$ and cell productivity was sustained throughout the duration of the experimental period (15 days), under such conditions. The average cell density registered at the highest light intensity was 4.1 $x10^5$ cells mL^{-1}. The cell productivity at the end of day 1 in which a maximum specific growth rate was observed (1.30 d^{-1}), was $3.1x10^5$ cells mL^{-1} d^{-1}. Cell size was affected significantly by light intensity, being higher at the lowest (10.92 ±1.26 µm) and medium (11.88 ±1.12 µm) levels tested , compared to the size observed at the highest level (5.25 ±1.26 µm). To the best of our knowledge, there are no other reports on cell size changes as a response to different irradiance levels for this type of microalgae. Furthermore, irradiance did affect lipid accumulation: cultures exposed at the highest light intensity, showed a higher percentage of stained cells with Sudan III. This lipophilic dye turned out to be a fast and easy to use screening dye for lipids presence in microalgae.

Key words: *Green microalgae, light intensity, biodiesel production, cell diameter, Sudan III.*

Efecto de la intensidad luminosa sobre la densidad, tamaño celular y acumulación de lípidos en *Neochloris oleoabundans*

Resumen

Neochloris oleoabundans (Chlorophyceae) ha demostrado tener potencial como fuente de lípidos para producción de biodiesel. Sin embargo, existe un mínimo de información sobre el efecto de la intensidad luminosa sobre el crecimiento y composición de esta microalga. Es más, no existe

ningún reporte sobre el efecto de este parámetro sobre su tamaño celular. El objetivo del presente trabajo fue evaluar el efecto de tres intensidades luminosas (50, 94 and 136 μmol $m^{-2}s^{-1}$), sobre la densidad y el tamaño celular de *N. oleoabundans,* cultivada en medio BBM modificado, así como la presencia de lípidos intracelulares usando Sudán III. Se encontró que la mayor densidad celular ocurrió a 136 μmol $m^{-2}s^{-1}$ y que la productividad celular fue sostenida durante el período experimental (15 días), bajo tales condiciones. La densidad celular promedio registrada a la mayor intensidad luminosa fue 4.1 $x10^5$ **c**élulas mL^{-1}. La productividad celular al final del primer día de cultivo durante el cual se observó la mayor velocidad específica de crecimiento (1.30 d^{-1}), fue 3.1$x10^5$ **c**élulas $mL^{-1}d^{-1}$. El tamaño celular fue afectado significativamente por la intensidad luminosa, siendo mayor a la menor (10.92 ±1.26 μm) y media (11.88 ±1.12 μm) intensidades probadas comparado con el tamaño observado al mayor nivel probado (5.25 ±1.26 μm). De acuerdo a nuestro conocimiento, este es el primer reporte que demuestra cambios en el tamaño celular como respuesta a diferentes intensidades luminosas en esta microalga. Además, la intensidad luminosa afectó la acumulación de lípidos: los cultivos expuestos al mayor nivel probado, mostraron un mayor porcentaje de células teñidas. El Sudán III mostró ser un colorante lipofílico de fácil uso para una rápida y preliminar determinación de la presencia de lípidos en microalgas.

Palabras clave: *algas verdes, intensidad luminosa, producción de biodiesel, diámetro celular, Sudán III.*

1. Introduction

Neochloris oleoabundans (Chlorophyceae) is a unicellular green microalga which was isolated the first time from the Rub al Khali desert in Saudi Arabia (Chantanachat and Bold, 1962). Despite the fact that this microalga has potential for industrial use, there is little knowledge about it, compared to many other green microalgae. Among the early work, the one carried out by Tornabene *et al*. (1983), has been very useful since it provided detailed information about its lipid composition. More recently, research with this microalga has been oriented towards its potential as source of lipids useful for biodiesel production (Li *et al*., 2008; Lopes da Silva *et al*., 2009; Gouveia and Oliveira, 2009; Gouveia *et al*., 2009; Pruvost *et al*., 2009, 2011), including the use of wastewater for its cultivation at a low cost (Levine *et al*., 2011; Yang *et al*., 2011).

Despite the fact that it is well established that light together with temperature is one of the most important abiotic factors regulating growth of microalgae in photobioreactors (Carvalho *et al*., 2011), there is scant information about the effect of light intensity on the growth and morphological and physiological changes of *N. oleoabundans*. There is only one recent report showing that a sequential increase of light intensity resulted in a twofold increase in biomass density under a continuous illumination regime (Wahal and Viamajala, 2010). On the other hand, no reports are available related to the effect of light intensity on the cell size of *N. oleoabundans.*

Regarding lipid content, most of the work related to the assessment of the potential of certain microalgae species as a source of lipids for their transformation in biodiesel, utilize gravimetric quantification for total lipids and chromatographic analysis to determine the lipid profile (Griffiths and Harrison, 2009). Nevertheless, a fast screening of strains and the use of lipids staining is necessary and very convenient. Nile red is a lipophilic dye which has been utilized for evaluation of lipid content in

microalgae (Chen *et al*., 2011; Yen Doan y Obbard, 2011). However, sophisticated equipment is required for detecting the fluorescence derived from neutral lipids stained inside the cells and its use is limited. On the contrary, Sudan III is a lysochrome (fat-soluble dye) of the diazo type, used for staining of triglycerides and some protein bound lipids and intracellular lipoproteins.

The aim of this work was to evaluate the effect of light intensity on the cell density and cell size of *N. oleoabundans* cultivated in a modified BBM, as well as to evaluate the presence of lipids using Sudan III.

2. Materials and methods

2.1 Strain and culture conditions

Neochloris oleoabundans (UTEX 1185) was cultivated in a Bold Basal Medium (Barsanty and Gualtieri, 2006), containing the following compounds (mg L^{-1}): $NaNO_3$, 250; $CaCl_2 \cdot 2H_2O$, 25; $MgSO_4 \cdot 7H_2O$, 75; K_2HPO_4, 75; KH_2PO_4, 175; NaCl, 25; N_3BO_3, 11.42; $ZnSO_4 \cdot 7H_2O$, 8.82; $MnCl_2 \cdot 4H_2O$, 1.44; $Na_2MoO_4 \cdot 6H_2O$, 1.42; $CuSO_4 \cdot 5H2O$, 1.57; $Co(NaNO_3)2 \cdot 6H_20$, 0.49; Na_2EDTA, 50; 3 KOH, 31 and $FeSO_4 \cdot 7H_2O$, 4.98. The modification of the original formula of the BBM consisted in using $NaNO_3$ industrial grade (Química Meyer), instead of reagent grade. *N. oleoabundans* was inoculated with approximately 1.0 x 10^5 cells mL^{-1} in 250 mL Erlenmeyer flasks containing 100 mL of modified BBM (mBBM). The pH was adjusted to 6.6 and it was sterilized at 121°C for 15 min before inoculation. The flasks were incubated at 33 ±2°C, under 50 (LL), 94 (ML) and 136 (HL) µmol $m^{-2}s^{-1}$ (cool-white fluorescent light) and a 16:8 (light:dark) cycle, without agitation. Cultures aimed at being used as inoculum, were incubated under the three different light intensities in order to perform experiments with photo-acclimated cultures. Two flasks were established per each experimental condition. Cultures were agitated manually three times daily.

2.2Cell density

Cell density was measured using a Neubauer hemocytometer, daily during 15 days. The specific growth rates (µ) were determined with the following growth equation:

$$\mu = \frac{lnW_2 - lnW_1}{t_2 - t_1}$$

where µ is the specific growth rate, t_1 is the time at the beginning of the exponential phase (day), W_1 is the cell number at the beginning of the exponential phase (cell mL^{-1}), and W_2 is the cell number at the end of such phase (t_2) (Guillard, 1973).

2.3 Staining of cells with Sudan III

Intracellular lipids were stained according to O'Brien and McCully (1981). For the preparation of the ethanolic solution, 0.5 g of Sudan III were dissolved in 100 mL of 70% ethanol (J.T. Baker), keeping the solution in a warm bath during 3 hours; afterwards, the solution was filtered with Whatman No. 4 filter paper (vacuum assisted filtration). To stain the cells, 150 µL of each treatment were harvested daily throughout the cultivation period and the samples were stained with 100 µL of the ethanolic solution of Sudan III. The samples were incubated a 4°C during 24 h. The cells were observed with a Nikon Eclipse E600 microscope. Photographs were taken with a Nikon Coolpix 950 photo camera using the 40X objective.

2.4 Cell diameter

For microscopic measurement of the cells, digital images of the samples were obtained by using the camera Nikon Coolpix 950 mounted on the same microscope (Nikon Eclipse E600). The measurements of the cells diameters were made using the ImageJ software. All photographs were taken using the 40x objective. Ten measurements were made for each sample.

2.5 Statistical analyses

One-way analysis of variance (ANOVA) was used to identify differences among the treatments, and Tukey's tests at reliability level of 5% when ANOVA indicated at least one significantly different result. Normality and homocedasticity was checked using Shapiro and Barlett tests respectively in order to test ANOVA's assumptions. Statistical analyses were carried out with the statistical program R 2.13.0 (R Development Core Team 2006).

3. Results

Cultures of *N. oleoabundans* were exposed to three different light intensities which in this work will be recognized as low and medium level (50 and 94 µmol $m^{-2}s^{-1}$) and high level (136 µmol $m^{-2}s^{-1}$). There was a clear effect of the light intensity on the cell density of cultures of *N. oleoabundans* (Fig. 1), since the cell density observed after 7 and 15 days was significantly higher (p = 0.0000) at the highest irradiance compared to the lower and medium light intensity tested.

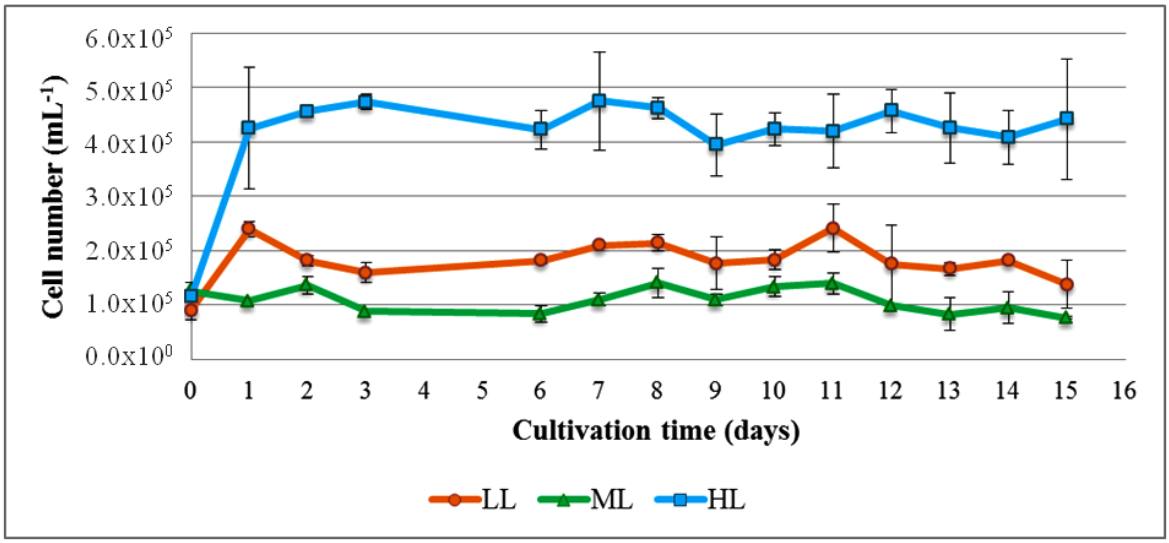

Figure 1. Effect of three light intensities (50, 94 y 136 µmol $m^{-1}s^{-1}$, LL, ML and HL, respectively) on the cell density of *Neochloris oleoabundans*.

In fact, cultures exposed to the medium irradiance level, did not show evidence of significant growth throughout the experimental period. Furthermore, it should be noticed that exponential growth only occurred at the lowest and highest irradiance tested during the first day of culture. The specific growth rate was significantly higher (µ = 1.30 d^{-1}) (p = 0.0000) at the highest light intensity tested, compared to the lowest irradiance level (µ = 0.98 d^{-1}).

In relation to the effect of the light intensity on the cell size, it is very interesting to note that this environmental factor had a strong effect on this parameter (Fig. 2).

The average cell size of the cells grown at the lower and medium light intensity tested (10.92 ±1.26 and 11.88 ±1.12 µm, respectively), was significantly higher (p = 0.0000) compared to the cell size observed at the highest level (5.25 ±1.26 µm), regardless whether the statistical analysis was performed with data from day 1 or day 15 of the experimental period (Fig. 3).

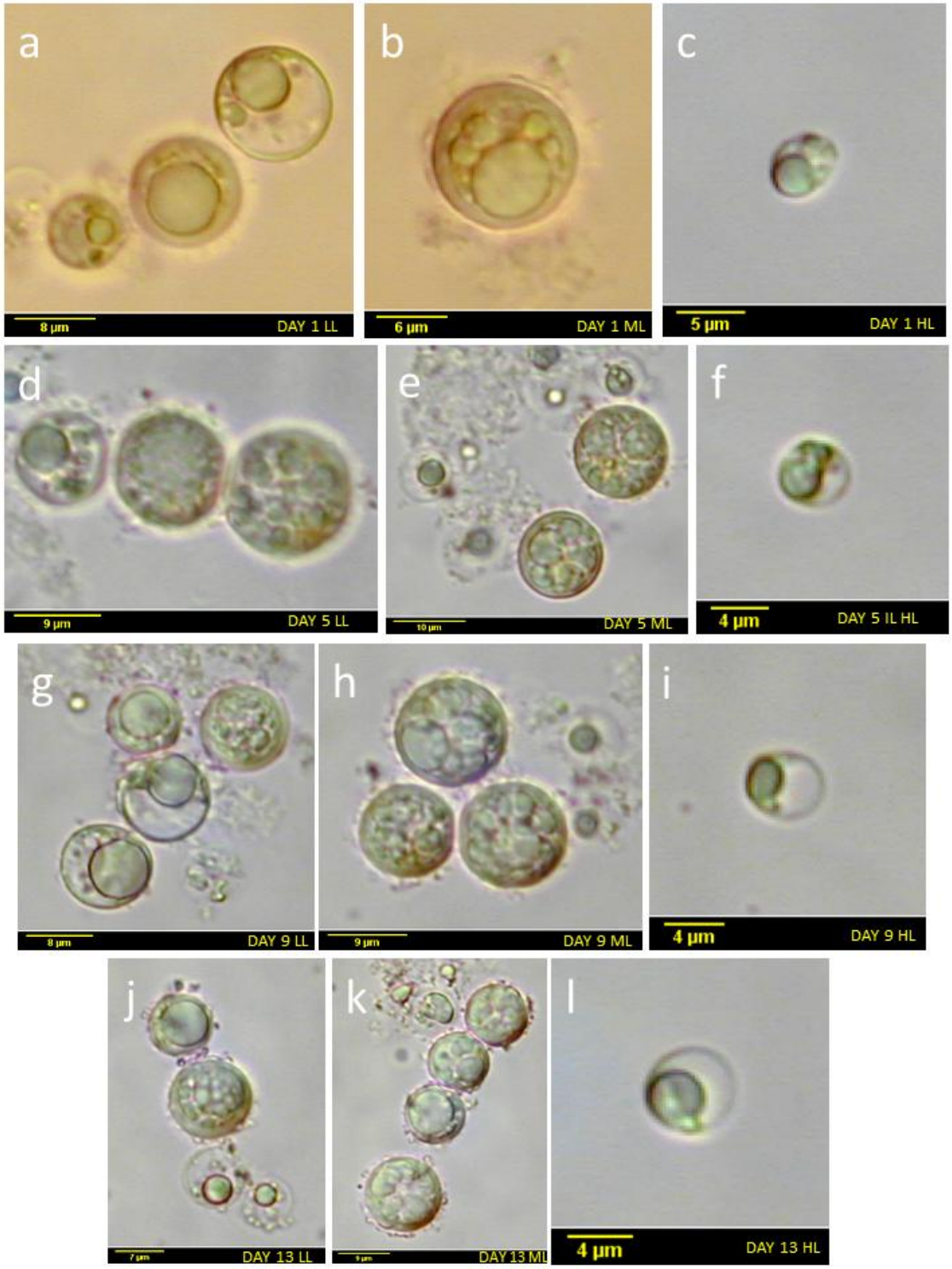

Figure 2. Effect of three light intensities (50, 94 y 136 µmol $m^{-1}s^{-1}$, LL, ML and HL, respectively) on the cell size of *Neochloris oleoabundans*. Photographs **a**, **b** and **c** were taken after 1 day of cultivation. Photographs **d**, **e** and **f**, were taken after 5 days of cultivation. Photographs **g**, **h** and **i** were taken after 9 days of cultivation. Photographs **j**, **k** and **l** were taken after 13 days of cultivation.

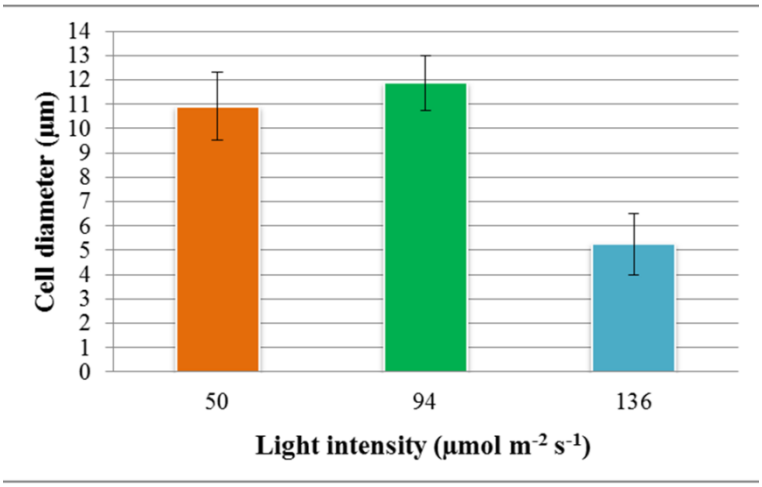

Figure 3. Average cell size of *Neochloris oleoabundans* measured at three light intensities (50, 94 y 136 µmol $m^{-1}s^{-1}$, LL, ML and HL, respectively) during 15 days. n = 10

The highest cell size recorded (12.6 µm) was observed at the lowest light intensity tested (50 µmol $m^{-2}s^{-1}$) and the smallest cell size registered (4.04 µm) was observed at the highest light intensity tested (136 µmol $m^{-2}s^{-1}$).

A preliminary assessment of the presence of lipids in the cultures of *N. oleoabundans* was carried out, staining the cells with Sudan III. The characteristic red-orange color that appears when lipids (especially triacylglycerides) are present in the cells was observed in all cultures from day 1 to day 13, regardless of the light intensity (Fig. 4).

In the case of the highest light intensity, a largest percentage of cells showed lipid accumulation (Table 1 and Fig. 4c, f, i, l), indicating a higher accumulation of lipids under this particular condition.

Table 1. Percentage of stained cells of *Neochloris oleoabundans* with Sudan III throughout the cultivation period.

Cultivation time (days)	50 µmol $m^{-1}s^{-1}$	94 µmol $m^{-1}s^{-1}$	136 µmol $m^{-1}s^{-1}$
1	44.4	57.1	74.5
3	58.5	52.2	87.5
5	71.4	57.7	80.0
7	73.3	55.2	76.5
9	62.5	55.5	100
11	61.1	52.6	75.0
13	68.3	68.9	93.2

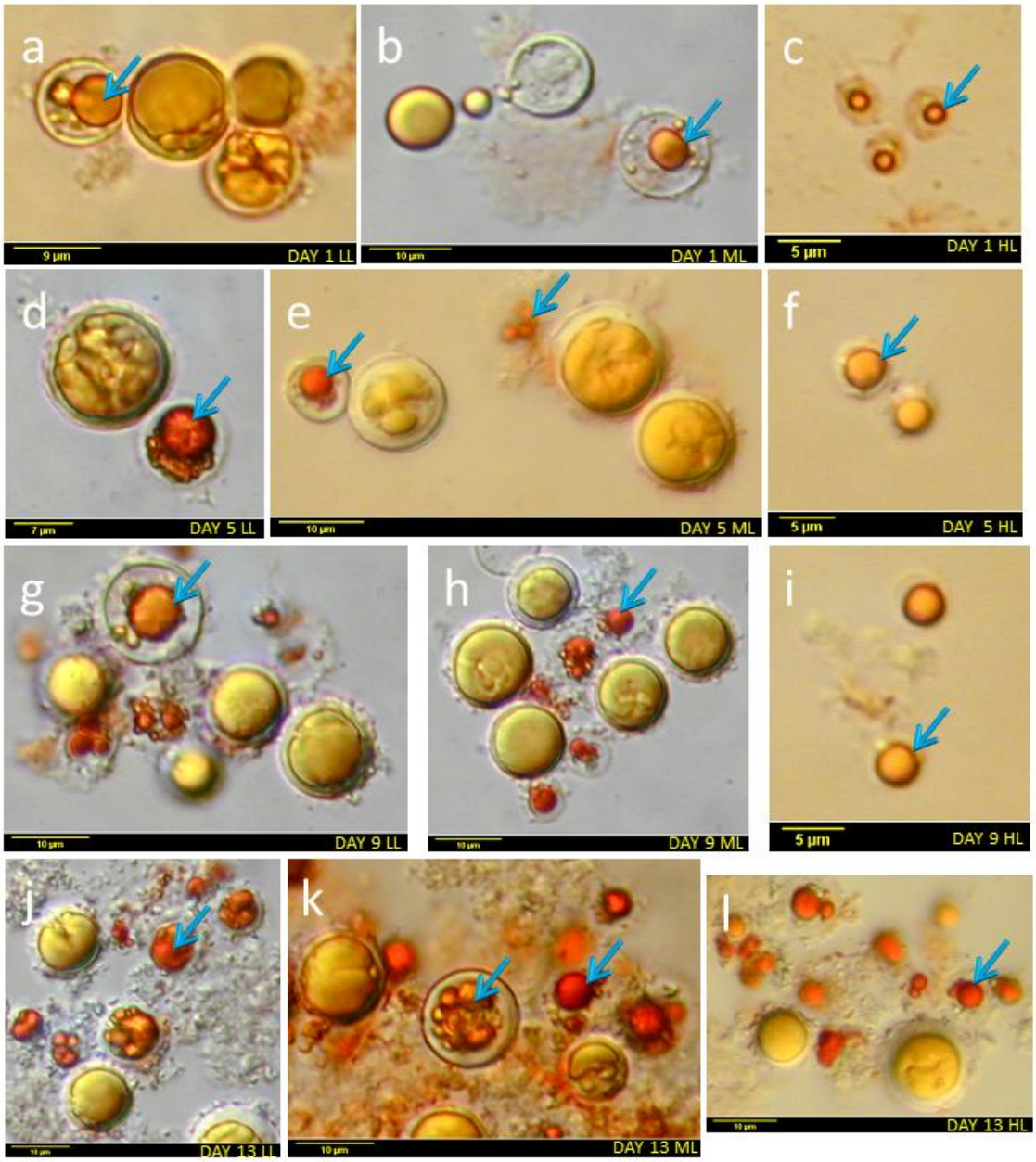

Figure 4. Presence of lipids in *Neochloris oleoabundans* cells stained with Sudan III, cultivated at three light intensities (50, 94 y 136 µmol $m^{-1}s^{-1}$, LL, ML and HL, respectively). Photographs **a**, **b** and **c** were taken after 1 day of cultivation. Photographs **d**, **e** and **f**, were taken after 5 days of cultivation. Photographs **g**, **h** and **i** were taken after 9 days of cultivation. Photographs **j**, **k** and **l** were taken after 13 days of cultivation. Arrows shows the lipid droplets stained with Sudan III.

Discussion

Despite the fact that there are a number of studies related to the effect of light intensity on the growth of cyanobacteria (Lu *et al*., 2001; Olguín *et al*., 2001) and microalgae (Pal *et al*., *in press*; Imagoglu *et al*., 2009), there is very little work performed with the green microalgae *N. oleoabundans* in this respect. In fact, to the best of our knowledge,

only in the work carried out by Wahal and Viamajala (2010), *N. oleoabundans* cultures were grown at six different levels of irradiance (in the range 70–273.1 μmol m^{-2} s^{-1}) and their effects on cell growth were evaluated. However, it is important to mention that such cultures were subjected to a continuous illumination regime. This latter factor provides artificial conditions, which are different from those encountered under natural light illumination. Furthermore, the circadian rhythm of photosynthesis, respiration, cell division, and the growth rates have been found to be influenced by the day length or period of exposure to light (Bouterfas *et al*., 2006). Thus, it is difficult to compare the results in the work reported herewith in relation to the effect of the light intensity on cell growth and maximum cell densities in *N. oleoabundans* cultures, in which the light regime was 16h light per 8h dark, with those carried out under continuous illumination. Nevertheless, comparison with research carried out with other microalgae may also provide useful discussion.

Under the conditions prevailing in the current work, the cell density of *N. oleoabundans* was significantly higher (p = 0.000) at the higher light intensity tested (136 μmol $m^{-2}s^{-1}$). These results are similar to those found by Wahal and Viamajala (2010) who reported a maximum cell density of *N. oleoabundans* at 177.8 μmol $m^{-2}s^{-1}$, observing a decrease at higher irradiance levels. Thus, it remains to be determined the optimum irradiance level to obtain the maximum cell density of *N. oleoabundans* under the conditions tested in this work. Furthermore, the lack of growth at the medium irradiance level should be explained. The observation that cell density increases directly with an increase in light intensity is also in agreement with some other reports, working with other microalgae. Tang *et al.* (2011) found that *Chlorella minutissima*, showed the highest cell density at the highest light intensity tested in the range 100 to 350 μmol $m^{-2}s^{-1}$. Likewise, an increase in the cell density of *Pseudochlorococcum* sp. was reported as the light intensity increased from 20 to 600 μmol $m^{-2}s^{-1}$ (Li *et al*., 2011).

In agreement with the results of cell density, the maximum specific growth rate of *N. oleoabundans* reported in this work (1.304 d^{-1} with a generation time of 0.53 d), during the first day of growth was significantly higher at the higher irradiance tested. It is important to note that such value compares favorably with those reported for *N. oleoabundans* (0.495 d^{-1} and 0.37 d^{-1}) by Gouveia *et al*. (2009) and Murray *et al*. (2011), respectively and is similar to the value reported for *N. oleoabundans* cultures (1.38 d^{-1}) when it is under a partial oxygen pressure of 0.24 bar (Sousa *et al*., 2012).

It should be emphasized that although the value of the maximum specific growth rate is important, the most relevant parameter related to biomass production is biomass productivity (i.e. number of cells per ml per day or dry weight per liter per day). The average cell density throughout the whole experimental period, registered at the highest light intensity tested in this work (136 μmol $m^{-2}s^{-1}$) was 4.1 $x10^5$ cells mL^{-1}. The cell productivity at the end of day 1 in which a maximum specific growth rate was observed, was $3.1x10^5$ cells $mL^{-1}d^{-1}$. This maximum value compares well with the data reported for *Ettlia oleabundans* (Yang *et al*., 2011), grown in a mixture of 2% anaerobic effluents from agricultural wastes after 35 days ($2.6 x10^5$ cells $mL^{-1}d^{-1}$).

Oleaginous microalgae should produce not only a high cell density but also sustained cell productivity in order to become attractive for lipid and biodiesel production. It is worth to note that *N. oleoabundans* maintained a sustained cell density throughout all the experimental period (15

days) at the higher light intensity tested in this work, in contrast with the other two lower intensities in which cell density started to decline after 11 days. Thus, it seems likely that intracellular storage compounds served as source of nutrients once the external sources were exhausted at this higher level of irradiance, compared to the other lower intensities. Further work is required to elucidate these aspects. It has been shown that nitrogen deficiency induces starch and lipid accumulation in *Chlamydomonas reinhardtii*. A first peak of starch accumulation appeared after only 2 days of cultivation, before the peak of lipid accumulation occurred after 5 days of growth under nitrogen deficiency conditions. Simultaneously, chlorophyll content decreased (Siaut *et al*., 2011). In the results reported herein, cultures appeared lacking chlorophyll as a pale yellow color predominated throughout the cultivation period.

The results shown in this work in relation to the effect of the light intensity on the cell size of *N. oleoabundans* are quite original, since to the best of our knowledge, there are no other reports showing a similar effect of irradiance for this microalga. Although very little is known in other microalgae about the effect of irradiance on cell size, other factors have been shown to have influence over this parameter. *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* exhibited restricted cell division and increased cell size following N-limitation (Dean *et al*., 2010). Thus, it could be possible that the larger cell size found in *N. oleabundans* cultures exposed to the low and medium light intensity tested in this work, could have been the result of a restricted cell division and as a result of this, a lower cell density was observed under these conditions in comparison with the cultures exposed to the highest light intensity tested.

Biochemical composition has been shown to be influenced by the irradiance in cyanobacteria (Olguín *et al*., 2001; Lu *et al*., 2001; Ravelonandro *et al*., 2008) and in microalgae (Seyfabadi *et al*., 2011; Imamoglu *et al*., 2009). In this study, preliminary work has been performed in order to have a rapid indication of the presence of lipids in the cell biomass of *N. oleoabundans* using Sudan III. Although further work is in progress to assess lipid accumulation by quantitative techniques, it is interesting to note that irradiance did affect lipid accumulation. Cultures exposed at the higher light intensity tested in this work, showed a higher percentage of stained cells. These results are in agreement with reports for other green microalgae. In cultures of *Nannochloropsis oculata*, it was shown that irradiance exhibited a significant influence on lipid production. The highest lipid productivity was obtained at an irradiance of 500 µmol photon $m^{-2}s^{-1}$ (Su *et al*., 2011). It has also been shown that nitrogen deficiency resulted in a lower biomass accumulation in *Chlorella minutissima* cultures, while lipid yields increased to 40–46% DW (Ördög *et al*., in press). Thus, it could be possible that in the *N. oleoabundans* cultures reported herein, a nitrogen deficiency together with a response to a high light intensity exposure, could have promoted lipid accumulation.

5. Conclusions

Novel aspects in relation to the effect of light intensity on growth and biochemical composition of *N. oleoabundans* have been described in this work. Cell density was highest at the highest light intensity tested and cell productivity was sustained throughout the duration of the experimental period (15 days), under such conditions. Cell size was affected significantly by light intensity, being higher at the lowest and medium levels tested. To the best of our knowledge, there are no other reports related

to morphological changes as a response to different levels of irradiance for this microalga.

Finally, Sudan III turned out to be a fast and easy to use screening dye for lipids presence in microalgae. Most interesting, it was found that irradiance did affect lipid accumulation: cultures exposed at the higher light intensity tested in this work, showed a higher percentage of stained cells.

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