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Salt Induced Changes in the Growth of *Chlorococcum humicolo* and *Scenedesmus bijugatus* under Nutrient Limited Cultures

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Due to climatic changes in tropics, saline water bodies are often subject to natural fluctuations of salinity through precipitation, dessication, drought conditions or anthropogenic interference. Growth and persistence of algae under these conditions require physiological tolerance and/or resistant stages in the life cycle. Salinity, which ranges from 95.9-174.7 mg/L as NaCl, is considered as an important parameter for the Indradyumna pond (485 x 396 sq ft, maximum depth 5 m) of Puri, Orissa for it is located at a distance of 1 km from the Bay of Bengal. The fluctuation of salinity level of the pond has been observed during 1988-1991 mostly due to excessive use by innumerable number of pilgrims all over the year (Dash 1992).

Increased salinity favors growth of cyanobacteria because this is the only group of photoautotrophic plankton taxa requiring Na^+ for growth (Allen and Arnon 1955). Green algal species like halotolerant planktonic algae (Hellebust and Le Gresley 1985), Chlorella, Ankistrodesmus and Scenedesmus (Kessler 1980) have also been found to be favored by increased salinity. However, the rationale of studying salinity tolerance of algae under nutrient limited conditions seems to be more appropriate in the bioassay method because it is established that the susceptibility / resistance characteristic of an algal species to external stress is nutritionally determined (Herbert and Bradley 1989; Mohapatra and Mohanty 1992). Accordingly, it was decided to see the effects of salinity stress on two indigenous phytoplankton species viz., Chlorococcum humicolo (Nag) Rabenh. and Scenedesmus bijugatus Kutz. in culture with differential nutrient enrichment using the sterile pond water as medium.

MATERIALS AND METHODS

The important variables of water quality as defined in this pond are : hardness as $CaCO_3$ (62.75-77.50 mg/l), pH (8.40-7.78), DO (3.34-7.58 mg/L) and salinity (95.9-174.7 mg/L NaCL). The pure

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monocultures of Chlorococcum and Scenedesmus were prepared after collecting the inoculum from the pond with the help of a plankton net (aperature 64 um:200 mesh). The pellets, on repeated washing with sterile distilled water through centrifugation at 1000 rpm, were combined in a small volume of sterile nutrient medium (Chu No. 10[+] medium modified by Safferman and Morris (1964) with A_6 (B, Mn, Zn, Cu, Co and Mo) micronutrients of Gerloff et al. (1950); homogenized by repeatedly drawing the content into and out of a 5-mL sterile syringe (1.5 mm opening); and delivered in 2.5-mL samples into 47.5 mL of the nutrient medium so as to get 50 mL final volume contained in a 250-mL borosilicate glass conical flask. The pure cultures of both the species were then prepared by following standard methods (Stein 1975). The stock cultures were grown in 250-mL conical flasks with 50-mL sterile pond water filtered through a 0.45 um porosity G-4 borosilicate Buchnner funnel (200-mL) applying 0.30 atm. pressure through a vacuum pump under aseptic condition. The experimental cultures were grown in triplicates in non-absorbent cotton stoppered 100-mL borosilicate glass conical flasks containing 25 mL of cultures. All the cultures were maintained under conditions described by Mohapatra and Mohanty (1992). Each experimental flask was inoculated with 1.0 mL algal stock in order to get an initial cell density of about 10^5 cells/mL for both the species.

Two separate sets of experiments were designed. In the first, salt tolerances of both species were evaluated by culturing the algal species at different levels of salinities for 15 d and by measuring the optical densities (O.D.) of the entire cultures (at 678 nm) on 7th and 15th d of incubation to record the change in growth. The initial salinity of the mixed water sample (in order to represent the entire pond water) used in the experiment was calculated to be 2.57 mM (in terms of NaCl) and has been referred as 'X' in the test. Varied salinities ranging from 0.64 mM (1/4 X) to 328.5 mM (128 X) were prepared by dilution of pond water (filtered through sterile G-4 glass filter) with sterile distilled water or by addition of sodium chloride salt (for elevated salinity). In the second experiment three salinity levels (normal, growth accelerating, and growth retarding) that varied with the species (X, 8X, 32X for Scenedesmus and X, 16X, 64X for Chlorococcum) were selected from the first phase of observation and were taken as the salinity variables. The cultures were divided into six groups each having three flasks. The first group enriched with 1.5 mM $NaNO_3$, 0.3 mM Na_2HPO_4, 0.2 mM sodium metasilicate, trace elements: 1.8 uM Fe^{3+}, 7.3 uM Mn^{2+}, 0.8 uM Zn^{2+}, 0.3 uM Cu^{2+}, 2.0 uM Mo_2^{2+}, 0.2 mM Co^{2+} and chelated with 25 uM NA_2EDTA per liter medium served as the control (C_1) while the second containing only filtered tank water served as another control (C_2). The remaining four groups were separately enriched with 1.8 mM HPO_4^{2-}, 0.2 mM SiO_3^{2-}, 1.8 mM NO_3^- and 0.2 mM $EDTA^{2-}$ at different levels of salinity gradient. The O.D. of the entire cultures (at 678 nm) and pigment extract (at 663 and 645 mm) were taken on 8th d of incubation with the help of a SPEKOL spectrophotometer (Carl Zeiss ZENA). The pigment contents after extraction in 80% acetone

(Talling and Driver method 1963) were measured in terms of O.D. and expressed in mg/L of culture (Arnon 1949). The standard deviations and the least significance differences (LSD) were calculated for the first experiment while in the second experiment the data were compared through Duncan's multiple range test (DMRT) at p = 0.05 (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

Of the different prepared salinities tested (0.64-328.5 mM NaCl), Chlorococcum and Scenedesmus showed optimum growth at 41.06 and 20.53 mM, respectively. However, compared to the control (pure pond water), the salinity of 5.13-82.12 mM caused growth enhancement of Chlorococcum while for Scenedesmus the range was 5.13-41.06 mM till the 7th d and 5.13-20.53 mM after 15 d in nutrient enriched cultures (Fig. 1a and b). The range was further reduced for Scenedesmus in normal tank water medium where it was 5.13-20.53 mM and 5.13-10.27 mM after 7 and 15 d, respectively, but it remained unchanged for Chlorococcum (Fig. 2a and b). The growth rates at all salinity levels, however, increased rapidly during the first phase of culture (0-7 d) in both normal nutrient enriched pond water.

In Scenedesmus the growth rates at different salinities were significantly different from each other in nutrient enriched medium (LSD = 0.013 and 0.018 after 7 d and 15 d, respectively, at p = 0.05) while in pond water the growth rates between 5.13-10.27 mM, and 41.06-82.12 mM were not significant. On the other hand, the growth of Chlorococcum varied significantly at all but 10.27-20.53 mM in nutrient added cultures (LSD= 0.007 and 0.004 on 7th, and 0.023 and 0.006 on 15th d in nutrient added and pond water medium, respectively). The inhibitory salinities were $\leq$1.28 mM and $\geq$164.24 mM for Chlorococcum while $\leq$ 1.28 and $\geq$82.12 mM for Scenedesmus, compared to growth at pond water salinity level.

Nutrient addition at the three selected salinities produced marked differences in growth and pigment concentration from that in the control (Table 1 and 2). The enhancement of growth and chlorophyll content was recorded in both the algae at all the three salinity levels with each combination of nutrient and pond water. Optimization of growth and pigment biomass was observed in C_1 culture, with certain exceptions (See Table 1 and 2). Scenedesmus, however, followed the trend- $C_1 > +NO_3^- > +HPO_4^{2-} > +SiO_3^{2-} > +EDTA^{2-} > C_2$ except that at 8X, $EDTA^{2-}$ added cultures experienced better growth and pigmentation than SiO_3^{2-} added cultures. This indicated the tendency of Chlorococcum to grow in phosphate rich medium while Scenedesmus favoring NO_3 rich medium.

Our results demonstrate that the range of salinity tolerance of the species is different and varies with concentration gradient. Growth optimization occured at 8X salinity in Scenedesmus while at 16X in Chlorococcum. At 328.5 mM concentration, growth of both the algae was significantly inhibited and salt induced reduction of pigment biomass was observed (Table 2) indicating that the salinity levels have radical effect on primary production in a habitat. Since both

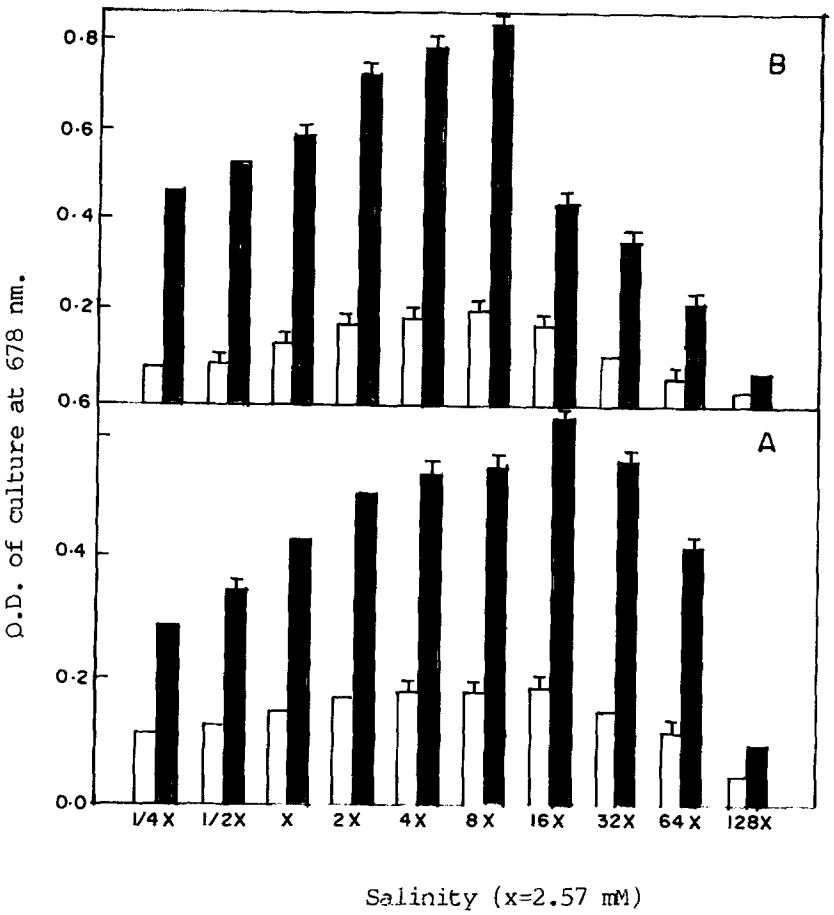

Figure 1. Response of (A) Chlorococcum and (B) Scenedesmus to different degrees of salinity gradient in nutrient enriched water of Indradyumna pond. Legends: □ growth after 7 d; ■ growth after 15 d.

test organisms have been isolated from the same environment, the difference in genetic adaptation between the species or physiological acclimatization of either of them cannot be considered as the principal cause for the better halotolerance of Chlorococcum. However, the morphological and biochemical differences can be considered, which warrants intensive study of growth physiology. Kessler (1977, 1980) has, however, observed that Scenedesmus exhibits lesser degree of halotolerance compared to other members of Chlorococcales.

Our studies emphasised the influence of nutrient levels on phytoplankton growth and the halotolerance nature of the species in response to nutrient enrichment. The cell growth, composition and density of natural phytoplankton assemblages under salt stress are enhanced by phosphorus and nitrogen enrichment, the former being important one (Sullivan 1976). We attempted to maximize opportunity for the amplification of growth of both the test algae at the three selected salinity levels on addition with nitrogen,

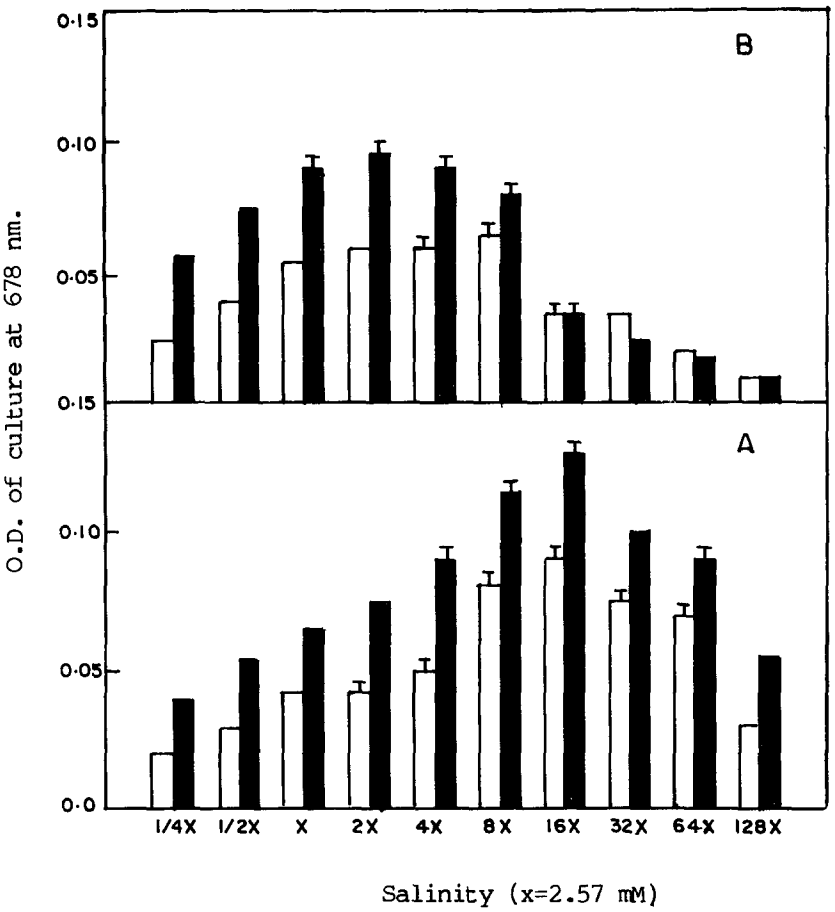

Figure 2. Response of (A) Chlorococcum and (B) Scenedesmus to different degrees of salinity gradient in Indradyumna pond water. Legends as for Figure 1.

phosphorus and some other macro- and micronutrients. At each of the salinities the nutrients, used separately or in combination, caused more or less acceleration of growth compared to that in C_2 though, however, the acceleration caused by SiO_3^{2-} and $EDTA^{2-}$ enrichment was insignificant in both the cases (Table 1 and 2): As expected , maximum growth and pigment biomass was noted in C_1 and for both the algae the results obtained in C_1 was significantly higher than the growth and pigment content observed either in nitrogen or phosphorus enriched cultures. However, in Chlorococcum the pigment concentration at 16X and 64X salinities with added $EDTA^{2-}$, was insignificantly less than C_2. It is probably due to the fact that some of the pigments were not extracted and remained cell bound making a very little difference between the two cultures.

The varied response of the algae to nitrogen and phosphorus enrichment indicates that the two test algae require differential N:P ratios in the medium for their optimum amplification.

Table 1. Effects of selective nutrient enrichment and the salinity levels on growth (OD at 678 nm) of Chlorococcum and Scenedesmus (cultures were in triplicates for each nutrient/salinity).

Nutrient	Chlorococcum			Scenedesmus		
	Salinity			Salinity		
	X	16X	64X	X	8X	32X
C_1	0.147^{a}	0.180^{a}	0.113^{a}	0.157^{a}	0.202^{a}	0.127^{a}
C_2	0.047^{e}	0.082^{d}	0.035^{c}	0.056^{d}	0.065^{d}	0.033^{e}
HPO_4^{2-}	0.095^{bc}	0.157^{b}	0.075^{b}	0.070^{c}	0.081^{c}	0.057^{bc}
SiO_3^{2-}	0.058^{d}	0.088^{d}	0.038^{c}	0.051^{d}	0.068^{d}	0.037^{de}
NO_3^{-}	0.074^{c}	0.118^{c}	0.067^{b}	0.087^{b}	0.105^{b}	0.063^{b}
$EDTA^{2-}$	0.48^{de}	0.089^{d}	0.037^{c}	0.064^{cd}	0.071^{cd}	0.041^{cd}

C_1 and C_2 are nutrient enriched (1.8 mM HPO_4^{2-}, 0.2 mM SiO_3^{2-}, 1.8 mM NO_3^{-} and 0.2 mM $EDTA^{2-}$) and normal sterile pond water, respectively. Values indicated by same letters are not significantly different from each other through Duncan's multiple range test (DMRT).

Table 2. Effect of selective nutrient enrichment and salinity levels on chlorophyll content (mg/L) of Chlorococcum and Scenedesmus (cultures were in triplicates for each nutrient/salinity).

Nutrient	Chlorococcum			Scenedesmus		
	Salinity			Salinity		
	X	16X	64X	X	8X	32X
C_1	1.00^{a}	1.19^{a}	0.69^{a}	1.23^{a}	1.46^{a}	1.01^{a}
C_2	0.23^{d}	0.58^{d}	0.20^{c}	0.41^{c}	0.56^{cd}	0.22^{d}
HPO_4^{2-}	0.40^{bc}	0.99^{b}	0.41^{b}	0.48^{bc}	0.60^{c}	0.39^{b}
SiO_3^{2-}	0.31^{cd}	0.56^{d}	0.19^{c}	0.39^{cd}	0.48^{cd}	0.25^{d}
NO_3^{-}	0.46^{b}	0.71^{c}	0.39^{b}	0.60^{b}	0.86^{b}	0.37^{bc}
$EDTA^{2-}$	0.29^{d}	0.56^{d}	0.20^{c}	0.34^{d}	0.57^{cd}	0.29^{cd}

Legends as for table 1.

Harrison et al. (1990) reported that nitrogen and phosphorus starvation significantly affect the carbohydrate, fatty acid and protein contents in halotolerant algal cells, which increases the sensitivity of the species to salt stress. Our results show that the inhibition of growth of both species might be due to nutrient starvation in nutrient limited medium or salinity stress restricting nutrient uptake and slowing down the cell division rate. It can also be suggested that the reduced growth is the result of (a) higher accumulation of sodium and/or chloride ions

(b) differences in ion compartmentation, or (c) greater access of ions to cellular metabolic pool. These possibilities are not mutually exclusive. On the other hand, enhancement of growth of Chlorococcum and Scenedesmus is probably due to the nutrient enrichment, especially of nitrogen and phosphorus, overcoming the nutrient starvation and increasing the halotolerance of algae. The maximum growth at certain salinity levels probably remaining within an optimal range causes more nutrients available for algal metabolism which would have been otherwise not possible under low salinity levels. This may also be attributed to salting out phenomenon vis-a-vis low oxygen solubility coupled with high photosynthetic activity.

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