CHAPTER I

EXTENT OF HALOTOLENCNE IN PHORMIDUM AMBIGUUM: GROWTH
CHARACTERISTICS AND PIGMENT LEVELS.

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Salinity and saline agricultural soils have assumed a lot of importance especially in developing countries of the third world and this fact need not be over-emphasised. In recent past, there has been an obvious spurt in studies involving plant growth as affected by salinity. Understandingly so, most of them are related to eucaryotic crop plants. Lately, biologists have realised the importance of procaryotic microorganisms like blue-green algae, and bacteria not only as bio-fertilisers but as possible natural agents for reclamation of arid, saline soils. The importance of terrestrial cyanophytes and the role they may play towards the fertility of the soil has already been discussed in the introductory remarks earlier. As compared to higher plants (Greenway, 1968; Prisco and O'Leary, 1970), studies involving effects of sodium chloride salinity on growth characteristics of blue-green algae are few.

Brown (1964) has stated that whereas for a given concentration of ions in a natural environment, some of them are biologically more effective than NaCl, they are not as obviously important as NaCl in defining a range of a natural habitat; of the inorganic salts that are readily soluble in water, NaCl is the only one to provide a natural habitat for microorganisms.
effectively over its entire concentration range (0 - 6.2 molal). Sodium chloride is also a predominant component of saline soils (Richards, 1954).

The importance of sodium to the nutrition of blue-green algae was earlier emphasised by several workers (Benecke, 1898). Singh (1950) observed that blue-green algae grew well on certain saline soils of India where most plants with the exception of halophytes fail to grow. Later, Allen (1952), Gerloff et al. (1952), Allen and Arnon (1955) and Kratz and Myres (1955) - all in varying manner - reported positive growth responses of blue-green algal cultures to the presence of relatively high concentrations of sodium. Provasoli (1959, c.f. Hans and Sarger, 1966) while examining the causes of algal blooms in Lake Washington attributed them to increasing salinity. Barnett and Jaffrey (1964)* reported stimulatory effects on growth of some algae by salts of some monovalent anions like Na⁺ and K⁺. After a lag of some years thereafter, the advent of seventees saw a burst of renewed interest in the subject. This was probably encouraged by the realisation of the importance of algae in general to the study of plant biochemistry and a simultaneous understanding of the importance of salinity effects on plant growth. Thus, it was in 1971 that growth responses of certain blue-green algae to NaCl concentrations were recorded and researched upon from a limited physiological view-point by

* c.f. Hans and Sarger, 1966
Datterton and Van Allen. Later, Apte and Thomas (1971) examined the ability of nitrogen-fixing blue-green algae to grow under saline conditions and the kinetics of sodium uptake in these organisms; both the species of Anabaena studied by them were found to be sodium-requiring and fairly halotolerant. Ward and Wetzel (1975) studied the growth of heterocystous blue-green algae in various concentrations of sodium nitrate. Kessler (1976) after studying the comparative physiology and taxonomy of Chlorella sp. observed that some physiological tolerances including salt tolerance could be species-specific.

Against this background and in the context of the present investigation, a study of growth characteristics of P. ambiguaum which was isolated from the arid, saline soils of Kachchh, India and the extent to which it tolerates external concentrations of NaCl cannot be precluded. In addition, prior to any study concerning environmental effects on the physiology of a microorganism, it is important to understand its growth characteristics in the same environment. Thus, as a prelude to studies involving effects of salt stress/some enzymes, amino acid and protein metabolism, the extent of halotolerance of a representative terrestrial cyanophyte P. ambiguaum, was investigated.
**MATERIAL AND METHODS**

*Phormidium ambiguus* Gomont was isolated from other microorganisms forming a dry crust over a patch of semi-arid, virgin soil near Bhuj, Kachchh, India. This was done after growing the soil organisms in sterile Kratz and Myres' 'C' medium (detailed below) and later by repeated plating and isolation on sterilised agar media.

The composition of Kratz and Myres' 'C' medium (1955; c.f. Venkatraman, 1969) is as follows:

- \( \text{KNO}_3 \) : 1.0 g/litre
- \( \text{K}_2\text{HPO}_4 \) : 1.0
- \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \) : 0.250
- \( \text{Ca(NO}_3)_2 \cdot 4\text{H}_2\text{O} \) : 0.025
- \( \text{Na}_2\text{citrate} \) : 0.165
- \( \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \) : 0.020
- \( A_5 \) solution : 1.0 ml
- pH : 8.0

Volume made to 1 litre with double, glass-distilled water (GDW).
Composition of A5 micro-nutrient solution is as follows:

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂PO₄</td>
<td>2.900 g/litre</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>1.810</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.110</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.080</td>
</tr>
<tr>
<td>2(NH₄)₂O₇MoO₃·4H₂O</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Volume made to 1 litre with GDW.

The medium was contained in 250 ml Erleynmeyer flasks which were later autoclaved at 15-20 lbs. for 20 minutes. These were inoculated with the isolated alga homogenised after vigorous shaking with glass beads. The cultures were incubated under continuous illumination providing a light intensity of 5000 lux and a constant temperature of 25°C. All procedures related to the culturing of the organism were carried out under aseptic conditions.

Prior to any further studies, it was necessary to obtain E. ambiguum in a bacteria-free state. After several lengthy, unsuccessful attempts, only a combination of the methodology employed by Vaidya and Mehta (1975) and application of very dilute combination of antibiotics suggested by Jones et al. (1973) succeeded; the former method involves repeated plating on a solid medium followed by a treatment with soap-nut (Sapindus laurifolius) solution to loosen the mucilage and
final low-dose, short-term irradiation with UV rays; the antibiotics employed were Penicillin, Chloromphenicol, Neomycin and Streptomycin (Jones et al., 1973).

The stock culture of *E. ambiguum* was regularly screened for any bacterial contamination. For this purpose, a loopful of algal cells were always inoculated into tubes containing sterilised bacterial growth media viz. nutrient broth, nutrient broth plus 1.0% glucose and thioglycollate medium.

Nutrient broth:

- Peptone : 1.0% (w/v)
- NaCl : 0.5% "
- Beef extract : 0.3% "
- pH adjusted to 7.6

Thioglycollate Medium:

- Yeast extract : 0.500 g
- Casein hydrolysate : 1.500
- Glucose : 0.550
- L-Cystein : 0.050
- Agar : 0.075
- NaCl : 0.25
- Sod. thioglycollate : 0.05
- 1.0% 
- Aqueous methylene blue : 0.02 ml

Volume made to 100 ml with double, glass-distilled water
If these media remained free from any bacterial growth after an incubation period of 10 days at 30°-35°C., then, the alga was considered bacteria-free.

For growing bacteria-free _P. ambiguus_ under saline conditions, sodium chloride was added to 'C' medium so as to obtain media with 0.5, 1.0 and 2.0% NaCl (w/v). Growth medium labelled 'Control' did not contain any NaCl. A bit of thallus was first broken-up into a homogenous mass using sterile glass beads; with the help of a sterilised platinum loop, algal cells (always from a common source) were inoculated into a series of autoclaved 6'' x 1'' test tubes, each containing 10 ml of 'C' medium, a set each for control and varying concentrations of NaCl. All procedures connected with inoculation were carried out in aseptic conditions.

Algal bio-mass from each test tube was harvested on 7th, 14th, 21st and 28th day, washed thoroughly and quickly with distilled water to remove adhering salts, dried at 60°C. and weighed on pre-weighed chromium planchettes. Results (as means of triplicates) were plotted against time and growth curves were established (Table 1; Fig. 1, Plate I).

It is pertinent here to note that the culture of _P. ambiguus_
was not a synchronous one. For this experiment as also for others in this study, 3 loop-fulls of algal cells were picked up from a common source (broken-up, homogenous mass) and inoculated into the growth media. The experiment concerned with growth characteristics was repeated several times to establish a trend. An ideal case, where results in triplicate did not show significant variations, was selected for further study.

The measurement of relative growth rate (RGR) was first contemplated by Blackmann (1919) to determine the growth behaviour of higher plants.

C. RELATIVE GROWTH RATE (RGR)

The method of Blackmann (1919) was adopted for expressing RGR values in case of P. ambiguum. RGR was determined as the difference between the Naperian logarithms of the values of successive dry weights obtained on 7th, 14th, 21st and 28th days. The formula of Blackmann adopted here is as follows:

\[ RGR = \frac{\log_e W_1 - \log_e W_0}{t} \]

where \( W_1 \) and \( W_0 \) represent the successive weights (e.g. 14th day and 7th day respectively). Results have been presented in Table 2 and Fig. 2 of Plate 1.
Chlorophyll 'a' was extracted following the method of Harborne (1973). *P. ambiguum* was grown under varying salt concentrations as detailed earlier; algal material was harvested on the 18th day during the log phase of growth and weighed. Fresh material was always crushed in mortar with the aid of acid-washed, sterilised sand. Cold, 80% acetone was used as an extraction medium and a pinch of CaCO$_3$ was added to prevent phaeophytin formation; the resultant slurry was filtered and after repeated washings, the clear extract was made to volume with cold, 80% acetone. Absorbance readings were taken at 663 nm on a digital, Beckmann DU -2 spectrophotometer. For calculations, the following formula was used:

\[
\text{Chl. 'a' (mg/g dry wt.)} = \frac{12.3 \lambda_{663} - 0.86 \lambda_{645}}{a \times 1000 \times W \times V}
\]

where, 
- \(a\) = length of the light path
- \(W\) = dry weight in grams (after conversion from the fresh wt. actually used for extraction) and
- \(V\) = volume

The value, \(0.86 \lambda_{645}\) in the formula pertains to chlorophyll 'b'. This is presumed to be zero in blue-green algae.
O-phycocyanin was extracted in a similar way and simultaneously from the same source as that for the extraction of chlorophyll 'a'. Cold phosphate buffer, 0.15 M, pH 7.0 was found to be an efficient extraction medium. For calculations, the following procedure (Myres and Kratz, 1956) was followed.

(a) \[ D_p = 1.016 \ D_{618\text{nm}} - 0.203 \ D_{677\text{nm}} \]

where, \( D_p \) = Corrected Optical Density (O.D.) after considering a possible error due to red chlorophyll seen at 677 nm. (DU-2, Beckmann Spectrophotometer)

(b) \( D_p / D_{677\text{nm}} = 0.079 \) = Percent phycocyanin based on the value of Svedberg and Katsurai (1929; c.f. Myres and Kratz, 1956).

All pigment-extraction procedures were carried out in dark at 0-4°C. Results of this experiment have been presented in Table 3, Plate II: each value represents an average of triplicate readings and has been expressed as percentages on dry weight basis.
The alga, *P. ambiguum* grew well in culture medium not containing any sodium chloride (hereafter designated 'Control').

With increasing NaCl concentrations, the lag phase lengthened. This was more pronounced for alga growing in 1.0 % and 2.0 % NaCl. After this initial adaptation period, the growth picked up quickly (Plate I, Fig. 1). It was interesting to note that although the algal bio-mass decreased with an increase in NaCl concentration, relative growth rates for any concentration increased; between 14th day and 21st day (Plate I, Fig. 2) cells growing in 1.0 %, 2.0 % and 0.5 % NaCl (in decreasing order) showed faster R.G.Rs when compared with those of control. Algal cells in control and 0.5 % NaCl showed signs of degeneration after about 30 days of growth while cells growing in 1.0 % and 2.0 % NaCl continued to survive for about 10 days more although no further increase in weight was noted (not shown in Plate).
Chlorophyll 'a' content was adversely affected by increasing concentrations of sodium chloride (Table 3; Plate II). In cells grown in control, this PIGMENT STUDIES was 1.9% decreasing slowly to 0.8% in cells grown in 2.0% NaCl. C-phycocyanin content however increased simultaneously but was lower in cells grown in 2.0% NaCl (1.71%) than in control (2.3%). The ratio of C-phycocyanin to chlorophyll 'a' was two-fold its value in control at 0.5% and three fold at 1.0% NaCl levels; the same was two-fold at 2.0% NaCl level (Table 3).
DISCUSSION

Blue-green algae comprise a pre-dominant flora on the semi-arid and saline soils of Kachchh (Anantani, 1970; Brahmkshtri, 1979). Phormidium ambiguum is one such cyanophyte which survives in these soils where there are drastic variations in seasonal temperatures and which receives a deficient, erratic rainfall. The alga must therefore be surviving sharp fluctuations in soil salinity. Growth characteristics of P. ambiguum grown in varying concentrations of sodium chloride under laboratory conditions gave ample evidence of its halotolerant nature, surviving salt concentrations as high as 2.0% per cent. Salt not only sustained but enhanced its growth after an initial lag period (Plate I; Fig. 2). Several investigators have recently shown positive growth responses of blue-green and green algae to varying concentrations of NaCl in culture media (Matterton and Van Sallen, 1971; Apte and Thomas, 1974; Kessler, 1976). Stam and Holleman (1975) while studying the influence of differing salinity on growth and morphological variability of several Phormidium species observed that all fresh-water species decrease in growth with an increase in salinity; they however did not agree with Drouet's statements (1968; c.f. Stam and Holleman, 1975) that variations in the morphology
appear when trichomes regenerate after catastrophic changes in an environment such as salinity. In the present study too, no significant morphological changes were observed after subjecting the alga to a salt stress. Trichomes grew well after the initial osmotic-cum-ionic shock and the resultant lag phase which increased with an increase in salinity (Plate I).

Salinity effects on plants apparently have a dual role; while effecting osmoregulation and osmoadaptation in an organism, salts change both, the electrolyte balance of the plant tissue and its water relations; these may result in stimulation or curtailment of plant growth depending upon the effects on the metabolic status of the plant. As far as the electrolyte balance is concerned, it would be pertinent here to note that most living cells accumulate potassium in preference to sodium when the external medium is poor in potassium and rich in sodium (Epstein and Hagen, 1952). Miller et al. (1976) studied the intracellular ion content of a halophilic blue-green alga, *Aphanathece halophytica* as a function of age, external sodium and potassium and got results similar to those reported by Ginzburg et al. (1970) for a bacterium, *Halobacterium* species; their data supports the hypothesis put forward by Christian and Waltho (1962) that proposes a correlation between the extent of $K^+$ accumulation in a bacterial cell and salt...
tolerance, suggesting Na\(^+-K^+\) transport mechanism. However, both the organisms cited above are halophilic ones and a halophilic organism is perhaps a different entity from a halotolerant one. No conclusive evidence has been established as to the actual process of Na\(^+-K^+\) transport across a cell membrane of a halotolerant, blue-green alga. Batterton and Van Allen (1971) failed to observe any activity of Mg-dependent, ouabain-sensitive, Na\(^+-K^+\) transport ATPase enzyme in the cell membranes of fresh-water cyanophyte undergoing salt stress of up to 2.0 percent. Such an enzyme ("Sodium-pump") has been found in higher plants (Tikhaya et al., 1976-77). Apte and Thomas (1974) who studied Na\(^+\) uptake in 0.5% NaCl-tolerant species of Anabaena, found evidence that uptake of sodium by such halotolerant blue-green algae is a multiphasic process possibly with more than one carrier site for sodium uptake—a phenomenon similar to the multiphasic K uptake sites in barley plants (Epstein, 1966). They also observed (c.f. Apte and Thomas, 1974) that some photosynthetically linked active phenomena regulate sodium fluxes in the algae they studied.

As far as the pigments of blue-green algae are concerned, Chlorophyll 'a' is often considered as an index of growth. In the present investigation, chlorophyll 'a' was adversely affected by increasing salinity (Table 3; Plate II). Kim
(1958; c.f. Melachlan, 1961) also showed that in general, in higher plants, there is an inverse relationship between chlorophyll and salt content of the culture solution. In a marine diatom *Cyclotella cryptica*, Liu and Hellobust (1976 a) observed that the chlorophyll content per cell does not vary much as function of salinity in the range of 33 % - 100 % artificial sea water but decreases sharply above and below this range.

During harvest-time (18th day), the relative growth rate value was highest for algae grown in 1.0 % NaCl, followed in decreasing order by cells grown in 2.0 % and 0.5 % NaCl and control (Plate 1, Fig. 2); at the same time, chlorophyll 'a' content decreases linearly with an increase in salinity; apparently, RGR values do not have any correlation with the chlorophyll 'a' content; again, although the chlorophyll 'a' content was measured only during the growth peak of *E. ambiguus*, it can be presumed that the decreasing tendency in its content with increasing salinity will be observed at every stage of the growth. While the algal bio-mass goes down with an increases in salinity, the corresponding relative growth rates are faster (Plate 1, Fig. 2) thus indicating a 'shooting-up' tendency in cells undergoing salt stress after the initial adaptation period (lag phase). Apparently therefore, photosynthetic processes were not disturbed despite a loss in chlorophyll 'a' content under salt
stress. A possible explanation lies in the fact that another pigment, c-phycoerythrin showed a sharp increase with an increase in salinity (Table 3; Plate II); these values were 2.3% in cells grown in control and 3.6% in cells grown in 0.5% NaCl and 1.0% NaCl; c-phycoerythrin content in cells grown in 2% NaCl showed nearly 50% decrease when compared to those of control (Table 3). Phycocyanin to chlorophyll 'a' ratio increased 3-fold under 0.5% and 1.0% salt concentration and two-fold under 2.0% salt concentration when compared with the ratio in cells grown under control conditions (Table 3).

C-phycoerythrin is a blue, bile pigment-protein conjugate (biliprotein) characteristically found in blue-green algae. It was shown by Haxo and Blinks (1950) that phycocyanin was a principal, photosynthetic accessory pigment in these algae. It is well established (Duysens, 1952; c.f. Chapman, 1973) that biliproteins transfer absorbed light energy to chlorophyll with very high efficiency. The adverse effects of salinity on the content of chlorophyll 'a' noted here thus seem to be counter-balanced by a sharp increase in the phycocyanin to chlorophyll 'a' ratio. This probably results in an improvement in the efficiency of the energy transfer reactions which in turn, contributes to the enhanced growth rates of P. ambiguum under saline conditions despite a simultaneous loss in chlorophyll 'a'. Lately, there has been a good deal of
work on the amino acids of C-phycoeyanin with regards to salt tolerance; while a detailed reference has been made to this aspect in the relevant chapter in this thesis, it can perhaps be assumed here that increased phycocyanin levels in *E. ambiguum* under salt stress could be playing an important role in its halotolerance.

Studies on *Na⁺-K⁺* transport mechanisms or intracellular ionic balance were not possible in this laboratory. However, it can be said with a fair degree of assurance that the good halotolerance observed for *E. ambiguum* is related to some such process/es whereby the intracellular ionic balance as also the turgor pressure is maintained. This should result in disturbances to the metabolic status of a cell leading to shifts in enzyme activities and protein metabolism in general. With this in view, an attempt has been made in the chapters to follow, to understand certain enzyme activities, their electrophoretic patterns and protein and amino acid metabolism of *E. ambiguum* vis-a-vis NaCl-salinity.