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Full Length Research Article

Effect of Salinity on the Growth Parameters of Halotolerant Microalgae, *Dunaliella spp.*

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ABSTRACT: *Dunaliella* strains (T35, T36 and T37) were isolated from saline river of Namibia. The effects of salinity stress on their growth and pigment content was investigated. The specific growth rate (µ) of *Dunaliella* was established in a controlled environment. NaCl concentration has a strong effect on the growth rate and generation time of these halotolerant microalgae. *Dunaliella* strains were shown to withstand large variations of salinity of the growth medium, 0.5 - 4.0 M NaCl. The optimum conditions for their growth were 1.0 M NaCl at 25 °C, pH 7.5 and 45 µmol m^{-2} s^{-1}. Increasing salinity from 1.0 to 4.0 M NaCl decreased cell density by 40 %. Increased cellular chlorophyll content was observed form 2 to 14 days of inoculation for all the three strains investigated. However, after day 10 of inoculation there was no significant difference in the pigment content per cell in all strains ($p \leq 0.05$). This study shows that there is a good relationship between growth rate and efficient photosynthetic apparatus during the cultivation.

Keywords: *Dunaliella,* salinity stress, chlorophyll, specific growth rate

INTRODUCTION

Halophytic microalgae, *Dunaliella* species, are ubiquitous microorganisms in hypersaline environments; they are able to survive in saline media ranging from 0.5 M NaCl to saturated salt solutions of around 5.0 M NaCl (Ben-Amotz and Avron, 1983; Ben-Amotz and Avron, 1990). The microalgal growth responses involve complex interactions to a number of variables such as temperature, pH, salinity and nitrogen concentration.

Carotene and chlorophyll production by mass cultivation of *Dunaliella* is one of the foremost successes in applied algal biotechnology as reported by Ben-Amotz and Avron, (1989). In order to promote mass production and reduce carotene production costs, it is essential to identify the set of conditions required for the cultivation of halotolerant microalgae.

The wide distribution of species of the genera *Dunaliella* may be attributed to their tolerance to a wide range of salinities and other environmental parameter such as temperature, light and pH. These factors influence the rate of growth of numerous halotolerant microalgae species (Borowitzka and Borowitzka, 1988). This study was aim to establish a laboratory cultivation system in which both species of *Dunaliella* obtained from Namibia could be grown at a high specific growth rate. This study presents results of an investigation on the effect of salinity on growth and chlorophyll content of *Dunaliella* (T35, T36 and T37).

MATERIALS AND METHODS

Algal strain and cell cultivation

Dunaliella strains were isolated and identified by the Marine Biological Association (MBA) United Kingdom. Samples were obtained from Swakopmund in the Erongo region of Namibia, Africa. In the west, Erongo has a shoreline on the Atlantic Ocean. On land, it borders the following regions: Kunene to the north, Otjozondjupa to the east, Khomas to the southeast and Hardap to the south. Dunaliella strains were maintained in a temperature controlled growth chamber at 25±2 °C. Illumination was provided under a 12/12 h light/dark cycle by cool white fluorescent lamps with a light intensity of 45 µmol m^{-2} s^{-1}. *Dunaliella* strains (T35, T36 and T37) were cultured in modified Johnson's medium (Johnson *et al.* 1968, Borowitzka 1988) containing the following

components (per litre): 1.5 g $MgCl_2 \cdot 6H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.2 g $CaCl_2 \cdot 2H_2O$, 1.0 g KNO_3, 0.043 g $NaHCO_3$, 0.035 g KH_2PO_4, 1.89 mg Na_2EDTA, 2.44 mg $FeC1_3 \cdot 6H_2O$, 0.41 mg $ZnCl_2$, 0.61 mg H_3BO_3, 0.015 mg $CoCl_2 \cdot 6H_2O$, 0.041 mg $CuC1_2 \cdot 2H_2O$, 0.41 mg $MnCl_2 \cdot 4H_2O$, and 0.38 mg $(NH4)_6Mo_7O_{24} \cdot 4H_2O$, with NaCl added to 2.0 M. Hydrochloric acid (HCl) was used to adjust the medium pH to 7.5. The medium was sterilized by autoclaving at 120 °C for 15 min before cultivation, with phosphate components being autoclaved separately. Stock cultures of *Dunaliella salina* were inoculated into fresh medium with a dilution ratio of 1/10 (v/v) and cultures were carried out in 250 ml flasks containing 100 ml medium.

Effect of salinity on growth parameters

Halotolerant microalgae *Dunaliella* strains (T35, T36 and T37) were maintained in the cultivation media containing 1.0 M NaCl as described above. A 1.0 ml (x10^6 cells) microalgal samples were taken at the exponential growth phase from the maintenance culture and added to Johnson's medium with different amounts of NaCl (0.5, 1.0, 2.0, 3.0 and 4.0 M NaCl).

Monitoring of microalgae cell culture growth

The culture flasks were agitated by hand and the cell numbers were determined by sampling each unit every 48 h by taking an aliquot of 400 – 500 µl from each culture flask. The aliquot samples were fixed with 2.0 % formaldehyde and counted using a 0.2 mm deep Neubauer haemocytometer, with a light microscope. The mean cell number were calculated and expressed as the number of cells per ml using equation 1.0.

$$\text{Number of cells/ml} = \frac{\text{Total cells}}{\text{Squares counted}} \ \text{X} \ \text{DF} \ \text{X} \ 10^4 \dashrightarrow \textbf{\textit{Eq}. 1.0}$$

Where DF = dilution factor and 104 = is the volume conversion factor for the haemocytometer

Determination of specific growth rate (µ)

Experiments were conducted using triplicate cultures. The cell density measurements started at day 4, to allow adjustment to ionic strength of the medium. The measurements were continued every 2 days for 28 days. Determination of the doubling time (i.e. the time taken for the number of cells in a population to double) and the specific growth rate (increase in cell mass per unit time) of the microalgae under the influence of varying salinity was calculated using the following equations (Eq. 2.0 and 3.0).

Harvesting microalgae

Dunaliella cells were routinely harvested by centrifugation at 3,000g for 8 min at 25 °C.

Determination of chlorophyll content

The chlorophyll content of *Dunaliella* cells was determined according to the procedure described by Ben-Amotz and Avron (1983). 10 ml of microalgae culture was centrifuged at 3000 g for 10 min; the supernatant was discarded and the pellet was resuspended in 100 % acetone

$$\text{Specific growth rate } (\mu) = \frac{\ln N_{t2} - \ln N_{t1}}{(t_2 - t_1)} \dashrightarrow \textit{Eq}.\ 2.0$$

$$\text{Doubling time (G)} = \frac{(t_2 - t_1)\log 2}{\log N_t - \log N_o} \dashrightarrow \textit{Eq}.\ 3.0$$

Where (t2 – t1) = time interval in days, No = number of cells at a starting point, Nt = the number of cells after the period of time.

The acetone served to extract the chlorophyll from the pellet during a 1 hr incubation period (in the dark). Thereafter, the samples were subjected to vortexing for a few minutes followed by centrifugation at 3000 g for 10 minutes. The supernatant was diluted to 80 % by the addition of distilled water to a final volume of 10 ml. Chlorophyll *a* and *b* was assayed on a Jenway 67 Series UV/Visible Spectrophotometer at 663 and 645 nm against acetone (blank). Concentration of chlorophyll *a* and *b* and total chlorophyll were calculated by equation 4.0, 5.0 and 6.0 respectively (Porra *et al*., 1989).

$$\text{Chl } a = 12.25 \text{ x } A_{663.6} \text{ - } 2.55 \text{ x } A_{646.6} \quad \text{-----} \rightarrow \quad \textbf{\textit{Eq}}. \ 4.0$$

$$\text{Chl } b = 20.31 \text{ x } A_{646.6} \text{ - } 4.91 \text{ x A}_{663.6} \quad \text{-----} \rightarrow \quad \textbf{\textit{Eq}}. \ 5.0$$

$$\text{Total Chl} = [\text{Chl } a + \text{Chl } b] = 17.76 \text{ x } A_{646.6} + 7.34 \text{ x } A_{663.6} \quad \text{-----} \rightarrow \quad \textbf{\textit{Eq}}. \ 6.0$$

Data analysis

Analysis of variance (ANOVA) was performed to test the differences between the means of growth rates at various NaCl concentrations. When the ANOVA results showed that the treatments were significant ($p < 0.05$), Tukey's multiple comparison tests was used to compare the value of the mean of each treatment.

RESULTS AND DISCUSSION

Effect of salinity on cell number growth, specific growth rate and doubling time

This study reported the effect of salinity on growth and chlorophyll content of *Dunaliella* (T35, T36 and T37). After inoculation of microalgae cells in modified Johnson's medium, the cultures undergo a lag phase. This is the time represented for adaptation of microalgae to the new environment; the cultures were clear and transparent (Figure 1.0). For *Dunaliella* the lag phase lasted approximately 5 days after inoculation. On approximately day 6-7, there was a visibly detectable increase in cell density (Figure 1.0). Development of green colour in the cultures indicated that the microalgal cells were in the exponential growth phase (increase in cell number, at a constantly growing rate). By day 25 the cultures were opaque and dark green in colour (Figure 1.0).

Under all conditions of NaCl concentrations there was an apparent higher rate of growth from day 3 to 5 compared to the later, exponential phase (Figure 2.0). The maximum cell density was observed at day 27 of the experimental period, which may indicate that under the specified conditions the amount of nutrients in the growth medium was able to support the cultures for 27 days. After 27 days of experiment the cells density declined at 4.0 M NaCl medium (Figure 2.0). The cell may not withstand constant exposure to high salinity, which may relate to drain of ATP in the starch to glycerol conversion (Kaplan *et al*., 1980). Previous research has shown that *D. viridis* grows optimally in 1.0 – 1.5 M NaCl and tolerates up to 4.0 M NaCl whereas *D. salina* grows best in 2.0 M NaCl and tolerates up to 6.0 M NaCl (Borowitzka *et al*., 1977). However *D. viridis* has been reported to grow optimally at 5.8 M NaCl and temperature of 30 °C (Jimenez and Niell, 1991). A comparison of the cell densities of *Dunaliella* strains (T35, T36 and T37) shows a decrease of the cell number with increasing salinity above 1.0 M NaCl and in salinity below 1.0 M NaCl. Furthermore, the results indicated that the growth performance for the three *Dunaliella* strains was better at 1.0 M NaCl.

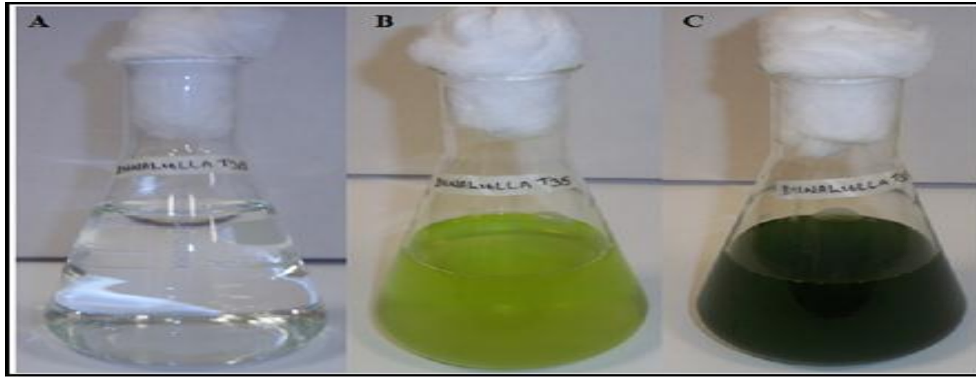

Figure 1.0 *Dunaliella* T35 cultures grown at 1.0 M NaCl concentration at 25 °C, pH 7.5, 45 µmol m-2 s-1 with 12 h light: 12 h dark photoperiod in a modified Johnson's medium; (A) Cultures after 24 hours of inoculation, (B) cultures on day 7 of inoculation and (C) cultures on day 25 of inoculation.

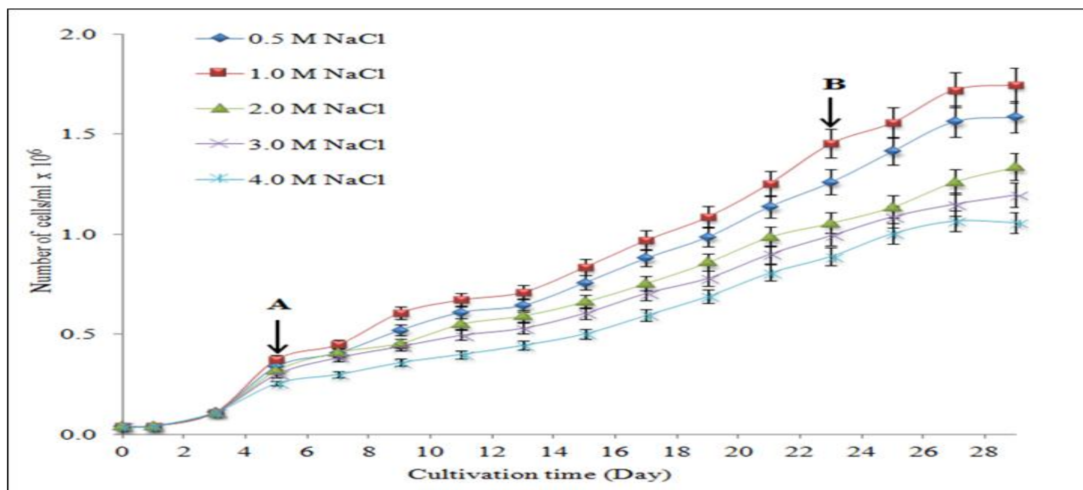

Figure 2.0 Effect of increasing NaCl (0.5 to 4.0 M) on the growth of *Dunaliella* T35 at 23°C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium. A to B are the data points used in estimating specific growth rate. Error bars represent the standard deviations (n≥3) of measured values of cell density

The specific growth rate and doubling time for each strain (Figure 3.0 and 4.0) were obtained by plotting the natural log of cell number against cultivation time for the period from day 5 to day 23. In culture medium with 1.0 M NaCl, the specific growth rate for all strains of *Dunaliella* was more rapid (0.119-0.123 Div.d^{-1}) than at any other salt concentrations investigated (Figure 4.0). Notably, *Dunaliella* T36 had a faster doubling time than either T35 or T37. It is considered the best strain in terms of rapid increase in cell number. *Dunaliella* have shown ability to grow in saturated brine (similar to natural conditions) (Johnson *et al*., 1968), but optimum growth always occurs at lower salinity (0.5 to 2.0 M NaCl). In this regard, optimal growth rates at 1.0 to 2.0 M NaCl by Namibian isolates (*Dunaliella*, T35, T36 and T37) are therefore typical of the species. Increasing NaCl

concentration of the growth medium from 1.0 to 3.0 and 4.0 M inhibited growth, but growth increased from 0.5 to 1.0 M which points to their obligate halotolerant nature.

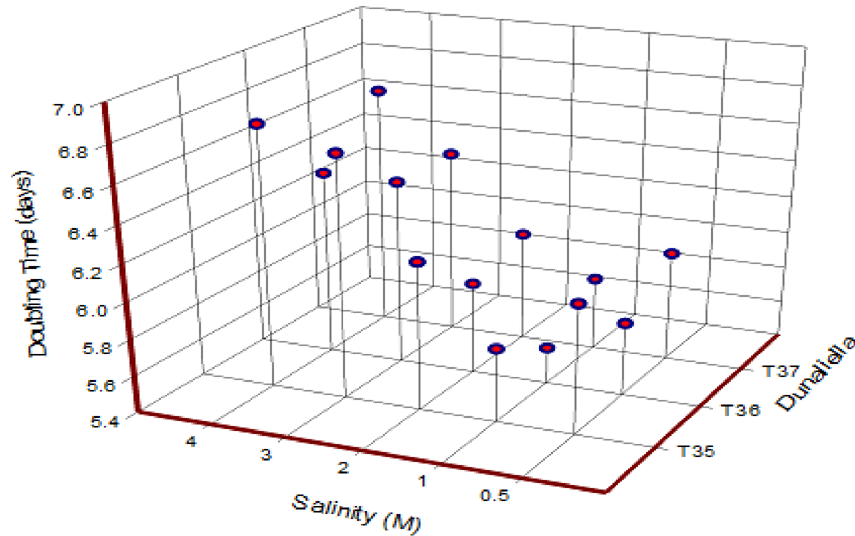

Figure 3.0: Doubling time of *Dunaliella* T35, T36 and T37 grown in a range of NaCl concentrations at 25 °C, pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium.

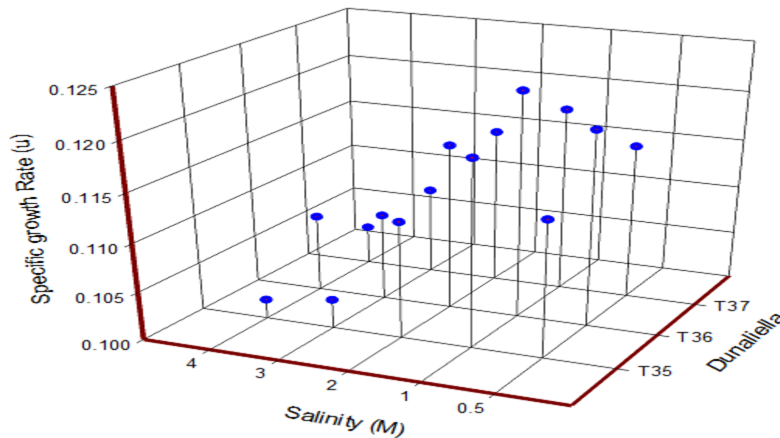

Figure 4.0: Specific growth rate of *Dunaliella* T35, T36 and T37 grown in a range of NaCl concentrations at 25 °C, pH 7.5 and 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in a modified Johnson's medium.

Chlorophyll composition and dry weight of *Dunaliella*

It is worth pointing out that the *Dunaliella* strains have chlorophyll *a* and *b* (Masuda *et al*., 2002); therefore, the extraction yields presented in this study represent the sum of both.

Chlorophyll content was used for estimating biomass of microalgae in culture and can be used to measure growth as well as productivity. The chlorophyll content per ml of culture for 1.0 M NaCl reached a plateau after 10 days of inoculation for *Dunaliella* strains but continued to increase in 4.0 M NaCl (Figure 5.0). However,

since the content of chlorophyll per cell at the end of 14 days was the same in both 1.0 M NaCl and 4.0 M NaCl for each strain investigated, the results show that lower chlorophyll levels per volume of culture in 4.0 M NaCl negatively affected by low cell number growth compared to 1.0 M NaCl. This study also shows that *Dunaliella* strain T36 had a significantly lower content of chlorophyll (6.4 ± 0.02 pg/cell) than either T35 or T37 (Figure 5.0). According to Ramaraj *et al*. (2013) the conventional method of chlorophyll measurement might not be a good index for biomass estimation: chlorophyll has been reported to be the primary target of salt toxicity and results in reduced photosynthesis and reduced growth (Rai 1990; Rai and Abraham 1993). The lower chlorophyll content of T36 and T33b may reflect a greater susceptibility to salt toxicity. According to Moradi and Ismail (2007) the lower amount of chlorophyll per ml at higher salinities is due to decrease in photosynthetic rate because of salt osmotic and toxic ionic stress. Therefore chlorophyll determination can be used to estimate photosynthetic production in cells.

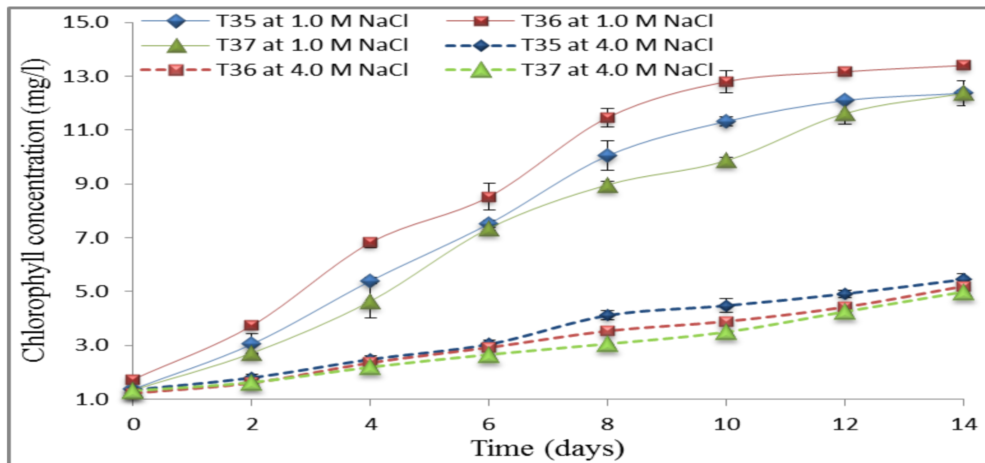

Figure 5.0 Chlorophyll content of *Dunaliella* T35, T36 and T37 cultivated at 25 °C, pH 7.5, 45 µmol m^{-2} s^{-1} with 12 h light: 12 h dark photoperiod in 1.0 and 4.0 M NaCl medium. Error bars represent the standard deviations (n=3) of the measured chlorophyll

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CONCLUSION

This study determined the optimum salinity for the growth of *Dunaliella* strains isolated from Namibia. These strains can withstand large variations in the external salinity of the growth medium from 0.5 to 4.0 M NaCl concentration. By investigating the cell growth kinetics of microalgae using cells density determination, it was demonstrated that the rate of cell growth (increase in cell number) was maximum at 1.0 M NaCl concentration. The highest mean specific growth rate was 0.168 division day^{-1}. This research highlights the remarkable diversity of halotolerant microalgal strains that exists even within the same physical location. The collection of *Dunaliella* strains, T35, T36 and T37 isolated from the saline river of Namibia differed in response to different NaCl concentration. Chlorophyll can be used to assess biomass of *Dunaliella*, measure growth as well as productivity. This study indicates that the cellular chlorophyll yield increased proportionally to the growth rate of *Dunaliella* strains.

REFERENCES

Ben-Amotz, A. and Avron, M. (1983) 'Accumulation of metabolites by halo-tolerant algae and its industrial potential', *Annual Review of Microbiology* **37**: 95-119

Ben-Amotz, A. and Avron, M. (1989) 'The Biotechnology of Mass Culturing *Dunaliella* for Products of Commercial Interest', In Algal and Cyanobacterial Biotechnology, RC Cresswell, T A V Rees and N Shah (eds.), Longmans, Harlow

Ben-Amotz, A. and Avron, M. (1990) 'The biotechnology of cultivating of the halotolerant alga *Dunaliella*', *Trends in Biotechnology* **8**: 121-126

Borowitzka, L. J, Kessly, O. S, and Brown, A. D. (1977) 'The Salt Relations of *Dunaliella*', *Archives of Microbiology* **113**: 131-l38

Borowitzka, M. A. (1988) 'Algal growth media and sources of cultures', In: Borowitzka M.A. and Borowitzka L.J. (eds.), Micro-algal Biotechnology. Cambridge University Press: Cambridge. pp. 456-465

Borowitzka, M. A. and Borowitzka, L. J. (1988) '*Dunaliella*. Microalgal biotechnology', page 27-58, *Cambridge University Press*. Cambridge, UK

Jimenez, C. and Niell, F. X. (1991) 'Growth of *Dunaliella viridis* Teodoresco: effect of salinity, temperature and nitrogen concentration', *Journal of Applied Phycology* **3**: 319-327

Johnson, M. K., Johnson, E. J., Mac Elroy, R. D., Speer, H. L. and Bruff, B. S. (1968) 'Effects of salts on the halophilic alga *Dunaliella* viridis', *Journal of Bacteriology* **95**: 1461-1468

Kaplan, A., Schreiber, U. and Avron, M. (1980) 'Salt-Induced Metabolic Changes in *Dunaliella salina*', *Plant Physiology* **65**: 810-813

Masuda, T., Polle, J. E. W. and Melis, A. (2002) 'Biosynthesis and Distribution of Chlorophyll among the Photosystems during Recovery of the Green Alga *Dunaliella* salina from Irradiance Stress', *Plant Physiology* **128**: 603-614

Moradi, M. and Ismail, A. M. (2007) 'Responses of Photosynthesis, chlorophyll fluorescence and ROS - Scavenging systems to salt stress. During seedling and reproductive stages of Rice', *Annals of Botany* **99**: 1161-1173

Porra, R. J., Thompson, W. A. and Kriedemann, P. E. (1989) 'Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy', *Biochimica et Biophysica Acta*, **975**: 384-394

Rai, A. K. (1990) 'Biochemical characteristics of photosynthetic response to various external salinities in halotolerant and fresh-water cyanobacteria. *FEMS Microbiology Letters* 69, 177-180

Rai, A. K. and Abraham, G. (1993) 'Salinity tolerance and growth analysis of the cyanobacterium *Anabaena doliolum*', *Bulletin of Environmental contamination and Toxicology* **51**: 724-731

Ramaraj, R., Tsai, D. D.W, and Chen, P. H. (2013) 'Chlorophyll is not Accurate Measurement for Algal Biomass', *Chiang Mai Journal of Science* **40**: 547-555