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*Research Article*

# Fatty acid profile and productivity variation during the growth of *Dunaliella* sp. under different photon flux densities and glycerol concentrations

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**ABSTRACT.** Microalgae can accumulate lipids during the stationary growth phase, but little information is available about fatty acid profile changes during this phase to determine the best harvesting time in respect to lipid content. In this study, *Dunaliella* sp. was cultured in F/2 medium at three different photon flux densities (380, 226 and 8.2 μmol photon $m^{-2}$ $s^{-1}$) and three different glycerol concentrations (0, 10 and 20 g $L^{-1}$). Samples were taken during the stationary phase to assess lipid content and fatty acid profile variations. Microalgal biomass production was higher at 380 and 226 μmol photon $m^{-2}$ $s^{-1}$ than at 8.2 μmol photon $m^{-2}$ $s^{-1}$ in accord to light limitation. The maximum lipid content (345.78 mg $g^{-1}$) was achieved at 8.2 μmol photon $m^{-2}$ $s^{-1}$ and 20 g $L^{-1}$ glycerol at day 12, similar to that achieved at day 9 (334.16 mg $g^{-1}$). The maximum polyunsaturated fatty acid amount (65.30 μg $mg^{-1}$) was achieved at day 7 of culture without glycerol addition, decreasing in proportion over time. So, the best conditions and harvesting time in respect to fatty acid quality would be at 380 μmol photon $m^{-2}$ $s^{-1}$ without glycerol addition and after 7 days of culture.

**Keywords:** fatty acids; lipids; microalgae; glycerol; cholesterol

## INTRODUCTION

Aquaculture has grown in recent decades, feeding of aquatic organisms has been based on fishmeal and fish oil, which has increased the need for wild fish, the feedstock for these products (Sfez et al. 2015), leading to economic, ethical and environmental concerns (Steinrücken et al. 2017). As fishing has reached its maximum sustainable yield (Shah et al. 2018), alternatives for fish oil are needed to cover the demand of the growing aquaculture industry (Sarker et al. 2016, Shah et al. 2018). Microalgae oils are an alternative, since they are the primary aquatic producers, capable of producing most of the nutrients needed for food production in the aquaculture industry (Shah et al. 2018). Fatty acids are accumulated by certain microalgal species, under specific culture conditions (Castilla-Casadiego et al. 2016).

Microalgae require energy and carbon sources for cell multiplication and lipid accumulation (Patel et al. 2020). Some microalgae store lipids, these cells are able to survive in adverse environmental conditions or in the presence of stressors (Mixson-Byrd & Burkholder 2017). Lipid accumulation in microalgae depends on diverse factors, such as growth conditions and metabolism (Chavoshi & Shariati 2019), which alter lipid metabolism in terms of total content and composition in response to environmental stressors

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(Mixson-Byrd & Burkholder 2017). Organic carbon sources influence lipid production, contributing to their accumulation and enhancing microalgal growth (Ahuja et al. 2020). Growth conditions also have an effect on sterol composition and concentration (Lu et al. 2014). Scarce information is available about synthesis and regulation of sterol in microalgae, which could be connected to that of triacylglycerol production (Scodelaro-Bilbao et al. 2020).

Microalgae of the *Dunaliella* genus are able to grow at high salinities, which inhibit the growth of other microorganisms that could damage or feed on the microalgae and thus avoiding contamination in open cultures (Mobin & Alam 2017, Hopkins et al. 2019). *Dunaliella* species lack rigid cell walls, making lipid extraction easier since cellular lysis is not difficult (Mobin & Alam 2017). *Dunaliella salina* is able to accumulate glycerol when cultured under high salinity conditions, accumulating up to 50% glycerol on a dry basis (Monte et al. 2020). The osmotic stress in high salinity environments triggers the regulation of glycerol, this osmolyte is used for maintaining enzyme activity in the presence of low water activity (Oren 2017, Polle et al. 2020). Glycerol production occurs via $CO_2$ fixation or starch degradation (Mixson-Byrd & Burkholder 2017).

Expression of the enzyme glycerol-3-phosphate dehydrogenase is induced by abiotic stress and involved in lipid synthesis since glycerol-3-phosphate is an intermediate in the lipid synthesis pathway (Wu et al. 2019). Glycerol metabolism depends on the microalgal growth phase, whether it needs energy for division or requires energy storage in the form of lipids, so glycerol can be synthesized or used to produce other metabolites, such as carbohydrates or pyruvate. Additionally, glycerol is the molecule onto which fatty acids are esterified to form triacylglycerides (Mixson-Byrd & Burkholder 2017).

Several studies have investigated the production of glycerol by *Dunaliella* species, but few studies have focused on the use of glycerol as an organic carbon source for *Dunaliella*. The research of Sohrabi et al. (2019) and Liang et al. (2019) is about how culture aading glycerol affected the lipid concentration in biomass but not fatty acids. Another study did not use glycerol as a carbon source and reported lipid concentrations but not fatty acids (Chavoshi & Shariati 2019). Several studies have investigated the influence of abiotic stress on lipid and other metabolite concentrations. In most of them, fatty acids were reported only at one point of the stationary phase (Lamers et al. 2012, Lee et al. 2014, Castilla-Casadiego et al. 2016, Mixson Byrd et al. 2017, Hopkins et al. 2019, Almutairi 2020, Chen et al. 2020, Da Silva et al. 2020, Hosseinzadeh-Gharajeh et al. 2020a,b, Salinas-Whittaker et al. 2020, Wu et al. 2020). Microalgae cultured under optimal growth conditions principally synthesize fatty acids for membrane lipids (Hu et al. 2008) but under unfavorable conditions, some accumulate energy in the form of neutral lipids (Singh et al. 2016). Unfavorable conditions impose stress on the culture via chemical or physical factors, as well as growth phase or aging of the culture. Neutral lipids are rich in triacylglycerols, but unlike glycerolipids that form membranes, triacylglycerols are not structural and are only a reservoir of carbon and energy (Hu et al. 2008). *De novo* lipid synthesis uses acetyl-CoA as a precursor (Lenka et al. 2016); microalgae have acetyl-CoA reservoirs in the chloroplast and in the cytosol. Cytosolic acetyl-CoA is used for polyunsaturated fatty acid (PUFA) elongation, forming long chain PUFAs (Garay et al. 2014). Under high light intensity, excessive electrons are generated via photosynthesis, producing reactive oxygen species (ROS) that trigger triacylglycerol accumulation, which acts as an electron sequestration mechanism (Lenka et al. 2016). In general, low light intensities induce membrane polar lipid formation, whereas high light intensity increases neutral lipid storage. Light intensities also alter fatty acid unsaturation; in most cases, low intensity favors PUFA formation, while high intensities favor saturated and monounsaturated fatty acid formation (Hu et al. 2008). Therefore, changes in the fatty acid profile of the microalga need to be assessed during the stationary phase (where neutral lipids accumulate) because information is scarce about the best harvesting time for a culture in which more saturated, unsaturated or PUFAs are produced based on the final use of lipids.

Moreover, very little information is available on sterol production in microalgae. In this regard, the purpose of this study was to culture *Dunaliella* sp. in two culture stages, first in autotrophic mode and then at different photon flux densities, adding two different glycerol concentrations to demonstrate how modifying these culture conditions could affect the lipid content and fatty acid composition of the microalgae during its stationary growth phase. Thus, lipid content and fatty acid profile were assessed during the stationary growth phase to determine the best harvesting time when better lipid quality could be obtained from microalga biomass.

## MATERIALS AND METHODS

### Microalgae culture

The microalga *Dunaliella* sp. is part of the culture collection of the Live Food Laboratory from the Multidisciplinary Research and Teaching Unit (UMDI-

Sisal), Faculty of Sciences, Universidad Nacional Autónoma de Mexico (UNAM). This microalga was donated by the Norwest Center of Biological Research (Centro de Investigaciones Biológicas del Noroeste S.C.), Baja California Sur, Mexico. The culture was unialgal since microalgae were cultured in solid F/2 medium at 32, where isolated microalgal colonies were selected as inoculants for liquid F/2 culture medium.

The cultures were performed in 2-L flasks with f/2 Guillard culture medium (Guillard, 1975) at a salinity of 32, 24°C and continuous photoperiod at photosynthetically active photon flux density (PAPFD) of 350 µmol photon $m^{-2}$ $s^{-1}$ with white fluorescent lamps (Phillips 32 Watt, Phillips & Son, USA) for six days. F/2 medium was sterilized at 121°C and 106 kPa. The cultures were stirred with a flow of filtered air at 0.4 L $min^{-1}$. At day 6, some cultures were supplemented with glycerol (10 and 20 g $L^{-1}$) at a PAPFD of 380, 226 and 8.2 µmol photon $m^{-2}$ $s^{-1}$, every culture was performed with a control without glycerol addition. Each treatment was made by triplicate. Cell counting was performed with a Neubauer improved haemocytometer (American Optical) under an optical microscope (Leica, CME) at 40x magnification by triplicate.

## Biomass determination

Biomass was harvested every two to three days, starting at day 6 of the culture, in a centrifuge (IEC centra MP4R) at 1760 g for 8 min, and subsequently lyophilized (Labconco Freezone 2) to obtain dry biomass. Finally, the microalgae were burned at 550°C to get ash-free dry weight biomass (Algal Biomass Organization, 2010). Only one sample was taken for flask.

Biomass, lipid, and fatty acids productivities, were calculated according to Zhu et al. (2016).

## Lipid extraction and total lipid determination

Lipid extraction of 100 mg of lyophilized microalgae was performed according to Folch's extraction procedure (Folch et al. 1957) with modifications. An ultrasound-assisted extraction was performed with dichloromethane:methanol solution (2:1 v/v). Lipid extracts were evaporated with a $N_2$ stream at 40°C; the total lipid percentage was determined by gravimetry (Magaña-Gallegos et al. 2018b).

## Fatty acid profile determination

Extracted lipids were saponified with potassium hydroxide/methanol (20% w/v) solution to separate the saponifiable fraction, obtaining free fatty acids by adjusting the pH to 1-2, and hexane was added to separate them from other lipidic fractions. Fatty acid methyl esters (FAMEs) were formed by esterification with 10% $BF_3$ in methanol (Fluka 15716) at 80°C for 60 min. FAMEs were analyzed by capillary gas chromatography in a Perkin Elmer Clarus 500 gas chromatograph equipped with a Zebron ZB-WAX capillary column (Phenomenex, 7FD-G007-08; 20 m of length, 0.18 mm I.D. and 0.18 µm film thickness) and a flame ionization detector (FID) (Cárdenas-Palomo et al. 2018, Magaña-Gallegos et al. 2018a).

## Cholesterol determination

Cholesterol was measured spectrophotometrically with a cholesterol plasma kit (ELITech Group) with a 5 min incubation time, during which the detection of other phytosterols was negligible according to Moreau et al. (2003). One determination was made by sample.

## Chlorophyll determination

Chlorophyll was extracted from biomass with 90% acetone and determined spectrophotometrically (BioRad x Mark Microplate Spectrophotometer) using the equations described by Yu et al. (2017). Chlorophylls *a* and *b* were determined; the values shown are total chlorophyll (the sum of both chlorophyll *a* and *b*). One determination was made by sample.

## Experimental design and statistical analysis

Biomass and fatty acid data were analyzed by a multifactorial linear model of mixed effect design to evaluate the effect of photon flux density, glycerol concentration, and culture time on lipid content and fatty acid profile. The model included three main variation sources: 1) photon flux density (fixed factor with three levels: 380, 226, and 8.2 µmol photon $m^{-2}$ $s^{-1}$), 2) glycerol concentration (fixed factor with three levels: 0, 10 and 20 g $L^{-1}$), and 3) time (fixed factor with five levels: 7, 9, 12, 14 and 16 days). All these factors had first- and second-order interactions. For each combination of photon flux density and glycerol concentration, three replicates were used. Every replicate was considered a random variation source nested in the first-order interaction of photon flux density and glycerol concentration. Lipid content was evaluated with univariate analysis of variance (ANOVA). Homogeneity of variances was evaluated with Levene's test, as well as normal distribution of residuals with the Shapiro-Wilk test (Quinn & Keough 2002).

In the case of the fatty acid profile, the analysis was similar to that for the lipid content but with a multivariate analysis of variances based on distances and permutations (Anderson, 2017). To guarantee that all fatty acids had the same contribution in the multivariate analysis, data were normalized (i.e. scaled to zero mean and unit variance). The Euclidean distance between each pair of replicates was calculated; the total variation in this distance matrix was partitioned with the linear mixed effects model described above. For each term in the model, the null hypothesis was created with 999 permutations of residuals under the reduced model. Each experimental treatment centroid was projected in a main component analysis. Then, a matrix of centroids was calculated for the second-order interaction time × photon flux density × glycerol and projected in an ordination using the first two components of a principal component analysis (PCA).

Each microalgal culture had three replicates, of which those without glycerol addition were the control groups. For lipid, biomass and fatty acid profiles, three measurements were also performed.

Almost all statistical analyses were performed with R (R Core Team 2020). Univariate ANOVA was performed with the R package GAD (Sandrini-Neto & Camargo 2014), and univariate charts were generated with ggplot2 (Wickham 2010). The multivariate analyses were performed with PRIMER v7 & PERMANOVA+ (Anderson et al. 2008).

Chlorophyll and cholesterol were assessed using a linear mixed effect model (LME) (Millar & Anderson 2004) with interaction terms for time, photon flux density and glycerol concentration. As a random effect, the intercept for flasks was considered. Model analysis and data exploration were carried out according to (Zuur et al. 2009, 2010). LME was fit using a restricted maximum likelihood. A structure variance ($\varepsilon ij \sim \sigma 2\delta_S^2$) with different dispersions by photon flux density stratum was included when the chlorophyll model was fitted, where S represents the ratio between the standard deviations of photon flux density at 226 µmol photon m$^{-2}$ s$^{-1}$ stratum ($\delta = 0.45$) and photon flux density at 8.2 µmol photon m$^{-2}$ s$^{-1}$ stratum ($\delta = 0.30$) with respect to photon flux density at 380 µmol photon m$^{-2}$ s$^{-1}$ stratum ($\delta = 1$). On the other hand, the cholesterol model included structural variance ($\varepsilon ij \sim \sigma 2[\text{Time}ij]2\delta$) with a constant parameter $\delta = 0.99$, which represents the variability of cholesterol concentrations with time. Model assumptions were validated by visual inspection of residual plots versus fitted values and residuals for temporal dependency. The pairwise intercept and slope comparisons were assessed with Tukey's test with Bonferroni correction using the *R* package *emmeans* (Lenth 2020). The LME model was fit using the R package *nlme* (Pinheiro et al. 2020). Data are shown as the mean ± standard deviation (n = 3). The analyses were performed with R (R Core Team 2020).

## RESULTS

### Cell density

The highest cell density (2.39×10$^6$ cells mL$^{-1}$) was obtained in the culture at 380 µmol photon m$^{-2}$ s$^{-1}$ without glycerol addition after 16 days of culture.

From day 0 to day 6, an exponential growth phase was observed in all cultures; from day 6 to 7, an adaptation phase corresponded to the addition of glycerol to the cultures. After day 7, new exponential growth was observed in cultures with and without glycerol addition, but this time, microalgal growth was slower (Fig. 1).

The cell density was very similar ($P > 0.05$) at the same photon flux density even with different glycerol concentrations, and the cell density was different only when the photon flux density changed. At 380 and 226 µmol photon m$^{-1}$ s$^{-1}$, cell density was similar ($P > 0.05$) and always higher than that at 8.2 µmol photon m$^{-2}$ s$^{-1}$ (Fig. 1).

Biomass productivity was higher after seven days of culture in all cases because it was the end of the exponential growth phase. In this case, the highest biomass productivity was achieved in the culture at 226 µmol photon m$^{-2}$ s$^{-1}$ without glycerol addition (247 mg L$^{-1}$ d$^{-1}$), followed by the culture at 380 µmol photon m$^{-2}$ s$^{-1}$ without glycerol addition (188 mg L$^{-1}$ d$^{-1}$). Due to low growth in cultures at 8.2 µmol photon m$^{-2}$ s$^{-1}$, the lowest biomass productivities were achieved under this condition.

### Total lipids

The second-order interaction time × photon flux density × glycerol concentration was statistically significant ($P < 0.05$) (Table 1), which implied that the light × glycerol interaction was not consistent over time.

The lipid content was always higher in cultures supplemented with 20 g L$^{-1}$ glycerol ($P < 0.05$). At seven days, the maximum lipid content was achieved at 226 µmol photon m$^{-2}$ s$^{-1}$ (267.64 ± 47.08 mg g$^{-1}$), but at nine days of culture, the highest lipid contents were achieved at 226 and 8.2 µmol photon m$^{-2}$ s$^{-1}$ (339.93 ± 47.81 and 334.16 ± 45.21 mg g$^{-1}$, respectively). The highest lipid content was achieved after 12 culture days at 8.2 µmol photon m$^{-2}$ s$^{-1}$ (345.78 ± 30.25 mg g$^{-1}$), similar to the content achieved at nine days of culture at 226 µmol photon m$^{-2}$ s$^{-1}$ (339.93 ± 47.81 mg g$^{-1}$) (Fig. 2). In this case, the highest lipid productivity was also achieved in the culture at 226 µmol photon m$^{-2}$ s$^{-1}$ with the addition of 20 g L$^{-1}$ glycerol after nine culture days (63.1 mg L$^{-1}$ d$^{-1}$). However, even when a high lipid content was achieved in cultures with the addition of 20

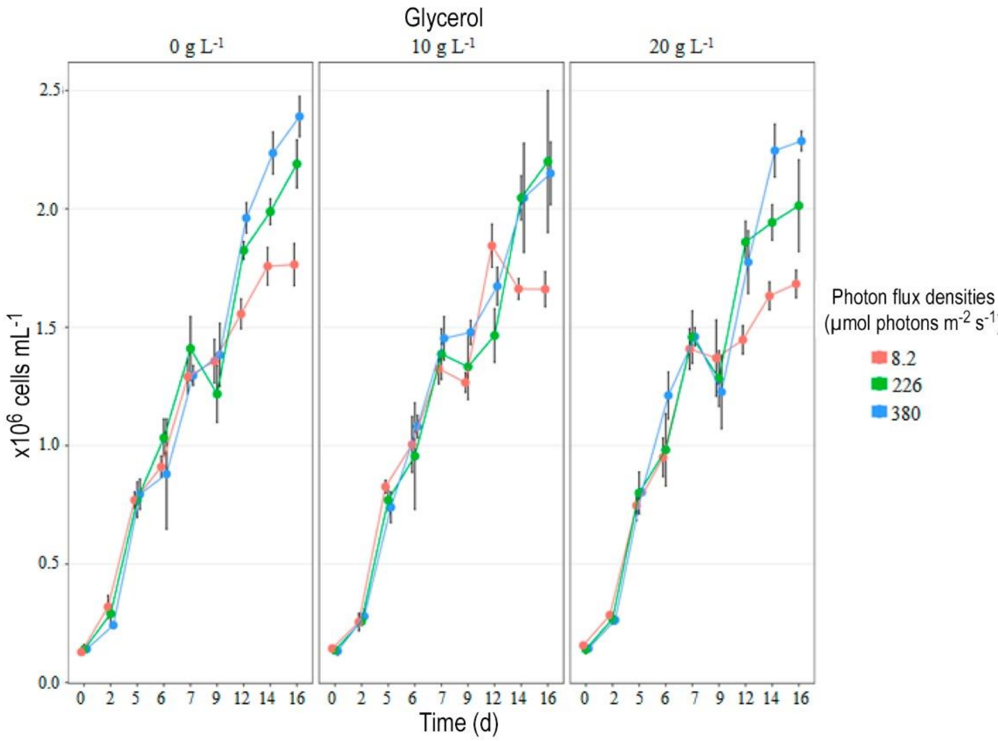


**Figure 1.** Growth curves of *Dunaliella* sp. at 8.2, 226, and 380 µmol photon $m^{-2}$ $s^{-1}$ and the control without glycerol addition and added 10 and 20 g $L^{-1}$ glycerol (mean ± standard deviation, n = 3).

**Table 1.** Analysis of variance (ANOVA) of mixed effects was used to evaluate the effect of photon flux density, glycerol and culture time on lipid production in *Dunaliella* sp. PFD: photon flux density, df: degrees of freedom, SS: sum of squares, MS: mean squares.

| Source | df | SS | MS | Pseudo-F | *P* (perm) |
|---|---|---|---|---|---|
| Time | 4 | 34915 | 8729 | 9.53 | 0.001 |
| PFD | 2 | 28308 | 14154 | 9.12 | 0.003 |
| Glycerol | 2 | 174990 | 87493 | 56.37 | 0.001 |
| Time × PFD | 8 | 13171 | 1646 | 1.8 | 0.094 |
| Time × glycerol | 8 | 16490 | 2061 | 2.25 | 0.033 |
| PFD × glycerol | 4 | 31473 | 7868 | 5.07 | 0.009 |
| Flask (PFD × glycerol) | 18 | 27939 | 1552 | 1.69 | 0.052 |
| Time × PFD × glycerol | 16 | 35225 | 2202 | 2.4 | 0.007 |
| Residuals | 72 | 65982 | 916 | | |

g $L^{-1}$ glycerol at 380 µmol photon $m^{-2}$ $s^{-1}$, this culture did not have high lipid productivities, although they were greater than those of the culture at 226 µmol photon $m^{-2}$ $s^{-1}$ and seven days of culture (38.5 mg $L^{-1}$ $d^{-1}$).

Only the lipid content at 8.2 µmol photon $m^{-2}$ $s^{-1}$ remained over 300 mg $g^{-1}$ until day 16. At 380 µmol photon $m^{-2}$ $s^{-1}$, the lipid content never exceeded 275 mg $g^{-1}$, and the cultures without glycerol addition did not have great variations in lipid content (Fig. 2).

**Fatty acid profiles**

The first-order interaction time × glycerol concentration was statistically significant ($P < 0.05$, Table 2), which indicated that the glycerol effect was not consistent over time. On the other hand, the photon flux density × glycerol interaction was not statistically significant ($P > 0.05$, Table 2), which meant that the photon flux density and glycerol effects (both with $P <$ 0.05, Table 2) on the fatty acid profile were significant and independent.

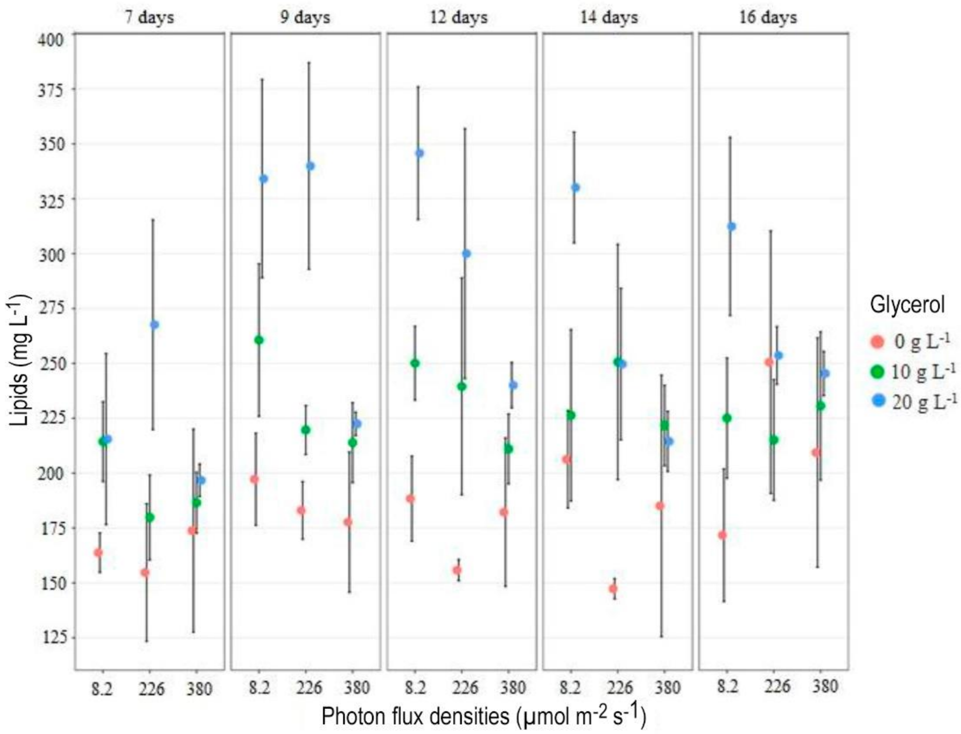


**Figure 2.** Lipid content of *Dunaliella* sp. at 8.2, 226, 380 µmol photon $m^{-2}$ $s^{-1}$ and the control without glycerol addition and added 10 and 20 g $L^{-1}$ glycerol (mean ± standard deviation, n = 3).

**Table 2.** Multivariate analysis of variance of mixed effects was used to evaluate the effect of photon flux density (8.2, 226, 380 µmol photon $m^{-2}$ $s^{-1}$), glycerol (0, 10 g $L^{-1}$ and 20 g $L^{-1}$) and culture time (7, 9, 12, 14 and 16 days) on the fatty acid fractions. PFD: photon flux density, df: degrees of freedom, SS: sum of squares, MS: mean squares.

| Source | df | SS | MS | Pseudo-F | *P* (perm) |
|---|---|---|---|---|---|
| Time | 4 | 317.70 | 79.43 | 5.07 | 0.000 |
| PFD | 2 | 239.67 | 119.83 | 5.19 | 0.000 |
| Glycerol | 2 | 368.97 | 184.49 | 7.99 | 0.000 |
| Time × PFD | 8 | 145.70 | 18.21 | 1.16 | 0.196 |
| Time × Glycerol | 8 | 192.40 | 24.05 | 1.54 | 0.017 |
| PFD × Glycerol | 4 | 116.50 | 29.13 | 1.26 | 0.187 |
| Flask (PFD × Glycerol) | 18 | 415.81 | 23.10 | 1.48 | 0.003 |
| Time × PFD × Glycerol | 16 | 291.89 | 18.24 | 1.17 | 0.135 |
| Residuals | 72 | 1127.30 | 15.66 | | |
| Total | 134 | 3216.00 | | | |

The fatty acids with the greatest contribution to the differences between glycerol concentrations were C20:1n9, C20:2, C20:0, C22:2, C18:1N9, C20:3n6, C14:0, C16:0, EPA, C14:1, and ARA (Fig. 3).

Although the highest lipid content (345.78 ± 30.25 mg $g^{-1}$) was achieved at 8.2 µmol photon $m^{-2}$ $s^{-1}$ with 20 g $L^{-1}$ glycerol addition at 12 days of culture, the highest amount of polyunsaturated fatty acids (65.30 µg $mg^{-1}$) was obtained in the culture without glycerol addition and at 380 µmol photon $m^{-2}$ $s^{-1}$ at seven days of culture. This value was similar to that achieved at 8.2 µmol photon $m^{-2}$ $s^{-1}$ with the addition of 10 g $L^{-1}$ glycerol (63.41 µg $mg^{-1}$) (Fig. 3).

In relation to the cultures at 380 µmol photon $m^{-2}$ $s^{-1}$, the proportion of saturated fatty acids in lipids decreased with no glycerol addition and with 10 g $L^{-1}$ glycerol. However, when 20 g $L^{-1}$ glycerol was added, the proportion of saturated fatty acids decreased until day 12 of culture and then started to increase again. A similar phenomenon occurred with PUFAs, which

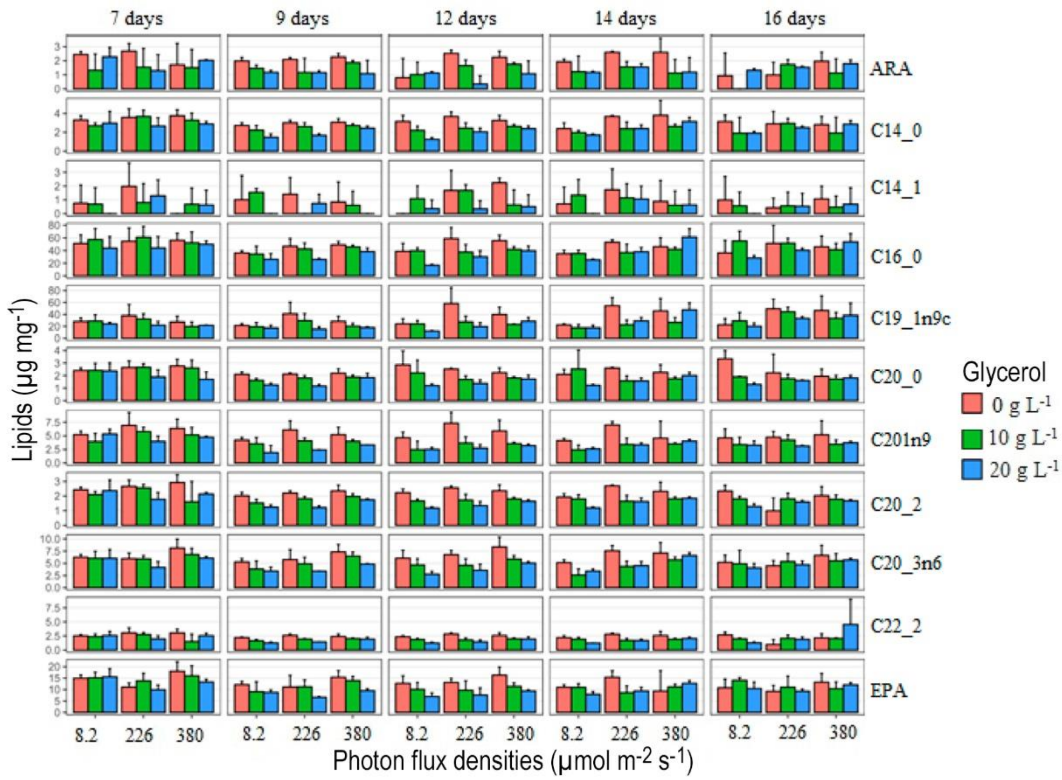


**Figure 3**. Fatty acid content of *Dunaliella* sp. cultures, expressed as μg of fatty acid $mg^{-1}$ of lipids (mean + standard deviation, n = 3).

tended to decrease in the cultures without glycerol addition and with 10 g $L^{-1}$ glycerol until day 12, after which an increase was observed. For the monounsaturated fatty acids (MUFAs) in the cultures without glycerol and with 10 and 20 g $L^{-1}$ glycerol, the MUFA proportion tended to increase. The opposite occurred with unsaturated fatty acids (UFAs), which in all cases tended to decrease during the stationary phase (Fig. 3).

In the cultures at 226 μmol photon $m^{-2}$ $s^{-1}$, MUFAs showed a similar behavior to those at 380 μmol photon $m^{-2}$ $s^{-1}$. The proportion of these fatty acids increased at all three glycerol concentrations. The opposite occurred with the other fatty acid fractions, where the fatty acid proportion decreased at the different glycerol concentrations (Fig. 3). The same behavior was observed for fatty acid productivity, where the tendency was a decrease in productivity over time.

In the cultures at 8.2 μmol photon $m^{-2}$ $s^{-1}$, where higher lipid concentrations were achieved, all fatty acid fractions showed the same behavior, decreasing their proportion over time at the three different glycerol concentrations (Fig. 3).

The fatty acids of major interest in aquaculture are the polyunsaturated fatty acids omega 3 and omega 6, such as eicosapentanoic acid (EPA), arachidonic acid (ARA) and docosahexanoic acid (DHA). DHA was found in only a few replicates of the experiment, but ARA and EPA were found in almost all cultures. Table 3 shows that in general, the highest fatty acid concentration was achieved at day seven, and then the fatty acid concentration started to decrease. In the case of ARA, the highest concentration was achieved at 226 μmol photon $m^{-2}$ $s^{-1}$ without glycerol addition, but at 380 μmol photon $m^{-2}$ $s^{-1}$, the highest EPA concentration was achieved without glycerol.

Considering that the highest lipid content was achieved at 8.2 μmol photon $m^{-2}$ $s^{-1}$, fatty acid behavior was evaluated at this photon flux density. A clear tendency of fatty acid concentration decrease was observed in all treatments, but it was more evident in the cultures with the addition of 20 g $L^{-1}$ glycerol (Fig. 4), where the lipid concentration was higher. This behavior might have been due to an increase in lipid concentration by another lipid fraction, for which fatty acids are not responsible.

### Cholesterol content

In terms of cholesterol production, an interaction was observed between photon flux density and glycerol concentration ($P$ < 0.05), so this interaction was not

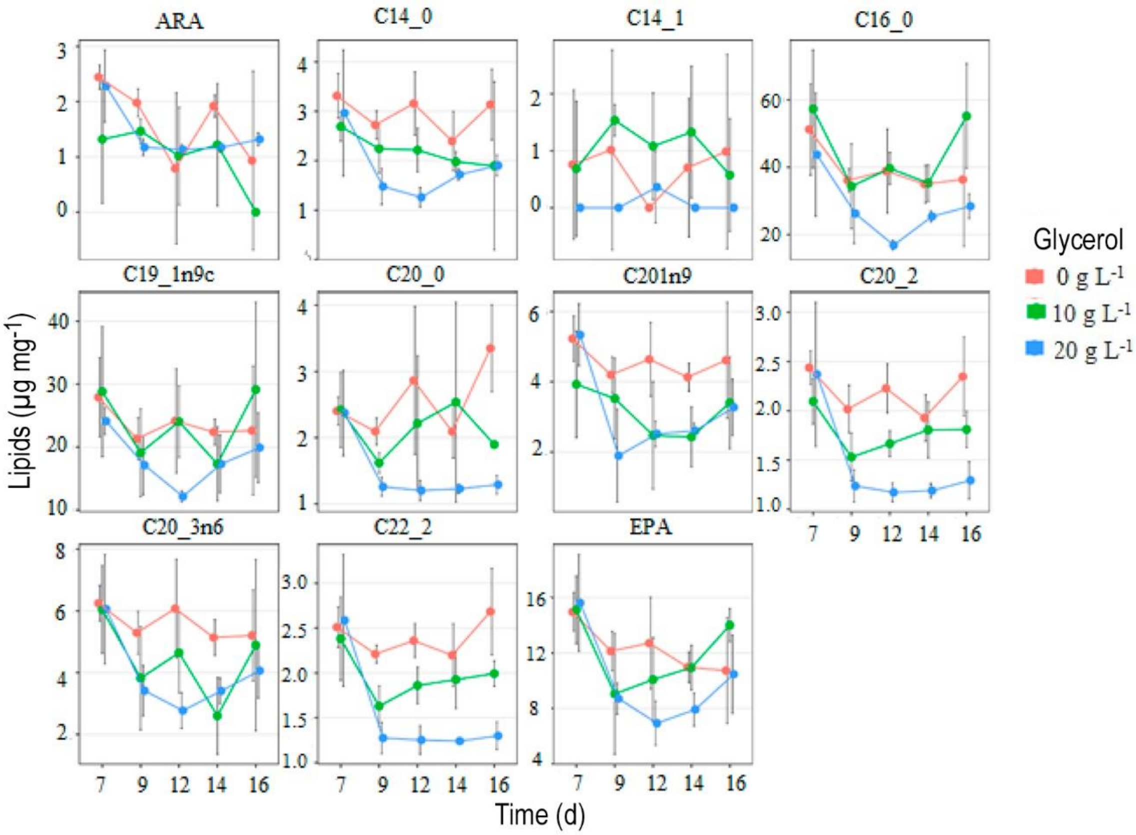


**Figure 4.** Fatty acid profile evolution over time in the cultures of *Dunaliella* sp. at 8.2 µmol photon $m^{-2}$ $s^{-1}$ and without glycerol addition and with 10 and 20 g $L^{-1}$ glycerol (mean ± standard deviation, n = 3).

consistent over time. At 380 µmol photon $m^{-2}$ $s^{-1}$ without glycerol addition and with 10 g $L^{-1}$ glycerol, cholesterol increased over time, achieving the greatest cholesterol concentrations, while at 20 g $L^{-1}$, the cholesterol concentration increased very slightly. At 226 µmol photon $m^{-2}$ $s^{-1}$, the cholesterol concentration increased only in the cultures without glycerol addition, since with 10 and 20 g $L^{-1}$ glycerol, no cholesterol content variation was observed. At 8.2 µmol photon $m^{-2}$ $s^{-1}$, a slight cholesterol content increase was appreciated without glycerol addition and with 10 g $L^{-1}$ glycerol, while with 20 g $L^{-1}$ glycerol, the opposite occurred, with a slight decrease in cholesterol content (Fig. 5).

The greatest cholesterol concentration was found in the culture without glycerol addition and at 380 µmol photon $m^{-2}$ $s^{-1}$ and 16 culture days (2.99 mg cholesterol $mg^{-1}$ biomass), similar to that achieved under the same conditions but at 12 culture days (2.87 mg cholesterol $mg^{-1}$ biomass). A greater concentration of cholesterol was observed in the cultures at 380 µmol photon $m^{-2}$ $s^{-1}$ without glycerol addition and with 10 g $L^{-1}$ glycerol than in the other two cultures. The cultures at 8.2 µmol photon $m^{-2}$ $s^{-1}$ had lower cholesterol concentrations than those at the other two photon flux densities. Additionally, at the three different photon flux densities, the cultures that achieved the lowest cholesterol content were those supplemented with 20 g $L^{-1}$ glycerol.

## Chlorophyll content

For chlorophyll, an interaction was observed among time, photon flux density and glycerol concentration ($P < 0.05$). In general, a tendency to decrease was observed for chlorophyll concentration, which could have been due to chlorophyll recycling because of a limited nitrogen concentration in the culture. No correlation was observed between the glycerol concentration of the culture and chlorophyll content, where at high glycerol concentrations, less chlorophyll content was expected. Lower chlorophyll content was observed in the cultures at 226 and 8.2 µmol photon $m^{-2}$ $s^{-1}$ than in those at 380 µmol photon $m^{-2}$ $s^{-1}$ (Fig. 6).

At 380 µmol photon $m^{-2}$ $s^{-1}$ and without glycerol addition, chlorophyll did not show a decrease. At 226 µmol photon $m^{-2}$ $s^{-1}$, the opposite occurred; without glycerol addition, a decrease in chlorophyll content was

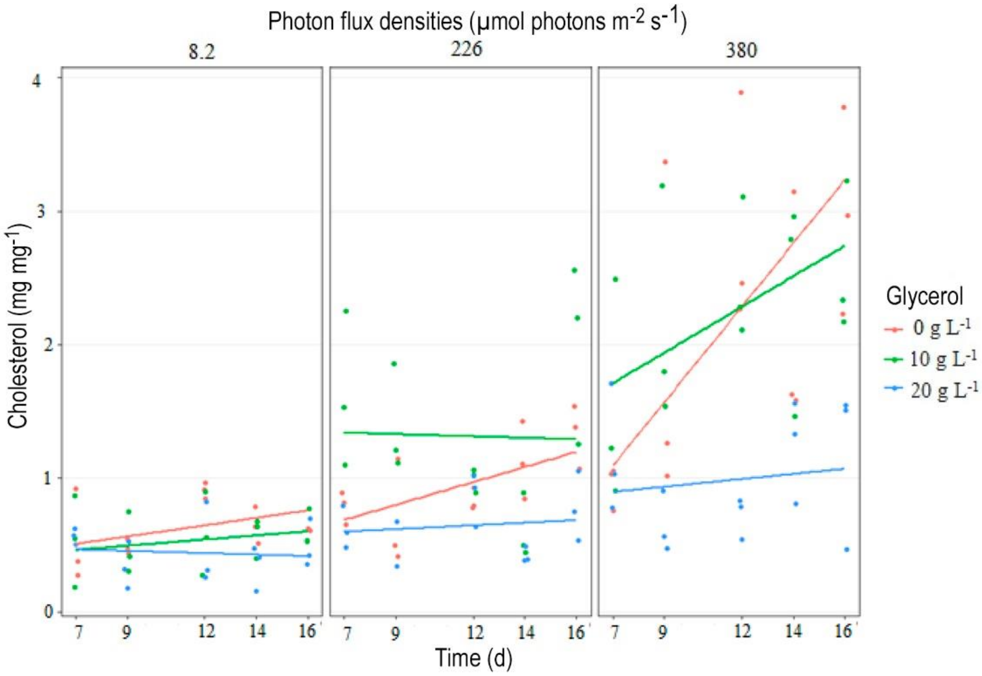


**Figure 5.** Fixed effects of the linear mixed effect model (LME) of *Dunaliella* sp. cholesterol content (mg cholesterol $mg^{-1}$ biomass) for photon flux density, glycerol concentration (fixed factors), and time (continuous variable). Every replicate was considered a random effect. LME was fitted using restricted maximum likelihood. The estimated effects are drawn with different colored lines and the points show the distribution of data for each variable.

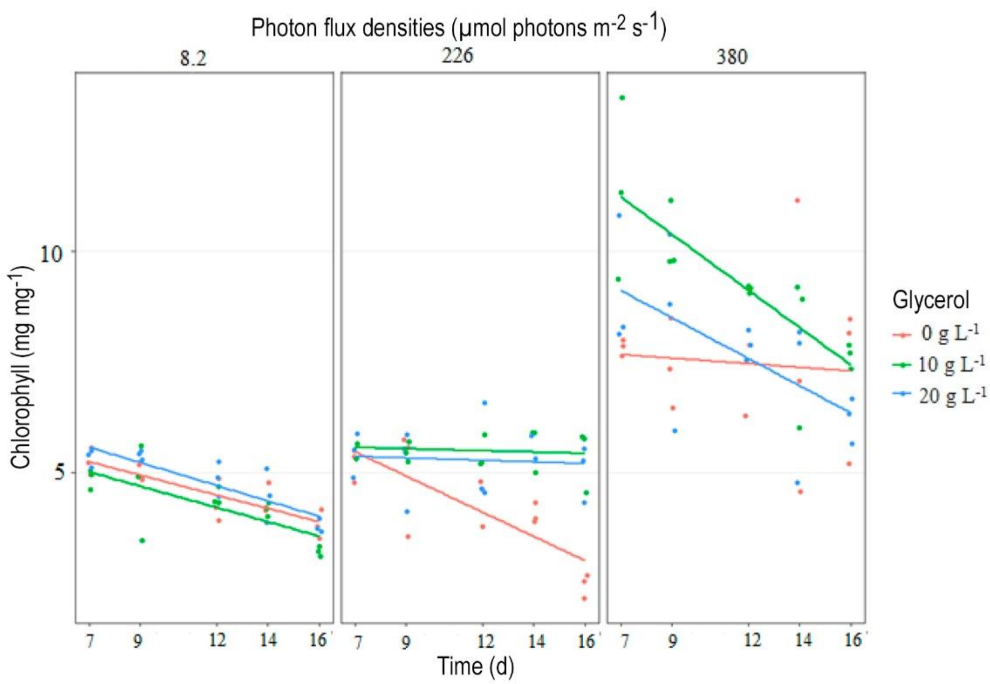


**Figure 6.** Fixed effects of the linear mixed effect model (LME) of *Dunaliella* sp. chlorophyll content (mg chlorophyll $mg^{-1}$ biomass) for photon flux density, glycerol concentration (fixed factors), and time (continuous variable). Every replicate was considered a random effect. LME was fitted using restricted maximum likelihood. The estimated effects are drawn with different colored lines and the points show the distribution of data for each variable.

observed. With the addition of glycerol, no significant decline in chlorophyll content was recorded. At 8.2 µmol photon $m^{-2}$ $s^{-1}$, a decrease in chlorophyll content was recorded over time in all treatments (Fig. 6).

## DISCUSSION

### Cell density

The highest cell density was $2.39\times10^6$ cells $mL^{-1}$ (380 µmol photon $m^{-2}$ $s^{-1}$ without glycerol addition, after 16 days of culture), it was similar to that obtained by Sohrabi et al. (2019) ($2.56\times10^6$ cells $mL^{-1}$) (Fig. 1), although the irradiance used was 75 µmol $m^{-2}$ $s^{-1}$, and the photoperiod was 16:8 (light:dark). The lowest cell density was obtained at 8.2 µmol photon $m^{-2}$ $s^{-1}$ that was the lowest PAPFD employed, which could be explained because *Dunaliella* is an obligate autotroph (Chavoshi & Shariati 2019), so light limitation affects its growth. At 380 µmol photon $m^{-2}$ $s^{-1}$ with 10 g $L^{-1}$ glycerol, the cell density was $2.29\times10^6$ cells $mL^{-1}$, which was less than the obtained by Sohrabi et al. (2019) ($4.96\times10^6$ cells $mL^{-1}$) with the same amount of glycerol, maybe because of the difference in PAPFD. .

In this study, cell density showed no significant variation at different glycerol concentrations at the same photon flux density, which was different from the results obtained by Choi & Lee (2015), where higher biomass was achieved with cultures supplemented with 10 g $L^{-1}$ glycerol than with those without glycerol addition. This difference may be due to the differences in the source of light, photoperiod and culture period, since in the research of Choi & Lee (2015), they used LED light (75 µmol $m^{-2}$ $s^{-1}$) with a photoperiod of 16:8 (light:dark), and the culture duration was 21 days.

### Total lipids

The highest lipid contents of the cultures supplemented with 20 g $L^{-1}$ glycerol may have been due to the changes in osmotic pressure induced by the high content of glycerol in the culture medium, which probably stressed the microalgae and triggered lipid synthesis.

Although the lipid content was similar at days 9 and 12 of culture, less energetic input was needed for a nine-day culture than for a 12 day culture, even if the photon flux density was greater for the 226 µmol photon $m^{-2}$ $s^{-1}$ culture. Achieving maximum lipid content at nine days of culture implies less microalga culture time, which may be reflected in lower production costs of oil derived from this microalga.

The lipid content achieved after nine days of culture at 226 and 8.2 µmol photon $m^{-2}$ $s^{-1}$ was similar to the lipid contents achieved by (Chavoshi & Shariati 2019), which were slightly more than 0.3 g $g^{-1}$ at seven culture days with the addition of acetate 100 mM or glucose 60 mM with *D. salina*.

The lipid content in cultures supplemented with 20 g $L^{-1}$ glycerol at 8.2 µmol photon $m^{-2}$ $s^{-1}$ was always higher than the obtained in cultures without glycerol addition, even when the cultures with glycerol addition had less biomass production than those without glycerol addition (Fig. 1). The reason might be that the osmotic pressure modification in the culture medium along with low photon flux density stressed the microalgae, triggering lipid production and limiting biomass production (Capa-Robles et al. 2021).

### Fatty acid profiles

The culture with the highest lipid content was not the same as the one in which the highest amount of polyunsaturated fatty acids (PUFAs) was obtained, which means that achieving high lipid content does not necessarily mean achieving higher amounts of fatty acids. Biomass can have high lipid content but poor-quality fatty acids, i.e., with high proportions of both long-chain PUFAs and omega 3 and 6 fatty acids.

The increase in MUFAs that only occurred in the cultures at 226 and 380 µmol photon $m^{-2}$ $s^{-1}$ was mainly due to the increase in oleic acid proportion (Table 3), which can be used by Nile tilapia as a good substrate source for growth metabolism (Liu et al. 2019). The rise in palmitic acid and oleic acid (Table 3) could be explained because these fatty acids result from *de novo* synthesis; then, elongases and desaturases transform them into PUFAs (Scodelaro-Bilbao et al. 2017), so there is a need to discover a mechanism to trigger the action of these enzymes to increase PUFA production. Additionally, high amounts of linoleic (LA) and α-linolenic (ALA) acids were observed, which may be used by fish as precursors in the production of long chain PUFAs (Monroig & Kabeya 2018).

The greatest production (15.23%) and productivity (1.06 mg $L^{-1}$ $d^{-1}$) of LA in this study were obtained at 380 µmol photon $m^{-2}$ $s^{-1}$ without glycerol addition after seven days of culture; these values were greater than those reported by Sajjadi et al. (2018), with 11.5% for *D. salina*, and Zhukova & Aizdaicher (1995), with 5.2 and 6.1% for *D. tertiolecta* and *D. salina*, respectively. Finally, Lv et al. (2016) obtained 11.47 and 12.30% for *D. salina* with media including nitrogen and lacking nitrogen, respectively, after 15 days of culture.

In this study, *Dunaliella* sp. produced 15.63% ALA acid with a photon flux density of 8.2 µmol photon $m^{-2}$ $s^{-1}$ and medium supplemented with 10 g $L^{-1}$ glycerol after seven days of culture. This production was lower than the reported in other studies, which obtained 38.7% for *D. tetriolecta* and 36.9% for *D. salina* (Zhukova & Aizdaicher 1995), as well as 38.1% in medium with nitrogen and 35.4% for nitrogen-depleted medium for *D. salina* after 15 days of culture (Lv et al. 2016). Although the highest production was achieved at 5.4 µmol photon $m^{-2}$ $s^{-1}$ with 10 g $L^{-1}$ glycerol, the greatest productivity was achieved at 380 µmol photon $m^{-2}$ $s^{-1}$ without glycerol addition at seven days of culture (0.94 mg $L^{-1}$ $d^{-1}$).

In the case of arachidonic acid (ARA), *Dunaliella* sp. produced 1.03% at 380 µmol photon $m^{-2}$ $s^{-1}$ without glycerol addition after seven days of culture, higher than the values obtained in other studies (Zhukova & Aizdaicher 1995, Sajjadi et al. 2018), in which ARA production by *D. salina* was not detected; moreover, Lv et al. (2016) did not detect ARA production in nitrogen-depleted medium. *D. tertiolecta* produced 0.3% ARA (Zhukova & Aizdaicher 1995), which was lower than the present study. However, Sajjadi et al. (2018) obtained 1.91%, which was higher for *D. salina* than in this study, and Lv et al. (2016) obtained 3.65% ARA after 15 days of culture. The greatest productivity of this fatty acid was achieved in the cultures at 226 and 380 µmol photon $m^{-2}$ $s^{-1}$ without glycerol addition at seven days of culture (86.77 and 90.17 µg $L^{-1}$ $d^{-1}$), which did not show significant differences between them.

The highest eicosapentanoic acid (EPA) production (8.2%) and productivity (0.56 mg $L^{-1}$ $d^{-1}$) were achieved at 380 µmol photon $m^{-2}$ $s^{-1}$ without glycerol addition after seven days of culture, and the production was greater than the obtained in other studies in which *D. salina* produced 0.05% EPA and *D. teriolecta* 0.4% EPA (Sajjadi et al., 2018), as well as 0.1% EPA for *D. salina* and 0.4% EPA for *D. tetriolecta* (Zhukova & Aizdaicher 1995). Thus, *Dunaliella* is a good source of EPA. The decrease in fatty acid proportion over time may be explained by the increase in total lipids due to another lipid fraction besides that containing fatty acids.

Although the highest lipid concentrations were found in the cultures at 8.2 µmol photon $m^{-2}$ $s^{-1}$ with the addition of 20 g $L^{-1}$ glycerol, the highest PUFA proportion was found in the culture at 380 µmol photon $m^{-2}$ $s^{-1}$ at day 7 (65.30 µg $mg^{-1}$), very similar to the value obtained at 8.2 µmol photon $m^{-2}$ $s^{-1}$ and 10 g $L^{-1}$ glycerol (63.41 µg $mg^{-1}$). Although the highest lipid concentration was achieved with the addition of 20 g $L^{-1}$ glycerol at day nine, under this condition, the highest fatty acid proportion was not achieved.

When *D. tetriolecta* was cultured on nitrogen-deficient medium, some fatty acids, such as C16:1, C18:1, and C18:2, also showed a decrease in concentration, as in this study; however, others, such as C18:3, demonstrated a very slight concentration increase, which was different from the results obtained in this study (Chen et al. 2011). *D. salina* was cultured in a medium where the salinity concentration increased the fatty acid concentration in general, but as the salinity concentration decreased, fatty acids also decreased in concentration, similar to the results obtained in this study (Rismani & Shariati 2017).

Some fatty acids tended to decrease as the stationary phase proceeded, while others did not (C16:0, C16:1, C18:1n9), so the accumulation of different fatty acids seems to be regulated by different mechanisms inside microalgal cells.

### Cholesterol content

A concentration of 2.99 mg cholesterol $mg^{-1}$ biomass was achieved without glycerol addition and at 380 µmol photon $m^{-2}$ $s^{-1}$ at 16 culture days, similar to 2.87 mg cholesterol $mg^{-1}$ biomass achieved under the same conditions, but at 12 days of culture, which is more economically feasible since there is less culture time. Cholesterol from microalgae could be used as a replacement for the cholesterol needed in shrimp diets for aquaculture (Iba et al. 2014).

### Chlorophyll content

The reduction seen in chlorophyll content at lower photon flux density may be due to light limitation in these cultures. At 380 µmol photon $m^{-2}$ $s^{-1}$ without glycerol addition, there was no decrease in chlorophyll content, probably because no stress was related to osmotic pressure, so there was no need to recycle nutrients, mostly nitrogen from chlorophyll. At 226 µmol photon $m^{-2}$ $s^{-1}$ without glycerol, the opposite occurred, perhaps because in this photon flux density, chlorophyll was less effective at maintaining microalgal functions. At this same PAPFD with the addition of glycerol, no significant decline in chlorophyll content was observed, perhaps because the microalgae did not need to recycle chlorophyll to obtain nutrients. At 8.2 µmol photon $m^{-2}$ $s^{-1}$, a decrease in chlorophyll content was observed over time (Fig. 6), probably due to low photon flux density where chlorophyll was not required to fix carbon, so maybe it was recycled as a nutrient source (Msanne et al. 2012).

## CONCLUSIONS

The highest PUFA concentrations were obtained at the early stationary phase and decreased throughout this phase, so harvesting should be performed at the beginning of the stationary phase to obtain the maximum PUFA content.

Glycerol could be used to modify the osmotic pressure in the culture medium to increase microalgae lipid content, since it was greater in the cultures supplemented with 20 g $L^{-1}$ glycerol. Additionally, lipid productivity was greater in cultures with glycerol addition, although biomass productivity was greater in cultures without glycerol addition.

Photon flux density had an effect on lipid content in microlagae since higher lipid content was recorded at lower photon flux densities. The lipid content was higher at the early stages of the stationary phase, and it was not reflected in the fatty acid profile since a decrease was generally observed in fatty acid concentration and productivity over time. The greatest fatty acid proportion and productivity were obtained at the beginning of the stationary phase.

Although greater lipid productivities were obtained in cultures with the addition of glycerol, the greatest production of fatty acids of interest (LA, ALA, ARA and EPA) was obtained in cultures at 380 µmol photon $m^{-2}$ $s^{-1}$ and without glycerol addition.

The best culture conditions in respect to lipid content would be with the addition of 20 g $L^{-1}$ of glycerol at a PAPFD of 226 µmol photon $m^{-2}$ $s^{-1}$ with the best harvesting time after nine days of culture where most lipids were produced by the microalga. Since biomass can have high lipid content but poor-quality fatty acids, the best culture conditions in respect to fatty acid quality were at a PAPFD of 380 µmol photon $m^{-2}$ $s^{-1}$ without glycerol addition with the best harvesting time after seven days of culture, where most PUFA's were obtained.

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