

Full Length Research Paper

Growth and photosynthesis response of the green alga, *Picochlorum oklahomensis* to iron limitation and salinity stress

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The extreme environment of the Great Salt Plains (GSP) results in low algal biomass, such that natural selection is likely driven by survival of multiple abiotic stresses rather than rapid growth and biotic interactions. The objective of this study was to determine the effect of combined salinity stress and iron limitation on growth and photosynthesis in the green alga, *Picochlorum oklahomensis* isolated from the GSP habitat. Algal cells were grown in batch cultures under iron sufficiency or iron limitation at salinities of 10, 50 and 100 ppt in artificial seawater (AS 100) medium. Cells were physiologically characterized by growth rates, cell density, photosynthetic light-response curves (oxygen evolution), pigment composition and the chlorophyll fluorescence parameters F_v/F_m , Φ_{PSII} , qP and NPQ. *P. oklahomensis* cultured in no added Fe exhibit smaller inhibition by 100 ppt salinity relative to 10 ppt. Thus, high salinity appears to be reduced under low nutrient conditions. Fe stress resulted in qualitative differences in physiological response. The interaction between salinity and low iron is consistent with a general stress response that concurrently protects against several abiotic stress factors.

Key words: Iron limitation, salinity stress, *Picochlorum* sp.

INTRODUCTION

Origin, characteristics and classification of *Picochlorum oklahomensis*

Picochlorum oklahomensis, a unicellular halotolerant green alga, was isolated from shallow evaporitic pools at the Salt Plains National Wildlife Refuge (SPNWR) in northwestern Oklahoma, USA. *Picochlorum* sp. is classified as belonging to the Domain Eukarya, Kingdom Protista, Division Chlorophyta and Class Trebouxiophyceae (Graham and Wilcox, 2000; Henley et al., 2002, 2004).

The SPNWR is characterized as being a hypersaline environment with a warm-temperate, semiarid, continen-

tal climate (Henley et al., 2002). Hypersaline environments, including the SPNWR usually have high salinity, exhibit fluctuations in their salt concentration over time because they are occasionally flooded by rain or seawater and are also subject to evaporation. Some minerals may precipitate as a result of the evaporation and this could lead to changes in the brine composition (Eugster and Jones, 1979; Nissenbaum, 1980).

Nutrient concentrations and ratios vary with salinity and anion composition in saline lakes and soils and these changes can potentially affect algal nutrient requirements (Schobert, 1974; Cole et al., 1986; Zamani et al., 2012). Organisms living in the SPNWR are exposed to these

environmental variations and may have developed ways to cope with these changes.

There is uncertainty about the effects of high and variable salinity on nutrient solubility and bioavailability. It is anticipated that high salinity would make nutrients less available to the organism because of precipitation and therefore organisms found in such environments may become adapted to low nutrient levels.

There have been various interests in looking at the effect of salinity on algal and plant growth as well as separate studies investigating the effect of low nutrients on algae and plants. However, there has been only very few studies looking at the effects of both salinity and low nutrients concurrently in algae or plants (Venova 2008; Yousfi et al., 2012). Recent efforts have focused on combinations of either salinity and temperature, temperature and light or salinity and light. However, no previous study has examined the effect of salinity and nutrients limitation relating to plants and algae particularly those from the SPNWR.

Salinity

Salinity refers to the salt content of water or soil. It is considered to be a very important environmental factor because of its role in reducing crop yields in most part of the world (Komori et al., 2003). Apart from challenges experienced by organisms in the natural habitats such as marine and hypersaline environment, increases in salinity are also encountered as a result of inappropriate irrigation techniques employed by farmers in soils considered to be arid or semiarid. Salinity stress may affect plants and algae through osmotic and ionic stress. Water deficit brings about osmotic stress while excess Na^+ and Cl^- and reduction in the uptake of other mineral nutrients can bring about ionic imbalances or stress (Ashraf and Harris, 2004; Mgbeze et al., 2011).

Plants and algae may differ in the way they respond to salt stress. Some are able to tolerate the salt stress within certain limits without any difficulty. Others are able to exclude the salts through their leaves or salt glands. Still others produce compounds making their tissues tolerant to the salt concentration. One of such common ways is the production of osmoprotectants or compatible solutes to lower the internal water potential of the cell thus enabling the cell to take up water from the environment. The compatible solutes include mannitol and proline. Proline is produced in the cell from glutamate and the synthesis requires ATP and NADPH. Trying to maintain proper osmotic conditions may therefore be at a high energy cost which may be manifested in reduced growth rates and decrease in photosynthetic electron transport activities (Gimmler et al., 1981; Kirst, 1989; Ashraf and Harris, 2004). Lawlor (2002) suggested that these osmoprotectants may also play other roles such as being nitrogen sinks during periods of reduced growth and photosynthesis. Salt stress also affects protein syn-

thesis, the functioning of some enzymes and membrane integrity. When salinity negatively affects membrane lipids, it leads to problems with membrane permeability, transporters and enzymes (Kerkeb et al., 2001).

Iron

Iron is considered to be a micronutrient that is very important for all organisms. It is commonly found in plants and algae as metalloproteins which are necessary for photosynthesis, respiration and nitrogen assimilation (Crosa, 1997; Bulter, 1998). Cytochromes, iron-sulphur proteins and ferredoxin contain iron (Guikema and Sherman 1983).

Raven (1990) estimated that 80% of the iron in phytoplankton forms part of the photosynthetic electron transport chain. Iron is also needed in the biosynthesis of chlorophyll and for nitrate and nitrite reductases activity. In saline water, iron occurs as ferric hydroxides which undergo precipitation due to the formation of complexes with organic ligands that alters its solubility and availability to algae (Motekaitis and Martell 1987, Millero et al., 1995; Sunda and Huntsman, 1995).

Some phytoplanktons have iron transporters which help with the uptake of iron from the environment. However a limitation encountered is that these are found to be responsive to only dissolved inorganic iron (Anderson and Morel, 1982; Hudson and Morel, 1990, 1993). Another mechanism for efficient iron uptake used by eukaryotic phytoplanktons is the production of siderophores which help to release the iron bound to organic ligands. The siderophores are usually produced under low iron and are thought to be low molecular weight chelators which form stable complexes with Fe (III) (Trick, 1989; Wilhelm and Trick, 1994).

It has been observed that in order to easily release the iron bound to the siderophores, microorganisms and plants make use of ligand exchange at the cell surface, hydrolysis and acidification and reduction of Fe (III) (Neilands, 1974; Guerinot and Yi, 1994). Since iron is part of almost all the components of the photosynthetic electron transport chain, it implies that photosynthesis would be greatly affected by iron deficiency. Doucette and Harrison (1991) have reported the decrease in the size and structure of chloroplast in iron limited grown cells.

The objective of this study was to determine the effect of combined salinity stress and iron limitation on growth and photosynthesis in the green alga, *P. oklahomensis*.

MATERIALS AND METHODS

Organism

The organism used in this study is *P. oklahomensis*, a unicellular green alga, which was isolated from the Salt Plains National Wildlife Refuge, Oklahoma, USA. This is maintained in the laboratory in AS

100 medium in flask. The study was done at Oklahoma State University.

Growth media

The batch cultures were maintained in artificial seawater medium, AS100 (Starr and Zeikus, 1993) but with some modification. The sodium chloride (NaCl) content was changed to give a concentration of 10, 50 and 100 ppt. The salinity was checked using a hand-held refractometer (A366ATC). In preparing the AS100 medium, MgSO₄, KCl, NaNO₃, CaCl₂, NH₄Cl and Tris buffer (pH of 8.08) were added to the NaCl solution and then after autoclaving, the vitamins, sodium thiosulphate, chelated iron, sodium bicarbonate and potassium dihydrogen phosphate solutions were added, using a syringe and Acrodisc 0.2-µm sterile syringe filters.

Iron limitation was induced by not adding iron to the medium (though minute quantities of impurities may be present in the chemicals used). The control (iron sufficient) medium had 11.7 µM chelated iron. 50 ml of each treatment was poured into a 125 ml Erlenmeyer flask made from optically clear polycarbonate and plugged with cotton wool surrounded with aluminium foil. There were three flasks maintained for each treatment giving three replicate cultures of *Picochlorum* for each treatment. The preparation of the medium and the inoculation procedures were carried out in a laminar flow hood under sterile conditions.

Growth conditions

The experimental cultures were kept in a growth room at a day/night temperature regime of about 27 to 35°C and a photon fluence rate (PFD) of about 200 µmol photons·m⁻²·s⁻¹. The source of light was a 1000 W metal halide lamp at a 14:10 -h light:dark (L:D) cycle. For the experiments, the inoculum from the stock culture in Salt Plain medium (prepared from salt brine from the salt plains) was pre-acclimated in a 50 ppt AS 100 medium for seven days before inoculating for the beginning of the two-stage experiment. Samples from the first culture were used to inoculate the second set of cultures in triplicate after ten days of growth. The first stage of culture enabled the *Picochlorum* cells to acclimate to the nutrient treatments and to ensure that most of the stored nutrients had been completely used. Each treatment had three replicates.

Determination of cell numbers and growth rate

Samples were taken daily from each of the cultures throughout the experiment and actual cell counts was performed. Total cell densities were determined by transferring 10 µl of each sample unto a hemocytometer and performing total cell count. The growth rate (µ, d⁻¹) was determined by fitting Jassby and Platt (1976) function of a nonlinear curve to the graph of cell density versus time for each replicate culture using Sigma Plot 2000 software. The mathematical function by Jassby and Platt (1976) is given below:

$$F = CDm \times \tanh \{(\mu \times T)/CDm\} + CDo$$

where, CDm is increase in cell density, CDo is initial cell density, µ is growth rate (in d⁻¹, which is the initial slope) and T is time in days.

This equation was used because there was no prolonged exponential phase giving a linear line for the determination of growth rate. The cell densities at the end of day 10 were used as the final cell yield. In order to further analyze the curvature of the graphs as a way of getting more information from the growth data, Bannister's function (1979) was used to obtain the convexity values.

$$F = CDm \times (\mu \times d / \{CDm^c + (\mu \times d)^c\}^{1/c}) + CDo$$

where, CDm is final cell density, CDo is initial cell density, µ is growth rate (in d⁻¹, which is the initial slope), T is time in days, and c is convexity.

Photosynthetic measurement

Six days old second stage cultures were used for this experiment. Some of the cells were concentrated in order to obtain cell densities almost equal to all treatments and spiked with 8 mM NaHCO₃. Photosynthetic light-response (P-I) curves were measured as whole cell oxygen exchange at 27°C in a Hansatech DW water-jacketed, 9-mL electrode chamber with continuous stirring and connected to a computer. Samples were exposed to a series of 12 increments of photon flux densities (PFD) from darkness to > 1500 µmol photons m⁻² s⁻¹ using a slide projector as source of light fitted with neutral density filters. Exposure to each PFD was 4 min after a 30-s equilibration period.

Photosynthetic rates (µmol O₂ hr⁻¹) were automatically calculated in real time by linear regression after each PFD. The calculated photosynthetic rates were also checked manually from the data generated and normalized to cell density. Light saturated photosynthesis (P_{max}), initial slope (α) and dark respiration (R_d) were determined by fitting individual curves to a Jassby and Platt (1976), function using Sigma Plot 2000 software.

$$F = Pm \times \tanh \{(\alpha \times I)/Pm\} - R_d$$

Where, Pm is light-saturated photosynthetic rate, R_d is rate of respiration in darkness, α is the initial ascending slope and I is light intensity.

Pigment content

In order to understand the effect of low iron and salinity stress on the pigments in *Picochlorum*, cultures in the second stage were harvested on day 6 and the chlorophyll content determined by filtering 3 mL of each culture suspension and transferring the cells together with filter paper into 3.5 mL dimethyl -formamide (DMF) and keeping them in the dark for 24 h. DMF was used because it is able to extract a lot of chlorophyll from samples as compared to other solvents commonly used (Porra et al., 1989). DMF has also been identified as being able to chemically stabilize chlorophyll and as well as preventing its oxidation (Hu et al., 2013). Extracts were centrifuged to remove cellular debris and the absorption spectra were measured from 400 to 750 nm using a spectrophotometer with 2-nm band pass. The concentrations of chlorophylls a and b were determined using the equations by Porra et al. (1989):

$$\text{Chlorophyll a} = 12.00 A_{664} - 3.11 A_{647}$$

$$\text{Chlorophyll b} = 20.78 A_{647} - 4.88 A_{664}$$

where, A is the absorbance at the specified wavelength, corrected for scattering by subtracting A₇₅₀.

Total carotenoids were calculated using Wellburn's equation (1994):

$$\text{Total carotenoids} = (1000 A_{480} - 0.89 C_a - 52.02 C_b)/245$$

Where, A₄₈₀ is the absorbance at 480 nm, and C_a and C_b are concentrations of chlorophyll a and b, respectively.

Chlorophyll fluorescence

Six days old second stage cultures were used for this experiment.

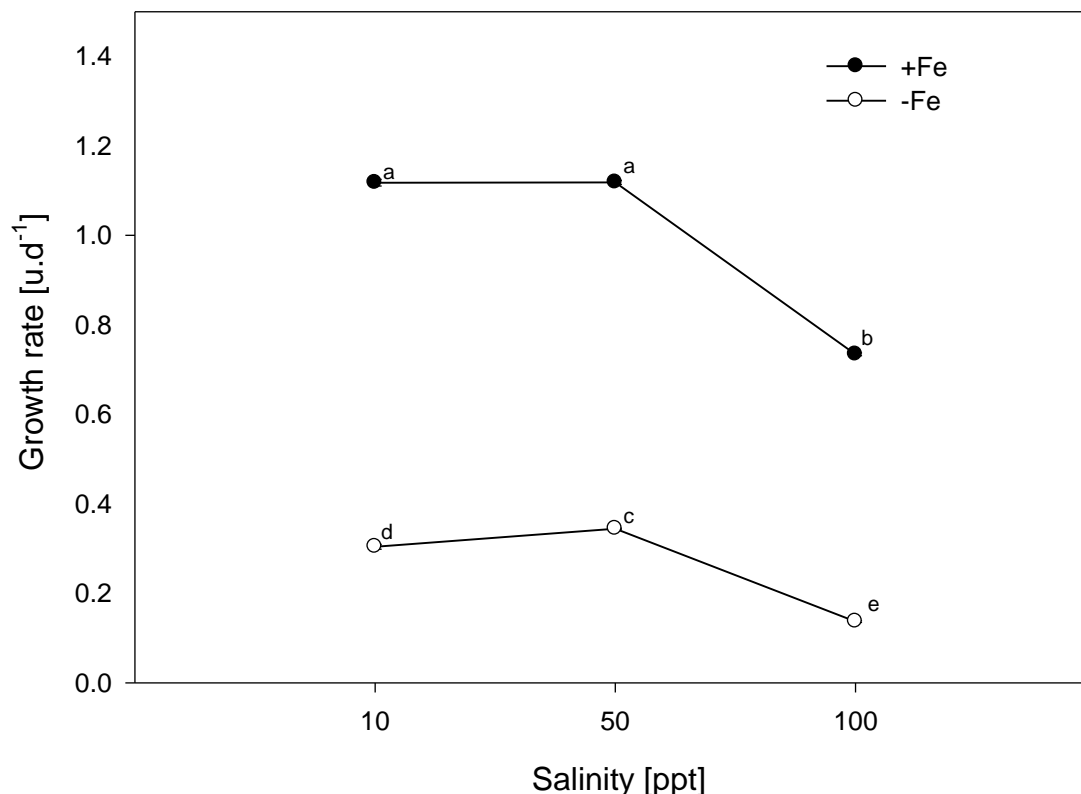


Figure 1. Growth rate of *Picochlorum* sp. with high iron (shaded symbols, +Fe) and low iron (open circles, -Fe) at different salinities of 10, 50 and 100 ppt. Mean values \pm standard deviation, $n = 3$ for all treatments (Error bars smaller than symbols). Different letters indicate significance differences between means.

Chlorophyll fluorescence quenching analysis was carried out at room temperature using a Dual-Modulation Kinetic fluorometer (PhotoSystems Instruments, Czech Republic). The fluorometer was connected to a computer with data acquisition software, Fluowin. Two milliliters of culture was put into a cuvette and the cuvette was placed into the sample compartment for the measurement to proceed.

Each sample was dark-adapted for 10 min before starting with the measurement. The minimal fluorescence level in the dark-adapted state (F_0) was measured by activating the modulated light (ML) which was very low in order to prevent the induction of significant variable fluorescence.

A 0.8 s flash of saturating white light ($>1000 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was then applied to determine the maximal fluorescence in the dark-adapted state, (F_m). After a lag phase of 20 s, the actinic light, AL (about $400 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) from light-emitting diode was turned on to provide continuous white illumination for 30 s. In order to determine the light-adapted maximal chlorophyll fluorescence (F_m'), saturating pulses were applied to the sample at different intervals during the period of actinic light driving photosynthesis.

Using both the dark and light fluorescence parameters, the following were calculated for the fluorescence analysis: the maximum efficiency also known as optimal quantum yield of PS II photochemistry in the dark-adapted state, (F_v / F_m); the actual quantum yield of PSII electron transport in the light-adapted state, $\Phi_{\text{PSII}} = (F_m' - F') / F_m'$; the photochemical quenching coefficient, $qP = (F_m' - F') / (F_m' - F_0')$, which gives a measure of the proportion of open PSII reaction centers and the non-photochemical quenching, $\text{NPQ} = (F_m - F_m') / F_m'$ (Genty et al., 1989; Bilger and Björkman, 1990). The fluorescence nomenclature used in this section follows van Kooten and Snel (1990).

Experimental design and statistical analysis

A factorial design was used for the experiments with salinity as one factor at three levels (10, 50 and 100 ppt) and iron as the other factor at two levels (control and low). Data of the initial growth rates, cell densities, photosynthetic parameters, pigment content and the fluorescence parameters were analyzed by using two-way analysis of variance (ANOVA) with replication at $p = 0.05$. The significance of the pairwise differences between treatments was analyzed using Tukey HSD multiple comparison. The software used for all the statistical analysis was SYSTAT version 10.

RESULTS

Growth rates and cell growth

Picochlorum growth rate is largely unaffected by salinity below 50 ppt, but is reduced by about one-third at 100 ppt in control (Figure 1). Low iron cultures exhibit the same pattern as cultures grown in added iron (+Fe) medium but were shifted to lower initial growth rates (Figure 1).

Primary effects of both salinity and iron were highly significant in two-way ANOVA ($p < 0.001$). All individual treatments were significantly different except +Fe at 10 and 50 ppt (Tukey, $p < 0.05$). In addition, iron-salinity interaction was significant, because growth rate was reduced at 10 ppt relative to 50 ppt in the Fe⁻ treatment,

Table 1. P-I parameters P_{max} (fmol O_2 .cell⁻¹.h⁻¹), α and R_d (fmol O_2 .cell⁻¹.h⁻¹) measured for *Picochlorum* sp. in high and low iron at different salinities. Mean \pm standard deviation, n = 3. Different letters indicate significant differences between means for that parameter.

| | High iron | Low iron |
|--|-------------------------------|-------------------------------|
| P_{max} (fmol O_2.cell⁻¹.h⁻¹) | | |
| 10 ppt | 21.51 \pm 0.32 ^a | 12.24 \pm 0.49 ^b |
| 50 ppt | 20.96 \pm 1.17 ^a | 11.26 \pm 0.95 ^b |
| 100 ppt | 13.31 \pm 1.37 ^b | 7.51 \pm 0.58 ^c |
| α fmol O_2.cell⁻¹ (μmol photons m⁻² s⁻¹)⁻¹ h⁻¹ | | |
| 10 ppt | 0.12 \pm 0.03 | 0.17 \pm 0.11 |
| 50 ppt | 0.14 \pm 0.05 | 0.19 \pm 0.08 |
| 100 ppt | 0.11 \pm 0.06 | 0.15 \pm 0.02 |
| Dark respiration (R_d)[fmol O_2.cell⁻¹.h⁻¹] | | |
| 10 ppt | -0.30 \pm 0.93 | -0.18 \pm 1.26 |
| 50 ppt | -0.40 \pm 0.49 | -0.36 \pm 0.86 |
| 100 ppt | -0.27 \pm 0.28 | -0.33 \pm 0.15 |

but not in the Fe⁺ grown cells. The effect of low iron was proportionately the same at all salinities (LFe/control = 0.3). The absolute reduction of 0.6 day⁻¹ at 100 ppt was the lowest as compared to 0.8 day⁻¹ at 10 and 50 ppt. Cell densities at day 10 exhibited different patterns from the growth rate.

Increasing salinity significantly decreased cell density in the +Fe medium however in -Fe grown cells, an increase in salinity from 10 and 50 ppt rather increased cell density at day 10 (Figure 2). Both primary effects of salinity and low iron as well as the interaction between these two factors were significant for cell yields (2-way ANOVA, p<0.01).

Photosynthetic measurements

In order to investigate the effect of low iron and salinity stress on photosynthesis in *Picochlorum*, oxygen evolution was measured and the following three fundamental parameters were calculated: The rate of respiration in darkness (R_d), the slope at limiting PFDs, α , (which comprises both light-harvesting efficiency and photosynthetic energy conversion efficiency), and the light-saturated photosynthetic rate (P_{max}). Photosynthetic rates (μ mol O_2 hr⁻¹) were automatically calculated in real time by linear regression after 270 s exposures to PFD. Photosynthetic light-response (P-I) curves are shown in Figure 3. These parameters were obtained for each replicate of a particular treatment (3 per treatment) and then were analyzed using ANOVA.

Only salinity affected light-limited photosynthetic efficiency, α (Table 1). Both salinity and iron treatment

significantly affected P_{max} (2-way ANOVA, p<0.01). In both +Fe and -Fe treatments, P_{max} was significantly lower at 100 ppt than at lower salinities (Tukey, P<0.01). P_{max} was significantly lower at each salinities in -Fe grown cells than those cultured in +Fe medium (Tukey, P<0.05). There was also a significant interaction between salinity and low iron interaction for this parameter (2-way ANOVA p<0.01).

Salinity, iron limitation and pigments

Total chlorophylls per cell were significantly affected by salinity, iron treatment and the interaction between these factors (Figure 4a; 2-way ANOVA, p<0.05). Chlorophyll decreased with increasing salinity in added iron cultures (+Fe), with most of the decrease (28%) occurring at 100 ppt.

Iron limitation significantly decreased total chlorophyll per cell by 18% at 10 ppt and 41% at 50 ppt, but significantly increased by 25% at 100 ppt (Tukey test, p<0.01) as compared to Fe+ grown cells. Chlorophyll a/ b ratio was significantly reduced by low iron (Figure 4b; 2-way ANOVA, p<0.01), but not salinity or the interaction between these factors. The reduction by low iron was about 43% at 10 and 100 ppt and 61% at 50 ppt.

Total carotenoids per cell were significantly affected by salinity and iron treatments as well as the salinity-iron interaction (Figure 5; 2-way ANOVA, p<0.01). In +Fe cultures, total carotenoids decreased with increasing salinity but this was different in -Fe grown cells. At 100 ppt, carotenoids decreased by about 30% relative to 50 ppt in +Fe cultures. Low iron significantly reduced carotenoids at 10 and 50 ppt (22 and 42%, respectively), but not at 100 ppt which had a significant increase of 22%. The total chlorophylls/total carotenoids ratio was not significantly affected by salinity or iron treatment (ANOVA, p>0.05).

Determination of fluorescence parameters

The determination of the chlorophyll a fluorescence parameters in *Picochlorum* by the saturation pulse method were calculated from the fluorescence measurements.

Salinity, iron and fluorescence

The maximum quantum yield of PS II photochemistry in the dark-adapted state (F_v/F_m) was significantly affected by salinity, iron treatments as well as the interaction between salinity and iron treatment (Figure 6a; 2-way ANOVA, p<0.05). Iron limitation treatment significantly decreased, F_v/F_m by 80% at 10 ppt, 77% at 50 ppt and 48% at 100 ppt as compared to the +Fe medium. Within the -Fe treatments, increasing salinity from 10 and 50 ppt

to 100 ppt increased F_v/F_m by about 150%. The effective quantum yield of PSII electron transport in the light-adapted state, Φ_{PSII} , was significantly affected by low iron treatment (Figure 6a; 2-way ANOVA, $p < 0.01$) but not salinity or the interaction between these factors. Low iron significantly decreased Φ_{PSII} by 89% at 10 ppt and about 80% at 50 and 100 ppt (Tukey test, $p < 0.05$) as compared to the controls.

The photochemical quenching coefficient, qP , was significantly affected by low iron treatment (Figure 6b; 2-way ANOVA, $p < 0.01$) but not salinity nor the interaction between salinity and low iron treatments. Low iron reduced qP by 80% at 10 ppt and 63% at 50 and 100 ppt as compared to the controls. The non-photochemical quenching, NPQ, was significantly affected by iron treatment (Figure 6; 2-way ANOVA, $p < 0.01$) as well as salinity and the interaction between these factors. Increasing salinity significantly decreased NPQ by 29 and 24% at 50 and 100 ppt, respectively, relative to 10 ppt (Tukey test, $p < 0.01$). Low iron treatment, significantly, increased non-photochemical quenching by 250% at 10 ppt (Tukey test, $p < 0.01$) but caused a non-significant decrease of 41 and 25% at 50 and 100 ppt, respectively as compared to the controls.

DISCUSSION

The hypothesis for this study is that the effect of salinity stress on growth and photosynthesis in *Picochlorum* will be different under sufficient iron and iron-limited conditions. In other words the pattern of response by *Picochlorum* to the three different salinities of 10, 50 and 100 ppt will not follow the same pattern at the two iron levels. This may be due to the influence of the amount of the nutrient present. If there is a change in the response pattern from 10 through 50 to 100 ppt for the two iron levels, then there is a salinity-nutrient interaction. Examining the two factors together may result in the intensification of the stress in organisms or possibly the effect of one may reduce the effect of the other factor. The response of *Picochlorum* to the three salinities and iron limitation was investigated.

Effect of salinity and iron

This study has shown that response to growth and photosynthesis is influenced by the interaction between salinity and iron concentration. The response to salinity is different under high and low iron present in the growing environment as evident in the data for growth rates (Figure 1), cell density at day 10 (Figure 2), total chlorophyll (Figure 4), total carotenoids (Figure 5) maximum quantum yield of PSII, F_v/F_m (Figure 6a) and non-photochemical quenching, qP (Figure 6b).

Low iron significantly reduced growth rate, cell density at day 10 and photosynthetic capacity in *P. oklahomensis*.

Increasing salinity did not affect growth rates and P_{max} at lower salinities in the +Fe medium but significantly decreased these parameters at 100 ppt in the +Fe medium (Figure 1) which led to a decrease in the cell density. The growth rates (μ , d^{-1}) were calculated using the equation by Jassby and Platt (1976) of a nonlinear curve fit of cell density versus time for individual cultures. This equation was used because there was no prolonged exponential phase giving a linear line for determination of the growth rates. However in order to carry out further analysis of the curvature of the growth curves as a way of studying the effects on growth in *Picochlorum*, the Bannister's equation (1979) was used, which includes a convexity parameter:

$$F = CD_m \times (\mu \times d / \{CD_m^c + (\mu \times d)^c\}^{1/c}) + CD_o$$

This concept has been applied to P-I curves, but never before to batch grown cultures. However, this unusual analysis was used because of the shape of the μ curves. When an alga or a plant is stressed, the curvature of P-I curves is very low relative to unstressed organisms (Bannister, 1979; Henley, 1993). It is unclear whether this would also apply to the semi log time course of cell densities. In the analysis, the growth curves of *Picochlorum* in +Fe had much higher convexity values than that of -Fe grown cells, indicating that they were much stressed growing in -Fe medium. The effect on growth rate and cell density by salinity were both different under +Fe and -Fe grown cultures.

The reduction in growth rate of *Picochlorum* at 100 ppt salinity in +Fe medium is expected. Salinity stress affects algae and plants through osmotic stress, ion toxicity and ionic imbalance. Organisms adapted to high salinity try to overcome these effects by producing compatible osmolytes which require high energy cost leading to reduced growth rates and decline in photosynthetic electron transport (Gimmler et al., 1981; Kirst, 1989; Gilbert et al., 1998). This is also consistent with the P_{max} values in +Fe medium. *Picochlorum* produces proline (Hironaka, 2000), a compound which has been reported to play a major role in salt tolerance in some organisms, and its production from glutamate requires energy and carbon. Salt stress can inhibit protein synthesis such as D1 protein which will in turn affects the rates at which damaged D1 proteins are replaced, which ultimately will cause excitation pressure and result in a decrease in electron transport (Allakhverdiev et al., 2002; Mohanty et al., 2007).

In -Fe medium, however, growth rate and day 10 cell densities were highest at 50 ppt; day 10 cell densities were similarly reduced at 10 and 100 ppt but growth rate was significantly more reduced at 100 than 10 ppt. This is unexpected and shows that the response of *Picochlorum* to salinity may be influenced by the level of iron present in the culture medium which is consistent with the primary hypothesis that the response to growth and yield response of *Picochlorum* at the different salinities will

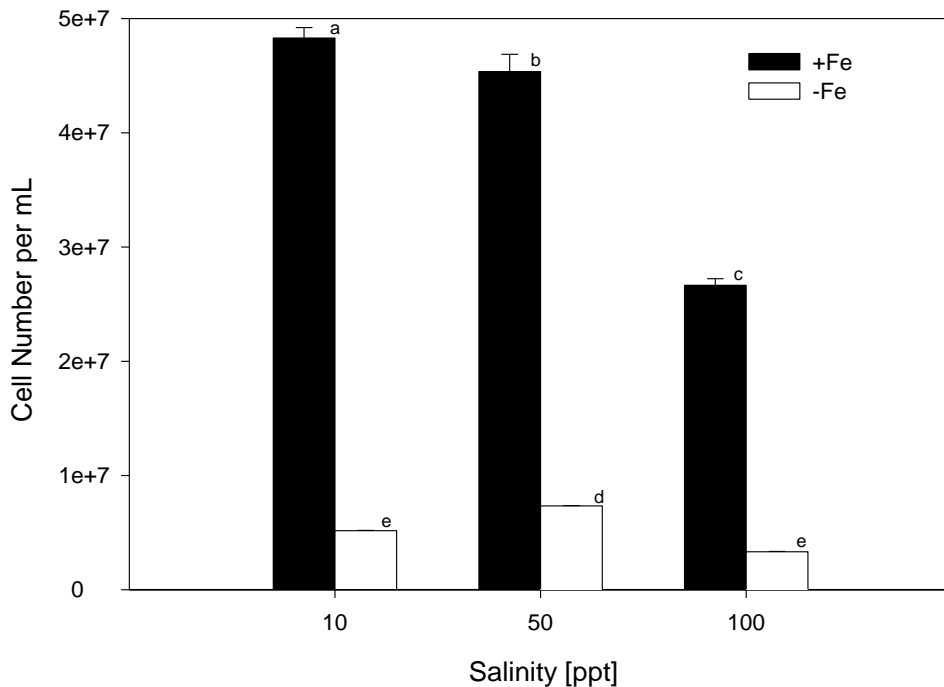


Figure 2. Cell density of *Picochlorum* sp. at day 10 in low iron (-Fe) when compared with the on added iron (+Fe) at different salinities. Mean values \pm Standard deviation, n = 3 for all treatments. Different letters indicate significance difference between means.

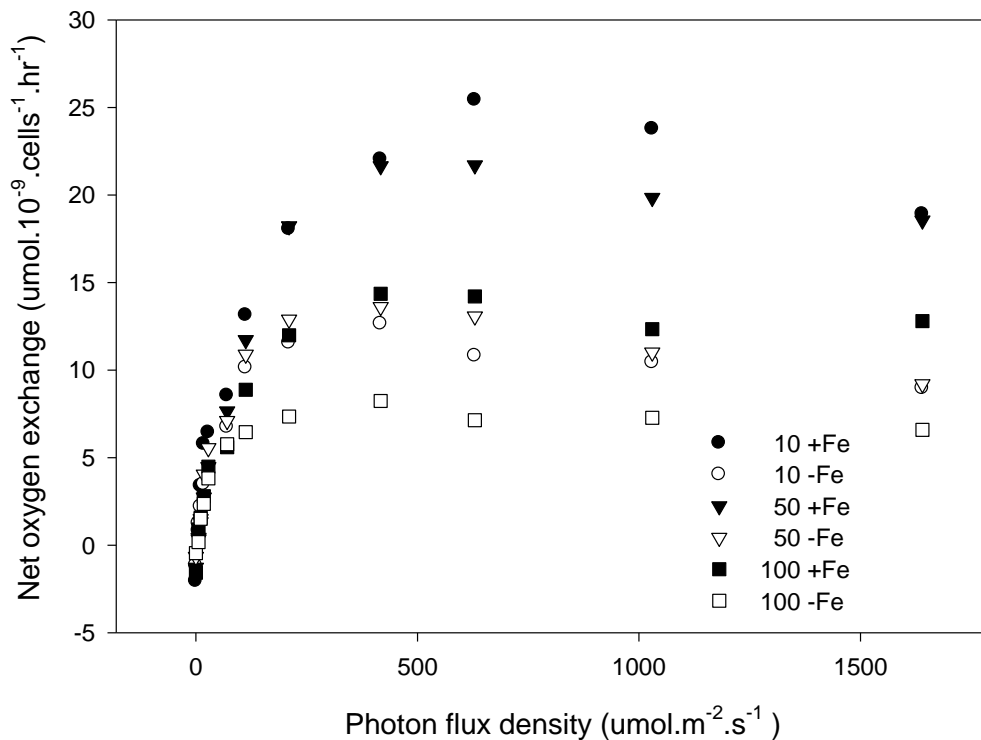


Figure 3. Photosynthetic light-response curves of *Picochlorum* sp. cultured in added iron medium (+Fe, shaded symbols) and in no added iron (-Fe, open symbols) at different salinities (indicated by the numbers next to symbols). Mean values, n = 3 for all treatments (Error bars not shown for clarity).

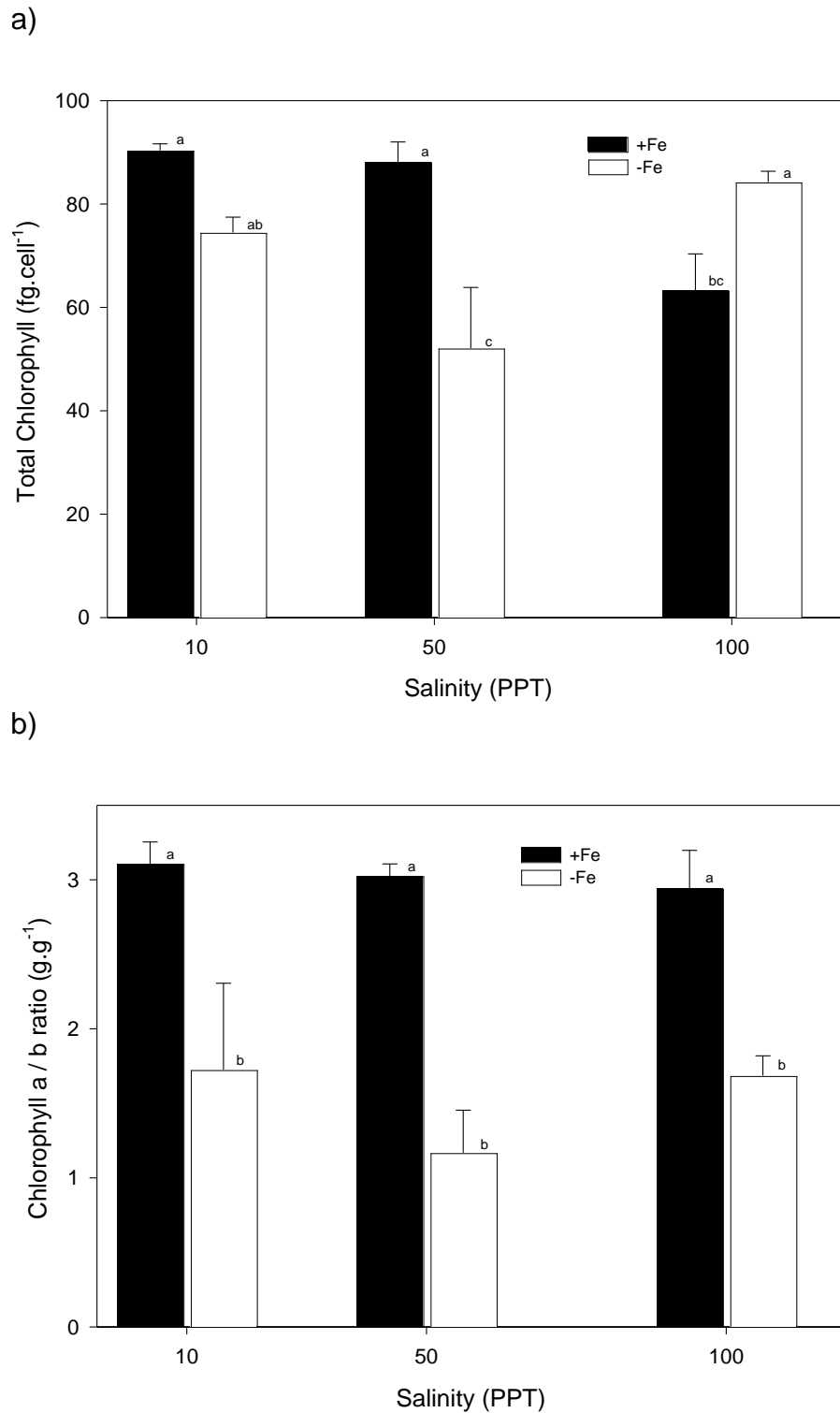


Figure 4. Average total chlorophyll (a) and chlorophyll a/b ratio (b) of *Picochlorum* sp. grown in added iron (+Fe) and no added iron (-Fe) and at different salinities. Mean values \pm Standard deviation, n = 3 for all treatments. Different letters indicate significant differences.

show different patterns under high and low iron. Even though P_{max} and growth rates were different at 10 and

100 ppt in -Fe media, the cell densities at day 10 were the same. This may be due to the fact that the physiology

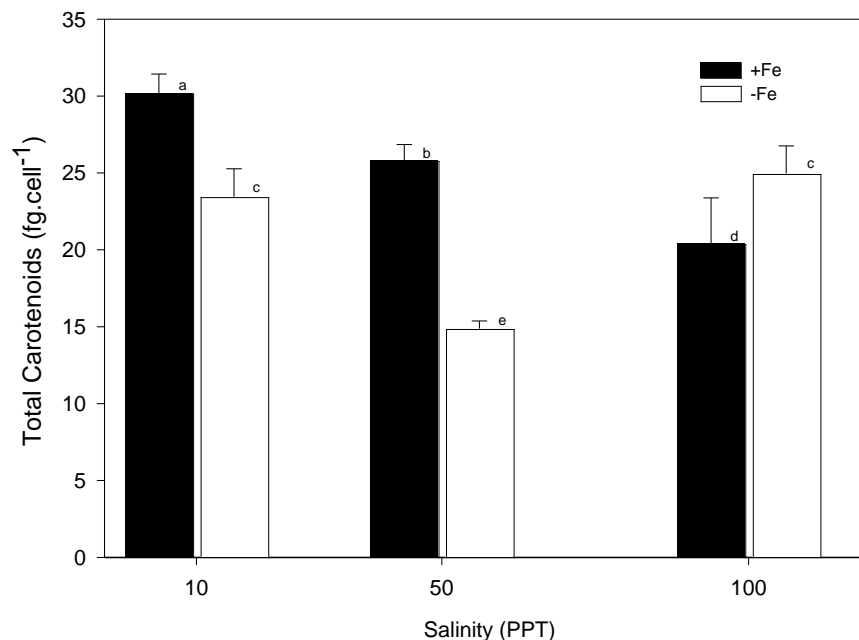


Figure 5. Average total carotenoids of *Picochlorum* sp. grown in added iron (+Fe) and no added iron (-Fe) and at different salinities. Mean values \pm Standard deviation, $n = 3$ for all treatments. Different letters indicate significant differences between means.

may have changed with time in the batch cultures considering declining nutrient availability. Iron stress in *Picochlorum* may affect the acquisition of other nutrients such as nitrate, as well as the assimilation of nitrogen in the chloroplast and therefore ultimately affecting growth and yield. Rueter and Ades (1987) reported decreased uptake of nitrate in the chlorophyte, *Scenedesmus quadricauda*, grown in iron-limited medium as compared to those in iron sufficient cultured cells. Further experiments may be performed to study the uptake of nitrate by *Picochlorum* in low iron medium.

The reduction in P_{max} in -Fe medium suggests that the photosynthetic capacity was affected with respect to electron transport and/or carbon fixation. Iron is an essential cofactor of some of the compounds found in the electron transport chain such as ferredoxin, cytochromes and the iron-sulphur proteins. Iron deficiency is likely to affect the amount of electron carriers available in the electron transport chain and the function they perform. If inadequate ATP and reductants are produced in the light reactions of photosynthesis, then assimilation of carbon in the Calvin cycle would be affected negatively.

Low iron reduced P_{max} in *Picochlorum* by about 45% at all salinities, but decreased growth rate by about 70% at lower salinities and 80% at 100 ppt, and decreased cell densities by more than 84% at all salinities. This indicates that photosynthesis may not be the only factor contributing to the reduced growth in *Picochlorum*. Iron is required for the functioning of nitrite reductase and nitrate reductase and therefore when iron is limited nitrogen, assimilation is affected as well as protein synthesis. Cell

division may be slowed down as a result of non availability of nitrogen for the formation of proteins and nucleic acids.

As evidenced in the fluorescence results (Figure 6), low iron massively decreased the maximum quantum yield of PS II photochemistry in dark-adapted state cells (F_v/F_m), which indicates that there is photodamage to some portions of PS II. The LHCII may have been decoupled from PSII due to iron deficiency (Morales et al., 2001) and may have contributed to the decrease. F_v/F_m was affected differently by salinity in *Picochlorum* grown in +Fe and -Fe media. Whereas there was no effect of salinity in +Fe medium, F_v/F_m increased at 100 ppt in -Fe medium, an indication of a salinity-nutrient interaction. A likely explanation is that high salinity reduces iron stress by reducing demand (lower growth rate at high salinity). Another interpretation for this decrease is that *Picochlorum* is adapted to coping with higher salinity at iron deficient conditions (Henley et al., 2002). This is consistent with the result that within the -Fe treatments, increasing salinity from 10 to 100 ppt significantly decreased non-photochemical quenching (NPQ) although this was not expected and was not seen in all the parameters measured.

Effective quantum yield of PS II electron transport in light-adapted state (Φ_{PSII}) and photochemical quenching (qP), which is an indication of actual open reaction centers as well as indicating the redox state of plastoquinone (Q_A), were reduced in *Picochlorum* by low iron as expected. The fewer open PSII reaction centers as indicated by qP, may be due to a reduced rate of

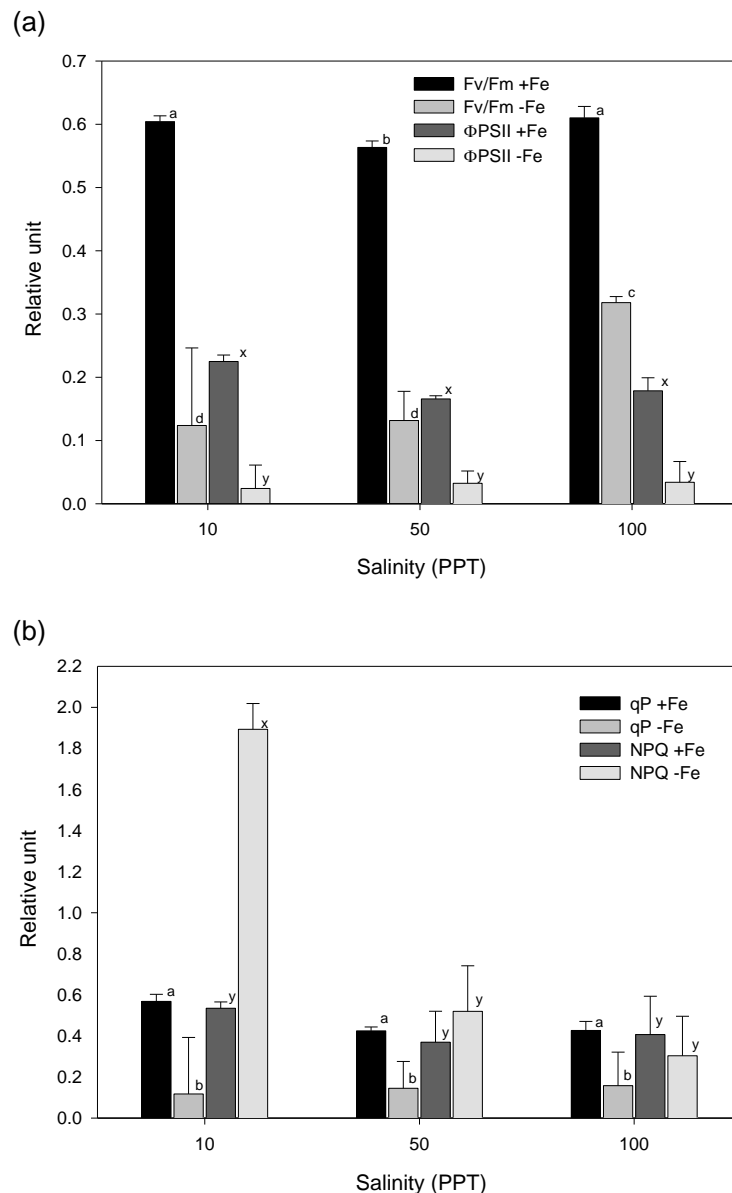


Figure 6. Changes in fluorescence parameters in *Picochlorum* sp. due to growth in no added iron (-Fe) when compared with added iron (+Fe) at different salinities. F_v/F_m is PSII maximum quantum efficiency, Φ_{PSII} is Quantum yield electron transport of PSII, qP, photochemical quenching and NPQ, non-photochemical quenching. Mean values \pm standard deviation, $n = 3$ for all treatments. Different letters indicate significant differences between means.

replacement of damaged D1 proteins as compared to the rate at which they were being destroyed (Allakhverdiev et al., 2002; Mohanty et al., 2007). As a result of iron deficiency, a reduction in electron carriers to accept and transfer electrons may cause excess excitation pressure with a concomitant decrease in qP. A reduction in the F_v/F_m may also indicate enhanced photoprotection to dissipate excess excitation pressure, for example through the xanthophyll cycle (Demmig-Adams and Adams, 1992; Gilmore, 1997; Lu et al., 2003). This is supported by the

significant increase in non-photochemical quenching caused by low iron at 10 ppt which was not evident in +Fe medium. Interestingly, this elevated NPQ was not evident at 50 and 100 ppt. The F_v/F_m data indicate that there was more photodamage to portions in PSII at 10 ppt than at 100 ppt. This may explain the higher NPQ values at 10 ppt as compared to 100 ppt under -Fe medium.

As expected, total chlorophyll and carotenoids per cell were decreased by low iron at 10 and 50 ppt, as iron is

required for the synthesis of chlorophyll in algae and plants. However, at 100 ppt, low iron increased total chlorophyll and total carotenoids per cell. This is unexpected considering that iron is required in the biosynthesis of chlorophyll and carotenoids. A deficiency in iron should result in a decrease in the total chlorophyll and carotenoids concentrations in the cells. It is possible that under -Fe at higher salinity, the low growth rate slows the "dilution" of chlorophyll even in the absence of chlorophyll synthesis. Although total chlorophyll per cell was not affected by salinity at 10 and 50 ppt in +Fe medium, chlorophyll content decreased at 50 ppt in -Fe treatment (Figure 4a), indicating salinity-Fe interaction. The mean cell density at day 6 for -Fe treatment was 4.4×10^6 at 50 ppt as compared to 3.5×10^6 and 1.9×10^6 for 10 and 100 ppt, respectively. The higher number of cells at 50 ppt may explain the decrease in the total chlorophyll per cell in the -Fe grown cells.

Salinity did not affect chlorophyll *a/b* ratio in +Fe or -Fe grown cells, but -Fe massively decreased chlorophyll *a/b* ratio (Figure 4b). The ratios of about 1.2 to 1.7 in -Fe are quite. It is possible that this may be due to an error of the sensitivity of the spectrophotometer used because of the relatively low absorption values of -Fe cultures. However, a possible explanation for the very low chlorophyll *a/b* ratios may in part be attributed to the decreases in chlorophyll *a* per cell caused by -Fe which indicates selective loss of reaction centers. The decrease may also be due to the increases observed in chlorophyll *b* per cell at all salinities in -Fe grown medium which suggests -Fe cultured cells had a larger light-harvesting antenna for absorbing at a high rate, though it appears they were not able to effectively convert the absorbed light into chemical energy as indicated by decreases in F_v/F_m , Φ_{PSII} and qP . The decoupling of the LHCII from PSII may account for this (Morales et al., 2001) inability to transfer the absorbed energy. There has been a report on an increase in the functional antenna size of PS II in the green alga *Chlamydomonas reinhardtii* grown in iron deficient medium Naumann et al. (2007). There was an earlier report on the formation of iron deficiency-induced protein A (*idiA*) and iron stress-induced protein A (*idiA*) in some cyanobacteria which tend to surround the photosystems under iron limitation (Burnap et al., 1993; Boekema et al., 2001).

The relatively high carotenoids at 100 ppt salinity in -Fe medium may be of importance to *Picochlorum* in protecting itself against the detrimental effects of high light. Pigments such as β -carotene help in the quenching of reactive species and violaxanthin, antheraxanthin and zeaxanthin are involved in the xanthophyll cycle in dissipating excess heat (Demmig-Adams and Adams, 1992; Gilmore, 1997; Lu et al., 2003).

A further study measuring the amount of these pigments may indicate their involvement or otherwise, because only crudely, the total carotenoids but not the individual pigments was measured. Also, the relatively

high total carotenoids at higher salinity may not be an increase in itself considering that in -Fe media the total carotenoids are the same at 10 and 100 ppt (Figure 5), but rather a lack of decrease as a result of the low growth rate as explained earlier for chlorophyll. The mean cell density at day 6 was 4.4×10^6 at 50 ppt as compared to 3.5×10^6 and 1.9×10^6 for 10 and 100 ppt, respectively, and therefore having more cells explains the decrease in the total carotenoids per cell in -Fe grown cells (Figure 1). In order to analyze the explanation for the relative increase, further studies may be performed by measuring the carotenoids and chlorophyll contents daily through the growth curve. If total pigments in the culture do not change, then the amount per cell must decrease with successive cell divisions.

In summary, some of the results in this study supported the hypothesis that response to the effect of salinity stress on growth and photosynthesis will be different under high iron and under no added iron salinity. The response pattern was different in initial growth rate, cell density at day 10, total chlorophyll, total carotenoids, F_v/F_m and NPQ. The different response in pigments and F_v/F_m seems to be due mainly to differences at 100 ppt, which may be explained by the extremely low growth rate (slow depletion of Fe) at 100 ppt which may be a survival mechanism. Since *Picochlorum* occurs in hypersaline environment characterized by fluctuations in salinity and possibly nutrients, it shows that at higher salinities and also during deficient nutrient period, it tends to exhibit low growth rates till more favourable conditions. This way it is able to survive and reproduce to continue its existence.

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