Photosynthetic and Antioxidant Activities in Extremophile Microalgae *Dunaliella Salina, Cylindrotheca Closterium and Phormidium Versicolor* According to NaCl Concentration and Irradiance

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Abstract

Dunaliella salina (Chlorophyceae), *Phormidium versicolor* (Cyanophyceae) and *Cylindrotheca closterium* (Bacillariophyceae) were isolated from three ponds in the solar saltern of Sfax (Tunisia) having an average salinity of 350, 100 and 90 respectively. Growth, pigment contents, photosynthetic and antioxidant enzyme activities were measured under controlled conditions: three light levels (300, 500 and 1000 µmol photons m^{-2} s^{-1}) and three NaCl concentrations (40, 80 and 140 g L^{-1}). The highest salinity reduced the growth of *D. salina* and *P. versicolor*, and strongly inhibited that of *C. closterium.* These results are in accordance with the species distibution in the salt marshes. Irradiance rise only induced a significant increase of net photosynthesis in *C. closterium* probably due to the efficient nonphotochemical quenching and antioxidative enzyme activities. According to $\square_{PSII}$ values, the photosynthetic apparatus of *P. versicolor* was stimulated by increasing salinity whereas that of *D. salina* and *C. closterium* was decreased by irradiance rise. The production of carotenoids in *D. salina* and *P. versicolor* was stimulated when salinity and irradiance increased whereas it decreased in the diatom. Antioxidant activity of carotenoids could compensate the low antioxidant enzyme activity measured in *D. salina*.

Introduction

Photosynthetic organisms are able to convert solar energy into biochemical compounds necessary for their growth and the development of trophic networks. In estuarine and coastal environments, photosynthetic organisms are often exposed to salt stress combined with light stress (*1*), particularly in saltworks composed of shallow ponds of increasing salinity (*2*). The combined effects of these two factors have a considerable impact on photosynthetic apparatus (*3*). So, organisms inhabiting these paralic ecosystems have developed osmotic adjustment mechanisms to cope with salt stress (*4- 6*).

Although primary producers rely on sunlight for photosynthesis, exposure to high levels of photosynthetic active radiation than those required for growth can lead to the inhibition of photosynthesis in algae and plants, particularly during a long period of exposure (*7*, *8*). This photoinhibition affects photochemical reactions by generating reactive oxygen species“ROS” (*9*), which can oxidize membrane proteins, lipids and pigments, resulting in membrane unstability and photobleaching of the photosynthetic pigments thus limiting photosynthesis efficiency and growth (*10*) and treaten survivability of organisms (*3*). To cope this critical situation, aerobic organisms develope defense mechanisms against ROS accumulation (*11*) that include antioxidant enzymes (superoxide dismutase, peroxidases, catalase, etc.), non-enzymatic system (carotenoids, ascorbate, glutathione, alpha-tocopherol, etc.) and DNA repair systems (*12*). Salt stress can also generate ROS (*13*). However, few studies have investigated the induction and the regulation of antioxidant defence system in microalgae under salt stress (*14*).

Under light stress, PSII repair appears to be common and involves the same components than in plants and cyanobacteria: proteolytic degradation and synthesis of new D1 protein, specific phosphorylation (in plants) of several proteins and PSII migrating damaged complex between the grana regions and stromal thylakoid which is accompanied by changes in the structure of these oligomeric complexes (*15*). The repair mechanisms triggered by salt stress is not well clarified in microalgae yet (*3*). When in combination with light stress, salt stress enhances the inhibition of PSII in *Chlamydomonas reinhardtii* (*16*), in leaves of *Hordeum*

vulgare and *Sorghum bicolor* (*17*) and in *Spirulina platensis* (*18*). According to (*3*), high light induces photodamage to PSII, whereas salt stress inhibits the photodamaged PSII repair and does not directly accelerate damage of PSII. The combination of light and salt stress appears to inactivate PSII very rapidly as a consequence of their synergistic effects. Chlorophyll fluorescence technique and photosynthetic oxygen production measured with a Clark-type probe have been regarded as very useful tools to measure the performance of the photosynthetic apparatus especially when microorganisms are under stress (*18*).

Several authors believe that the repair mechanism of PSII in green algae looks like the mechanisms described in land plants, although this aspect is not well studied in algae (*19*). In brown algae and diatoms, the mechanisms of photoprotection and repair of PSII have only recently begun to be revealed (*20*, *21*). According to (*7*), microalgae minimize light effects by developing short- and long-term mechanisms to tune the balance between energy utilization and dissipation. Carotenoids play a crucial role in these processes. Indeed the photosynthetic apparatus is protected against photoinhibition either by thermic dissipation of excess excitation energy in PSII antenna due to xanthophylls cycle (non photochemical quenching) or by transferring electrons from the PSII to different receptors within the chloroplast (photochemical quenching) (*22*) and finally by dissipating energy as fluorescence (*23*). The xanthophyll cycle acts as a photo-protective process that regulates the dissipation of excess light energy (*24*). In Chlorophyceae and Phaeophyceae violaxanthin is de-epoxidazed into antheraxanthin and zeaxanthin under excess light (*25*) and diadinoxanthin is converted into diatoxanthin in diatoms (*26*).

In order to better understand the physiological and biochemical mechanisms of light and salt tolerance in two microalgae (*Dunaliella salina*, *Cylindrotheca closterium*) and the cyanobacterium *Phormidium versicolor* isolated from an extreme environment like a solar saltern, we investigated in controlled conditions growth rate, photosynthetic pigments, photosynthetic and antioxidative enzyme activities in these three phytoplankonic species. The statistical analysis has allowed to highlight for each species what is the most stressfull factor.

Material And Methods

The solar saltern of Sfax (Tunisia, 34° 39’ 0.1” N and 10° 42’ 35” E) consists of artificial interconnecting ponds that cover 1,500 ha along 13 km stretch of the Mediterranean coast. Sea salt precipitates under evaporation and is harvested in crystallizing ponds for human consumming. Three autotrophic species: *Dunaliella salina* (Chlorophyceae)*, Cylindrotheca closterium* (Bacillariophyceae) and *Phormidium versicolor* (Cyanophyceae) were isolated from water samplings collected fromTS (mean salinity 346), C41 (95.5) and C21 (88.6) ponds, respectively (*2*). Species identification was carried out using morphological criteria and various identification keys (*27*, *28*).

Monoclonal cultures of *D. salina, C. closterium* and *P. versicolor* were carried out into 500 mL artificial seawater (*29*) under controlled conditions at 24 ± 1 °C under 300 µmol photons m^{-2} s^{-1} provided by white fluorescent tubes Philips LTD, 18 W) with a light/dark cycle 14 h/10 h. The algal cultures were axenized with anantibiotic–antimycotic (10,000 units mL^{-1} penicillin G, 10 mg mL^{-1} streptomycin sulphate, 25 mg mL^{-1} amphotericin B) treatment (Sigma–Aldrich, St. Quentin Fallavier, France). Initial density of algal cultures was 10^6 cells mL^{-1} for *D. salina*, 50,000 cells mL^{-1} for *C. closterium* and the initial chlorophyll *a* (Chl*a*) concentration of *P. versicolor* cultures was 5 ng mL^{-1}. All the experiments were carried out in triplicate. Growth was measured

by cell counting using Neubauer haemocytometer, growth of the filamenteous cyanobacterium *P. versicolor* was assessed by determining spectrophotometrically Chl *a* concentration after extraction with dimethyl formamide (*30*). After an acclimation period of 30 days in the conditions previously cited, the threee species were grown in artificial seawater (ASW) containing 40, 80 and 140 g L^{-1} of NaCl and exposed to an irradiance of 300, 500 and 1,000 µmol photons m^{-2} s^{-1} (E300, E500 and E1000) provided by white fluorescent tubes (Philips LTD, 18 W) for 6, 12 or 13 days to reach the stationnary growth phase depending on culture conditions and the species.

$$\mu \max = \frac{Log\ x2 - x1}{t2} - t1$$

Maximum specific growth rate(day^{-1}) was determined during the exponential growth phase (*31*) where *x1 and x2* are cell concentrations at *t1* and *t2*. The maximum cell density or Chl*a* content was obtained at stationary phase.

Photosynthetic pigments in *D. salina* and *C. closterium* were extracted with 90% acetone from 20 mL of algal culture. Photosynthetic pigments in *P. versicolor* were extracted with DMF from 10 mL of culture. Chl*a*, *b* and *c* were calculated for the Chlorophyceae and the Bacillariophyceae (*32*) and for the Cyanophyceae (*30*). Carotenoids contents were calculated for the Cyanophyceae (*33*) and for the Chlorophyceae (*34*). The following equation was used to determine fucoxanthin (*Fuco*) content in *C. closterium* (*35*):

$$Fuco\ (\mu g\ mL^{-1}) = [(DO_{443nm} - DO_{750nm}) - Chla\ \varepsilon_{Chla}\ R_{Chla} - Chlc\ \varepsilon_{Chlc}\ R_{Chlc}]$$

$\varepsilon_{Fuco}\ L$

ε_{Chla}= 88.1510^{-3} L mg^{-1} cm^{-1}, extinction coefficient of Chl*a* in acetone at 663 nm

R_{Chla} = 0.57, absorbance report for Chl*a* in acetone between 443 nm and 663 nm

ε_{Chlc}= 38.210^{-3} L mg^{-1} cm^{-1}, extinction coefficient of Chl*c* in acetone at 630 nm

R_{Chlc}= 8.14, absorbance report for Chl*c* in acetone between 443 nm and 630 nm

ε_{Fuco}= 166 10^{-3} L mg^{-1} cm^{-1}, extinction coefficient of fucoxanthin in acetone at 443 nm

L= 1 cm, optical path

The size of light harvesting antennas was evaluated by calculating Chl*a*/ Chl*b* ratio in *D. salina* and Chl*a*/ Chl*c* ratio in *C. closterium* (*25*, *36*).

The rate of net oxygen evolution (P_N) of intact cells during exponential growth was monitored with a Clark-type oxygen electrode (Hansatech LTD, UK) under growth conditions as previously described (*37*). The oximeter calibration was performed using ASW with NaCl 40, 80 and 140 g L^{-1}, the maximum dissolved oxygen concentrations were calculated (*38*).

Modulated fluorometry is a non-intrusive method providing fastly reliable and reproducible informations on PSII (*39*). Chl*a* fluorescence was measured with 2 mL of algal culture maintained at 24 ± 1 °C with the modified fluorometer FMS-1 (Hansatech Ltd., Cambridge, UK) (*40*). The sample is stirred with a magnetic bar placed in the cuvette to ensure the homogeneity of the suspension. Before fluorescence measurement, a period of dark adaptation of samples is applied for 10 minutes. This period is necessary for a complete re-oxidation of PSII electron acceptors. Then, the measurement of the minimum fluorescence level (F_0) and the maximum fluorescence level (F_m) made it possible to calculate the variable fluorescence $F_{v=} F_m - F_0$ and the maximum quantum efficiency of PSII (F_v/F_m). The steady-state fluorescence (F_s) was measured after 10 min at 300, 500 or 1000 µmol photons m^{-2} s^{-1}. A saturating flash induced the maximum fluorescence level of the light acclimated sample (F'_m) and the effective quantum yield efficiency of PSII (Φ_{PSII}) was calculated: $\Phi_{PSII} = (F'_m - F_s) /F'_m$. The non-photochemical quenching (*NPQ*) of fluorescence was determined: $NPQ = (F_m - F'_m)/F'_m$.

Algae and cyanobacteria were harvested by centrifugation (900×g, 4 °C) and then immediately freezed in liquid nitrogen. Pellets were transferred into a mortar previously cooled with liquid nitrogen and grinded with 1 mL of extraction buffer (sodium phosphate 50 mM pH 7, EDTA-Na_2 1 mM, ascorbic acid1 mM). The homogenate was centrifuged (10,000×g, 15 min, 4 °C), and the supernatant was used for spectrophotometric determination of antioxidative enzyme activities and total protein content. Catalase (CAT) activity was performed (*41*). The reaction mixture (phosphate buffer 50 mM, pH 7.5 and 100 µL of extract) was placed in a quartz cuvette at 20 °C. The addition of 100 µL of H_2O_2 (200 mM) allowed to measure CAT activity by monitoring H_2O_2 reduction at 240 nm for 1 min. The molar extinction coefficient of H_2O_2 at 240 nm is 0.04 mM^{-1} cm^{-1}. One catalase enzymatic unit corresponds to the quantity of enzyme that degrades one µmol H_2O_2 per min. Ascorbate peroxidase (APX) activity was assessed for 3 min by the decrease of absorbance at 290 nm due to ascorbate consumption in the presence of H_2O_2. The reaction mixture containing phosphate buffer 50 mM, pH 7.5, H_2O_2 0.5 mM and 100 µL of microalgal extract was placed in a quartz cuvette at 25°C. The addition of 50 µL of ascorbate (250 µM) triggers the reaction. An unit of APX is defined as the amount of enzyme needed to consume one µmol ascorbate mg^{-1} proteins for 1 min. Superoxide dismutase (SOD) activity was determined by measuring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) which absorbs at 560 nm (*42*). The reaction mixture (sodium phosphate buffer 50 mM pH 7.8, NBT 0.57 mM, methionin 5 mM, EDTA 10 mM, Trixton X-100 0.03% and 100 µL of extract or 100 µL of buffer for the control) was maintained at 25 °C. Riboflavin 10 µM was added to the reaction mixtures that were immediatly illuminated with 600 µmol photons m^{-2} s^{-1}. Absorbance was measured after 7 min of illumination. One unit of SOD activity was calculated as the enzyme amount required to induce 50% inhibition of the NBT photoreduction (*42*). Protein concentration of each microalgal extract was determined by standardizing with bovine serum albumin (*43*).

Statistical analysis

Data are the average ± SE of three independent replicates performed with independent cultures. The data were analyzed by three-way analysis of variance (ANOVA) with two factors: irradiance and NaCl concentration as independent variables. For multiple comparisons, tests of Tukey were used. Differences were considered to be

significant at a probability P<0.05, 0.01 and 0.001 depending on experience and species. The computational program used was IBM SPSS Statistics version 20.

Results

In this study, the growth of *D. salina, C. closterium* and *P. versicolor* grown in nine experimental conditions was monitored for 6, 12 or 13 days depending on the light level and the species. An exponential growth pattern was observed at each salt concentration except for *C. closterium* in the presence of NaCl 140 g L^{-1} under E500 and E1000 (Fig. 1). Growth curves of *D. salina*, cultivated with NaCl 40 and 80 g L^{-1}, were similar whatever the irradiance and the growth was reduced with NaCl 140 g L^{-1}. The Tukey test shows that the different salinities used dereased the maximum cell density (P< 0.001) and the maximum growth rate (P< 0.01). The maximum cell density increased concomitantly with illumination level from E300 to E500 with NaCl 40 and 80 g L^{-1} (Fig. 1, P< 0.001, Table 1). Maximum growth rate of *D. salina* was increased when irradiance rose from E500 with NaCl 40 and 80 g L^{-1} (P<0.001). Cell densities obtained with *C. closterium* were lower than those recorded with *D. salina*. The growth of *C. closterium* was slightly higher with 40 than with NaCl 80 g L^{-1} under E300 (Fig. 1) and was almost absent at 140 g L^{-1} under the three light levels (Fig. 1). Maximum growth rate was null with NaCl 140 g L^{-1} from E500 (Table 1). Maximum cell density decreased with increasing salinity under the three light levels. Maximum cell density of *C. closterium* increased significantly when irradiation reached E1000 with 40 and 80 g NaCl L^{-1} (P<0.001, Table 1). The highest growth of *P. versicolor* was recorded with NaCl 80 g L^{-1} under E300, the lowest with NaCl 140 g L^{-1} under E1000.

In *D. salina,* the Post Hoc test (Tukey) shows a significant increase of Chl*a* content with NaCl 140 g L^{-1} under E500 and E1000 compared to E300 (P<0.05) (Table 1). The Chl*b* content was about three times lower than that of Chl*a*, the highest concentration was obtained with NaCl 140 g L^{-1} under E1000 like this of Chl*a*. A significant increase was observed with NaCl 140 g L^{-1} under E500 and E1000 (P<0.05, Table 1). The light harvesting antenna size stayed unchanged under the different salt concentrations and light levels. Carotenoid content increased concomitantly with salt concentration (P<0.001). The Tukey test shows a significant increase of these pigments with the highest salinity and the highest irradiance (Table 1). It was not possible to detect photosynthetic pigments in *C. closterium* cells grown in the presence of NaCl 140 g L^{-1} under E500 and E1000. In *C. closterium*, Chl*a* content decreased significantly (P<0.001) with NaCl 140 g L^{-1} under E300. Chl*a* concentration decreased significantly (P<0.001) from E300 to E500 with NaCl 40 and 80 g L^{-1} (Table 1) and decreased significantly when NaCl reached 140 g L^{-1}under E300 (P<0.01). Chl*c* content significantly decreased under higher light levels (E500 and E1000) (P<0.01). Fucoxanthin content followed the same trend as Chl*c* (Table 1). Chl*a*/ Chl*c* ratio was almost unchanged under the different conditions allowing the growth of this species (P<0.001). Chl*a* content in *P. versicolor* showed a significant decrease with NaCl 140 g L^{-1} under each light level tested (P<0.001, Table 1). Under E300, the accumulation of carotenoids increased when the salinity increased (P<0.001). A higher irradiance led to a significant increase of carotenoids (P<0.001). The maximum carotenoid content was measured with NaCl 80 g L^{-1} under E1000.

The photosynthetic O_2 emission (P_N), on a Chl*a* basis, was higher in *D. salina* than in *P. versicolor* and *C. closterium* (Table 2). In *D. salina*, P_N was almost unchanged up to NaCl 80 g L^{-1} and significantly decreased

(P<0.001) with NaCl 140 g L^{-1} under the three light levels. In *C. closterium*, NaCl was the main factor which significantly reduced (P<0.01) P_N (Table 2). The addition of NaCl 140 g L^{-1} in the culture medium led to a significant decrease (P<0.001) of P_N of *P. versicolor* under the three light levels.

In *D. salina*, the maximum quantum yield (F_v/F_m) was equal to about 0.7 whatever the culture condition. The effective quantum yield (Φ_{PSII}) remained the same in the range of 0.3 whatever the salinity under E300; a higher light level (E500 and E1000) induced a significant decreased (P< 0.001) of this parameter. *NPQ* increased concomitantly with the NaCl concentration and the light level (P< 0.001, Table 2). A significant increase (P< 0.001) of *NPQ* of about 9 fold between E300 and E1000 was observed with the highest NaCl concentration. Due to the absence of growth, fluorescence parameters of *C. closterium* could not be determined in cells cultivated with NaCl 140 g L^{-1} under E500 and E1000. In the other conditions, F_v/F_m was about 0.7 like in *D. salina*. Under E300, a significant decrease of Φ_{PSII} value (P<0.001) of approximately a half was observed with NaCl 140 g L^{-1} compared to the lowest salinity (Table 2). Φ_{PSII} did not exceed the value of 0.37 ± 0.02 under E500 and E1000 and was significantly reduced (P<0.001) compared to values obtained under E300. Irradiance and NaCl interacted on this parameter as indicated by the Tukey test (F= 0.81, *ddl* = 9, P<0.001). *NPQ* increased when NaCl concentration and light level increased (P<0.05, Table 2). In *P. versicolor*, F_v/F_m was lower than values recorded in both microalgae with an average value of 0.4 (Table 2). As in *D. salina*, no significant variation was observed whatever the experimental conditions. Φ_{PSII} tended to increase with NaCl rising under the three light levels (P<0.001). No significant variation of *NPQ* was assessed in the cyanobacterium (P<0.001, Table 2) under the different growth conditions.

Activities of APX, CAT and SOD were only detected and measured when cells were grown under E1000 (Fig. 2). SOD activity was about twice higher in *C. closterium* and *P. versicolor* than in *D. salina*. This enzyme activity increased significantly in *D. salina* (F = 24.68, *d.d.l* = 6, P< 0.01), *C. closterium* (F = 8.67; *d.d.l* =6; P< 0.05) and *P. versicolor* (F = 29.78, *d.d.l* = 6, P <0.001) when the salinity increased. The highest CAT activity was measured in *C. closterium* and the lowest was recorded in *D. salina* whatever the salinity. CAT activity increased significantly in each species when the salinity increased (*D. salina*: F = 8.68; *d.d.l* = 6; P< 0.05; *C. closterium*: F = 6.20; *d.d.l* = 6; P< 0.05, *P. versicolor*: F = 8.21; d.d.l = 6; P<0.05). APX activity was not detected in *P. versicolor*. APX activity significantly increased in *C. closterium* when salinity increased (F = 23.76; *d.d.l* = 6; P< 0.01) and it stayed almost at the same level in *D. salina* whatever the NaCl concentration (Fig. 2).

Discussion

This study evaluated the growth and the photosynthetic and antioxidant activities of three phytoplanktonic species under nine experimental conditions. Their biomolecular signatures have confirmed the determination based on morphological traits we did previously (*44*). The growth of the three species studied was differently affected by increasing salinity. The different levels of salt tolerance measured experimentally were in accordance with the distribution of the three species in the salt marshes. Indeed, previous studies (*2*) have shown that in ponds with a salinity ranges from 42 and 96, Bacillariophyceae, among which *Cylindrotheca closterium*, dominate the other taxa since they represent more than 60% of the total phytoplankton; Chlorophyceae, represented mainly by *D. salina*, and Cyanophyceae, including *P. versicolor*, represented 13 % and 3 %, respectively. In ponds in which salinity was ranged from 190 to 340, Chlorophyceae and

Cyanophyceae were relatively abundant (31% and 70%, respectively) (*2*). Our results confirmed that NaCl 140 g L^{-1} decreased at different light levels the growth of *D. salina* and *P. versicolor* and inhibited the growth of *C. closterium*. Moreover, the maximum growth rate in *D. salina* decreased significantly at NaCl 140 g L^{-1} when light level increased whereas a significant increase was showed with NaCl 40 and 80 g L^{-1} under E500 and E1000. Salinity and irradiance were the main determining factors in growth rate variation (*45*). Our results confirm that *D. salina* and in *P. versicolor* resist to salt stress and that the diatom *C. closterium* is salt sensitive as it is observed in saltworks. Net photosynthesis values of the tree species studied are in accordance with growth curves. Against NaCl stress, each species develop different physiological mechanisms more or less efficient. So, *D. salina*, except other Chlorophyceae, is devoid of rigid polysaccharide cell wall, giving it the ability to adapt to high NaCl concentrations reaching saturation (*46*). This adaptability is due to plasma membrane plasticity (membrane reservoir) which prevents break and apoptosis of the cells (*6*). An increase of the degree of fatty acid saturation and hence, a reduction of the membrane fluidity and permeability of *Dunaliella* sp. isolated from an Antarctic hypersaline lake were observed (*47*). Intra-cellular Na^+ in *D. salina* remains unchanged up to 2.0 M NaCl (117 g L^{-1}) and thereafter a significant increase was observed (*5*). Glycine betaine and glycerol contents increase concomitlantly with salt concentration. Calcium acts as a second messenger in the osmoregulation system of this halotolerant species (*4*). Other species as *Chlamydomonas* sp. have depigmentented cells following lipid peroxidation of plasma membrane in the presence of 165 g L^{-1} NaCl (*48*). In cyanobacteria, the synthesis of osmolytes depends on their ability to tolerate salt (*49*): species with low salt tolerance (up to 0.7 M NaCl) accumulate sucrose and trehalose, species such as *Synechocystis* sp. PCC 6803 with moderate salt tolerance (up to 1.8 M NaCl) accumulate glycosylglycerol (*50*) and species that tolerate high salt concentration (up to 2.7 M NaCl) such as *Synechococcus* sp. PCC 7418 and *Aphanothece halophytica* (51) accumulate glycine betaine or betaine-glutamate. The frustule of *Cyclotella meneghiniana* contained less silica when cells were exposed to increasing salinity (NaCl 4 to 18 mg L^{-1}) (*52*). Such demineralization process could contribute to NaCl sensitivity of *C. closterium*.

On the other hand, carotenoids synthesis is stimulated in adapted cells against high irradiation or high salt concentration (*34*, *53*). These pigments dissipated light energy excess via the xanthophyll cycle and they act as filters that protect photosynthetic apparatus from photo-oxidation (*34*). Moreover, they have antioxidant properties that avoid lipid peroxidation in the photosynthetic apparatus by scavenging singlet oxygen (*54*, *55*). Our results show that high irradiance and high salinity stimulated carotenoids synthesis, especially in *D. salina* (15.64 ± 1.46 µg 10^{-6} cells) and in *P. versicolor* only in the presence of NaCl 80 g L^{-1} (Table 1). These results are consistent with those of other authors (*5*, *46*). An enhanced carotenoid production in *Nostoc muscorum* and *Phormidium faveolarum* when light and salinity increase whereas Chl*a* were measured and phycocyanin content is significantly affected (*56*).

In microalgae like *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta* the light harvesting antenna size is adjusted according to light and salinity (57). Our results showed that the number of photosystems increased significantly in *D. salina* when light level and NaCl increased, their size remaining unchanged. On the contrary, Chl*a* and Chl*c* contents tended to decrease in *C. closterium* leading to a decrease of photosynthesis rate and a lower growth under salt stress. The photosynthetic apparatus adjusts not only the number of photosystems but also its activity according to the light level. (*9*) showed that the photosynthetic apparatus in *D. salina* was

stimulated by high irradiance (2,000 µmol photons m^{-2} s^{-1}). We only observed this trend between E300 and E500. On a Chl*a* basis, the net photosynthesis rate in *D. salina* was about 2 fold than that in *P. versicolor* and in *C. closterium*. Antenna truncation in the cyanobacterium *Synechocystis* sp. strain PCC6803 results in decreased productivity (*58*). The photosynthetic activity also depends on salt concentration. It appears that the photosynthetic apparatus of *D. salina* and *P. versicolor* is more protected against salt stress than in *C. closterium*. NaCl increasing from 0.5 to 1M (from 29 to 58 g L^{-1}) leads to the decrease of photosynthesis in *Spirulina platensis* under different light levels (80, 100, 200 and 3,500 µmol photons m^{-2} s^{-1}) (*59*, *60*). This decrease is a regulation of the photosynthetic activity rather than a real damage (*59*). Berry et al. (*61*) suggested that *Spirulina platensis* adapts itself under high salinity by different mechanisms in thylakoid and cytoplasmic membranes like the regulation of intracellular Na^+ concentration via a Na^+-ATPase, ATP being generated by respiration and the cyclic electron transport around PSI (*62*). Na^+-ATPases belonging to the family of P-type ATPases have also been found in marine microalgae, *Tetraselmis viridis* (*63*), *Heterosigma akashiwo* (*64*) and *D. maritima* (*65*). Adaptation of *Synechocystis* to light and salt stress can be associated to the balance between the rate at which damage was induced and the rate of repair of PSII (*3*). To estimate the state of photosystems, especially PSII, fluorescence of Chl*a* was measured with a modulated fluorometer.

The ratio F_v/F_m has been widely used to assess the extent of the photo-inhibition in microalgae (*66*). A decrease of F_v/F_m can both be an indicator of PSII damage or a regulation index of electron transport at the PSII level, which leads to heat dissipation of light energy exces. F_v/F_m was almost constant (about 0.7) in both microalgae but it was lower (about 0.55) in *P. versicolor* (*67*). This ratio was defined as an index of maximum photochemical efficiency of PSII (*68*) which depends on both F_0 and F_v. In cyanobacteria, phycobiliprotein fluorescence interfers with chlorophyll fluorescence which leads to an increase in F_0 value. As a consequence F_v/F_m value decreases (*69*). Moreover, the saturating flash detaches phycobiliproteins from the photosynthetic apparatus causing fluorescence decrease (*70*). This reaction is considered as a photo-protective mechanism that protects photosynthetic apparatus against high light levels in cyanobacteria. Aquaporins in the cytoplasmic membrane of *Synechocystis* sp PCC6803 might be necessary for the repair of PSII and PSI photodamage (*71*).

When photochemistry is working, the effective quantum yield (Φ_{PSII}) decreased since a part of PSII centres are reduced (or closed). Under salt stress, the reduction of PSII activity in *D. maritima* leads to an immediate reduction of Φ_{PSII} values (*72*). Under our experimental conditions, a decrease of Φ_{PSII} in *D. salina* was measured when irradiance increased and in *C. closterium* when it was summitted to a high salinity and a high light level. We can notice that P_N and Φ_{PSII} did not always have the same trend in the diatom and the cyanobacterium (for example: *C. closterium* NaCl 80 g L^{-1}, E1000). This absence of positive correlation between these two parameters is due to salt and/or light impacts on the other components of photosynthetic activity. (*3*) showed that *Synechocystis* sp. (PCC 6803) cells exposition to light (E500) or salt stress (NaCl 29 g L^{-1}) leaded to partial inactivation of PSII. Moreover, the combination of these two stresses induced a complete PSII inhibition. We observed a similar phenomenom with *C. closterium* that was unable to survive in the presence of NaCl 140 g L^{-1} beyond E500. According to Zakhozhii et al. (*72*), the reduction of PSII activity is due to structural as well as functional disturbances of PSII and electron transport chain in *D. maritima.* Despite these disruptions, photosynthetic apparatus continued to operate and produce energy required for

physiological and bio-chemical processes (*5*). Bukhov and Carpentier (*73*) showed that PSI has a crucial role by producing the energy needed for defence mechanisms against stress. Net photosynthesis as Φ_{PSII} decreased in both the microalgae while Φ_{PSII} values increased and net photosynthesis decreased in response to NaCl rising under the three light levels in *P. versicolor.* In this latter species, PSII could be less affected by NaCl than carbohydrate synthesis. Liska et al. (*74*) showed that photosynthesis activity was over 2-fold (from 96.8 to 193.6 µM O_2 mg^{-1} Chl*a* h^{-1}) in cells grown in 3 M NaCl than in 0.5 M NaCl in *D. salina*. According to these authors, this improvement serves the synthesis of organic solutes and osmolytes. So, cyanobacteria like *Aphanothece* sp., *Phormidium* or *Oscillatoria* sp hilled up glycine betaine or betaine glutamate in the presence of NaCl 156 g L^{-1} (*75*). The effect of salt stress on PSII in cyanobacteria could be attributed to a direct interaction between salt and PSII via cellular components still unknown (*69*). Zeng and Vonshak (*60*) observed that Φ_{PSII}in *Spirulina platensis* decreases by 15% after a 25 h-exposition to NaCl 29 g L^{-1} under 100 µmol m^{-2} s^{-1}, whereas Φ_{PSII} decreased by about 75% under 200 µmol m^{-2} s^{-1} at the same salinity. However, PSII activity regained its original level after an 80 h-exposition showing that, after an initial acclimation phase during which photosynthetic activity was inhibited, a new steady state was established with a recovery of the photosynthetic activity. Our results showed that light level had no significant effect on *P. versicolor* PSII activity.

NPQ increase acquaints about the dissipation of light excess energy as heat when cells are subjected to stress (*26*). Our results are in accordance with those of other works (20, 66, *76*) who reported that *NPQ* increases when microalgae are subjected to salt and / or light stress. Under the most stressful condition, *NPQ* was 24-fold the value measured in *D. salina* under control condition, 80-fold in *C. closterium* and 10 fold in *P. versicolor*. In *C. closterium* that was the most NaCl sensitive species, *NPQ* reached the value of 21 in the presence of NaCl 80 g L^{-1} and E1000. The xanthophyll-dependant *NPQ* appeared as an efficient photoprotective mechanism in diatoms (*24*) since the net photosynthesis of *C. closterium* was stimulated under E1000. Thaipratum et al. (*77*) precised that *NPQ* in *D. salina* is a multi-component process as it was also shown in the diatom *Phaeodactylum tricornutum* (*7*).

Reactive oxygen species (ROS) generated by abiotic stresses are scavengered by antioxidative molecules and antioxidative enzyme activities in species having physiological mechanisms to cope with ROS (*78*). APX, CAT and SOD activities were only detected when the three species were cultivated under E1000, except the APX activity in the cyanobacterium. *Nostoc flagelliforme* (*79*) and *Cyanobium bacillare* (*80*) are also devoided of APX activity. The salinity rise stimulated ROS production and the three enzyme activities studied. Similar results were obtained in *Ulva fasciata* after a 12 h exposure to NaCl 90 g L^{-1} since CAT, Fe-SOD, Mn-SOD and APX activities were stimulated (*81*). Rijstenbil (*82*) showed that salt stress (60 PSU) stimulates the production of ROS in *C. closterium* regardless of light irradiance since SOD and APX activities attained 400 and 35 enzyme units per mg proteins, respectively. These values are clearly higher than those obtained in the strain isolated from the Sfax saltern under the most stressful condition (NaCl 140 g L^{-1} and E1000). It is probable that strains living in salt marshes have acquired adaptative mechanisms to salt that are more efficient than in marine strains. Among the salt adaptative mechanisms, species living in saltern can have a non-enzymatic antioxidative system particularly active and / or an efficient NaCl exclusion system and / or an efficient photoprotective system. We also noticed enhanced SOD and CAT activities in *P. versicolor* when the salinity

increased whereas antioxidative enzyme activities in *D. salina* weakly varied when salinity increased. In this latter species, the carotenoid accumulation could play a major role in the antioxidative defence. The raise of SOD, CAT and APX activities in relation with salt concentration was higher in *C. closterium* than in the two other species. This biochemical response could be related to growth inhibition as in *Chlamydomonas reinhardtii* and *Peridinium gatunense* in which a highest antioxidative activity preceds cell death (*83*), these authors suggest that the high antioxidative activity or a metabolite generated by stress triggers cell death cascade.

Despite the stimulation of antioxidative enzyme activities in *C. closterium*, this diatom was more affected by NaCl 140 g L^{-1} than *D. salina* and *P. versicolor*. Salt and high irradiance triggered protective mechanisms that were more efficient in *D. salina* and *P. versicolor* than in *C. closterium*. The maintain of photosynthetic activity allowed the production of energy required for physiological and bio-chemical processes necessary for cell survival (eg. osmolytes and carotenoids synthesis, antioxidative enzyme activities). In *C. closterium*, antioxidative enzyme activities were triggered but this defence mechanism was not sufficient to cope with NaCl and light stress.

Declarations

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Ethical approved

This is an research study made on extremophile microalgae isolated from solar saltern of Sfax (Tunisia). The Faculty of Sience of Sfax and University of Le MAns Research Ethics Committee has confirmed that no ethical approval is required.

Consent to Participate

This study not include human subject

Consent to publish

All data presented her belong to this study

Competing Interests

There is no conflict of interest

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Tables

Table 1 Maximum growth rate, maximum cell density (or Chl*a* content), pigment contents and light harvesting antenna size in *Dunaliella salina*, *Cylindrotheca closterium* and *Phormidium versicolor* grown in artificial seawater containing NaCl 40, 80 and 140 g L^{-1} under an irradiance of 300, 500 and 1000 µmol m^{-2} s^{-1}. Means ± SD (n = 3), $P < 0.01$ or $P < 0.001$ (depending on the species and the parameter)

Species	*Dunaliella salina*								
Irradiance (μmol m^{-2} s^{-1})	300			500			1000		
NaCl (g L^{-1})	40	80	140	40	80	140	40	80	140
Maximum growth rate (day^{-1})	0.53 ± 0.15^{a1}	0.61 ± 0.15^{a1}	0.57 ± 0.23^{a2}	1.06 ± 0.08^{b1}	0.95 ± 0.01^{b1}	0.36 ± 0.05^{b2}	0.91 ± 0.04^{a1}	0.80 ± 0.01^{a1}	0.27 ± 0.06^{a2}
Maximum cell density (10^6 cells mL^{-1})	2.02 ± 0.15^{a1}	2.00 ± 0.12^{a2}	1.61 ± 0.10^{a3}	2.89 ± 0.16^{b1}	2.57 ± 0.02^{b2}	1.13 ± 0.07^{b3}	2.37 ± 0.05^{a1}	2.09 ± 0.01^{a2}	0.95 ± 0.01^{a3}
Chl*a* (μg 10^{-6} cells)	1.26 ± 0.10^{a1}	0.96 ± 0.05^{a1}	1.01 ± 0.11^{a2}	1.19 ± 0.10^{b1}	1.13 ± 0.06^{b1}	1.47 ± 0.17^{b2}	1.21 ± 0.07^{b1}	0.97 ± 0.13^{b1}	1.64 ± 0.10^{b2}
Chl*b* (μg 10^{-6} cells)	0.29 ± 0.05^{a1}	0.18 ± 0.03^{a1}	0.16 ± 0.02^{a2}	0.24 ± 0.03^{a1}	0.22 ± 0.06^{a1}	0.27 ± 0.05^{a2}	0.30 ± 0.03^{a1}	0.28 ± 0.06^{a1}	0.36 ± 0.09^{a2}
Carotenoids (μg 10^{-6} cells)	0.79 ± 0.41^{a1}	1.30 ± 0.22^{a1}	2.89 ± 0.28^{a2}	1.15 ± 0.16^{a1}	1.56 ± 0.64^{a1}	7.06 ± 1.51^{a2}	1.83 ± 0.42^{b1}	3.51 ± 0.94^{b1}	15.64 ± 1.46^{b2}
Antenna size (Chl*a*/ Chl*b*)	4.4 ± 0.93^{a1}	5.31 ± 0.95^{a1}	6.29 ± 1.09^{a1}	4.90 ± 0.68^{a1}	5.57 ± 1.96^{a1}	4.95 ± 0.58^{a1}	4.10^{a1} ± 0.79	3.53 ± 1.06^{a1}	4.65 ± 1.32^{a1}
Species	*Cylindrotheca closterium*								
Maximum growth rate (day^{-1})	0.46 ± 0.07^{a1}	0.22 ± 0.05^{a1}	0.04 ± 0.02^{a2}	0.40 ± 0.05^{a1}	0.35 ± 0.02^{a1}	0.00 ± 0.00^{a2}	0.22 ± 0.12^{a1}	0.22 ± 0.02^{a1}	0.0 ± 0.0^{a2}
Maximum cell density (10^6 cells mL^{-1})	0.42 ± 0.04^{a1}	0.30 ± 0.02^{a2}	0.22 ± 0.02^{a3}	0.53 ± 0.07^{a1}	0.49 ± 0.05^{a2}	0.06 ± 0.01^{a3}	0.72 ± 0.06^{b1}	0.62± 0.05^{b2}	0.06 ± 0.01^{b3}
Chl*a* (μg 10^{-6} cells)	2.28 ± 0.38^{a1}	3.21 ± 0.11^{a1}	2.49 ± 0.20^{a2}	1.81 ± 0.27^{b1}	1.75 ± 0.16^{b1}	0 ± 0^{b2}	1.57 ± 0.14^{b1}	1.44 ± 0.12^{b1}	0 ± 0^{b2}
Chl*c* (μg 10^{-6} cells)	0.95 ± 0.08^{a1}	0.96 ± 0.13^{a1}	0.58 ± 0.17^{a2}	0.39 ± 0.08^{b1}	0.34 ± 0.10^{b1}	0 ± 0^{b2}	0.38 ± 0.04^{b1}	0.38 ± 0.04^{b1}	0 ± 0^{b2}
Fucoxanthin (μg 10^{-6} cells)	1.23 ± 0.17^{a1}	1.94 ± 0.28^{a1}	1.33 ± 0.07^{a2}	0.92 ± 0.35^{b1}	0.87 ± 0.11^{b1}	0 ± 0^{b2}	0.63 ± 0.24^{b1}	0.83± 0.18^{b1}	0 ± 0^{b2}
Antenna size (Chl*a*/ Chl*c*)	2.38 ± 0.19^{a1}	3.39 ± 0.61^{a1}	2.29 ± 0.55^{a2}	4.63 ± 0.36^{a1}	5.33 ± 1.22^{a1}	0 ± 0^{a2}	4.20 ± 0.58^{a1}	3.78 ± 0.16^{a1}	0 ± 0^{a2}

Species	*Phormidium versicolor*								
Maximum growth rate (day^{-1})	0.61 ± 0.07^{a1}	0.66 ± 0.04^{a1}	0.29 ± 0.01^{a1}	0.45 ± 0.04^{ab1}	0.27 ±0.02^{ab1}	0.31 ±0.03^{ab1}	0.35 ± 0.06^{b1}	0.32 ± 0.04^{b1}	0.27 ± 0.03^{b1}
Chl*a* (µg mL^{-1})	1.55 ± 0.23^{a1}	0.77 ± 0.23^{a1}	0.71 ± 0.16^{a2}	1.38 ± 0.49^{a1}	1.31 ± 0.41^{a1}	0.17± 0.06^{a2}	0.94 ± 0.26^{a1}	2.12 ± 0.03^{a1}	0.46 ± 0.10^{a2}
Carotenoids (µg mL^{-1})	0.13 ± 0.01^{a1}	0.19 ± 0.01^{a2}	0.30 ± 0.01^{a2}	0.25 ± 0.01^{b1}	0.41 ± 0.03^{b2}	0.21 ± 0.13^{b2}	0.14 ± 0.05^{b1}	0.56 ± 0.08^{b2}	0.34 ± 0.01^{b2}

1, 2, 3: subsets of NaCl levels generated by the TUKEY test; different numbers indicate a significant difference

a, b: subsets of light levels generated by the TUKEYtest; different letters indicate a significant difference

Table 2 Effect of NaCl and irradiance on net photosynthesis (P_N), maximum quantum yield (F_v/F_m), effective quantum yield of PSII (Φ_{PSII}) and non-photochemical quenching (*NPQ*) in *Dunaliella salina*, *Cylindrotheca closterium* and *Phormidium versicolor* grown in artificial seawater containing NaCl 40, 80 and 140 g L^{-1} under 300, 500 and 1000 µmol m^{-2} s^{-1}. Means ± SE, $P < 0.001$ except for Φ_{PSII} and net photosynthesis in *Cylindrotheca closterium*: $P < 0.05$ and $P < 0.01$, repectively

Species	*Dunaliella salina*								
Irradiance (µmol m^{-2} s^{-1})	300			500			1000		
NaCl (g L^{-1})	40	80	140	40	80	140	40	80	140

P_N (µmol O_2 h^{-1} mg^{-1} Chl*a*)	728^{a1} ± 35	649^{a1} ± 53	467^{a2} ± 32	1017^{a1} ± 36	711^{a1} ± 120	447^{a2} ± 27	480^{a1} ± 27	668^{a1} ± 52	367^{a2} ± 28
F_v/F_m	0.71^{a1} ± 0.02	0.75^{a1} ± 0.01	0.74^{a1} ± 0.04	0.75^{a1} ± 0.02	0.67^{a1} ± 0.12	0.75^{a1} ± 0.01	0.78^{a1} ± 0.03	0.75^{a1} ± 0.01	0.64^{a1} ± 0.02
Φ_{PSII}	0.32^{a1} ± 0.09	0.28^{a1} ± 0.13	0.37^{a1} ± 0.17	0.31^{b1} ± 0.02	0.22^{b1} ± 0.01	0.23^{b1} ± 0.01	0.23^{b1} ± 0.02	0.16^{b1} ± 0.01	0.11^{b1} ± 0.01
NPQ	0.23^{a1} ± 0.11	0.23^{a2} ± 0.06	0.49^{a2} ± 0.19	0.74^{b1} ± 0.12	1.01^{b2} ± 0.07	0.71^{b2} ± 0.12	1.3^{c1} ± 0.07	2.48^{c2} ± 0.44	5.97^{c2} ± 0.39
Species	*Cylindrotheca closterium*								
P_N (µmol O_2 h^{-1} mg^{-1} Chl*a*)	387^{a1} ± 69	343^{a2} ± 19	243^{a3} ± 32	427^{b1} ± 39	365^{b2} ± 53	nd	425^{b1} ± 34	287^{b2} ± 71	nd
F_v/F_m	$0,71^{a1}$ ± 0.02	$0,74^{a1}$ ± 0.09	$0,76^{a2}$ ± 0.11	$0,68^{b1}$ ± 0.04	$0,76^{b1}$ ± 0.02	nd	0.75^{b1} ± 0.03	0.73^{b1} ± 0.04	nd
Φ_{PSII}	0.51^{a1} ± 0.05	0.39^{a1} ± 0.13	0.20^{a2} ± 0.08	0.25^{b1} ± 0.03	0.37^{b1} ± 0.02	nd	0.20^{b1} ± 0.03	0.30^{b1} ± 0.01	nd
NPQ	0.51^{a1} ± 0.10	0.31^{a2} ± 0.18	8.05^{a3} ± 2.24	4.18^{b1} ± 0.19	10.89^{b2} ± 2.47	nd	7.76^{c1} ± 0.15	21.31^{c2} ± 4.63	nd
Species	*Phormidium versicolor*								
P_N (µmol O_2 h^{-1} mg^{-1} Chl*a*)	552^{a1} ± 66	432^{a1} ± 26	278^{a2} ± 42	421^{a1} ± 52	370^{a1} ± 79	274^{a2} ± 28	474^{a1} ± 46	372^{a1} ± 33	279^{a2} ± 37
F_v/F_m	0.39^{a1} ± 0.04	0.46^{a1} ± 0.08	0.50^{a1} ± 0.07	0.35^{a1} ± 0.05	0.33^{a1} ± 0.04	0.45^{a1} ± 0.09	0.55^{a1} ± 0.07	0.55^{a1} ± 0.10	0.50^{a1} ± 0.19
Φ_{PSII}	0.28^{a1} ± 0.04	0.26^{a1} ± 0.03	0.52^{a2} ± 0.03	0.15^{a1} ± 0.05	0.16^{a1} ± 0.01	0.21^{a2} ± 0.05	0.14^{a1} ± 0.05	0.38^{a1} ± 0.04	0.47^{a2} ± 0.09
NPQ	0.12^{a1} ± 0.03	0.19^{a1} ± 0.13	0.21^{a1} ± 0.14	0.14^{a1} ± 0.07	0.15^{a1} ± 0.02	0.67^{a1} ± 0.24	0.65^{a1} ± 0.35	1.5^{a1} ± 0.87	0.33^{a1} ± 0.21

1, 2: subsets of NaCl levels generated by the TUKEY test, different numbers indicate a significant difference

a, b, c: subsets of light levels generated by the TUKEY test, different letters indicate a significant difference

nd: not determine

Figures

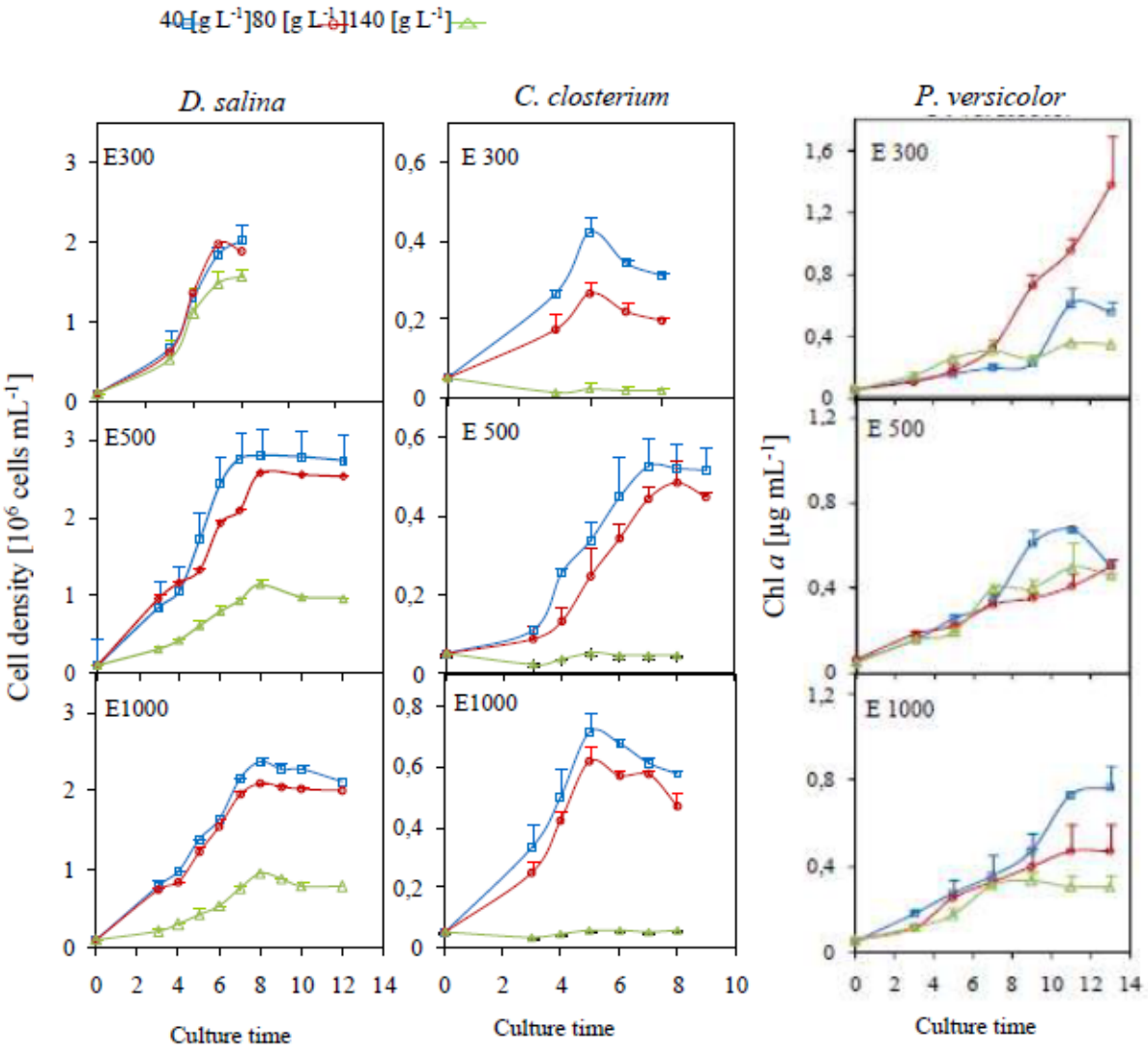

Figure 1

Growth kinetics of Dunaliella salina, Cylindrotheca closterium and Phormidium versicolor grown in artificial seawater containing NaCl 40, 80 and 140 g L-1 under 300, 500 and 1000 µmol m-2 s-1 (E300, E500 and E1000). Means ± SE

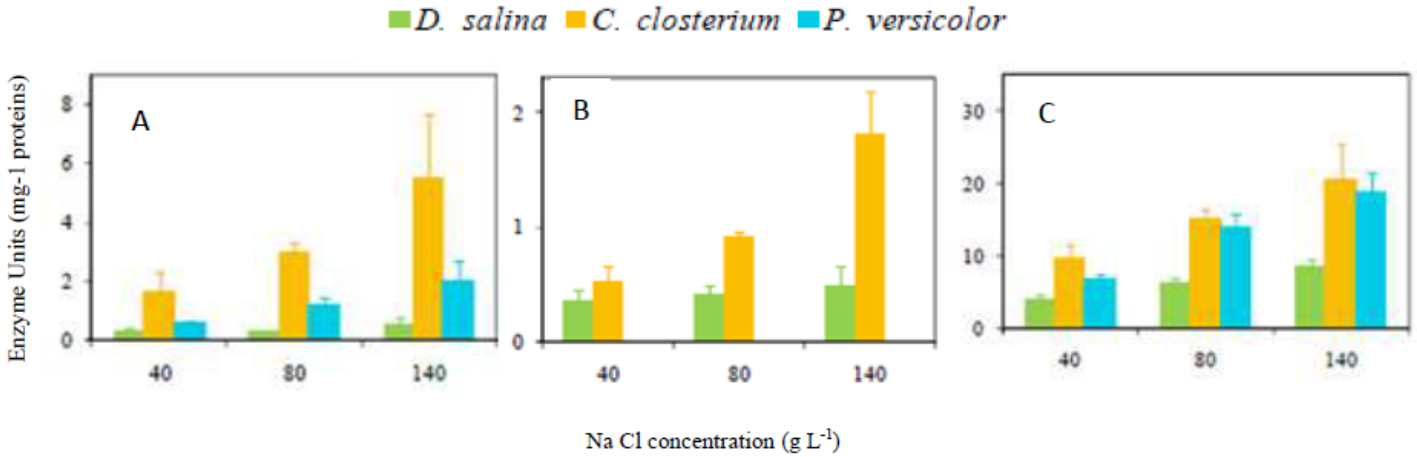

Figure 2

Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities in Dunaliella salina, Cylindrotheca closterium and Phormidium versicolor grown in artificial seawater containing NaCl 40, 80 and 140 g L-1 under 1000 µmol m-2 s-1. Means ± SE