http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (14): 2286-2291, 2007
ISSN 1028-8880

Chlorophylls, Proteins and Fatty Acids Amounts of Arthrospira Platensis Growing under Saline Conditions

[1]Samah Ayachi, [2]Amor El Abed, [1]Wissal Dhifi and [1]Brahim Marzouk
[1]Unité des Plantes Aromatiques et Médicinales, Centre de Biotechnologie,
Technopole de Borj-Cedria, BP 901, 2050 Hammam-Lif, Tunisie
[2]Institut National des Sciences et Technologies de la Mer, 28,
rue 2 mars 1934, 2025 Salambo, Tunisie

Abstract: *Spirulina platensis* (*Arthrospira platensis*) is a Tunisian strain isolated for the first time, in Tunisia, in Oued Essed (Sidi Bou Ali, in Sousse region). Evolution of biomass, proteins, chlorophylls and fatty acids (FA) has been followed during *Spirulina* growth. Experiments were carried out by varying sodium chloride concentrations in the culture medium in a range from 1 g L^{-1} (natural environment) to 60 g L^{-1}. Results analysis showed an increase in chlorophyll amounts at 15 g L^{-1} NaCl in 10 days old cultures but a decrease at high NaCl concentrations. Optimal proteins amounts was observed at 15 g L^{-1} NaCl in young cultures (5 and 10 days). FA composition was modified by NaCl and depended on culture age. Cultures exposed to high salinity concentrations showed not only a decrease in growth rate but also a loss in total fatty acids TFA quantities. Samples cultured over 15 days at 30 g L^{-1} NaCl rendered optimal quantities of lipids and γ-linolenic acid.

Key words: *Spirulina*, salinity, chlorophylls, proteins, fatty acids, γ-linolenic acid

INTRODUCTION

Spirulina platensis, renamed as *Arthrospira platensis*, is a planctonic blue green alga, dominating the flora of alkaline saline waters with pH of up to 11. It is widespread and the main species can exist in various types of habitats, namely ground, brackish waters, fresh waters, sea waters and waters of industrial and domestic use (Padhi *et al*., 1992; Sankaran and Rdharukman, 1992; Santra, 1992; Laliberte *et al*., 1997). In Tunisia, a stump of *Spirulina platensis* was identified in the waters of Oued Essed (Sidi Bou Ali, Sousse) that was purified and cultivated at INSTM Monastir pilot centre, in basins or in erlens.

Spirulina species have been widely used as a source of natural products for human or animal food. Furthermore, they contain high levels of pigments such as carotenoids and chlorophylls. *Spirulina* is also rich in proteins (60 to 70% of dry matter weight), vitamins and unsaturated fatty acids. The latter account for 6 to 7% of dry matter weight.

Compared to other sources, this Cyanobacterium is rich in gamma linolenic acid (18:3 ω 6 or GLA), a precursor of long chain FA biosynthesis and prostaglandins. GLA has been used in several medical applications such as the treatment of schizophrenia, multiple sclerosis, dermatitis, pre-menstrual syndrome, atopic eczema, diabetes and rheumatoid arthritis (Kennedy *et al*., 1993; Nakahara *et al*., 1992). It is mainly concentrated in Galactolipids (GL). Moreover, the GLA content accounts for 25% of total fatty acids, which is much higher than in evening primrose seed (7%) and in morterela (8%) (Eichi *et al*., 1992).

Physiological studies showed when natural sun light is high in outdoor *Spirulina* production, salinity stress is usually accompanied by photosynthesis inhibition (Vonshak and Guy, 1992). *Spirulina platensis* adapted to salinity by increasing sugars metabolism in cells (Warr *et al*., 1985; Vonshak *et al*., 1988; Martel *et al*., 1992). As this alga constituted one of the best sources of polyunsaturated fatty acids and mainly GLA, it was necessary to optimize its culture parameters in order to improve its FA production.

The aim of this study was to determine the optimal sodium chloride concentration leading to the highest production of TFA, GLA and proteins by a Tunisian strain of *Spirulina platensis* under laboratory controlled conditions (temperature, light).

MATERIALS AND METHODS

Organism and growth conditions: *Spirulina platensis* used in this work was originated from Oued Essed (Sidi Bou Ali, Sousse, Tunisia) (Medhioub *et al*., 1999).

Corresponding Author: Brahim Marzouk, Unité des Plantes Aromatiques et Médicinales, Centre de Biotechnologie, Technopole de Borj-Cedria, BP 901, 2050 Hammam-Lif, Tunisie
Tél: 216 71 430 855 Fax: 216 71 430 330 or 216 71 410 740

This study was conducted during 2004 at INSTM Monastir where *Spirulina* was cultured and at CBBC (Centre de Biotechnologie de Borj Cedria) for all experiments and analysis.

Algae were grown on autoclave-sterilized Zarrouk's (1966) medium, in the laboratory, at 30°C and kept in suspension by bubbling air. Illumination was provided by neon tubes with light intensity of 1300 Lux. Growth rate was estimated by dry weight measurement after filtration. Exponentially growing cells were inoculated in the previous medium using different NaCl concentrations.

To determine NaCl effect, a set of 9 Erlenmeyer flasks, each 3 L was used. Erlenmeyers were put into an air-conditioned room at 22°C and kept at 36°C with the help of a thermostat. Three series of varied NaCl concentrations cultures were prepared (15, 30 and 60 g L^{-1}). The effect of salinity on the evolution of FA composition was followed over 15 days of culture, every five days, starting from the day of inoculation; temperature, pH, dry matter weight and proteins amount were determined.

Dry weight determination: The sample containing 20 mL algal suspension was filtered through a stainless steel cloth filter (25 mesh) which was dried in an oven for 24 h at 80°C.

While filtering, 20 mL of phosphate buffer (KH_2PO_4 0.01 M, pH 7) was added in order to remove insoluble salts. The filter was then put in a glass Petri dish in the oven under the above conditions. After cooling, the filter was weighed again to evaluate the dry matter.

Extraction of total lipids: The biomass was filtered, rinsed in a phosphate buffer (pH 7), then divided into lots of 0.2 g of *Spirulina* and then frozen. *Spirulina* total lipids were extracted with chloroform-methanol (2:1, v/v) (Bligh and Dyer, 1959) using a magnetic stirring for 30 min in an ice bath. After centrifugation at 2000 x g over 15 min, the chloroform layer which contains total lipids was kept and evaporated under nitrogen and kept in a known volume of the mixture of Vorberck and Marinetti (1965) for further analyses.

Analysis and quantification of fatty acids: Total Fatty Acids (TFA) were methylated according to the method described by Cecchi and Biagini *et al.* (1985) using sodium methylate at 3% in methanol. Heptadecanoic acid (C17:0) was used as an internal standard in order to quantify FA. Their relative composition was subsequently determined as percentage of TFA using an HP 6890 gas chromatograph (Palo Alto, CA) equipped with a Flame Ionization Detector (FID) and an Electronic Pressure Control (EPC) injector. A polyethylene glycol fused silica capillary column (30×0.25 mm×0.25 μm film thickness) purchased from Agilent (Wilmington, DE), with Polyethylene Glycol (PEG) was used as polar stationary phase.

The analyses conditions were: carrier gas, nitrogen (U); flow rate, 1.6 mL min^{-1}; split ratio, 60:1. The detector and injector temperatures were held at 275 and 250°C, respectively. The oven temperature was programmed as follows: Isotherm at 150°C for 1 min; programmed at 15°C min^{-1} to 200°C and then held there for 3 min and finally ramped at 2°C min^{-1} to 242°C.

Protein quantification: Proteins were measured according to the method of Lowry *et al.* (1951) using bovine serum albumin as a calibration standard. *Spirulina* proteins were extracted using a Tris HCl (0.1 M) buffer.

For color reaction Folin-Ciocalteu reagent was diluted with water 1:1 (v/v), mixed well and kept for 30 min; then the absorbance was read at 750 nm.

Chlorophyll quantification: Chlorophylls were extracted using ethanol at 80% and their quantities determined spectrophotometrically at 645 nm according to the method described by Arnon (1949).

Statistical analysis: All analyses were carried out in triplicate and the results presented as mean values. The statistical comparison of data was performed by a SAS (Proprietary Software Release). All results shown in this work represent means of three determinations±Standard Error (SE), which did not exceed 5%. For better clarity, SE were removed from figures.

RESULTS AND DISCUSSION

Growth of spirulina: The culture of *Spirulina platensis* was carried out at four different concentrations of sodium chloride (1, 15, 30 and 60 g L^{-1}), at 36°C and 1300 Lux.

Figure 1 showed that an increase of NaCl concentration caused a reduction of growth of *Spirulina*.

At 1 and 15 g L^{-1}, the curves are sigmoid; therefore, in the first five days, a latency phase is noted followed by an exponential phase of growth for other five days. At least, a stationary phase is spread from 10 days to 15 days of culture.

At 30 g L^{-1} NaCl, the growth is exponential during the period of culture of *Spirulina*.

At 60 g L^{-1}, there is a latent phase during the first five days followed by a decreasing phase of growth until 15 days of culture.

The evolution of proteins amounts (mg g^{-1} of dry weight-DW) in the three cultures (Fig. 2) showed that the response to salt was accompanied by reduced

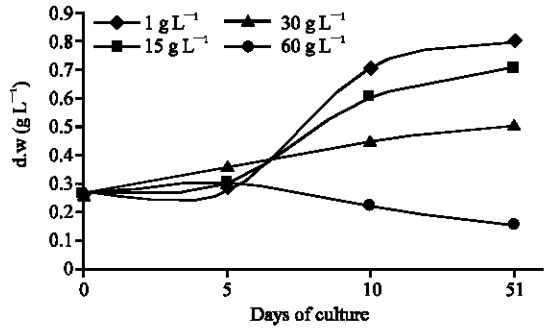

Fig. 1: Growth curves of *Spirulina* under saline conditions (g L^{-1} dry weight). Data are expressed as the mean of three samples±SE

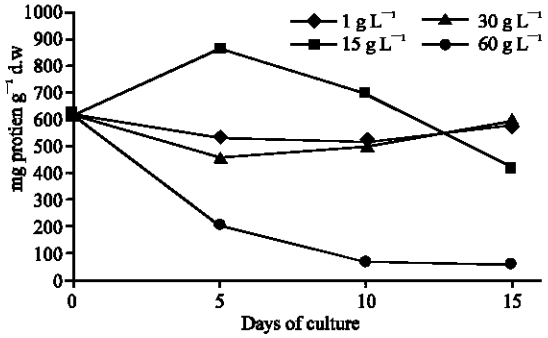

Fig. 2: Growth curves of Spirulina under saline conditions proteins (mg g^{-1} dry weight). Data are expressed as the mean of three samples±SE

biosynthesis of proteins. We also noted a growth rate reduction that is due to the salinity of sea-water as explained by Vonshak *et al.* (1988). These authors attributed this growth decrease to an energy shortage caused by pumping out the entering sodium ions and by the synthesis of sugars as osmoticum.

Chlorophylls: Under salt effect (30 or 60 g L^{-1}), chlorophyll biosynthesis dropped after 5 days of treatment (Fig. 3). At 60 g L^{-1}, there was practically a total inhibition of chlorophyll biosynthesis after 10 days while at 30 g L^{-1}, a partially and temporary inhibition was observed after the 5th day. So that, chlorophylls biosynthesis restarted after the 10th day. These results indicate that *Spirulina* cells growing under high concentrations of NaCl show a reduced ability to use light energy absorbed by their photosynthetic pigments.

The immediate inhibition of the photosynthetic and respiratory systems after exposure to salt was explained by Ehrenfeld and Cousin (1984) and Reed *et al.* (1985). They showed that short-term increase in the cellular sodium concentration was due to a transient-increase in

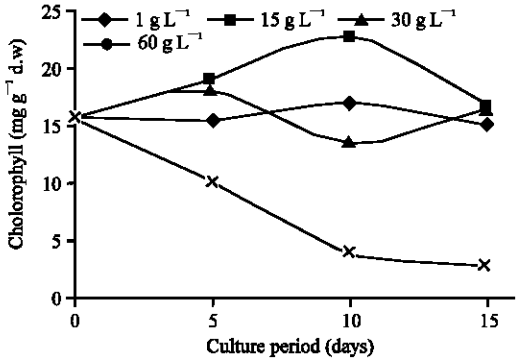

Fig. 3: Chlorophyll amounts during *Spirulina* growth at different NaCl concentrations (data are expressed as the mean of three samples±SE)

Table 1: Evolution of TFA amounts (mg g^{-1} d.w) in relation with salt concentration during *Spirulina* growth (data are expressed as the mean of three samples)

NaCl (g L^{-1})	5 days	10 days	15 days
1	37.50	37.90	36.32
15	44.64	65.00	28.86
30	40.36	33.58	114.12
60	14.91	8.06	8.69

the permeability of the plasma membrane during the first seconds of exposure to high salt concentrations. It has been suggested that the inhibition of photosynthesis arising from rapid entry of sodium might be the result of the detachment of phycobilisomes from the thylakoid membranes (Blumwald *et al.*, 1984). Elevated activity of dark respiration in *Cyanobacteria* after saline stress has previously been reported (Vonshak and Richmond, 1984; Fry *et al.*, 1986; Molitor *et al.*, 1986). This high activity might serve to maintain the required energy for pumping out the toxic sodium ion.

Fatty acids

Quantity of fatty acids: Table 1 shows accumulation of TFA when algae were treated with 30 g L^{-1} of NaCl solution, the concentration corresponding to that of sea water. The optimal quantity of total fatty acids was obtained after 15 days of culture.

At 15 g L^{-1}, FA amounts reached 65 mg g^{-1} DW after 10 days of culture, which represents almost the doubling of the quantity obtained at 30 g L^{-1} during the same period of culture. At the same NaCl concentration the optimal TFA amount was obtained after 15 days of culture, reaching 114.12 mg g^{-1} DW which is triple of the quantity obtained after 10 days of culture.

The exposure of *Spirulina* cultures to high NaCl concentrations (60 g L^{-1}) led to reduced lipid biosynthesis as shown in Table 1.

Main fatty acids quantities: Figure 4 shows that the amounts of most of FA increased steadily until the

15th day (30 g L^{-1} NaCl). However, the amount of palmitic and α-linolenic acids decreased slightly on the 10th day of culture, except for stearic acid whose amount remained constant during the entire culture period.

At 15 g L^{-1} NaCl (Fig. 5), the amounts of all fatty acids increased steadily until the 10th day, except for stearic, palmitic and oleic acids. Beyond 10 days, fatty acid quantities declined, except for C16:0.

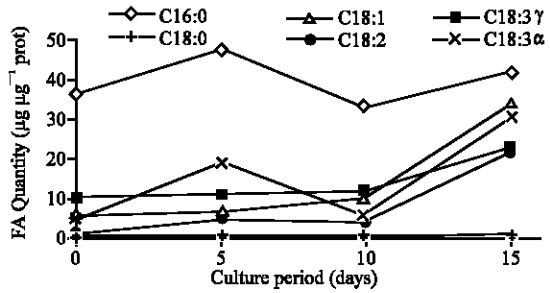

Fig. 4: Quantitative evolution of main fatty acids of *Spirulina* cultivated at 30g L^{-1} NaCl during growth. (data are expressed as the mean of three samples±SE)

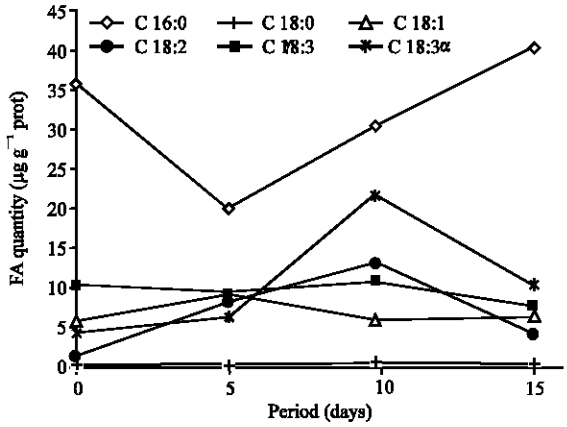

Fig. 5: Quantitative evolution of main FA of *Spirulina* cultivated at 15 g L^{-1} NaCl during growth. (data are expressed as the mean of three samples±SE)

Therefore, *Spirulina* cell is capable of adapting its function to high NaCl concentration. This adaptation is associated with an increase in the respiratory activity of cells (Vonshak *et al*., 1988). Such an increase, associated with salt tolerance, has also been observed in a marine *Spirulina* strain (Gabbay-Azaria *et al*., 1992).

Main fatty acids proportions: Among unsaturated fatty acids, C18:3γ showed the highest percentage exceeding 18% of TFA. Its highest percentages were obtained at 15 NaCl on the 5th day (17%) and 30 g L^{-1} NaCl on the 10th day (18.2%) (Table 2). Palmitic acid content reached a maximum of 56.5% at 15 g L^{-1} NaCl in the 15 days old cultures. Oleic acid content doubled and even tripled, at 30 and 60 g L^{-1} NaCl, respectively, on the 15th day of culture. As a consequence, the Unsaturated/Saturated Fatty Acids Ratios (UFA/SFA) decreased from 1.02 to 0.85 on the 5th day. According to Tomaselli *et al*. (1993), it has been reported that fatty acid composition displayed an increased degree of saturation under 0.54 M NaCl (31.5 g L^{-1}) in *Spirulina platensis*. In fact, the saturated/unsaturated fatty acid ratios (SFA/UFA) increased in *Spirulina maxima* and *Spirulina platensis*. There were also specific changes in long-chain FA (C18). In particular, the oleic acid amount doubled in the presence of 0.54 M NaCl (Tomaselli *et al*., 1988a).

CONCLUSIONS

No data were available on salinity impact on *Spirulina* fatty acids and proteins.

At the end of this study, the effects of various degrees of salinity on *Spirulina platensis* cells revealed that salt adaptation is characterized by a modified biochemical composition in cells. Cultures exposed to salt showed a reduced protein and total chlorophyll amounts, mainly at 30 and 60 g L^{-1} NaCl. As for TFA amounts, after

Table 2: Evolution of FA composition (% of TFA and UFA/SFA ratio) according to the salinity during *Spirulina* growth

Parameters		C16:0	C18:0	C18:1	C18:2	C18:3γ	C18:3α	UFA/SFA
Culture stock	NaCl g L^{-1}	61.2	0.8	9.6	2.6	17.7	17.9	0.77
5th day	1	48.5	0.8	9.1	15.2	11.2	15.0	1.02
	15	41.0	0.6	14.3	14.2	17.0	12.6	1.39
	30	53.3	0.6	5.3	5.5	13.2	21.9	0.85
	60	33.9	0.3	17.2	6.8	16.6	25.0	0.52
10th day	1	51.6	0.9	13.6	8.6	12.2	13.0	0.90
	15	40.0	0.7	7.0	14.2	14.1	23.9	1.45
	30	48.5	0.7	15.6	7.9	18.2	8.8	1.02
	60	36.3	0.9	28.0	8.8	18.1	7.7	1.68
15th day	1	56.3	1.3	9.3	7.0	12.5	13.4	0.73
	15	56.5	0.7	7.7	6.7	12.2	13.9	0.70
	30	33.1	0.8	18.8	14.3	15.0	17.8	1.90
	60	36.1	3.1	25.0	9.7	6.5	19.3	1.54

15 days of culture and in the presence of 30 g L^{-1} NaCl, a value reaching 114.12 mg g^{-1} DW was noted. At this concentration, the highest GLA amounts reached 13.29 mg g^{-1} DW (22.58 μg g^{-1} protein). So, these results suggest that *Spirulina platensis* is capable of adapting to high NaCl concentrations (30 g L^{-1}).

REFERENCES

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxydase in *Beta vulgaris*. Plant Physiol., 24: 1-15.

Bligh, E.G. and W.J. Dyer, 1959. A rapid method of total lipid extraction and purification. Canadian. J. Biochem. Physiol., 37: 911-917.

Blumwald, E., R.J. Mehlhorn and I. Paker, 1984. Salt Adaptation Mechanisms in the *Cyanobacterium synechcoccus* 6311. Advances in Photosynthesis Research. Sybesma, S. (Ed.), The Hague: Maritinus Nijhoff/Dr. W. Junk, pp: 627-630.

Cecchi, J.D. and C. Biagini, 1985. Fast Methanolyse of oils in solvents. Revue Française des Corps Gras, 32: 163-164.

Ehrenfeld, J. and J.L. Cousin, 1984. Ionic regulation of the unicellular green alga *Dunaliella* teritolecta: Response to hypertonic shock. J. Member Biol., 77: 45-52.

Eichi, M., H. Morio, T. Hiroyuki, I.D. Naoko, S. Koji and M. Tadashi, 1992. Influence of ammonium chloride on growth and fatty acid production by *Spirulina platensis*. Applied Biochem. Biotechnol., 34/35: 273-281.

Fry, I.V., M. Huflejt, W.W.A. Erber, G.A. Peschek and L. Paker, 1986. The role of respiration during adaptation of the freshwater cyanobacterium *Synechcoccus* 6311 to salinity. Arch. Biochem. Biophys., 244: 686-694.

Gabbay-Azaria, R., M. Shonfeld, R. Messinger and E. Tel-Or, 1992. Respiratory activity in the marine cyanobacterium *Spirulina subsalsa* and its role in salt tolerance. Arch. Microbiol., 157: 183-190.

Kennedy, M.J., S.L. Reader and R.J. Davies, 1993. Fatty acid production characteristics of fungi with particular emphasis on gamma linolenic acid production. Biotechnol. Bioenerg., 42: 625-634.

Laliberte, G., J. Eugenia and J. De Nouë, 1997. Mass Cultivation and Water Treatment *Spirulina*. In using: *Spirulina platensis* (*Arthrospira*) Physiology, Cell-Biology and Biotechnology. Avigad Vonshak (Ed.), pp: 159-173.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Rendall, 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Martel, A., S. Yu, G. Garcia-Reina, P. Lindbald and M. Pedersen, 1992. Osmoic-adjustement in the cyanobacterium *Spirulina platensis*: Presence of a-glucosidase. Plant Physiol. Biochem., 30: 69-74.

Medhioub, A., A. El Abed and H. Ben Wada, 1999. Présentation de la spiruline en Tunisie (Oued Sidi Bou Ali). Premières Journées Franco-Tunisiennes de zoologie. Nabeul, Tunisia, September, pp: 20-27.

Molitor, V., W. Erber and G.A. Peschek, 1986. Increased levels of cytochrome oxidase and sodiumporton antiporter in the plasma membrane of anasystis nidulans after growth in sodium enriched media. FEBS Lett., 204: 251-257.

Nakahara, T., T. Yokocki, Y. Kamisaka and O. Suzuki, 1992. Gamma-Linolenic Acid from Genus *Mortierella*. Kyle, D.J. and C. Ratledge (Eds.), Industrial Applications of Single Cell Oils. Champaign: AOCS, pp: 61-97.

Padhi, B., B. Rautary and P. Balliarsing, 1992. Ecological studies one *Spirulina* sp. has polluated in lays Berhampur of (Orissa). In: *Spirulina* ETTA Nat. Symp. MCRC, Madras, India, pp: 52-56.

Reed, R.H., D.L. Richmond and W.D.P. Stewart, 1985. Na^+ uptake and extrusion in the cyanobacterium *Synechcystis* PCC 6714 in response to hyper-saline treatment. Evidence for transent changes in plasmalemma Na^+ permeability. Biochim. Biophys. Acta, 814: 347-352.

Sankaran, V. and I. Rdharukman, 1992. *Spirulina platensis* (Nordset) Geitler. Has Comparison Between Liquid Cultures and Soil Cultures. In: Spirulina ETTA National Symposium, MCRC Madras, 1992. Seshadri, C.V. and N. Jeeji Bai (Eds.), pp: 119-124.

Santra, S.C., 1992. An Approach to Future *Spirulina* Research. In: *Spirulina* ETTA Nat. Symp. MCRC, Madras, Seshadri C.V. and N. Jeeji Bai (Eds.), pp: 119-124.

Tomaselli, L., L. Giovanetti, F. Bocci and A. Sacchi, 1988. Effetto della temperatura e della salinita sulla composizione biochemica di Spirulina. Atti Conv. Produzione di Biomasse Microbiche da Residui e Surplus Agro-alimentari: 5 anni di attivita IPRA. Monografia n°38 CNR.

Tomaselli, L., L. Giovanetti and G. Torzillo, 1993. Physiology of stress response in *Spitulina* sp. Bulletin de l'institut océanographique. Monaco, n° Special, 12: 65-75.

Vonshak, A. and A. Richmond, 1981. Photosynthetic and respiratory activity in *Anacystis nidulans* adapted to osmotic stress. Plant Physiol., 68: 504-510.

Vonshak, A., R. Guy and M. Guy, 1988. The response of the filamentous cyanobacterium *Spirulina platensis* to salt stress. Arch. Microbiol., 150: 417-420.

Vonshak, A. and R. Guy, 1992. Photoadaptation, photoinhibition and productivity in the blue-green alga, *Spirulina platensis* grown outdoors. Plant Cell Environ., 15: 613-616.

Vorberck, M.L. and G.V. Marinetti, 1965. Separation of glycosyl diglycerides from phosphatides using silicic acid column chromatography. J. Lipid Res., 6: 3-6.

Warr, S.R.C., R.H. Reed, J.A. Chudek, R. Foster and W.D.P. Stewart, 1985. Osmotic adjustment in *Spirulina platensis*. Planta, 163: 424-429.

Zarrouk, C., 1966. Contribution à l'étude d'une cyanophycée-influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de la *Spirulina maxima* (Setch et Garder) Geitler» Thesis, Faculté des Sciences de l'Université de Paris.