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Effect of Seawater Salinity Concentrations on Growth Rate, Pigment contents and Lipid Concentration in *Anabaena fertilissma*

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ABSTRACT

Salinity imposes significant stresses in various living organisms including microalgae. Under favourable and unlimited growth conditions microalgae produce primarily polar lipids, which enrich chloroplast and cellular membranes. However, under unfavourable growth conditions microalgae accumulate neutral lipids in lipid droplets located in the cytoplasm. The study focuses on the effect of salinity concentrations on the cell growth, photosynthetic pigments and some metabolic activities as carbohydrate, protein, lipid, total amino acids, proline, choline, glycine betaine and individual's fatty acids in *Anabaena fertilissma* by setting up different ranges of salinity (2.5, 5, 10, 15 and 20‰). The results were as follows. The microalga was able to tolerate the salinity levels, algal cell division showed a good adaptation to the highest salinities and cells would not go into osmotic shock and die, where the changes in salinity have significant effects on the growth. The growth and photosynthetic pigments tends to decline as salinity increases. While increased of protein, carbohydrate, lipid, proline, choline and glycine betaine. The composition of fatty acid methyl ester has been identified as C16:0, C18:0, C20:0, C16:1, C18:1, C18:2, C18:3, C20:4, C20:5 and C22:6. It is clear that the effects of salt stress on the proportional amounts of fatty acids.

Keywords: Growth rate, Individual fatty acids, Microalga, Protein, Pigments, Salinity variations.

INTRODUCTION

Salinity is one of the most important abiotic environmental factors for aquatic organisms; by surging, coastal run-off, and rains and may have different durations and ranges. For example, seawater salinity in coastal habitat may drop, especially during summer typhoons, to 2‰ and remain at this level from several hours to three days (Luchin *et al.,* 2005). Exceptionally salt tolerant (halotolerant) organisms could enrich our knowledge in knowing basic physiological mechanisms that may lead to enhance salinity tolerance in crop. Algae are inhabitants of biotopes characterized by varying salinities, and as a result, they have attracted considerable attention in salt tolerance studies domain. They have served as model organisms for better understanding of salt acclimation in more complex physiological processes of higher plants (Alkayal *et al.,* 2011). Among algal species, the unicellular green algae; *Dunaliella salina*, due to its remarkable ability to adapt to highly saline conditions, could act as a valuable model for the identification of such mechanisms (Chen and Jiang, 2009). This organism can practically adapt to the entire range of salinities, well above the maximal salinity range for growth of most plant species. The adaptation to extreme salinity involves short-term and long-term responses in *Dunaliella sp.*, the former include osmotic adjustment by accumulation of large amounts of intercellular glycerol and efficient elimination of Na^+ by plasma membrane transporters. Rapid alterations in the cell volume donated by lacking a rigid cell wall in this genus makes it possible to respond to changes in salt concentration by intercellular ions and glycerol concentration adjustments (Kacka and Donmez, 2008). As a matter of fact, increases in external concentrations of inorganic ions impairs the osmotic balance between the cell and their surrounding medium and forces water efflux (ex-osmosis) from the cells, leading to the loss of turgor pressure (Frick and Peters, 2002); in this respect, plants, including species of chlorophyta, response to high concentrations of salt by assimilation metabolites like those of fructose, sucrose and trehalose, which possess an osmolyte function, or those of charged molecules, such as proline and glycine betaine in order to read just osmotic equilibrium by preventing water loss (Banu *et al.*,2009; Ahmad *et al.,*2013). The lipid and fatty acid composition of algae differs according to the culture conditions. The lipid accumulation in algae usually occurs during environmental stress, including growth under nutrient deficient conditions. The algae adapt themselves to stress by undergoing changes in morphological and developmental pattern as well as physiological and biochemical processes. The increase in salt concentration affects the rate of respiration, distribution of minerals, ion toxicity, photosynthetic rate and permeability of the cell membranes (Sudhir, 2004). The percentage of saturated fatty acid in algae decreased as the concentration of NaCl increased, while the percentage of highly unsaturated fatty acid increased (Kirrolia *et al.,* 2011). Observations of algal cultures exposed to different water salinities give some insight into the mechanisms of survival and adaptation in algae. Up to now, however, the influence of this factor on algae has been studied using only several species (Dzhafarova, 1992; Fujii *et al.,* 1995; Fu *et al.,* 2003; Radchenko *et al.,* 2006).

The main objective of this study was to investigate the effects of various salinity conditions on the growth and the content of some metabolites including pigment fractions, proteins, lipids, carbohydrates, total amino acids and fatty acids of *Anabaena fertilissma*.

MATERIAL AND METHODS

The filamentous cyanobacterium *Anabaena fertilissma* was obtained from National Institute of Oceanography and fisheries, Alexandria. Stock culture were maintained on 1.5‰ agar slants of modified (G) of Barakat *et al.,* 1975. Slant cultures were grown at 30°C

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at a distance of 50 cm from a bank of 3 day-light, 20w fluorescent tubes.

Experimental design

The alga was cultured in conical Erlenmeyer flasks on (G) medium. When the cultured reached the exponential growth stage, they were inoculated into nutrient medium prepared with sterilized sea water diluted with distilled water to the salinity concentrations of 20, 15, 10, 5, and 2.5‰ of sea water. Each treatment consisted of three replicates. The growth rates under various salinity conditions were measured every two days for the 20 days of experimental period. However, those used for chemical analysis were grown for eight days.

Determination of total protein

Protein was determined by the method described by Hartree, (1972) which is modification of original method of Lowery, (1951). The intensity of blue color developed was measured using spectrophotometer at 650 nm.

Carbohydrates estimation

The total carbohydrate was estimated by the following phenol-sulphouric acid method of Dubois *et al*. (1956), using glucose as standard.

Lipid estimation

The lipid was estimated by using chloroform methanol mixture as described by Northcote *et al.* (1958).

Preparation of fatty acids methyl ester for gas chromatographic analysis of algal extract

Preparation of fatty acids methyl ester was performed according to the procedure of Radwan, (1978).

Estimation of chlorophylls

The amount of chlorophyll present in the algae was estimated by the method of Arnon, (1949). Absorbance was measured at 645nm and 663nm. The chlorophyll content was determined by using the following formula:

$$\text{Chlorophyll a } (\mu g/ml) = 12.7 \times A663 - 2.69 \times A645$$

$$\text{Chlorophyll b } (\mu g/ml) = 22.9 \times A645 - 4.68 \times A663$$

Where, A = Absorbance at respective wave length.

Estimation of carotenoid

The amount of carotenoid was estimated by the method of (KirK and Allen, 1965). The chlorophyll extract was measured at 480 nm in spectrophotometer to estimate the carotenoid content.

$$\text{Carotenoid } (\mu g/ml) = A480 + (0.114 \times A663) - (0.638 \times A645)$$

where, A= Absorbance at respective wave length.

Estimation of phycocyanin

The phycocyanin was determined according to the method described by Bennett and Bogorad (1973).

$$\text{Phycocyanin } (mg/l) = A_{615} - 0.474\ A_{652}/5.34$$

Estimation of total free amino acids

The total free amino acids were determined by using ninhydrin method described by Ya and Tunekazu (1966).

Determination of Proline

Free proline concentration was determined using ninhydrin method described by Bates *et al*. (1973). The absorbance was read at 520 nm using toluene as a blank.

Determination of quaternary ammonium compounds (QAC) (Choline and Glycine betaine (Gb)

Quaternary ammonium compounds were determined by modified periodide method described by Grieve and Grattan (1983). Gb was estimated from difference between total QAC and choline. QAC were precipitated as the periodide complex at low PH, from the water extract.

RESULTS

Effect of salinity on the growth rate of *Anabaena fertilissma*

Anabaena fertilissma was exposed to five different salinities, 2.5, 5, 10, 15, and 20‰ as compared to the controlled (G) medium. The growth and growth rate as seen in figures (1 & 2), where growth rates under various salinity conditions were measured every 2 days. Under most the salinity levels, a marked and linear increase in the algal growth phase. The maximum algal growth rate was obtained eight days under the most of salinity levels investigated.

However, the salinity level 2.5‰ was the best one, which revealed maximum growth and growth rate as compared to control.

Effect of salinity levels on the algal contents

a) Photosynthetic pigments

The pigment fractions content in *Anabaena fertilissma* (chlorophyll a, chlorophyll b, carotenoid and phycocyanin) were in figure (3). The pigment fractions were decreased gradually below its content at control. The minimum values were recorded at salinity 20‰. While the content of carotenoids increased in lower salinities 2.5 and 5‰ which recorded 4.47 and 4.8 µg/ml respectively, then it decreased gradually by increasing salinity levels as compared to control.

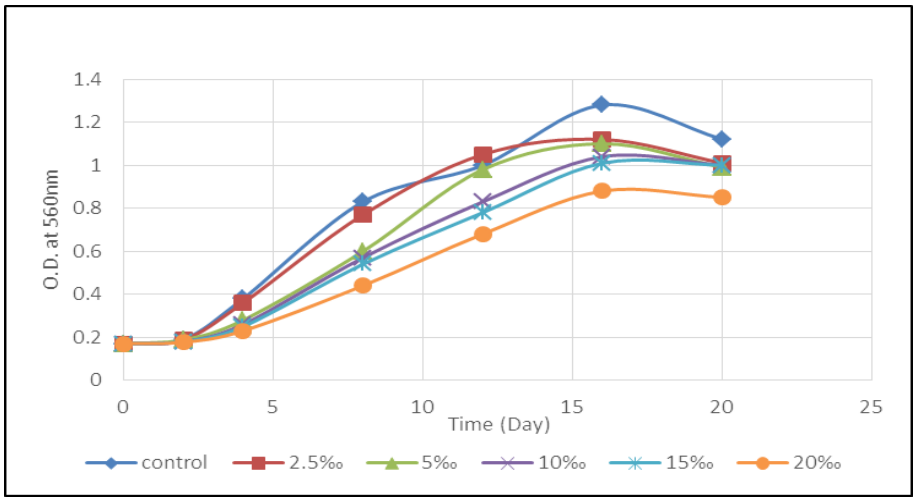

Figure (1): Growth curves of *Anabaena fertilissma* under different salinities, Each value is a mean of five replicates.

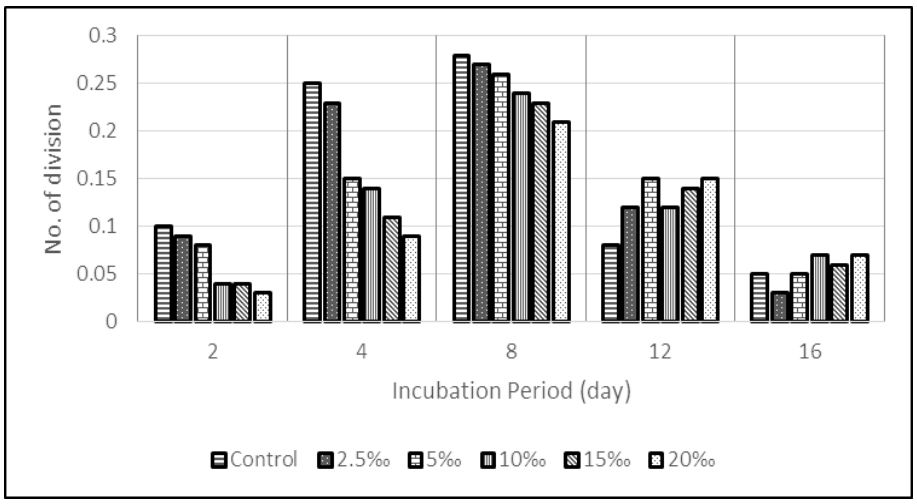

Figure (2): Growth rate of *Anabaena fertilissma* under different Salinities for different incubation periods, each value is a mean of five replicates.

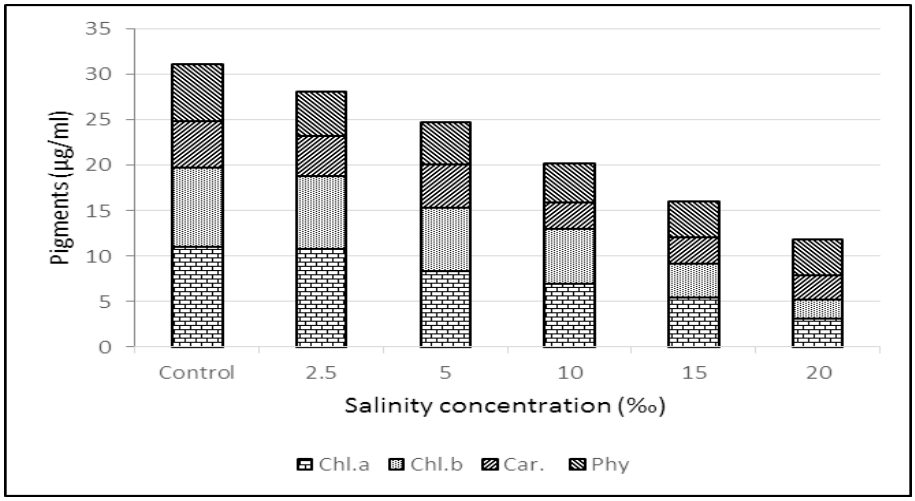

Figure (3): Photosynthetic Pigments Content in *Anabaena fertilissma* for eight days under different Salinities, Each value is a mean of five replicate.

b) Biochemical Composition

The biochemical compositions of *Anabaena fertilissma* under different salinities at eight days were recorded at in table (1). The total carbohydrate contents increased with salinity increased where the maximum carbohydrate content 52.06 µg/ml at salinity 20‰. In addition, the contents of protein, lipids, proline, choline and glycine betaine had the same trend of carbohydrate content, where, the maximum values: 128.57, 131.00, 0.201, 9.06 and 18.32 µg/ml respectively, recorded at salinity level 20‰. Only the total amino acid was decreased with increased salinity levels, where the lowest value 2.152 µg/ml obtained at salinity 20‰.

c) Effect of salinity on the individual fatty acids

The results of individual fatty acids recorded in table (2) which explain the effect of different salinity levels (2.5, 5, 10, 15, and 20‰) on the content of the individual fatty acids. From these results it clear that the total saturated fatty acids were increased as 4.599, 5.668, 6.598, 7.484 and 8.261**,** with increased the salinity levels, 2.5, 5, 10, 15 and 20‰, respectively, as compared to the control 4.522. It is clear that C16:0 (palmitic acid) and C18:0 (stearic acid) were more sensitive to the effect of salinity than C20:0 (arachidic acid), where at salinity 20‰ the C16:0 was increased nearly three folds: 1.140 as compared to the control: 0.50 and C18:0 was raised about two folds as compared to control. The total monounsaturated and individual monounsaturated fatty acids C16:1 (palmitoleic acid) and C18:1 (oleic acid) had the same trends of total saturated fatty acids under the effect of salinity stresses. The levels of the polyunsaturated C18:2 (linoleic acid) under different salinity levels were recorded lower values as compared to the value of control. While, polyunsaturated C18:3 (linolenic acid), C20:4 (arachidonic acid), C20:5 (pentaenoic acid) and C22:6 (docosahexaenoic acid) increased with increased salinity levels.

Table 1: The biochemical composition of *Anabaena fertilissma* at eight days of culturing at normal conditions and different salinity levels, each value is a mean of five replicates.

Biochemical composition	Salinity levels (‰)					
(µg/ml)	control	2.5	5	10	15	20
Total carbohydrates	47.20	47.95	49.52	50.43	51.96	52.06
Total proteins	110.35	113.05	119.67	122.80	126.98	128.57
Total lipids	104.22	110.36	117.01	121.45	132.07	131.00
Total amino acid	3.354	3.315	2.208	2.189	2.172	2.152
Proline	0.085	0.085	0.100	0.122	0.194	0.201
Choline	3.84	4.03	5.38	6.78	7.98	9.06
Glycine betaine	2.88	6.24	7.36	12.25	15.78	18.32

Table 2: The individual fatty acids of *Anabaena fertilissma* at eight days for culturing in normal conditions and different salinity levels. each value is a mean of three replicates.

			Salinity levels (‰)				
Fatty acids (‰)		Control	2.5	5	10	15	20
Saturated	C16:0	0.501	0.589	0.894	0.984	1.071	1.140
	C18:0	3.000	3.100	3.820	4.560	5.300	6.000
	C20:0	1.021	0.910	0.954	1.054	1.113	1.121
	Total	4.522	4.599	5.668	6.598	7.484	8.261
Mono-unsaturated	C16:1	0.614	0.602	0.656	0.674	0.685	0.698
	C18:1	5.021	4.880	5.121	5.437	5.642	5.780
	Total	5.635	5.482	5.777	6.111	6.327	6.478
Poly-unsaturated	C18:2	0.831	0.518	0.501	0.534	0.577	0.591
	C18:3	12.543	13.043	17.654	20.010	22.530	21.031
	C20:4	25.34	25.045	26.023	27.012	27.870	27.000
	C20:5	1.321	1.340	1.550	1.875	1.898	1.921
	C22:6	3.017	3.077	3.120	3.431	3.650	3.880
	Total	43.052	43.023	48.848	52.862	56.525	54.423
Total		53.209	53.104	60.293	65.571	71.336	69.162

DISCUSSION

Salinity represents one of the most important factors, which exert stress, or even injury of the metabolism of plant cell. The effects of salinity on the metabolism and activity of algae are mainly due to the specific effects of the various ions found in seawater. Algae are found in habitats of widely different water potentialities which have accordingly evaluated osmoregulatory mechanism to deal with environment (Reed, 1980). The decline in growth observed in many plants subjected to salt stress is often associated with a decrease in their photosynthetic activity (Gong *et al.*, 2008; Asulabh *et al.*, 2012). Our results obtained for the growth of alga and the photosynthetic pigment fractions chlorophyll

"a", chlorophyll "b", carotenoids and phycocyanin under all salinity levels lower than control and the maximum growth rate and pigment fractions were obtained at salinity 2.5‰. These results are similar with results obtained by Aizdaicher and Reaktsiya (1995); Koru and Cirik (2003); Gong *et al.* (2008).

Although many species of micro-algae are tolerant to great variations in salinity, their chemical composition can be affected (Lanping *et al*., 2013). The difference in the growth response with in the treatments suggests the existence of *Anabaena fertilissma* in the ability to cope with extremely low salinity and corroborates genetic and physiological heterogeneity. The increase in total protein content in algae subjected to salinity stress conditions have reported by many authors (Ashraf, 1989; Cowan and Rose, 1991). This may be due to suggestions that salinity stresses enhance protein synthesis (Langdale *et al*., 1973; Mishra *et al*., 2008). According to Rafiqul *et al*. (2003); Ghezelbash *et al*. (2008) suggested that the algae adapted to salinity stress by increasing carbohydrates in the cells.

Similar to our results, where the carbohydrate contents in *Anabaena fertilissma* are increased in different salinity levels over control. Hasaneen *et al.* (2008) reported that compatible osmolytes such as glycine betaine, choline and proline are synthesized in response to salt stress. These osmotic adjustments protect subcellular structure and reduce oxidative damage caused by free radicals produced in response to stress of high salinity (Hong *et al*., 2000). Our results support mentioned reports that accumulation of the intercellular glycine betaine, choline and amino acid proline increased gradually with increase in salinity levels. According to Fabregas *et al*. (1985) salinity stress triggers the production of lipids. Renaud *et al*. (1994) reported a 24.2‰ total lipid content for marine *Navicula* sp. Three fresh water *Navicula* sp. attained an average lipid content of 24 to 51‰ of dry weight (Griffiths and Harrison, 2009), for nutrient replete and deficient conditions. Algae differ in their adaptability to salinity and based on their tolerance extent, they are grouped as halophilic (salt requiring for optimum growth) and halotolerant (having response mechanism that permits their existence in saline medium). In either case, the algae produce some metabolites to adapt to salt injury and also to balance the surrounding osmotica (Huflejt *et al*., 1990). The results obtained for the effect of different levels of salinity on the content of fatty acids in *Anabaena fertilissma* cleared that considerable variations have been reported in the fatty acid content. These wide variations reported by (Ciferri, 1983 and Asulabh *et al*., 2012).

Nearby all of the individuals saturated, mono and polyunsaturated fatty acids were increased under the salinity stress. Except the polyunsaturated fatty acid C18:2 (linoleic acid) under different salinity levels were recorded lower values as compared to the value of control. Differences in the relative proportion of different fatty acids in salinity cultures could be attributed to the physiological state of the alga as reported by Rao *et al*. (2009). A similar salinity tolerance has been reported previously for *Isochrysis sp*. (Ben-Amotz *et al*., 1985; Fabregas *et al*., 1985; Mathad and Hiremeth, 2009). An increase in fatty acids with increasing salinity has been reported previously for *Navicula oculata* (Khamutov *et al.*, 1990; Hodgson *et al*., 1991). Many reports have suggested that lipids might be involved in the protection against salt stress (Talebi *et al*., 2013). When photosynthetic organisms are exposed to salt stress, the fatty acids of membrane lipids are desaturated. Hence, in the given study, the increase in salinity is perceived as an unfavorable condition resulting in a rise in the total and individuals fatty acid.

Biodiesel is composed mainly of palmitic (C16:0), stearic (C18:0), Oleic (C18:1) and linolenic (C18:3) acids. Definitely, the proportions of fatty acid methyl esters contribute strongly to the quality of biodiesel fuel (Knothe, 2008). Moreover, increasing palmitic acid is desirable for good quality biodiesel (Mandal and Mallick, 2011). The data in this research demonstrated a significant effect of salinity on the proportions of fatty acid methyl esters of *Anabaena fertilissma*.

CONCLUSIONS

Biofuels from microalgae is a viable alternative for replacing the global demand for petro-diesel. The two important desirable characteristics considered in a species to be used for biodiesel production are high biomass and lipid production. Salt stress is a major abiotic environmental factor that limits plant growth and productivity. The salinity stress and unfavorable light conditions are the main limiting factors of plant productivity both in aquatic and terrestrial, natural and anthropically modified environments. Microalgae differ in their adaptability to salinity and other stress conditions.

The ability of cells to survive and flourish in saline environment under the influence of osmotic stress has received considerable attention. Salt stress significantly affects cells growth and biochemical composition act as protein, carbohydrate, lipid, proline, glycine betaine and choline. Such biochemical composition act as secondary metabolite for algae and stress or unfavorable condition as nutrients, growth phase, temperature, light, etc., lead to the accumulation of more lipids, proline, choline and glycine betaine. Increase in the salinity impaired algal growth. As the increase or decrease of salinity-generated stress inside the algae, total lipid content increased acting as a storage reserve energy material till favorable conditions arise.

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تأثير تركيزات ملوحة ماء البحر على معدل النمو ومحتوى الصبغات وتركيز الدهون في الانابينا فرتيليزما

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تعتبر الملوحة من الضغوط الكبيرة على الكائنات الحية المختلفة بما في ذلك الطحالب. فيظل ظروف النموالمواتية وغيرمحدودة تنتج الطحالب الدهون القطبية في المقام الأول، والتي تتواجد في البلاستيدات الخضراء والأغشية الخلوية. ومع ذلك، في ظل الظروف الغيرمواتية لنموالطحالب تتراكم الدهون على هيئة قطرات في السيتوبلازم. وتركزالدراسة على تأثير تركيزات الملوحة المختلفة (٢.٥، ٥، ١٠، ١٥، ٢٠ ‰ من ملوحة البحر)على نموالخلايا والأصباغ وبعض الأنشطة الأيضية مثل الكربوهيدرات والبروتين والدهون،والأحماض الأمينية والبرولين والكولين وجليكاينالبيتين وافراد الأحماض الدهنية في طحلب الانابينا وكانت النتائج على النحو التالي: ان الطحلب له القدرة على التحمل و التكيف مع التغيرات المختلفة في درجات الملوحة حيث قل معدل النمو ولكن زادت بعض المكونات مثل الكربوهيدرات والبروتين والدهون والبرولين والكولين وجليكاينالبيتين وافراد الأحماض الدهنية .وأوضحت الدراسة تناسب التأثير الملحي مع كمية الأحماض الدهنية المتكونة.