

# Effect of salinity on growth, pigmentation, N<sub>2</sub> fixation and alkaline phosphatase activity of cultured *Trichodesmium* sp.

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**ABSTRACT:** *Trichodesmium* sp. isolated from the Great Barrier Reef lagoon was cultured in artificial seawater media containing a range of salinities. *Trichodesmium* sp. actively grew over a wide range of salinities (22 to 43 psu) and hence can be classed as euryhaline. Maximum growth occurred with salinities in the range 33 to 37 psu. Chl *a* content and alkaline phosphatase activity were found to increase with salinity over the range 22 to 43 psu, but the N<sub>2</sub> fixation rate was reduced at salinities below and above the range for maximum growth. Growth in media exhibiting maximum growth was characterised by well-dispersed cultures of filaments, while significant aggregations of filaments formed in other media. It is proposed that the tendency for *Trichodesmium* filaments to aggregate in media with salinities outside the range for maximum growth is an opportunistic response to a deficiency of cellular nitrogen, which results from the reduced N<sub>2</sub> fixation rates, and the aggregation occurs in order to enhance the uptake of combined N released within the aggregates and/or the N<sub>2</sub> fixation within the aggregates.

**KEY WORDS:** *Trichodesmium* sp. · Salinity · N<sub>2</sub> fixation · Alkaline phosphatase activity

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## INTRODUCTION

The diazotroph *Trichodesmium* is common in tropical and subtropical waters (Creagh 1985, Villareal & Carpenter 1990). In the Great Barrier Reef (GBR) lagoon, *Trichodesmium* blooms occur regularly, and on occasions blooms can extend for many hundreds of kilometers along the Queensland coast, extending from the shoreline to the outer barrier reef (Marshall 1933, Revelante & Gilmartin 1982). Recent studies have shown that active growth occurs at depth within the lagoon prior to bloom formation, which demonstrates that the observed surface aggregations are not simply blown in from the open sea (Bell et al. 1999). However, little is known about the causative factors of such blooms.

Several workers have observed that higher concentrations of *Trichodesmium* occur during periods of prolonged calm weather, i.e. during periods of relatively low wind activity (Marshall 1933, Revelante &

Gilmartin 1982, Bell et al. 1999). Some workers have noted that high concentrations of *Trichodesmium* tend to correlate with periods of low salinity i.e. 19.6 and 27 psu (Voltolina 1975, Eleuterius et al. 1981). However, other workers observed that high abundance of *Trichodesmium* occurred in higher salinities ranging from 32 to 36 psu (Dunstan & Hosford 1977, Jones et al. 1982, Revelante & Gilmartin 1982). Thus it would appear that *Trichodesmium* can grow over a wide range of salinities but the role of salinity *per se* on the growth of *Trichodesmium* is unknown.

It has been suggested that the most important effects of salinity on algal growth are the osmotic consequences of movements of water molecules along water-potential gradients, and the flow of ions along electrochemical gradients (Lobban & Harrison 1994). When submitted to any change in salinity, algae respond with an osmo-acclimation process or turgor pressure regulation. Some organisms are known to be osmotically adaptive and perform little actual work in

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adjusting to the external environment, while others are more sensitive to osmotic changes (Blinks 1951).

The salinity of open-ocean water is generally in the range of 34 to 37 psu, though lower in coastal waters and estuaries (Kirst 1989). Local salinity changes in near-shore regions such as the GBR lagoon can be far ranging, as they are affected by evaporation processes and the influx of rainfall and freshwater runoff: typical values are in the range of 27 to 36 psu (Revelante & Gilmartin 1982). A correspondence of low salinities with high silicate levels in the northern and central GBR lagoon indicates that the reduced salinity is due to the river discharge, and not simply to precipitation (Revelante & Gilmartin 1982, Bell & Gabric 1990, Bell 1992).

It is hypothesised that variations in salinity, which would occur within the GBR lagoon from time to time, could be a factor in the bloom-forming potential of *Trichodesmium*. This paper investigates the response of *Trichodesmium* to hypo- and hypersaline conditions. In particular, we examine the effects of salinity on the growth rate, N<sub>2</sub> fixation rate and chl *a* content of recently established laboratory cultures of *Trichodesmium* GBRTRLI101 under various external salinities. The ability of *Trichodesmium* to utilise organic forms of phosphorus has previously been demonstrated (Yentsch et al. 1972, Stihl et al. 2001, Mulholland et al. 2002), and hence the effect of salinity on alkaline phosphatase activity (APA) is also investigated.

## MATERIALS AND METHODS

**Media and growth experiments.** The *Trichodesmium* sp. GBRTRLI101 used in the present study was isolated from waters near to Low Isles in the Northern GBR lagoon. The initial culture was established in an enriched seawater medium and subsequent cultures of this strain were established in an artificial seawater medium (Bell et al. unpubl.). The artificial seawater medium used is a modified version of the Aquil medium (Morel et al. 1979, Bell et al. unpubl.). In particular, Si and N are not added and the phosphorus concentration (added as K<sub>2</sub>HPO<sub>4</sub>) is reduced from 10.0 to 3.0 µM. A suitable range of lower salinities (18, 22, 26, 29, and 33 psu) was obtained by diluting the principal salt solution (37 psu) with appropriate amounts of deionised Milli-Q (MQ) water prior to the addition of trace components, namely phosphorus, vitamins (B<sub>12</sub>, Biotin, Thiamine HCl) and trace metals (Mn, Zn, Fe, Mo, Cu, Co). The high salinity medium (43 psu) was produced by evaporation of the principal salts solution in a glass beaker at approximately 70°C prior to the addition of trace components. Thus any observed

effects of varying salinity are attributable to changes of salinity *per se* and not to the availability of trace components. All glassware was soaked in 0.1 M HCl for at least 1 d, rinsed with MQ water and autoclaved at 120°C for 30 min. There were 3 replicates for each treatment. The growth experiments were carried out in 100 ml conical flasks (stoppered with cotton wool) at a temperature of 25 ± 3°C, under an external photosynthetically available radiation (PAR) of 45 ± 2 µmol quanta m<sup>-2</sup> s<sup>-1</sup> using cool white fluorescent lights with a light:dark cycle of 14:10 h. All transfers were conducted under a laminar-flow hood and all media were filter sterilised (0.2 µm) to minimise microbial contamination. Examination of cells under ×1000 magnification revealed no other microorganisms and hence it was concluded that bacterial contamination was minimal. Time-course of growth curves in different salinities was constructed over a growth cycle based on cell yields.

**Determination of specific growth rate and pigment analysis.** Biomass was estimated by counting the number of filaments per ml (using at least 3 sub-samples of 0.25 ml) on a counting slide and determining the average length per filament with an eyepiece micrometer. The average cells per filament were counted under a 400-fold magnification on a microscopic slide with cover slip. Specific growth rate (µ) was calculated from the initial cell yields (C<sub>0</sub>) on Day 0 and the cell yields (C<sub>6</sub>) on Day 6 using the equation:  $\mu = (\log_e C_6 - \log_e C_0)/6$ . Chl *a* was determined with a Hitachi U-1100 spectrophotometer using the procedure given by Jeffrey & Humphrey (1975).

**Measurement of alkaline phosphatase activity (APA).** APA was determined in 3 replicates using p-nitrophenyl phosphate (pNPP) as a dissolved organic substrate (Li et al. 1998). Ten ml of cultures were concentrated on the Whatman GF-C filter and immersed in 6 ml of artificial seawater. AP activity was determined after addition of 0.3 ml of 10 mM pNPP, 0.81 ml trisglycine buffer (50 mM, pH 8.5) and 0.081 ml of 1 mM MgCl<sub>2</sub>. Samples were incubated for 3 to 5 h at 25°C and the absorbance read at 410 nm against a blank (buffer and substrate in artificial seawater solutions without *Trichodesmium*) in the spectrophotometer. Changes of the colour in the incubation solution were directly related to the enzymatic hydrolysis of pNPP. Enzymatic activity was expressed as µmol of paranitrophenol (pNP) released cell<sup>-1</sup> h<sup>-1</sup>. This assay method permits the incubation of the whole algae, so it is possible to estimate the overall enzymatic activity.

**Measurement of N<sub>2</sub> fixation rate.** N<sub>2</sub> fixation was measured on samples after 7 d growth using the acetylene reduction assay (Capone 1993). Sub-samples of the cultures contained in media of different salinities were treated in 15 ml wide-mouth serum bottles. After

sealing with red silicone-rubber serum stoppers, 1 ml of acetylene was injected and the sample was then shaken gently. A gas phase sample (0.1 ml) was extracted from each bottle at zero time using a gas-tight syringe, and was analysed immediately for ethylene concentration using a Photovac 10s Plus portable gas chromatograph (GC) fitted with a photoionisation detector (PID). The bottles were incubated under cool fluorescent lights ( $45 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) for 3 h at room temperature ( $25^\circ\text{C}$ ). Gas samples were then extracted and analysed for ethylene concentration on the GC. The gas phases of several control blanks (without *Trichodesmium* but with acetylene) were also analysed at zero time and after the incubation. Cell numbers were determined by microscopic examination. A 4:1 ethylene:nitrogen molar ratio was assumed in the calculations of the  $\text{N}_2$  fixation rates (Capone 1993).

## RESULTS AND DISCUSSION

### Effect of salinity on growth

Active growth of *Trichodesmium* GBRTRLI101 was observed over a range of salinities from 22 to 43 psu with maximum growth rates and cell yields occurring in the range 33 to 37 psu (Figs. 1 & 2A). However no active growth was observed in the lowest salinity medium (18 psu) and the exponential growth period in the 22 psu medium was severely curtailed. Overall the results suggest that *Trichodesmium* sp. could be classed as euryhaline, as it tolerated a wide range of

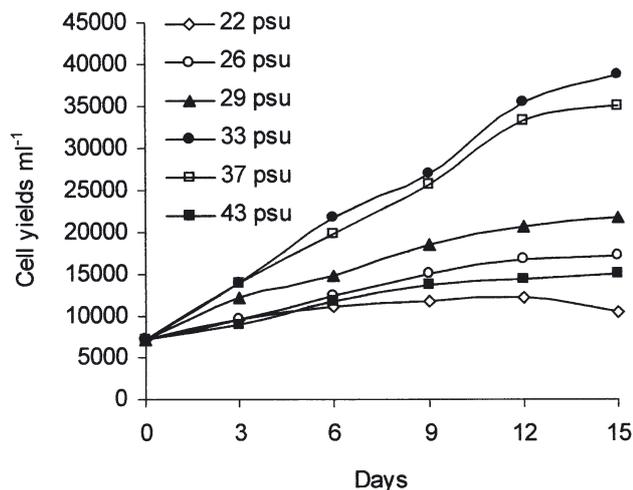


Fig. 1. *Trichodesmium* GBRTRLI101. Time-course of growth at different salinities. Values were the average of 3 replicates and reproducible within 5 to 10%

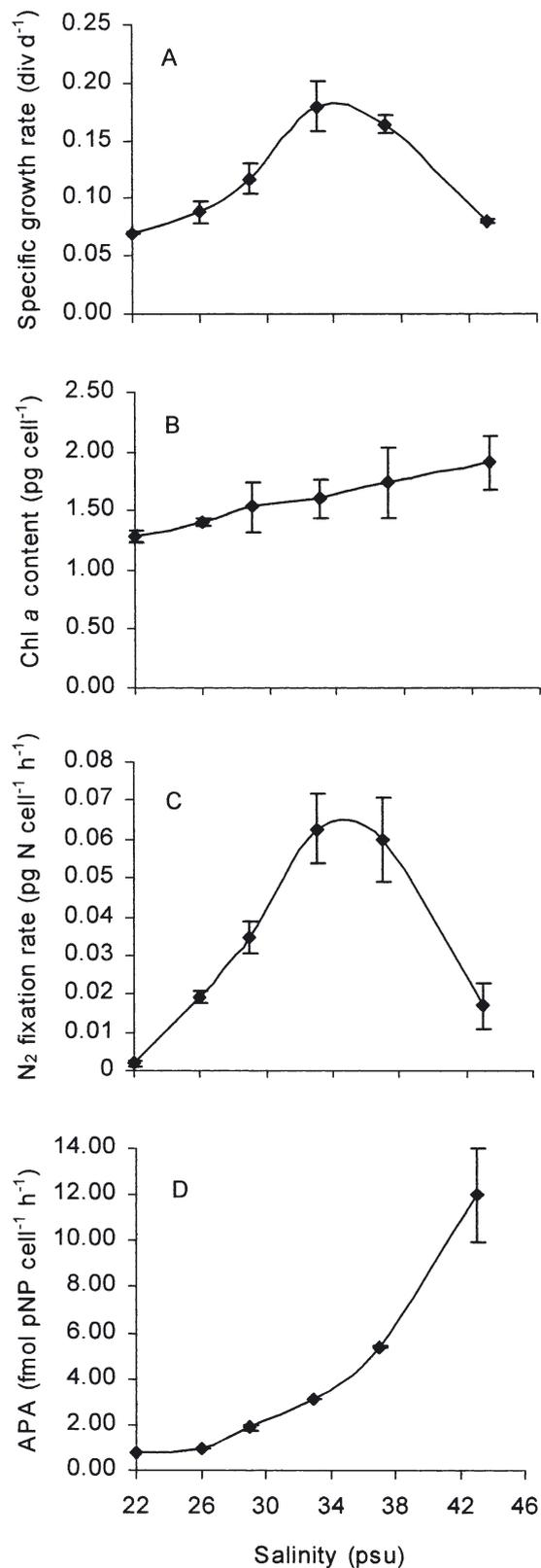


Fig. 2. *Trichodesmium* GBRTRLI101. Variation at different salinities of (A) specific growth rate, (B) cellular chl *a*, (C)  $\text{N}_2$  fixation rates and (D) alkaline phosphatase activity (APA). Values shown are means ( $n = 3$ )  $\pm$  SD

salinities with maximum growth occurring in salinities characteristic of normal marine waters. Maximum growth occurring in the range 33 to 37 psu is in agreement with the historical findings in the GBR lagoon. Jones et al. (1982) reported that the main pulse of *Trichodesmium* trichomes was detected in the water column from late August to mid October when salinities were high (36 psu) and rainfall was negligible (<30 mm). Revelante & Gilmartin (1982) documented that *Trichodesmium* reached its highest densities during periods of low to no precipitation with salinities in the range of 34 to 35.5 psu. *Trichodesmium* GBRTLI101 also exhibited active growth in the lower salinities ranging from 22 to 29 psu. This observation is consistent with the finding that blooms of *T. erythraeum* occurred in the northern Gulf of Mexico with salinities around 27 psu (Eleuterius et al. 1981). The reduction in growth rate of *Trichodesmium* sp. at low (<30 psu) and high (>40 psu) salinity supports the conclusions of Kirst (1989), who notes that the energy expended by algae in attempting to maintain turgor pressure will ultimately be reflected as a decrease in productivity. However, salt stress influences the physiology of cyanobacteria in other ways. For example, stress could be due to ion imbalance and/or induced nutrient deficiencies (Sellner et al. 1988, Shukla et al. 1997), or as some workers have suggested, a decline in photosynthesis under hyperosmotic stresses (Reed 1983, Wright & Reed 1985) could be due to changed fine structure of the chloroplasts (Wiencke 1982), causing a disruption of energy transfer between the 2 photosystems (Karsten & Kirst 1989). The results given below show that the N<sub>2</sub> fixation rate is also reduced in low and high salinities, which suggests the reduction in growth rate at high and low salinities could be largely due to the corresponding reduction in the N<sub>2</sub> fixation rates.

It is noted that large irregular filament aggregates or bundles (~160 filaments per bundle) formed early within the exponential growth phase (within 3 or 4 d) in all media with low salinities (<33 psu), while the filaments remained dispersed as individual filaments or aggregates of only a few filaments in the cultures in the media exhibiting the maximum growth rates (salinity range 33 to 37 psu Fig. 3A–D). Aggregations eventually formed in older cultures grown in the 33 to 37 psu salinity range (Fig. 3E), but these aggregations had a form that is normally attributed to *T. erythraeum*. Significant aggregation also occurred in the highest salinity medium (43 psu) during the exponential growth phase, but these aggregates contained far fewer filaments than those formed in the low-salinity media. Relative to the aggregates forming in the older cultures, the aggregates in the low salinities were compacted tightly and could not be broken apart with

moderate agitation. Prufert-Bebout et al. (1993) observed filament aggregations in the late stage of growth of *Trichodesmium*, but these aggregations were only loosely held together.

#### Why does *Trichodesmium* aggregate in low salinities?

While it is common knowledge that *Trichodesmium* does at times tend to form aggregations/bundles/colonies, the phenomenon observed here, namely that changes in salinity can lead to aggregation, has not previously been documented for *Trichodesmium*. However, Sellner et al. (1988) did observe that increasing salinity caused the aggregation of *Microcystis* sp. in estuarine waters. They suggested that this aggregation phenomenon probably resulted from decreased photosynthetic ability and substitution of divalent cations between hydrophilic side groups of mucilage surrounding the cells.

A review of the literature on conditions promoting microbial aggregation indicates that a common condition inducing aggregation is cell starvation (Calleja 1984). Logan & Hunt (1988) note that, for a pure culture of organisms to biofloculate when substrate is nearly depleted, the implication is that cell aggregations confer some advantage over freely dispersed cells, or in other words, the metabolic efficiency of aggregated cells is higher than that of cells in a dispersed state. Tsao & Hsu (1990) propose that a possible driving force for microbial aggregation is that the metabolic reaction of microorganisms is of an autocatalytic nature when there is a shortage of nutrients. For example, growth within an aggregate may be higher because nutrient uptake is higher than that of freely dispersed cells.

Now as discussed below it was found that N<sub>2</sub> fixation was reduced at salinities below and above the range for maximum growth (33 to 37 psu), and hence it is proposed that the tendency for *Trichodesmium* filaments to aggregate at salinities outside the range for maximum growth is an opportunistic response to a deficiency of cellular nitrogen, and the aggregation occurs in order to enhance N<sub>2</sub> fixation and/or the uptake of combined N released within the aggregates. Several studies have suggested that colony or aggregate formation promotes growth and survival of cyanobacteria by providing an increase in N<sub>2</sub> fixation rates; the increased rates being attributed to the occurrence of low partial pressures of O<sub>2</sub> within the aggregates where photosynthetic O<sub>2</sub> production is reduced, its consumption is enhanced, or both (Paerl & Bebout 1988, Carlton & Paerl 1989). Paerl et al. (1989) have confirmed, using antinitrogenase polyclonal antibodies, that most cells in trichomes distributed in aggregates

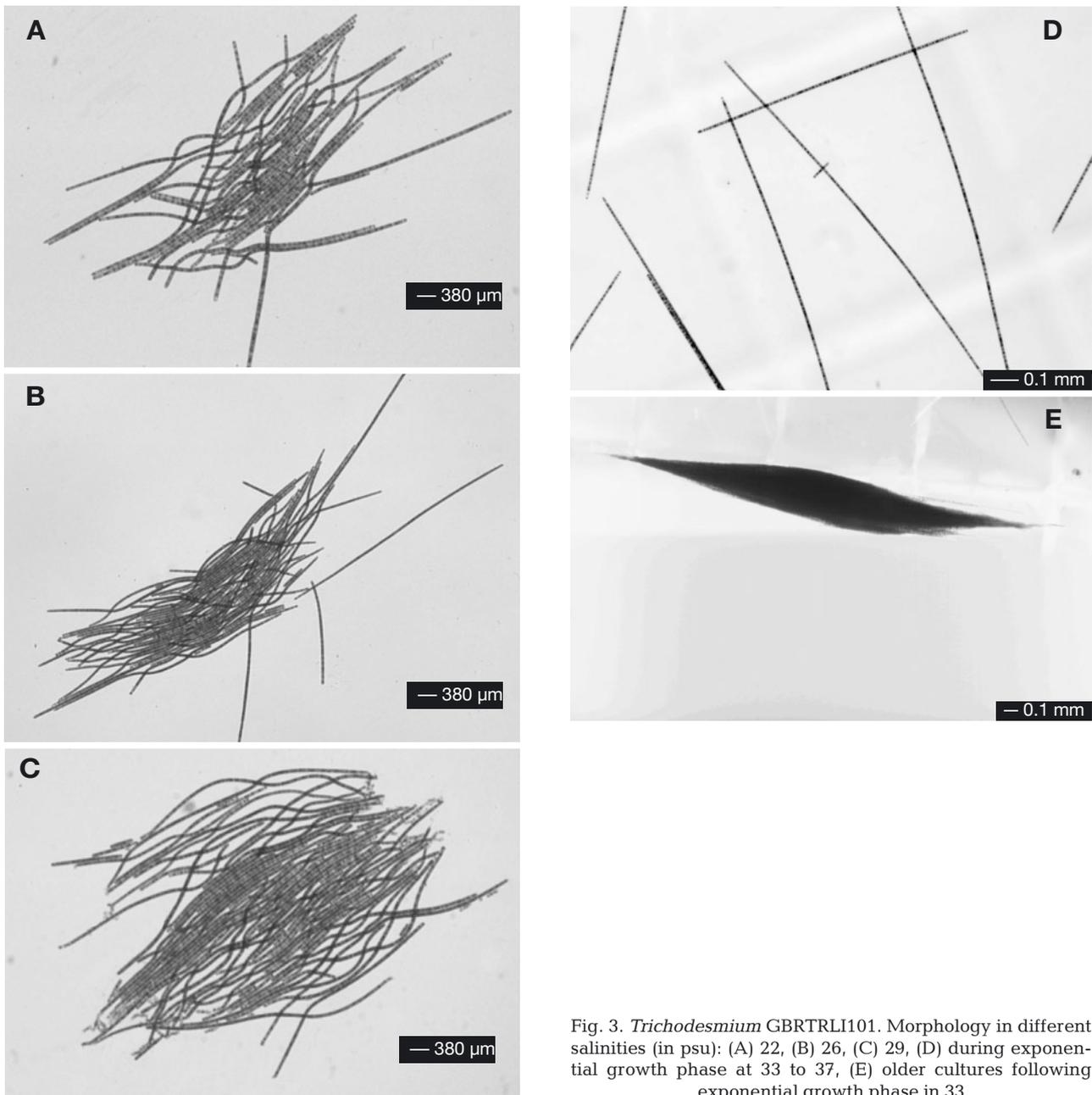


Fig. 3. *Trichodesmium* GBRTLI101. Morphology in different salinities (in psu): (A) 22, (B) 26, (C) 29, (D) during exponential growth phase at 33 to 37, (E) older cultures following exponential growth phase in 33

can fix  $N_2$ , and in a later study Paerl (1994) reported that chl *a*-specific  $N_2$  fixation was generally higher in large aggregates than single filaments.

#### Influence of salinity on the chl *a* contents of cells

There was a general trend of increasing chl *a* content with increasing salinity (Fig. 2B) which agrees with the results from other studies on salt tolerant algae e.g. studies on *Dunaliella salina* (Al-Hassan et al. 1987), the red alga *Bostrychia radicans* (Karsten &

Kirst 1989) and green alga *Ctenocladus* (Herbst & Castenholz 1994). The lower cell concentration of chl *a* at lower salinities (<29 psu) could have contributed to the lower growth rates at these lower salinities, while the higher value recorded for the high salinity (43 psu) could be a salinity induced response of the alga that allows it to maintain a reasonable growth rate. Another possible reason for the decrease is that proposed by Apte & Alahari (1994) in their studies on *Anabaena* sp., namely that  $K^+$  and  $Na^+$  deficiency leads to a decrease in chl *a* (and phycocyanin) with decreasing salinity.

### Effect of salinity on N<sub>2</sub> fixation rate

The variation in N<sub>2</sub> fixation rate follows the same general trend as that observed for cell yields, namely the maximum N<sub>2</sub> fixation rate occurs around normal marine salinities and that the rate drops off significantly with increasing and decreasing salinity (Fig. 2C). The reported N<sub>2</sub> fixation rates are for Day 7 of the growth cycle, and hence were measured during the exponential growth phase for all cultures except the 22 psu culture (Fig. 1). Thus the results do suggest that N<sub>2</sub> fixation rate of *Trichodesmium* sp. is particularly susceptible to osmotic stress. As noted by Blumwald & Tel-or (1982), the sensitivity of N<sub>2</sub> fixation rate to salinity suggests it would be a more useful measure of the degree of salt adaptation than productivity. Overall, the results are in agreement with those of Huber (1986) and Nordin (1974) (as reported by Huber 1986) who showed the salinity for maximum N<sub>2</sub> fixation rate by *Nodularia spumigena* was similar to that of maximum growth. Tel-or (1980) examined the effects of salinity on nitrogenase and related enzyme activities in the cyanobacterium *Calothrix scopulorum* in some detail, and found that electron transfer via the ferredoxin: ferredoxin-NADP reductase was very salt sensitive.

Work on *Anabaena torulosa* showed that nitrogenase proteins were synthesized under Na<sup>+</sup> deficiency but these were inactive, and that Na<sup>+</sup> is necessary for diazotrophic growth (Apte & Alahari 1994). It has been suggested that this requirement for Na<sup>+</sup> is related principally to the transport, and hence uptake, of P. Any reduction of P uptake would limit the availability of ATP and other nucleotide triphosphates, which would lead to a reduction in nitrogenase activity (Apte & Alahari 1994). Apte & Alahari (1994) conclude that observed reductions in photosynthetic activity of diazotrophs due to Na<sup>+</sup> deficiency could be a direct effect of nitrogen starvation.

Further work is required to determine the actual cause of the reduction in nitrogenase activity in hyper (>37 psu) and hypo (<33 psu) salinity for *Trichodesmium* sp. However, the conclusion of Apte & Alahari (1994) that reductions in photosynthetic activity are a direct effect of nitrogen starvation is consistent with the obvious correlation between N<sub>2</sub> fixation rates and on the observed aggregation phenomenon.

### Effect of salinity on alkaline phosphatase activity

Salinity significantly changed APA in *Trichodesmium* sp. (Fig. 2D). The general tendency was, as with chl *a* content, that APA increased with salinity. Overall, the results are in agreement with the work of Wilson

et al. (1964) in their studies on *Escherichia coli* and that of Hernández et al. (1992) with their work on *Porphyra umbilicalis*. Hernández et al. (1992) did find that salinities above 45 psu inhibited the enzymatic activity.

It is documented that the influence of salinity on APA is partly because of the increased ionic strength (Wilson et al. 1964). However, it is unclear whether the changes in APA with salinity are due to the influence of salinity itself or to the influence of particular ions. For example, Mahasneh et al. (1990) have shown that there was a marked rise in cell-bound APA with increased Ca<sup>2+</sup>, but a decrease in APA at higher concentrations of Mg<sup>2+</sup> in the blue-green alga *Calothrix viguieri* D253. The enhancement of APA in response to increased salinity could be due to a reduction in phosphate uptake. For example, it is possible that a change in external Na<sup>+</sup> concentrations or other ions, or the imbalance of an ion ratio, could reduce the uptake of phosphate by *Trichodesmium*, and this would then lead to an increase in APA. Further specific studies are required to delineate the effects of Na<sup>+</sup> and other cations (i.e. Mg<sup>2+</sup>, Ca<sup>2+</sup> or Zn<sup>2+</sup>) on the regulation of phosphate uptake in *Trichodesmium*.

### CONCLUSIONS

The present study has demonstrated that salinity is an important parameter in controlling the growth of *Trichodesmium* sp. through its effects on the N<sub>2</sub> fixation rates, APA and chl *a* synthesis. The results show that maximum growth and N<sub>2</sub> fixation rates occur for salinities in the range of 33 to 37 psu. Chl *a* content and APA were found to increase with salinity over the range of 22 to 43 psu. Growth in media exhibiting maximum growth was characterised by well-dispersed cultures of filaments, while significant aggregations of filaments formed in other media. It is proposed that the tendency for *Trichodesmium* filaments to aggregate in media with salinities outside the range for maximum growth is an opportunistic response to a deficiency of cellular nitrogen. This is thought to result from the reduced N<sub>2</sub> fixation rates, and the aggregation occurs in order to enhance the uptake of combined N released within the aggregates and/or N<sub>2</sub> fixation rate within the aggregates.

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