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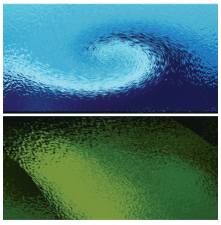

RESEARCH ARTICLE

Salinity and nitrate concentration on the growth and carotenoids accumulation in a strain of *Dunaliella salina* (Chlorophyta) cultivated under laboratory conditions

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Abstract

1 - The effect of salinity (9, 14, and 22 % NaCl w/v) and nitrate concentration (882, 435 and 212 µmol L^{-1}) on the growth and production of carotenoids was studied in a wild strain of *Dunaliella salina* Teod. isolated from solar salterns on the Tyrrhenian coast (Tarquinia – Central Italy).

2 - The alga was grown in batch culture at relatively low illumination (40 µmol photon $m^{-2}s^{-1}$). The total content of carotenoids produced from the alga and the amount of all-*trans*-β-carotene, the isomer showing a relevant commercial value, were determined by UV-Vis spectrophotometry and HPLC analysis.

3 - The highest growth rate in this strain of *D. salina* was obtained at salinity of 22% NaCl w/v and elevated nitrate concentration (882 µmol L^{-1}); a relatively high cell density was also observed at higher salinity and reduced nitrate concentrations. Low nitrate concentration negatively affected growth, but enhanced the carotenoids accumulation in the cells.

4 - The highest concentration of carotenoids (17 pg/cell) was observed in the 28 day old cultures in the late stationary phase at 22% NaCl w/v and 212 µmol L^{-1} nitrate concentration. Also the average of all-*trans*-β-carotene on total carotenoids was enhanced by low nitrate concentration changing from 5% in 28 day old cultures at 22% salinity and 882 µmol L^{-1} N, to 37% in 28 day old cultures at 22% NaCl w/v and lower nitrate concentration (212 µmol L^{-1}).

Keywords: *Dunaliella salina*; low irradiance; carotenoids; nitrate; salinity.

Introduction

The unicellular green alga *Dunaliella salina* Teodoresco (Chlorophyceae, Volvocales) is a halophilic organism observed in salt lakes worldwide from tropical to temperate to polar regions (Javor, 1989). This alga is one of the most environmentally tolerant eukariotic organisms know, it can live with salinity ranging from 3% NaCl w/v to NaCl saturation and temperatures from 0°C to > 38°C (Ginzburg, 1987; Borowitzka & Borowitzka, 1988). *D. salina* can produce massive amounts of a class of naturally occurring pigments (carotenoids) in the form of oily droplets in the chloroplast stroma in response to abiotic stress. Among them, β-carotene is a compound of particular importance representing the main natural source of vitamin A (or retinol). Vitamin A has long been known to be necessary to the biochemistry of vision and to the proper function of the epithelial tissues. Deficiencies of vitamin A may lead to reduced visual sensitivity, such as, night blindness,

or reduced resistance to infection through epithelial tissues. In addition, β-carotene plays a chemopreventive action in several types of cancer. Among the possible isomers depicted in figure 1 (Gomez *et al.*, 1999; Hu *et al.*, 2008), all-*trans*-β-carotene is of the highest commercial value. As already observed by Oren (2005) "the commercial cultivation of *Dunaliella* for the production of β-carotene throughout the world is one of the success stories of halophile biotecnology". At the present time, there are commercial-scale cultures of *Dunaliella* in Australia, Israel, China and India (Tseng, 1993; Ben-

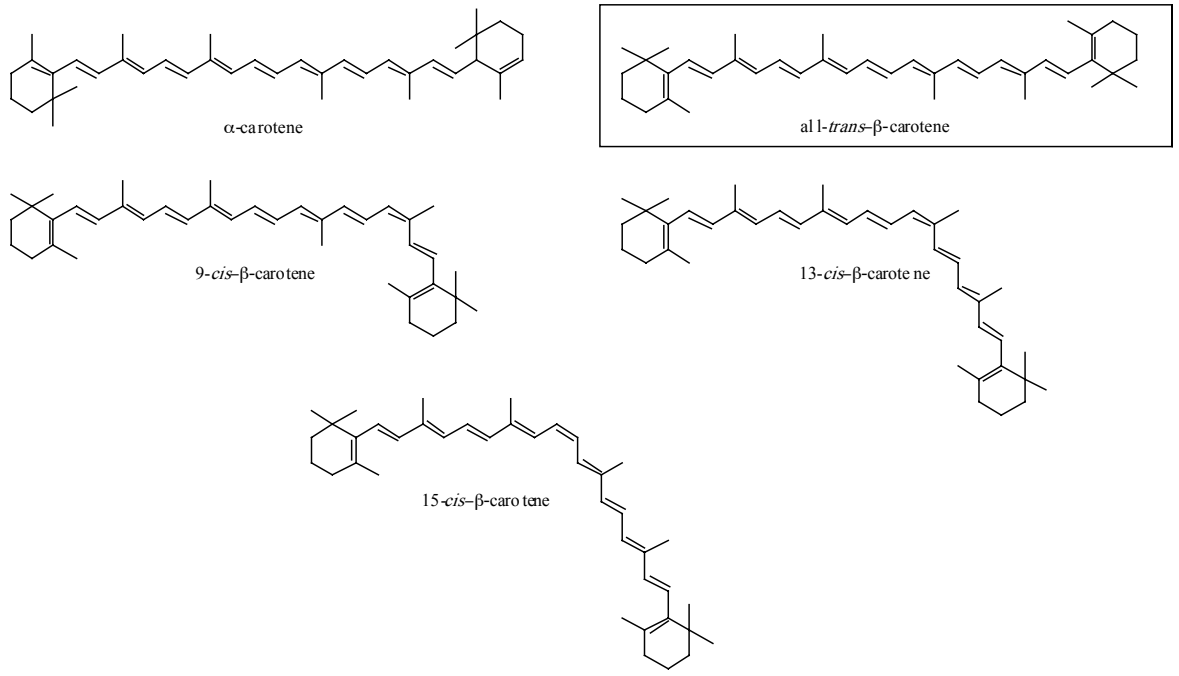

Figure 1. Trans and cis isomers of β-carotene.

Amotz, 1999; Borowitzka, 2005). Open ponds represent the conventional method used in commercial production plants for *Dunaliella* (Ben-Amotz, 2004; Del Campo *et al.*, 2007). The massive accumulation of β-carotene in *D. salina* is triggered by environmental stress such as intense irradiance, high salinity, nutrient starvation, and extreme temperatures (Semenenko & Abdullayev, 1980; Loeblich, 1982; Ben-Amotz, 1987; Borowitzka *et al.*, 1990; Gòmez *et al.*, 2003; Garcia *et al.*, 2007). In this alga β-carotene seems to act as a photo-protective 'sun-screen' that absorbs a substantial part of the excess light (in the range of 330 to 500 nm) before this light is able to damage the photosynthetic machinery of the cells (Ben-Amotz *et al.*, 1989; White & Jahnke, 2002). The cellular location of the β-carotene containing globules in the interthylakoid space of the chloroplast in close vicinity to the plasma membrana is consistent with the previous hypothesis (Hejazi *et al.*, 2004; Lamers *et al.*, 2008). Borowitzka and Borowitzka (1988) have also proposed that β-carotene also acts as a carbon sink that stores the excess carbon produced during photosynthesis when growth is limited but the photosynthetic carbon fixation must continue. Under stress conditions the physiological cell balance is disturbed, and formation of excessive free radicals occurs. In order to protect themselves and continue

their growth, cells produce additional β-carotene. Nutrient limitation seems to be the main regulatory factor for carotenoid accumulation, in controlling messenger RNA levels of the first two enzymes committed to carotenoid biosynthesis phytoene synthase (Psy) and phytoene desaturase (Pds) in *D. salina* (Coesel *et al*., 2008). Genes codifying for these two enzymes are also considered to play a key role in regulation of carotenogenesis in higher plants (Campisi *et al*., 2006; Salvini *et al*., 2005), in the chlorophytes *Haematococcus pluvialis* (Steinbrenner & Linden, 2001) as well as in the cyanobacterium *Synechococcus* sp. (Schaeffer *et al*., 2006). Several studies have demonstrated that the nature of the strains themselves plays a fundamental effect on the quantity and quality of the carotenes produced (Markovitis *et al*., 1993; Cifuentes *et al*., 1996 a, b; Gòmez & Gonzàlez, 2005; Gòmez *et al*., 1999). In this contest the physiological characterization of unreported native strain of *D. salina* would be desirable. In the present study a wild strain of *D. salina* was isolated from Tarquinia salterns and cultivated using moderate light levels. The aim was to examine the effects of different combinations of salinity and nutrient concentration on the growth and pigment accumulation.

Materials and Methods

Strain isolation and cultivation

The *D. salina* strain was isolated from solar evaporation salt-ponds at Tarquinia's salterns located on the Tyrrhenian coast of Central Italy. Using micropipettes and serial dilution of a reddish water sample with high microalgal concentrations, a single cell was eventually isolated (Guillard & Ryther, 1962; Beker, 1994). After isolation, stock cultures were established under controlled laboratory conditions (25±3 °C, 40 µmol photon $m^{-2}s^{-1}$, photoperiod of 12:12) in f/2 Guillard's seawater medium at 20% NaCl (w/v). When grown under laboratory conditions, the alga turned green. It was identified as *D. salina* on the basis of morphological characteristics and molecular analysis by Tempesta *et al*. (2010).

Experimental culture conditions

The alga was grown initially in modified f/2 seawater medium at different salinities to acclimatize the *D. salina* strain. Three batch cultures (700 ml) with salinities of 10, 15 and 24% (NaCl w/v) were prepared and algae were grown for ten days in 1000 mL Erlenmeyer flasks. The flasks were maintained under photon flux density of 40 µmol $m^{-2}s^{-1}$ (Phillips cool daylight) at 25±3 °C, without aeration and manually shaken twice a day. Following salinity acclimation, culture media (700 ml) were prepared combining three different salinities (9, 14 and 22% NaCl w/v) and three different nitrate concentrations (882, 435 and 212 µmol L^{-1} N, corresponding to 100, 50 and 25% of the nitrate concentration of Guillard f/2 medium). Three replicates of each experimental combination were set up. All the experiments were started by inoculating the flasks with exponentially growing cells of salinity acclimatized cultures in order to have an initial concentration of 1.6 x 10^4 cells ml^{-1}. The alga was grown for 28 days at the same culture conditions as in the acclimatization step.

Monitoring growth and pigment quantification

Growth was monitored by sampling three times every three days and by counting the cells using a haemacytometer. Pigments were extracted from algae collected after 15 (Period I) and 28 (Period II) days cultivation. Cells were pelleted by centrifugation of 10 mL of culture medium at 3000 rpm. Total carotenoids and chlorophyll, extracted from algal pellets using a 90% (v/v) acetone/water mixture (Wegmann and Metzner, 1971), were determined by UV-vis spectrophotometric analysis according to the method described by Strickland and Parsons (1972) using a

Perkin-Elmer Lambda 20 instrument. All-*trans*-β-carotene concentration was estimated in 28 day old cultures (late stationary phase) grown at 24% NaCl w/v (not stationary phase was observed in cultures grown at 10 and 15% NaCl w/v) by High Performance Liquid Chromatography (HPLC) analysis. The determinations were performed using a Varian ProStar instrument equipped with a C-18 column (150 mm × 4.6 mm × 5 μm) and a UV detector selected at λ= 460 nm. The mobile phase was a gradient of water/acetone (to 30/70 until 10/90 in 30 minutes). Solvents were filtered and degassed prior to use. Commercial samples of all-*trans*-β-carotene were purchased from Sigma-Aldrich ad used as standard. Quantitative analysis were performed by using a calibration curve built with known concentration of all-*trans*-β-carotene.

Statistical analysis

Data were tested for normality (Kolmogorov-Smirnoff) and homocedasticity (Cochran-Barlett). An analysis of variance (ANOVA) was used to determinate differences between treatments. A Tukey HSD multiple comparisons of means test was used when significant differences were found. Differences were considered to be significant at a probability of 5% ($P<0.05$). The computation program used was SYSTAT version 8.0.

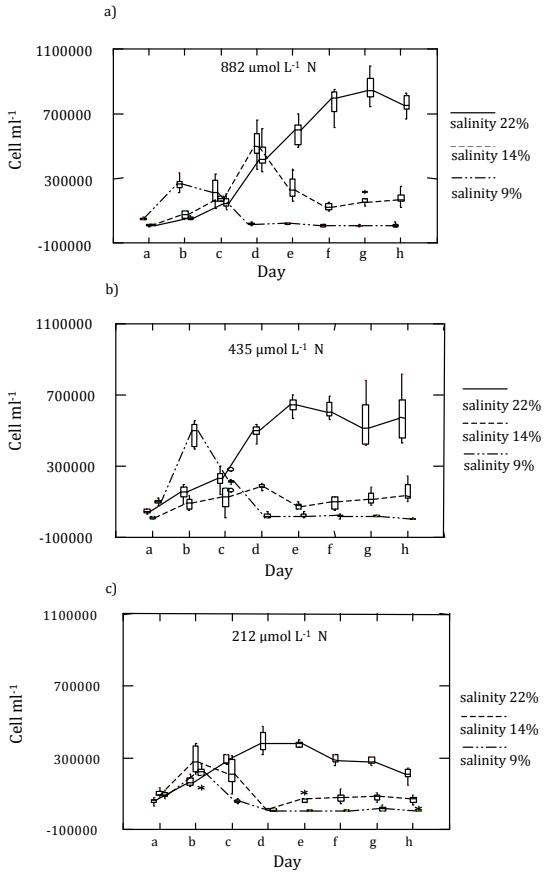

Figure 2. Changes in cell concentration of *D. salina* grown for 28 days at different salinities (9, 14, 22% NaCl w/v) in a medium initially enriched with a) 882, b) 435 and c) 212 μmol L^{-1} N. Small letters indicate times when cultures were monitored (a-7days; b- 10 days; c- 13 days; d- 16 days; e- 19 days; f- 22 days; g- 25 days; h- 28 days).

Results

Laboratory conditions

During the first ten days of the experiments a rapid exponential growth of the population that reached up to 2 x 10^5 cells/ml in all cultures was observed (Figure 2). After 19 days, the 22% salinity cultures approached a stationary phase and the growth rate decreased. In contrast, there was no stationary phase in others cultures and cell density of alga grown at 9% and 14% salinity began to decline after 10 and 16 days, respectively, in cultures with 882 and 435 μmol L^{-1} N (Figures 2a-b), and after 10 days in cultures with 212 μmol L^{-1} N (Figure 2c). Taking all treatments into account, the highest cell concentrations were observed when the alga was grown at 22% salinity.

Chlorophyll a and carotenoids

There were significant differences (ANOVA $P<0.05$) of Chl a concentration per unit volume among the treatments combining the three factors considered in this

study. Significant differences were found between the different factors ($P<0.05$) with the exception of the interaction between salinity and nitrate concentration ($P>0.05$). Significant differences of Chl a concentration were found between sampling times (Figure 3; Scheffé F-Test $P<0.05$): Chl a concentration per unit volume decreased in all cultures over time (with the exception of treatments combining a salinity of 22% whith higher nitrate concentrations, 882 and 435 µmol L^{-1} N). The highest Chl a concentration per unit volume was observed in cultures with the highest initial nitrate concentration at 22% salinity in period II. The treatments at different salinity showed significant differences of Chl a concentrations (Scheffé F-Test $P< 0.05$). Nitrate concentration affects treatments in progressively reducing concentrations of Chl a in cultures with 882, 435 and 212 µmol L^{-1} N at all salinity levels, no significant differences were observed in period I between the treatments at 14 and 22% salinity.

ANOVA analysis showed significant differences in carotenoid concentration per unit volume for the factors salinity and time of harvesting but not for nitrate concentration. Significant differences were found only between salinity and time of harvesting ($P<0.05$). Carotenoid concentration per unit volume decreased in period II in all cultures grown at lower salinity levels (9, 14%), on the contrary, in cultures grown at highest salinity, the behaviour changed in function of the initial nitrate concentration (Figure 4), carotenoids increased in cultures supplemented with 882 µmol L^{-1} N, were constant in cultures with 435 and were reduced in cultures with 212.

No significant differences were observed in carotenoid concentrations per unit volume in period I between cultures combining higher salinity (22, 14%) and 882 µmol L^{-1} N and, cultures combining lower nitrate with 9 and 14% salinity. As to the content of carotenoids

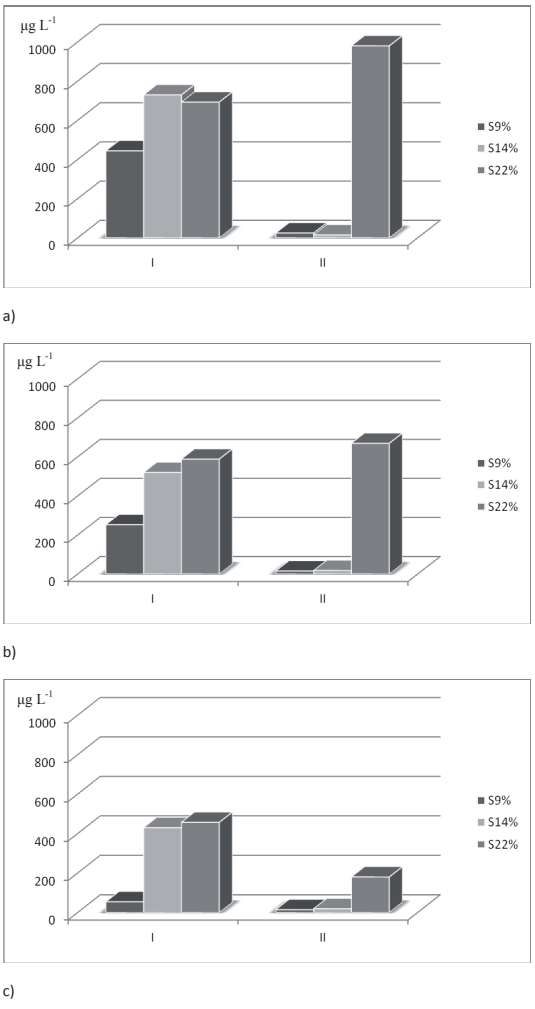

Figure 3. Concentration of Chl a per unit volume (µg L^{-1}), measured in period I (15 days) and period II (28 days) of *D. salina* cultivated at different salinity (9, 14, 22% NaCl w/v) in a cultured medium initially enriched with a) 882, b) 435 and c) 212 µmol L^{-1} N. Not significant differences were observed in period I between concentration Chl a values of salinity 14 and 22% at all nitrate concentration.

per cell, a significant increase was observed in cultures with lower nitrate concentrations and higher salinity between period I and II (Figure 5). The all-*trans*-β-carotene concentration was measured in period II in cultures at 22% salinity.

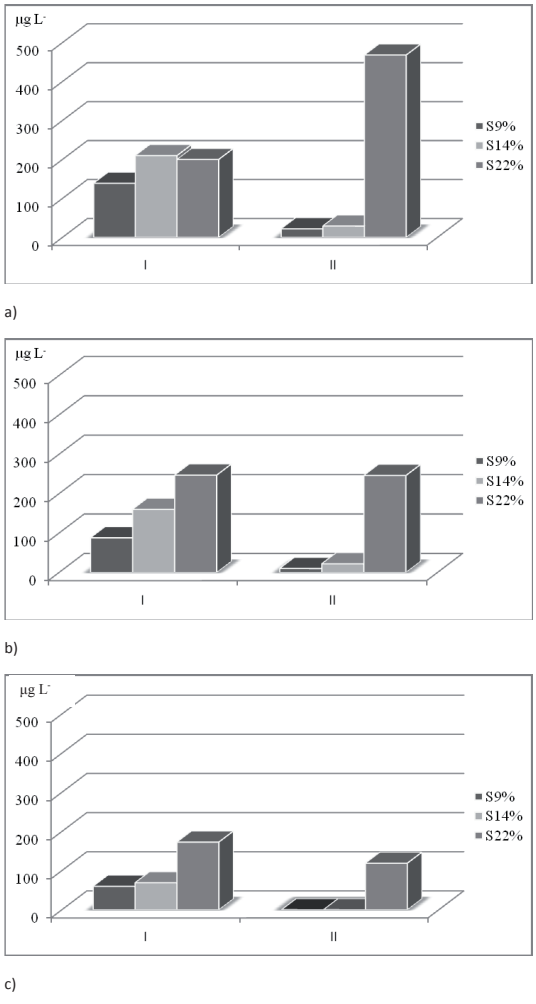

Figure 4. Concentration of carotenoid per unit volume (µg L^{-1}) measured in period I (15 days) and period II (28 days) of *D. salina* cultivated at different salinity (9, 14, 22% NaCl w/v) in a cultured medium initially enriched with a) 882, b) 435 and c) 212 µmol L^{-1} N.

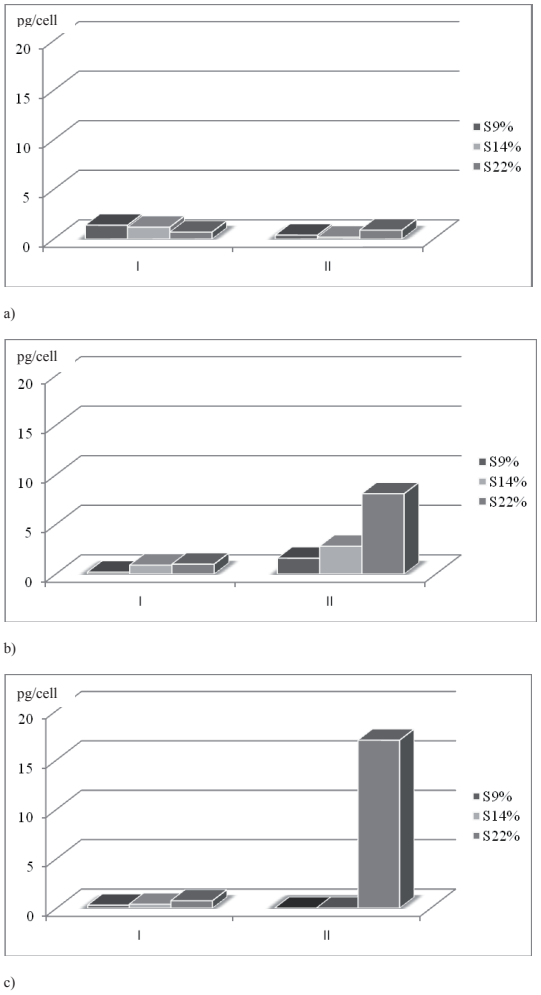

Figure 5. Concentration of carotenoid per cell (pg/cell) measured in period I (15 days) and period II (28 days) of *D. salina* cultivated at different salinity (9, 14, 22% NaCl w/v) in a cultured medium initially enriched with a) 882, b) 435 and c) 212 µmol L^{-1} N.

The average of all-*trans*-β-carotene and total carotenoids was enhanced by low nitrate concentration changing from 5% in cultures with 882 µmol/L N to 37% in cultures with 212 µmol L^{-1} N (Figure 6).

Conclusions

D. salina strain collected in Tarquinia salterns seems prefer high salinity conditions. In natural pools no growth was observed during spring-summer 2009, when salinity in the ponds was low (< 10% NaCl). In the previous summer period (2008) natural populations of *D. salina* were observed in ponds and the cell density was strongly related with salinity, with a maximum at higher salinity

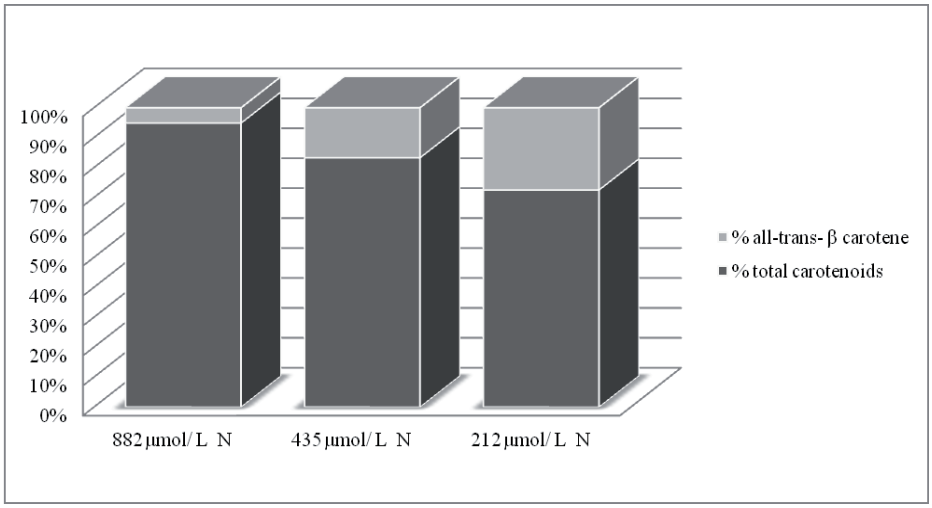

Figure 6. Percentage of all-*trans*-β carotene isomer on total carotenoids in cultures at different nitrate concentrations (882, 435 and 212 µmol L^{-1} N).

(Pasqualetti *et al.*, 2009). In laboratory conditions this strain exhibited higher specific growth rates at 22% salinity and it seemed particularly sensitive to reduced salinity, since the cell number (as well as Chl a content per unit volume) strikingly declined in all cultures at 14 and 9% salinity after the exponential growth phase. *D. salina* can tolerate a salinity of about 5% up to saturation, with an optimum around 20-25% NaCl (Borowitzka and Siva, 2007). Nevertheless, distinct geographical strains exhibited very different growth patterns in relation to salinity, i.e. *D. salina* from Yucatan showed an optimum salinity growth at 10% NaCl (Garcia *et al.*, 2007), *D. salina* from Chile at 5.8% NaCl (Gòmez *et al.*, 2003). The higher or lower halophytism is intrinsic to each strain and seems also related to other growth factors such as light intensity. Araujo *et al.* (2009) showed that the specific growth rate under high light intensity (300 µE/m^2/s) was not influenced by salinity, on the contrary this parameter determined under low light intensity (50 µE/m^2/s) showed salinity dependence. The growth rate of our strain is strongly influenced by salinity at low light level and natural blooms were observed only in the summer period in pools with a salinity greater than 18%, nevertheless, further research is needed to define its behaviour at high light levels. Microscopic observation showed that, during the exponential growth phase, cells grown in cultures at 9, 14, and 22% NaCl w/v were morphologically similar. At the beginning of the exponential growth phase the algae cultivated at 9% NaCl w/v growed faster but declined very quickly after 10 days (Figure 2). The decline of the microalgal population was observed after the exponential growth in cultures combining lower salinity at all nitrate concentrations. Marin *et al.* (1998) observed similar behaviours in a *D. salina* strain isolated from Araya salt ponds, but the decline of the microalgal populations was observed after the post-exponential growth in cultures combining 9% salinity and 212 µmol L^{-1} N; the authors considered the lack of substrate as the main factor of population decline. In our experiments the decline of algal populations seems to be mainly related

to salinity: it is possible that one or more metabolic processes are slowed down by low salinity, the use of radiant energy by the chlorophyll molecule may be inhibited and the chlorophyll may become oxidized and bleached, leading to a lethal photodynamic reaction (Loeblich, 1982). In this work we examined the combined effect of nutrient limitation and NaCl in *D. salina* cell growth and carotenoid accumulation at non-stressing irradiance. Several hypothesis have been made on the physiological role of carotenoids in *D. salina* (Levin & Mokady, 1994; Ben-Amotz & Levy, 1996), but the reasons for the accumulation of carotenoids in cells grown at non-stressing irradiance remain unclear. The carotenoid accumulation obtained in this experiment (up to 400 µg L^{-1} and 16 pg $cell^{-1}$) was relatively high if compared to similar experiments carried out at low irradiance. Marin *et al.* (1998), in a similar study, reported total carotenoid concentrations of 250-300 µg L^{-1} (0.8-1.6 pg $cell^{-1}$) and Loeblich (1982) reported a carotenoid concentration of 4.6 pg $cell^{-1}$ in *D. salina* grown at low irradiance and 21 pg $cell^{-1}$ for the same strain grown at high irradiance (> 290 µmol photon $m^{-2}s^{-1}$). Several authors analyzed the effect of salinity on the accumulation of carotenoids. Cifuentes *et al*. (1996 a) observed a significant increase of this parameter at the stationary phase only above 15% NaCl (2.6 M). Orset and Yung (2000) and Gòmez *et al*. (2003) did not observed clear influence of salinity on carotenid biosynthesis in *D. salina* between 0.4 to 3.5 M NaCl. Borowitzka *et al*. (1990) observed that optimum salinity for the rate of carotenoid increase is at about 25% NaCl and, despite large difference in geographical origin of the strains used, they observed little differences in their response to salinity changes. Coesel *et al*. (2008) observed that carotenoid accumulation was stimulated by nutrient depletion but neither high NaCl or high light intensity in the presence of nutrients led to significant carotenoid accumulation. In our study salinity influenced mainly cell concentration: only cultures growth at 22% NaCl reached a stationary phase. The highest carotenoid concentration per unit volume was obtained in cultures grown at high salinity and high nitrate concentrations in the late stationary phase and seemed related mainly to biomass increase. On the contrary, enhanced accumulation of carotenoids occurred in cells stressed by nutrient starvation (Figure 5). The chemical composition of the culture medium (especially N availability) significantly affects the quality and quantity of carotenoids (Gòmez *et al*., 2003). In our study at 22% salinity the average of all-*trans*- β carotene isomer/total carotenoids ranged from 5% to 37%, in relation to nitrogen availibility (Figure 6). The strains of *D. salina* collected in Tarquinia salterns showed an high halophytism and great physiological plasticity in response to both salinity and nitrate availability. Although the results of this study are promising for the carotenogenic potential of this *D. salina* strain, further research is needed in order to evaluate the real biotecnological value of this strain.

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