

Original Research Article

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Production in Cell Biomass and Carotenoids under the Effect of a Saline Stress in Microalgae *Dunaliella* spp. Isolated from Moroccan Saharian Saline

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ABSTRACT

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Microalgae contain a battery of bioactive molecules such as carotenoids used in various industrial sectors. This is the case of the microalga *Dunaliella* spp., whose cells, under stress conditions, accumulate remarkable levels of carotenoids. In this study, three native strains of *Dunaliella* (*D. viridis* (DUN4), *D. salina* (DUN5) and *D. salina* (DUN6)) isolated from the Moroccan saharian saline on the Atlantic coast were subjected to different salinity regimes (0.5, 1.5, 2.5, 3.5 and 4.5 M NaCl) to evaluate the ability to produce biomass and carotenoid biosynthesis in this species. Total carotenoids were determined by spectrophotometry. The strains are cultured for 15 days on F/2 Guillard medium and aerated at 25±1°C. Strain DUN5 showed, in the presence of 2.5 M NaCl, a maximum of carotenoids of the order of 3.267µg.ml⁻¹, 3,206µg.ml⁻¹ in the strain DUN 4 to 3.5 M NaCl, and 2,314µg.ml⁻¹ in the strain DUN 6 at 0.5 M NaCl. The highest cell densities are recorded in the strain DUN 4 being of the order of 3.199.10⁶ Cells.ml⁻¹, the concentrations remains lower in the strains DUN5 and DUN6. These results indicate that each strain has a different behavior and physiological response to salt stress. Moreover, these microalgae have remarkable capacities and can constitute a potential source of biochemical molecules of industrial interest.

Introduction

The microalgae *Dunaliella* spp. is a chlorophycea predominant in hypersaline environments, characterized by its accumulation of large amounts of carotenoids; making it a potential source of natural β-carotene, 10 times more active than synthetic and which is of remarkable importance for its use in the food and pharmaceutical industries

(Borowitzka et Borowitzka 1988; Cifuentes *et al.*, 1992; Gómez *et al.*, 1992; Masuda *et al.*, 2002; Guevara *et al.*, 2005). Carotenoids are pro-vitamins A which, thanks to their high antioxidant power, promote night vision and inhibit free radicals produced by ultraviolet radiation. They therefore offer protection to the membrane phospholipids of the skin cells

(Krinsky and Johnson, 2005, Riyahi *et al.*, 2007 Charioui *et al.*, 2017).

Several studies have shown that the production of carotenoids in *Dunaliella spp.* can be optimized by variations in salinity, light intensity, temperature and nutrient limitation (Guevara *et al.*, 2005).

One of the most important factors for carotenoids production is salt; it is therefore important to evaluate the influence of this element on the growth and the capacity of the microalgal cells to the accumulation of carotenoids. The purpose of this investigation is to evaluate cell growth and carotenoids production in native Moroccan strains of *Dunaliella spp.* under laboratory conditions in the presence of a salt stress regime and their ability to produce large cellular and pigment biomass. This can be a source of antioxidant compounds.

Materials and Methods

Microalgae

Three carotenogenic strains of *Dunaliella* were used for this experiment: *Dunaliella viridis* (DUN4), isolated from the continental saline of Tislatine in Boujdour region (Latitude 26.6905316°, Longitude -13.5562407°); *Dunaliella salina* (DUN5) isolated from the marine saline of Tazgha at Akhfenir (Latitude 27, 9477594°, Longitude -12,2852711 °); and *Dunaliella salina* (DUN6) from the continental saline of Oum Dbâa at Tarfaya (Latitude 27.577745°, Longitude -12.9553731°) in Morocco.

Isolation and culture of strains

The strains of *Dunaliella spp.* are harvested from three different salt production tables located in southern Morocco (Figure 1).

The algal strains are isolated by two methods of cell isolation: the successive dilution method and the plate dilution method in order to obtain monoalgal strains. After isolation, the cultures were established under laboratory conditions on the F/2 Guillard medium (Guillard and Rhyther, 1962) at 0.5 M NaCl under continuous white light at T=25±1°C. After turning the color of the crops towards the green, we then identify the strain of *Dunaliella spp.* on the basis of the morphological characteristics under optical microscope (Borowitzka and Siva, 2007).

Experimental culture conditions

Three microalgal strains of the genus *Dunaliella spp.* are cultured on the F/2 culture medium prepared from sea water at various salt concentrations. Five batch cultures (150 ml) with salinities 0.5; 1.5; 2.5; 3.5 and 4.5 M NaCl are prepared. The triplicate experimental cultures are maintained under continuous white light at a T=25±1°C for a period of 15 days. All cultures are inoculated from microalgal cells taken in exponential phase.

Cell and pigment quantification

The cell growth kinetics is monitored every two days by direct counting under an optical microscope using a hematometer (Thoma cell). The pigment analysis is carried out every two days by extracting carotenoids with 80% acetone (Charioui *et al.*, 2015; Fazeli *et al.*, 2006), and then assaying the pigment content by UV-Vis spectrophotometry and quantification following the equations described by Linchtenthaler (1987).

Statistical analysis

The graphic illustrations were made using the Microsoft Excel program. In addition, statistical analysis was based on ANOVA at

one factor performed by the LSD test ($p > 0.05$).

Results and Discussion

Effect of salt diet on cell growth in *Dunaliella* spp

Microalgae are a very promising plant resource for the production of cell biomass and high value added molecules. Certain strains have an important capacity to accumulate large quantities of carotenoids, in particular in the form of β -carotene.

The application to bioactive molecules requires indeed taking into account an important set of characteristics in the strain. The quantity of carotenoids, the quality and kinetics of cell biomass production must be taken into consideration.

The growth profiles of the cultured microalgae show the three phases of growth. A phase of latency or adaptation to the new culture conditions, facing different concentrations of salt of the medium; an exponential phase with rapidly increasing the number of cells; a stationary phase where there is a maximum number of cell division. In this case, there may be a phase of decline following the depletion of nutrient salts in the medium or the aging of the algal cells.

The evolution of the cell growth of all the strains showed a relatively short latency phase, the duration of which is between 2 and 3 days with a percentage of cell evolution $> 30\%$; with the exception of the strain DUN 5 cultivated at a salinity of 4.5 M which exhibits a stationary cell multiplication. All strains have easily adapted to the new growing conditions in the presence of a salt diet. The trend of the growth curves of the continental strain DUN 4 clearly shows an exponential growth displayed on all tested salt

concentrations. Cell division is noted for all variations in salt of the medium but with different densities. Moreover, it can be said that reproducibility of the growth is obtained whatever the concentration of NaCl. It is noted that the maximum cell density for this strain was obtained on the last day of culture at a salinity of 0.5 M in the order of $3,199.10^6$ Cells.ml⁻¹.

The marine strain DUN 5, on the other hand, has a negative correlation with respect to the concentration of salt in the medium. There is then a sort of inhibition of cell growth with increasing saline concentration. Nevertheless, this strain, cultivated at a salinity of 4.5 M, proved to have a low initial growth and the cells maintained an almost stable growth throughout the culture period. In addition, it tends to show a relatively low cell growth compared to the other strains studied. Maximum concentrations recorded in cell biomass are of the order of $0,519.10^6$ cells.ml⁻¹ and observed on media with a concentration close to the salinity of the sea water (0.5M).

The continental strain DUN 6 exhibits a similar behavior as that of the strain DUN4. There is an upward growth from the first days of culture on all salt concentrations, with maximum cell densities reached as early as the 12th day of culture of the order of $2,438.10^6$ Cells.ml⁻¹ recorded on a medium with a high concentration of NaCl (4.5M). The difference between strains DUN4 and DUN6 resides in a maximum cell biomass reached for DUN4 of $3,199.10^6$ Cells.ml⁻¹ at a salinity of 0.5M close to the salinity of sea water; and DUN6 of the order of $2,438.10^6$ Cells.ml⁻¹ at a salinity of 4.5 M. Moreover, the strain DUN4 recorded a growth peak at a low salinity, while the strain DUN6 showed a peak of growth at high NaCl concentration. The results of the biological tests of *Dunaliella* strains cultured at different salt concentrations clearly indicate the influence

of this parameter on the growth of microalgae. All strains except the marine strain DUN 5 showed good growth in the various concentrations of NaCl. The highest cell biomass was noted in strain DUN 4 at a salt concentration of 0.5M.

Depending on their batch culture behavior, the strains can be subdivided into two categories; the first category includes strains with rapid cell division, including the DUN 4 and DUN 6 strains of continental nature, although they are two different species. The second slow-growing category includes the DUN 5 strain of marine origin.

These tests showed that there was a difference in the growth behavior of the strains exposed to the same culture conditions. This difference is correlated with the specific characteristics of each species of microalgae tested and its original habitat. These differences show the value of taking into account the nature and origin of the strains in the selection and culture of strains especially for industrial production.

The results of strains DUN 4 and DUN 5 are in agreement with those of Cifuentes *et al.*, (2001), which reported that the maximum growth of *Dunaliella* is reached at saline concentrations of 0.5M NaCl. Similarly, Massart *et al.*, (2010) reported that it is preferable to decrease the concentration of NaCl to promote growth.

Other authors such as Arash Rad Faraz *et al.*, (2015), Fazeli *et al.*, (2005) and Gomez *et al.*, (2003) observed that the production of cell biomass is rather slow at concentrations greater than 3M NaCl, the highest cell biomass productions are recorded at a concentration of 1M and 2M NaCl, and vary from $0,23.10^6$ Cells.ml⁻¹ at $1,68.10^6$ Cells.ml⁻¹. Consistent with the work of Jahnke *et al.*, (2003), cell growth decreases from 30 to 55%

at high salt concentrations. These observations correspond exactly with the results of the two strains *D. viridis* (DUN4) and *D. salina* (DUN5) with cell densities which remain respectively of the order of $3,199.10^6$ Cells.ml⁻¹ and $0,519.10^6$ Cells.ml⁻¹ to Low salt concentration (0.5M). Nevertheless, there is a decrease in cell production with increasing saline concentration (Fig. 2).

Effect of saline diet on carotenoids production in *Dunaliella spp*

It was observed from figure 3 that the production of the carotenoids is variable for each strain tested, and against each concentration of NaCl. For the DUN 4 strain, we recorded an accumulation of carotenoids throughout the growing period with significant production recorded at a high salt concentration of between 3.5 and 4.5 M on the 14th day of culture; And thus show a productivity of the order of $3.206 \mu\text{g.ml}^{-1}$ and $3.067 \mu\text{g.ml}^{-1}$ at salt concentrations of 3.5M and 4.5M; respectively.

For the DUN 5 strain, the biosynthesis of carotenoids is considerable only from the 8th day. This strain was able to produce a better amount of carotenoids on all salt concentrations at the end of the cycle culture and showed a maximum concentration of productivity at 2.5 M NaCl on the last day of culture of the order of $3,267 \mu\text{g.ml}^{-1}$.

For the DUN 6 strain, the best potential for carotenoids biosynthesis was recorded on the 12th day of culture at low salt concentrations, with productions of the order of $2,314 \mu\text{g.ml}^{-1}$ (0.5 M) and $2,686 \mu\text{g.ml}^{-1}$ (1.5M); respectively.

Based on these results, we can say that each strain has a preferendum in saline concentration for the production of

carotenoids. The best productivities are obtained for the continental strain *D. viridis* (DUN 4) with a maximum concentration of $3,206 \mu\text{g.ml}^{-1}$ to 3.5 M of NaCl; and thus has a very marked response to salt stress.

In addition, the marine strain *D. salina* (DUN5) recorded eminent productivities of the order of $3,267 \mu\text{g.ml}^{-1}$ especially for mean salt concentrations at 2.5 M.

Conversely, the continental strain *D. salina* (DUN 6) exhibits a contrary effect under the same culture conditions; whose maximum carotenoids production of the order of $2,686 \mu\text{g.ml}^{-1}$ is observed at a low salt

concentration of 1.5M. The two different strains of *D. salina* (DUN5 and DUN6) have different behavior and response. This difference is a function of their different origin. The strain DUN5 is a microalgae isolated from marine saline, while the strain DUN6 is isolated from continental origin.

Based on data from the scientific literature in the field of microalgae, salinity significantly affects the accumulation of total carotenoids at the end of the growing period under a salt regime. The strains tend to show a different physiological behavior in relation to each NaCl concentration (Yanilka Karenia Lopez, 2008).

Fig.1 Graphical representation of the isolation salts of local microalgal strains

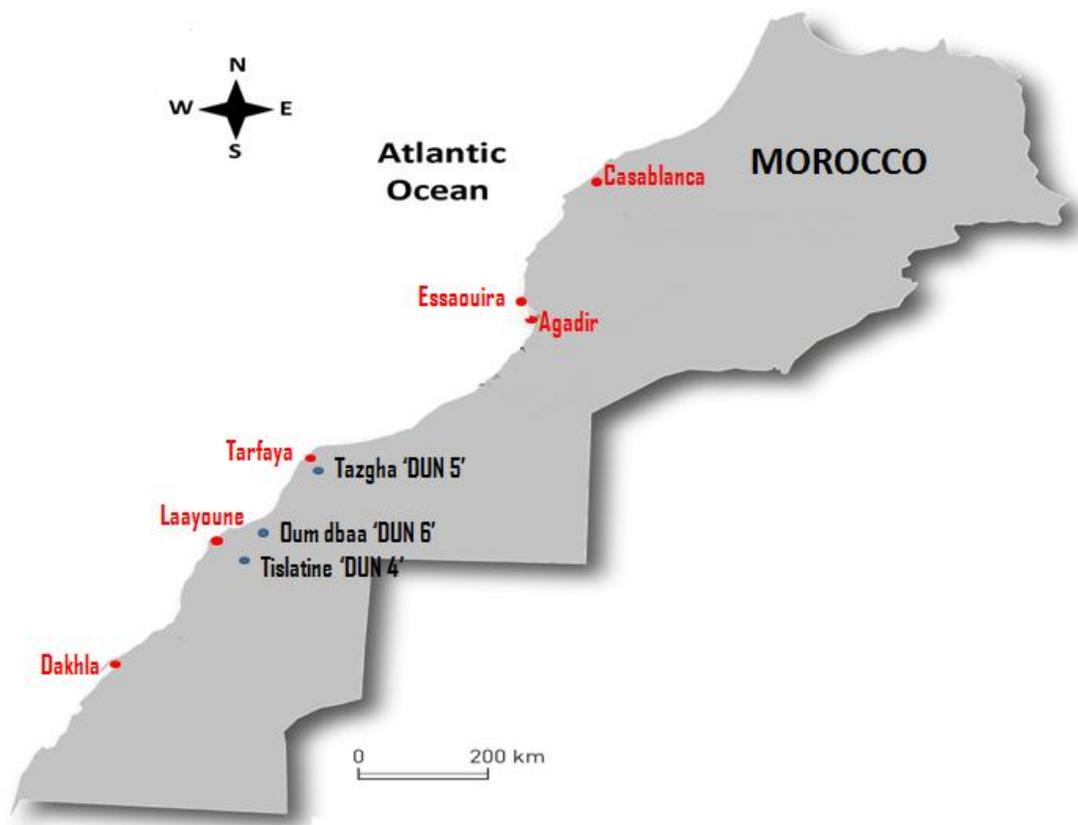


Fig.2 Growth kinetics of the microalgal strains DUN4 (a), DUN5 (b) and DUN6 (c) under different salt concentrations during 15 days of culture at T=25±1°C (p>0,05)

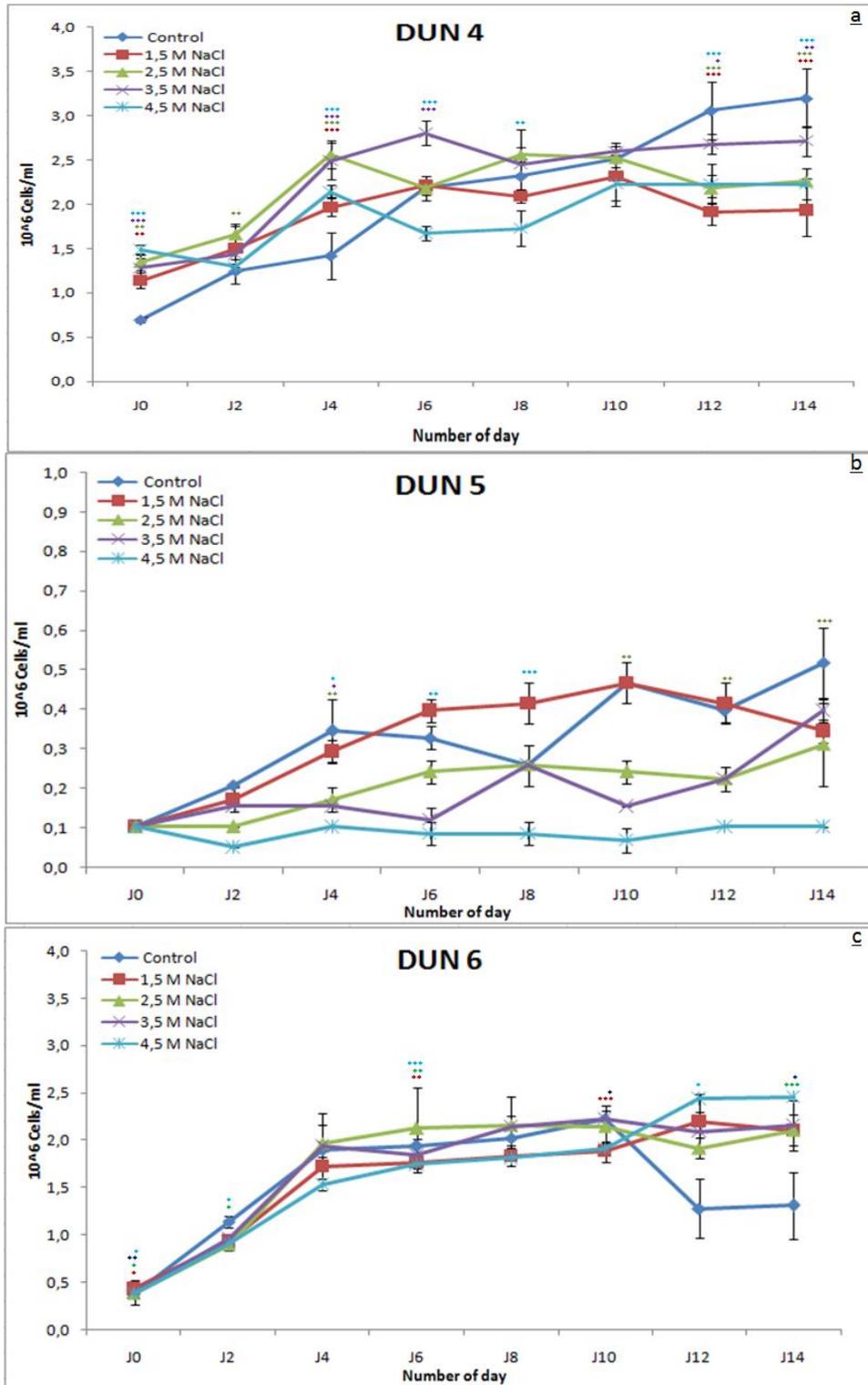
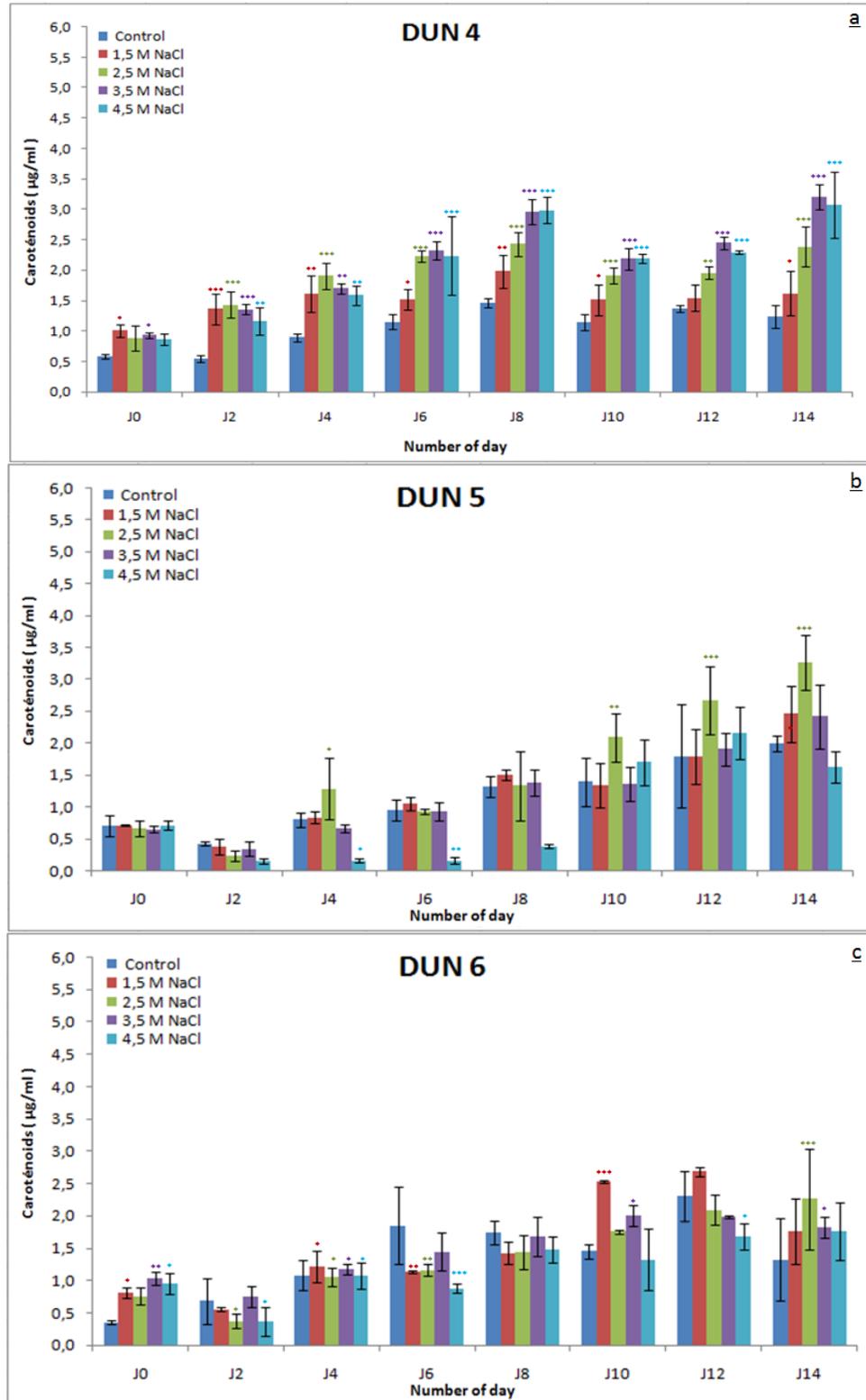


Fig.3 Carotenoids production of the microalgal strains DUN4 (a), DUN5 (b) and DUN6 (c) under different salt concentrations during 15 days of culture at T=25±1°C (p>0,05)



Similarly, Cifuentes *et al.*, (1992) reported that the environmental conditions of a particular habitat may be responsible in part for the development of carotenogenesis of a particular *Dunaliella* strain. This observation is also noted by Guevarra (2005), who showed that high concentrations of carotenoids are recorded in some *Dunaliella* strains at a salt concentration of more than 30%, while other strains tested have reached their maximum biosynthesis at concentrations below 20%. Similar observations are reported in our study, noting that the *D. viridis* (DUN 4) strain of continental nature showed carotenoids productivity at high salt concentrations 4,5 M de NaCl ($\approx 30\%$). On the other hand, the other cultivated strains of *D. salina* (DUN5 and DUN6) recorded productivities at concentrations which are still less than 3, 5 M de NaCl (20%).

Nevertheless, the results obtained showed the influence of salinity on growth inhibition in favor of carotenoids production in the marine strain *D. salina* (DUN5) and the continental strain *D. viridis* (DUN4). Nevertheless, the continental strain *D. salina* (DUN 6), which exhibits cell production combined with carotenoids biosynthesis, is a good candidate for the production of carotenoids (beta-carotene).

In conclusion, in this context, we conclude that each strain has a distinct response to a salt concentration. These results suggest that other physical and chemical factors prove useful in establishing a relevant strains comparison; and thus discern the factor for maximizing both biomass and carotenoids production for each strain.

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