Marine Biology 88, 1–9 (1985)

Marine Biology
International Journal on Life in Oceans and Coastal Waters
© Springer-Verlag 1985

# Osmotic adjustment and organic solute accumulation in unicellular cyanobacteria from freshwater and marine habitats

**R. H. Reed and W. D. P. Stewart**

**Department of Biological Sciences, University of Dundee; Dundee DD1 4HN, Tayside, Scotland**

## Abstract

The intracellular concentrations of low-molecular weight carbohydrates and quaternary ammonium compounds present in 26 axenic isolates of unicellular cyanobacteria have been studied over a range of external salinity from freshwater up to 300% seawater (100% = 35‰ S). In all cases, a single carbohydrate, either sucrose or glucosylglycerol, was identified as the principal organic osmoticum, showing major variation in response to the external salt concentration; quaternary ammonium compounds were present in osmotically insignificant amounts. Glucosylglycerol was accumulated as primary osmoticum by nine of the isolates from saline habitats and by five of the freshwater isolates; trace amounts of sucrose were also present. The remaining twelve freshwater strains accumulated sucrose as sole osmoticum. Glucosylglycerol-accumulating strains grew over the widest salinity range (up to 200 to 250% seawater), whether isolated from saline or non-saline habitats. Sucrose-accumulating strains were more stenohaline, growing only in up to 50 to 100% seawater and showing no sustained growth in hypersaline media (> 100% seawater). The data suggest that (1) glucosylglycerol accumulation is not unique to marine cyanobacteria, and (2) the upper salinity limit for growth may be linked to organic solute accumulation, rather than habitat, with glucosylglycerol-accumulating isolates having a greater potential for growth in salt-stressed conditions than sucrose accumulators.

## Introduction

Cyanobacteria (blue-green algae) occur in aquatic habitats of varied ionic composition and salinity, ranging from freshwater, through brackish and marine conditions, to hypersaline environments (Fogg *et al.*, 1973; Carr and Whitton, 1982). Recent studies have shown that long-term growth and osmotic adjustment in saline media may be linked to the accumulation of specific low-molecular weight organic solutes to achieve osmotic balance (see Reed *et al.*, 1984 b). To date, the carbohydrates glucosylglycerol (Borowitzka *et al.*, 1980), sucrose (Blumwald and Tel-Or, 1982; Erdmann, 1983) and trehalose (Reed and Stewart, 1983) have been identified as major components of intracellular osmotic pressure in isolates from freshwater and marine habitats. In addition, the quaternary ammonium-compound glycine betaine (tri-methyl glycine) has been shown to function as the principal organic osmoticum in several unicellular isolates from hypersaline environments (Mohammad *et al.*, 1983; Reed *et al.*, 1984 a), while the novel osmoticum glutamate betaine (tri-methyl glutamate) has recently been reported in two halotolerant isolates of filamentous, $N_2$-fixing cyanobacteria belonging to the genus *Calothrix* (Mackay *et al.*, 1984). Inorganic ionic solutes (primarily $K^+$) have also been implicated in the osmotic adjustment processes of an isolate of *Aphanothece halophytica* (*Synechococcus*) from a hypersaline solar evaporation pond in California, USA (Miller *et al.*, 1976; Yopp *et al.*, 1978).

In a previous study, Mackay *et al.* (1983) investigated the salt tolerances and organic solute accumulation profiles of 28 isolates of unicellular and filamentous cyanobacteria from freshwater, marine and hypersaline habitats. These isolates were classified into three physiological groups on the basis of their organic osmotica coupled with their upper salt tolerance limits, using visual estimation of growth in saline media. Eleven of these strains were found to accumulate glucosylglycerol in response to salt stress, growing in media containing up to 60–110 g $dm^{-3}$ NaCl; of this group, only one isolate was not of marine origin (*Synechocystis* PCC 6714), and these strains were thus designated as "marine". In contrast, the remaining eleven isolates from freshwater habitats grew in media containing up to 14–45 g $dm^{-3}$ NaCl, accumulating simple sugars (rather than the heteroside glucosylglycerol) in response to osmotic stress, and were designated as "freshwater" forms.

A third group (the most halotolerant isolates) grew up to 130–158 g $dm^{-3}$ NaCl, containing quaternary nitrogen compounds as organic osmotica. On the basis of these observations, Mackay *et al.* (1983) proposed that glucosylglycerol-accumulating strains showing growth under laboratory conditions at NaCl concentrations up to 60–110 g $dm^{-3}$ be classified as "marine", regardless of their origins. Furthermore, they have suggested that each of the three groups of cyanobacteria recognised in their study has a biosynthetic pathway for the formation of specific organic osmotica that is absent from the other two groups, and that the functioning and control of these pathways can be used as a distinguishing feature in classifying cyanobacteria. This hypothesis has been re-stated in a more recent paper (Mackey *et al.*, 1984), which gives details of the organic solute profiles of a further eight isolates, with the conclusion that the chemical class of the solute accumulated in response to salt stress was correlated with the original habitat of the strain.

In contrast, Reed *et al.* (1984b) have published data for the major low-molecular weight carbohydrates accumulated by 71 strains of cyanobacteria from freshwater, brackish and marine habitats with the observation that only 41% of the isolates from brackish and marine habitats accumulated glucosylglycerol, while 59% accumulated either sucrose or trehalose. From a total of 49 freshwater-derived strains, 94% accumulated sucrose or trehalose and 6% (three isolates) accumulated glucosylglycerol in response to hyperosmotic stress. Thus, while the *trend* was towards glucosylglycerol production in marine isolates and sucrose/trehalose accumulation in freshwater forms, no *absolute* differences were observed between cyanobacteria isolated from each habitat. On the basis of these results, Reed *et al.* (1984b) have suggested that glucosylglycerol production is not a unique characteristic of marine cyanobacteria, rejecting the hypothesis of Mackay *et al.* (1983).

In both of these studies, a limited amount of quantitative data was presented to support each proposal. In particular, no values were given for the intracellular levels of carbohydrates in each isolate, nor of any variation with external salinity. The latter character is common to all osmotica that function as turgor-regulating agents, since changes in the intracellular level of these compounds upon upshock and/or downshock bring about a recovery of cell turgor towards its original level (Cram, 1976; Wyn Jones and Gorham, 1983). Data for the intracellular concentrations of low-molecular weight carbohydrates in cyanobacteria are essential in order to assess the osmotic significance of these compounds and their quantitative role in turgor generation and turgor-regulation; to date, these data have been provided for a limited number of strains, including *Synechococcus* RRIMP-N-100 (Borowitzka *et al.*, 1980), *Synechocystis* PCC 6803 (Richardson *et al.*, 1983), *Rivularia atra* (Reed and Stewart, 1983) and *Nodularia harveyana* (Warr *et al.*, 1984a). Furthermore, the salinity ranges for growth have not been described in detail in all previous studies, with generalised statements replacing data for salinity tolerances. The use of some non-axenic isolates (Mackay *et al.*, 1983; Reed *et al.*, 1984b) may also have added further to experimental variation. Mackay *et al.* (1984) have also suggested that the data of Reed *et al.* (1984b) may not provide a comprehensive picture of organic solutes, since only low-molecular weight carbohydrates, rather than quaternary ammonium compounds were analyzed; they have suggested that some of the strains may also accumulate betaines in addition to sucrose or trehalose, and that carbohydrate content alone may not permit a distinction between "hypersaline" and other strains.

The present study represents a synthesis of our recent research on the accumulation of carbohydrates and quaternary ammonium compounds in 26 axenic isolates of unicellular cyanobacteria belonging to the genera *Synechococcus* and *Synechocystis* (Rippka *et al.*, 1979). Unicellular strains have been used, in preference to filamentous forms, because determination of their sizes, using an electronic particle size analyzer (Parsons, 1973), provided data for cell volume that could be used to estimate the intracellular concentrations (and, hence, the osmotic significance) of low-molecular weight carbohydrates. This has enabled us to investigate the effects of varying external salinity upon the intracellular concentration of low-molecular weight carbohydrates, expressing the data in terms of cell volume (Richardson *et al.*, 1983). The results presented below indicate that the ability of cyanobacteria to withstand salinity stress may be governed by the type of organic solute produced in response to salt stress, with no *absolute* relationship between habitat and organic-solute accumulation-profile.

## Materials and methods

### Cyanobacteria and growth conditions

All isolates were obtained from the culture collection of the Institut Pasteur (PCC; 28, Rue du Dr. Roux, Paris, France) and were maintained in axenic culture throughout the present study. Upon arrival, aliquots from slope cultures were incubated in BG-11, a freshwater-based growth medium (Rippka *et al.*, 1979) and BG-11SW, a marine medium (i.e., BG-11 medium plus the following additional salts: NaCl, 467 mmol $dm^{-3}$; $MgSO_4$, 28 mmol $dm^{-3}$; $MgCl_2$, 25 mmol $dm^{-3}$; KCl 10 mmol $dm^{-3}$; and $CaCl_2$, 10 mmol $dm^{-3}$; to give a final salinity equivalent to that of full-strength seawater, i.e., 35‰ S) at 25 °C and under constant illumination (at a photon fluence rate of 15 to 20 $\mu$mol $m^{-2}$ $s^{-1}$. Strains were categorized according to their subsequent growth in BG-11 only (Category A), BG-11SW only (Category B) or BG-11 and BG-11SW (Category C), as shown in Table 1. Isolates were subsequently maintained in BG-11 medium where possible (i.e., Category A and C strains); BG-11SW was used only for strains showing no growth in freshwater BG-11 medium (i.e., *Synechococcus* PCC 7003 and PCC 7335). Details of the origins of each isolate are also given in Table 1 (see also

**Table 1.** Marine, brackish and freshwater isolates of unicellular cyanobacteria: origins and growth in freshwater/marine media. +: growth; –: no growth. Designation: F: strains isolated from freshwater habitats, using freshwater-based culture media; S: strains isolated from saline/marine habitats in seawater-based media. Category – three categories of cyanobacteria have been recognised: A, growing in BG-11 and not in BG-11SW; B, growing in BG-11SW and not in BG-11; C, growing in both media

| PCC strain No. | Origin[a] | Growth in BG-11 | Growth in BG-11SW | Designation | Category |
|---|---|---|---|---|---|
| Genus: *Synechococcus* | | | | | |
| 6301 | Freshwater, Texas, USA | + | – | F | A |
| 6307 | Lake water, Wisconsin, USA | + | – | F | A |
| 6311 | Freshwater, California, USA | + | + | F | C |
| 6907 | Pond water, Cambridge, UK | + | – | F | A |
| 6908 | Source unknown (freshwater?) | + | – | F | A |
| 7002 | Marine mud, Puerto Rico | + | + | S | C |
| 7003 | Clam bed (marine), Connecticut, USA | – | + | S | B |
| 7117 | Low-salinity brine pond, W. Australia | + | + | S | C |
| 7202 | Alkaline saline pond, Chad | + | + | S | C |
| 7335 | Intertidal marine, Mexico | – | + | S | B |
| 73109 | Sea water, New York, USA | + | + | S | C |
| 7424 | Rice paddy, Senegal | + | + | F | C |
| 7502 | Bog, Kastianenbaum, Switzerland | + | – | F | A |
| 7943 | Freshwater | + | – | F | A |
| Genus: *Synechocystis* | | | | | |
| 6308 | Lake water, Wisconsin, USA | + | – | F | A |
| 6701 | Freshwater, California, USA | + | + | F | C |
| 6702 | Freshwater, California, USA | + | + | F | C |
| 6714 | Freshwater, California, USA | + | + | F | C |
| 6803 | Freshwater, California, USA | + | + | F | C |
| 6806 | Freshwater, California, USA | + | + | F | C |
| 6808 | (Freshwater?) California, USA | + | – | F | A |
| 6902 | Brackish water, Oregon, USA | + | + | S | C |
| 6905 | Low-salinity brine pond, California, USA | + | + | S | C |
| 7008 | Pond water, California, USA | + | + | F | C |
| 7201 | Above high water, California, USA | + | + | S | C |
| 7509 | Rock surface, Switzerland | + | – | F | A |

[a] See Rippka *et al.* (1979) for details of strain histories and isolation procedures

**Table 2.** Organic solute accumulation in unicellular cyanobacteria, according to revised classification of Rippka and Cohen Bazire (1983). Designations as in Table 1. +: present; –: absent; t: trace quantities

| PCC Strain No. | Revised classification | Designation | Organic solute accumulated | |
|---|---|---|---|---|
| | | | Sucrose | Glucosylglycerol |
| 6301 | *Synechococcus* I | F | + | – |
| 6307 | *Cyanobium* I | F | + | – |
| 6311 | *Synechococcus* I | F | + | – |
| 6907 | *Cyanobium* I | F | + | – |
| 6908 | *Synechococcus* I | F | + | – |
| 7002 | *Synechococcus* III | S | t | + |
| 7003 | *Synechococcus* VI | S | t | + |
| 7117 | *Synechococcus* III | S | t | + |
| 7202 | *Cyanobacterium* I | S | t | + |
| 7335 | *Synechococcus* V | S | t | + |
| 73109 | *Synechococcus* III | S | t | + |
| 7424 | *Cyanothece* I | F | + | – |
| 7502 | *Cyanobacterium* II | F | + | – |
| 7943 | *Synechococcus* I | F | + | – |

Rippka *et al.,* 1979). Table 2 shows the revised classification of *Synechococcus* isolates, according to the proposals of Rippka and Cohen-Bazire (1983).

For osmotic adjustment experiments, cells from the stock-culture flasks were centrifuged at $3\,000 \times g$ for 15 min and resuspended, at biovolumes up to 0.15 $mm^3$ $cm^{-3}$, in 100 $cm^3$ of fresh, sterile BG-11 medium containing varying amounts of sea salts (as described above), to give a graded series of salinity from freshwater (BG-11) up to 300% seawater. These cell suspensions were counted and sized, as described below, and incubated for 10 d at 25 °C under constant illumination (at a photon fluence rate of 20 $\mu$mol $m^{-2}$ $s^{-1}$). A temperature of 25 °C was used in order to minimize the production of secondary carbohydrates, since we have recently shown that some cyanobacteria grown under conditions of high temperature (and low salinity) may produce additional carbohydrates (i.e., disaccharides in glucosylglycerol-accumulating strains; Warr *et al.,* 1985 a, b). Those treatments which remained viable and showed an increase in cell number of more than 100% during this time period were deemed to

be capable of sustained growth and were then assayed for low-molecular weight carbohydrates and quaternary ammonium compounds; all other samples were discarded.

Measurement of intracellular volumes

Aliquots (50 to 200 $mm^3$) of cell suspensions were transferred to 25–30 $cm^3$ of an electrolyte solution (9.0 g $dm^{-3}$ NaCl) prior to particle-size analysis using a Coulter Electronics (Luton, UK) Model ZB (industrial) unit, together with a C 1000 channelyzer and linked to an Acorn microcomputer for direct estimation of mean cell number ($cm^{-3}$), cell size ($\mu m^3$) and biovolume ($mm^3$) per $cm^3$ of culture medium. Preliminary experiments have shown that transfer from freshwater and saline media to this electrolyte solution resulted in minimal variation in volume due to cell shrinkage or swelling, over this range (Reed *et al.*, 1985). All values for intracellular solutes have been expressed in terms of total cellular volume (Richardson *et al.*, 1983).

Analysis of low-molecular weight carbohydrates

Aliquots of cell suspensions (20 to 100 $cm^3$) were filtered onto Whatman GF/C filter discs (2.5 cm diam) and extracted in hot 80% ethanol followed by overnight extraction in 80% ethanol at room temperature. Samples were then dried and stored in a vacuum desiccator for 48 h prior to derivatization. Trimethyl-silyl derivatives were prepared as described previously (Holligan and Drew, 1971; Reed *et al.*, 1980) and analyzed by gas-liquid chromatography, as before (Warr *et al.*, 1984a) using a temperature programme from 140° to 280 °C at 20 C° $min^{-1}$, holding the initial and final temperatures constant for 2 min; this programme was found to be sufficient to allow identification of glucosylglycerol, monosaccharides and disaccharides.

Quaternary ammonium compounds were quantified using the periodide precipitation assay, as described previously (Reed *et al.*, 1984a), with glycine betaine standards to provide a calibration curve.

## Results

Fig. 1 shows data for carbohydrate accumulation in eight isolates of *Synechococcus* of freshwater origin (see Table 1). All of the isolates accumulated sucrose, rather than glucosylglycerol or trehalose, as their major intracellular low-molecular weight carbohydrate in response to upshock, with minimal amounts (< 20 mmol $dm^{-3}$) of this disaccharide in cells grown in BG-11 medium and maximum levels (up to 232 mmol $dm^{-3}$) in saline media. Trace amounts of monosaccharides were also observed (< 10 mmol $dm^{-3}$), although these showed no change with salinity. Only two of the strains showed any capacity for

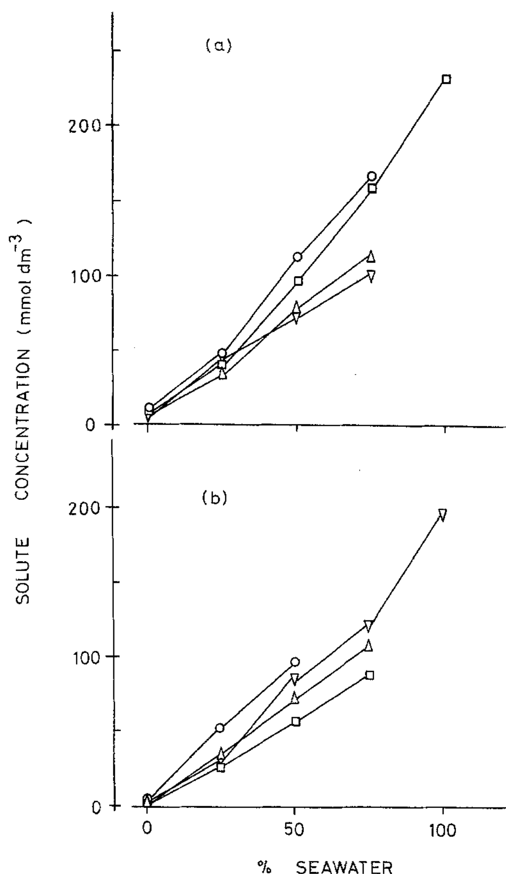


**Fig. 1.** *Synechococcus*. Solute (sucrose) accumulation in freshwater strains. (a) PCC 6301 (*Synechococcus* I; ○), PCC 6307 (*Cyanobium* I; ▽), PCC 6311 (*Synechococcus* I; □), and PCC 6907 (*Cyanobium* I; △); (b) PCC 6908 (*Synechococcus* I; ○), PCC 7424 (*Cyanothece* I; ▽); PCC 7502 (*Cyanobacterium* II; □) and PCC 7943 (*Synechococcus* I; △). Three replicates per treatment. (100% seawater = 35‰ S)

sustained growth in full-strength seawater (*Synechococcus* PCC 6311 and PCC 7424; Fig. 1a and b, respectively), while the other freshwater isolates grew only in freshwater BG-11 and in hyposaline media (< 100% seawater).

The six isolates from marine and saline habitats (Table 1) all accumulated glucosylglycerol when grown in BG-11SW (Fig. 2). Despite their saline origins, four of these strains grew in all hyposaline media and in freshwater BG-11 medium (i.e., *Synechococcus* PCC 7002, 7117, 7202 and 73109), while the remaining two isolates (PCC 7003 and 7335) showed no growth below 25% seawater (Fig. 2). The upper salinity limit for sustained growth of the six saline isolates was higher than for any of the freshwater isolates, at 200 to 250% seawater. Maximum intracellular concentrations of glucosylglycerol (up to 615 mmol $dm^{-3}$) were observed in the most extreme hypersaline media (*Synechococcus* PCC 7117: Fig. 2a) and minimal levels (< 15 mmol $dm^{-3}$) in BG-11 medium. No correlation was observed between organic solute accumulation profiles and the revised classification of *Synechococcus*, according to Rippka and Cohen-Bazire (1983) (see Table 2).

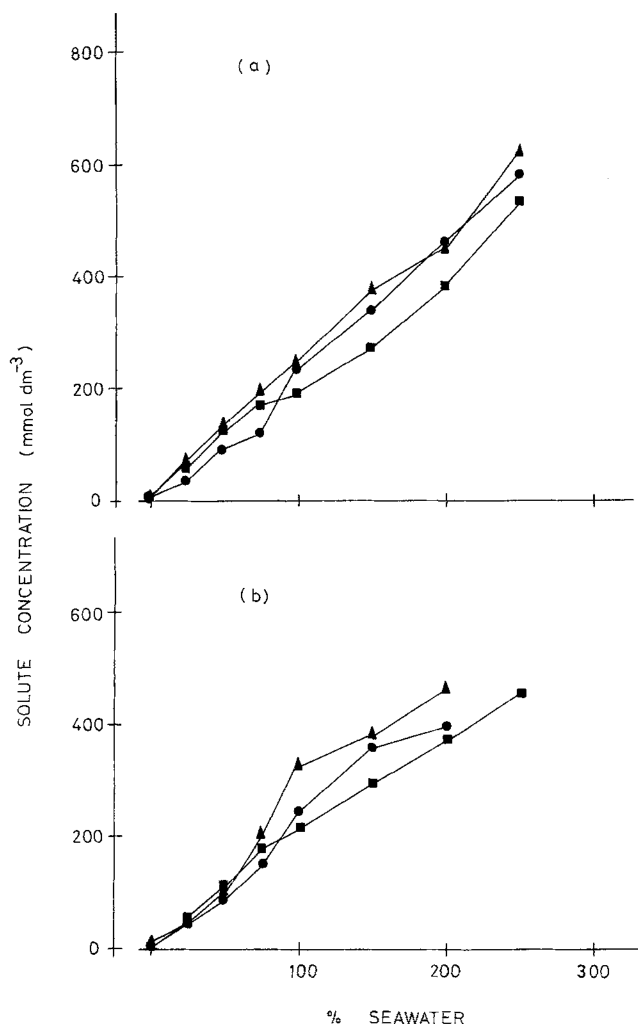


**Fig. 2.** *Synechococcus*. Solute (glucosylglycerol) accumulation in marine strains. (a) PCC 7002 (*Synechococcus* III; ●), PCC 7003 (*Synechococcus* VI; ■), and PCC 7117 (*Synechococcus* III; ▲); (b) PCC 7202 (*Cyanobacterium* I; ●), PCC 7335 (*Synechococcus* V; ■), and PCC 73109 (*Synechococcus* III; ▲). Three replicates per treatment

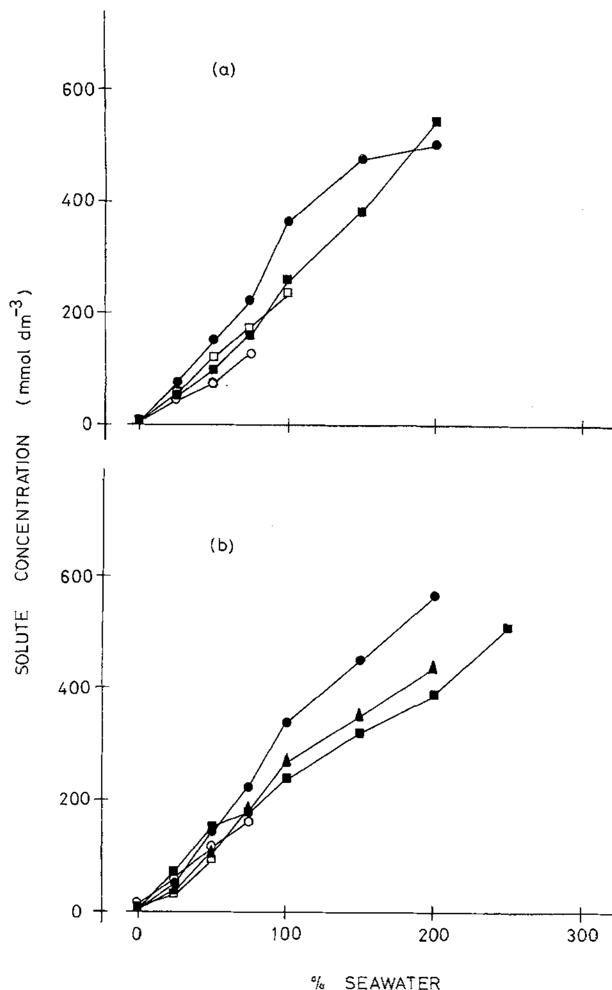


**Fig. 3.** *Synechocystis*. Solute accumulation in freshwater strains. (a) PCC 6308 (○), PCC 6701 (□), PCC 6702 (●) and PCC 6714 (■); (b) PCC 6803 (●), PCC 6806 (■), PCC 6808 (○), PCC 7008 (▲) and PCC 7509 (□). Open symbols: sucrose; filled symbols: glucosylglycerol. Three replicates per treatment

Fig. 3 shows data for carbohydrate accumulation in nine freshwater isolates of *Synechocystis*, while data for marine and saline strains are shown in Fig. 4. Of the freshwater isolates, four were found to be sucrose-accumulators, growing in media up to 50–100% seawater, while the remaining five isolates showed accumulation of glucosylglycerol with increasing salinity, growing in up to 200–250% seawater (Fig. 3). Thus, the maximum glucosylglycerol concentration (in *Synechocystis* PCC 6803 growing in a hypersaline medium; 200% seawater) exceeded the highest values for intracellular sucrose accumulation (as shown in Fig. 3 a) by more than 120%.

All three isolates of *Synechocystis* of marine or saline origin (Table 1) were also capable of sustained growth in freshwater BG-11 (Fig. 4), with upper salinity limits of 200 to 250% seawater. In all cases, glucosylglycerol was accumulated in response to salinity stress, forming the major low-molecular weight carbohydrate, with intracellular con-

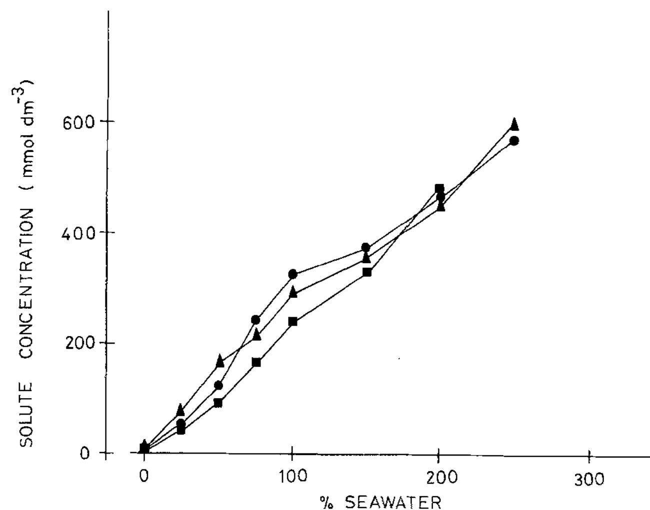


**Fig. 4.** *Synechocystis*. Solute (glucosylglycerol) accumulation in marine strains: PCC 6902 (●), PCC 6905 (■) and PCC 7201 (▲). Three replicates per treatment

centrations in seawater-grown cultures which compared favourably with freshwater-derived strains grown under similar conditions (cf. Fig. 3). Maximum levels of glucosylglycerol (595 mmol $dm^{-3}$) were recorded for cells of the marine isolate *Synechocystis* PCC 7201 grown in 250% seawater. Glucosylglycerol-accumulating strains of *Synechococcus* and *Synechocystis* also contained osmotically insignificant quantities of monosaccharides and trace amounts of sucrose (at < 10% of the corresponding glucosylglycerol concentration when grown in hyperosmotic media), showing that the biochemical pathways required to synthesize this disaccharide are present in these isolates (see Warr *et al.,* 1985 b). In contrast, glucosylglycerol was never observed in strains which accumulated sucrose as a major organic osmoticum.

Periodide precipitation assays showed that none of the strains used in the present study accumulated quaternary ammonium compounds to osmotically significant amounts when grown in saline media; all isolates contained < 30 mmol $dm^{-3}$ quaternary ammonium compounds, showing no major variation with external salinity.

Fig. 5 shows data for the upper salinity limit at which sustained growth occurred for sucrose-accumulating and glucosylglycerol-accumulating isolates. These two groups of cyanobacteria show dissimilar upper limits for growth, with glucosylglycerol-accumulating strains growing in hypersaline media, regardless of their origins, while sucrose accumulators grew only in up to 50–100% seawater.

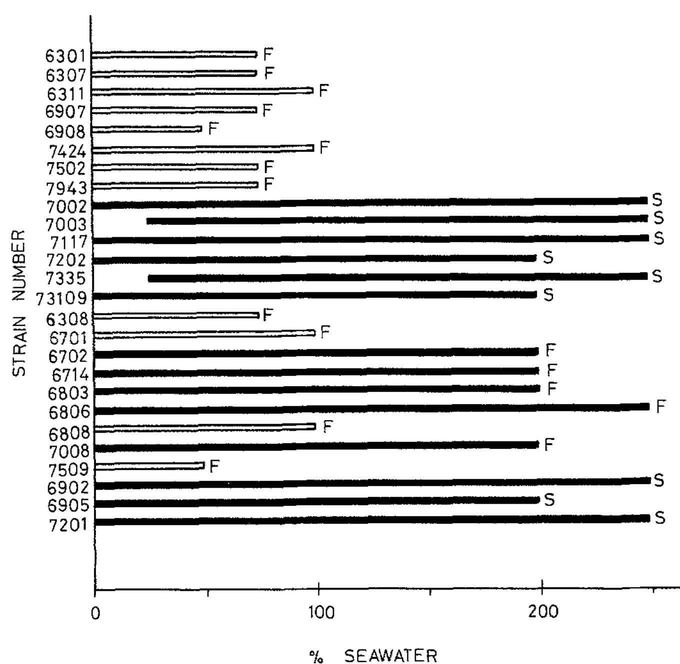


**Fig. 5.** Salinity ranges for sustained growth of unicellular cyanobacteria. F: freshwater strains; S: strains from saline habitats. Open symbols: sucrose-accumulating strains; filled symbols: glucosylglycerol-accumulating isolates

## Discussion

The values for intracellular carbohydrate accumulation shown in Figs. 1–4 suggest that either sucrose or glucosylglycerol acts as the major intracellular osmoticum in these cyanobacteria, with maximum values for cells grown in hyposaline media (< 100% seawater) balancing 29 to 35% of the external salt concentration, and falling to 23 to 31% in hypersaline media (> 100% seawater). Thus, increases in low-molecular weight carbohydrates will serve to compensate for up to one-third of the change in external salinity, as shown in previous studies (Borowitzka *et al.,* 1980; Reed and Stewart, 1983; Richardson *et al.,* 1983). Furthermore, these values are expressed in terms of total cellular volume, measured using a particle-size analyzer, with no correction for non-osmotic volume. If the non-osmotic volume of these cyanobacterial unicells is large, as has been suggested for microscopic algal cells (see Kirst, 1977; Wyn Jones and Gorham, 1983), then the osmotic significance of the data shown in Figs. 1–4 will be increased considerably.

The above data also provide us with sufficient information to consider whether salinity tolerance and organic solute accumulation in cyanobacteria are habitat-linked. Out of a total of seventeen isolates of freshwater unicells, twelve were sucrose accumulators (including all eight of the freshwater isolates of *Synechococcus*), while five freshwater isolates of *Synechocystis* accumulated glucosylglycerol (Figs. 1 and 3). Three of these freshwater-derived sucrose accumulators also showed a capacity for sustained growth in full-strength (100%) seawater. All five of the freshwater isolates of *Synechocystis* that accumulated glucosylglycerol also showed sustained growth in up to 100% seawater and beyond (Fig. 3); in contrast, none of the sucrose-accumulating isolates showed any growth in hypersaline media (Fig. 5). All nine of the isolates from marine and saline habitats accumulated glucosylglycerol (Figs. 2, 4) with seven strains also showing sustained growth in freshwater BG-11 medium; only two strains had an absolute requirement for elevated levels of sea salts (present Fig. 2; and Rippka *et al.,* 1979). None of the isolates showed accumulation of monosaccharides (e.g. glucose) in response to osmotic stress, in contrast to the report of Blumwald *et al.* (1983) for the cyanobacterium *Synechococcus* PCC 6311. This is in agreement with studies of osmotic adaptation in eukaryotic algae, which show accumulation of other saccharide derivatives, rather than monosaccharides, in response to osmotic stress (see Hellebust, 1976; Ben Amotz and Avron, 1983). Furthermore, none of the isolates accumulated quaternary ammonium compounds, in sharp contrast to halotolerant forms (Mohammad *et al.,* 1983; Mackay *et al.,* 1984; Reed *et al.,* 1984 a).

It is clear that glucosylglycerol-accumulating isolates grew over the widest range of salinity, whether they were isolated from saline or non-saline habitats, with an upper salinity limit of 200 to 250% seawater, in contrast to disaccharide-accumulating forms, which grew in up to

50–100% seawater (Fig. 5). Mackay *et al.* (1983, 1984) give slightly higher values for some of the upper salinity tolerances of several glucosylglycerol-accumulating isolates used in the present study (e.g. *Synechococcus* PCC 7002 and *Synechocystis* PCC 6714); this may be due to differences in medium composition (Mackay *et al.,* 1984 varied only NaCl, keeping the external $Mg^{2+}$, $Ca^{2+}$ and $K^+$ constant), and in method of salinity increase (a stepwise salt increase, at 5 to 20 g $dm^{-3}$ was used by Mackay *et al.,* 1984, whereas the present study used a single-step upshock).

The observation that heteroside (glucosylglycerol) accumulators are capable of growth in hypersaline media is in agreement with the previous findings of Richardson *et al.* (1983) for the euryhaline freshwater isolate *Synechocystis* PCC 6803. Heterosides are also known to function as osmotica in several groups of eukaryotic algae; these include the production of isofloridoside (galactosylglycerol) in osmotically-stressed cells of the freshwater chrysophyte *Poterioochromonas* (Kauss, 1967, 1978), and of floridoside (galactosylglycerol) or digeneaside (mannoglycerate) in several marine red algae (Kauss, 1968; Bisson and Kirst, 1979; Kirst and Bisson, 1979; Reed *et al.*, 1980, Reed, 1983 a), and there is no indication that these compounds are restricted solely to *marine* algae (Craigie, 1974; Kremer, 1981; Reed, 1985). In contrast, sucrose is known to be accumulated by a limited number of freshwater green algae under conditions of limited osmotic stress; these include the genera *Scenedesmus* (Wetherall, 1963), *Chlorella* (Setter and Greenway, 1979) and the brackish-water charophyte *Lamprothamnium* (Bisson and Kirst, 1980). Several marine green algae, including species of *Dunaliella* (Craigie and McLachlan, 1964) *Chlamydomonas* (Hellebust, 1976), and *Stichococcus* (Brown and Hellebust, 1980), accumulate polyols (glycerol or sorbitol) rather than sucrose, in response to osmotic stress. A further class of organic osmotica has been reported for euryhaline marine green algae belonging to the genera *Ulva* and *Enteromorpha,* which accumulate substantial quantities of the tertiary sulphonium compound dimethylsulphoniopropionate as an organic osmoticum (Dickson *et al.,* 1980; Reed, 1983 b), while intracellular sucrose levels are osmotically insignificant (Brown and Hellebust, 1980). Overall, the data for eukaryotic algae are in agreement with the findings of the present study, since heteroside-accumulating forms generally show a greater range of salt tolerance than sucrose accumulators. A plausible explanation of the reduced upper-salinity limit of sucrose-accumulating cyanobacteria comes from our recent studies of the effects of this disaccharide upon enzyme activity in cell-free extracts of *Nodularia harveyana* (Warr *et al.,* 1984 b). Sucrose was found to be a potent inhibitor of glutamine synthetase activity at concentrations in excess of 300 mmol $dm^{-3}$; similar findings have also been reported for a variety of soluble and particulate enzymes (Hinton *et al.*, 1969; Brown and Simpson, 1972). This is in agreement with the "compatible solute" hypothesis, advanced by Brown and co-workers (see Brown, 1976, 1983), i.e., that compounds which are accumulated as internal solutes in osmotically-stressed cells must show little or no detrimental effect on enzyme activity and metabolic function at physiologically relevant concentrations. Since sucrose is known to be an "incompatible solute" *in vitro* at high concentrations due to steric hindrance of enzyme molecules caused by the sugar rings (see Borowitzka, 1981), it seems likely that the maximum intracellular concentration of this compound *in vivo* may be governed by this detrimental effect. It thus follows that sucrose-accumulating cells, whether algal or cyanobacterial, may be unable to withstand the elevated levels of sucrose required to generate the positive turgor needed for cell expansion growth in high-salt media. The upper salinity limit for growth of these organisms may well be linked to (cytoplasmic) sucrose toxicity at high intracellular concentration (see Warr *et al.,* 1984 b). It is noteworthy that the brackish-water charophyte *Lamprothamnium* sp. is able to accumulate most of the intracellular sucrose fraction within its large central vacuole (Bisson and Kirst, 1980), thus reducing the problems of sucrose toxicity to (cytoplasmic) enzymes. However, cyanobacteria are essentially non-vacuolate and compartmentation of sucrose on this scale is not feasible.

Polyols are known to function as compatible solutes, showing minimal enzyme inhibition at high concentration (Brown and Simpson, 1972; Brown, 1976), and it seems likely that glucosylglycerol and other heterosides will also prove to be more compatible than sucrose, due to the presence of a glycerol rather than a saccharide moiety (see Borowitzka, 1981), although conclusive experimental proof is lacking. We propose that the reduced toxicity of heterosides and polyol-derivatives to enzyme activity provides an explanation that may account for the growth of glucosylglycerol-accumulating cyanobacteria in hypersaline media, while disaccharide-accumulating strains show restricted, stenohaline growth responses (Fig. 5). The third major class of organic solutes known to be accumulated in halotolerant cyanobacterial cells from hypersaline habitats includes the compounds glycine betaine (see Reed *et al.,* 1984 a) and glutamate betaine (Mackay *et al.,* 1984). Betaines are well-known for their lack of inhibition of enzyme activity and metabolic function in halophytic higher plant cells (see Wyn Jones and Gorham, 1983) and bacteria (Bouillard and Le Rudulier, 1983) and may also function in the protection of enzyme activity against NaCl inhibition when present at high concentrations (Pollard and Wyn Jones, 1979). We have recently confirmed that glycine betaine functions in a similar manner in cell-free extracts of the halotolerant cyanobacterium *Synechocystis* DUN 52; glycine betaine concentrations up to 2.0 mol $dm^{-3}$ were found to have no inhibitory effect upon glutamine synthetase activity (Warr *et al.,* 1984 b). Furthermore, glycine betaine (at 1.0 mol $dm^{-3}$) was found to reduce NaCl inhibition of glutamine synthetase by up to 30% at NaCl concentrations above 0.8 mol $dm^{-3}$. Overall, these data support the hypothesis that the upper salinity limit for growth of cyanobacteria may be linked to

the organic osmoticum, with sucrose accumulators showing a restricted, stenohaline growth range (at least up to full-strength seawater: present Fig. 5; and Reed *et al.*, 1984 b) while heteroside accumulators are capable of growth at higher salinities and betaine-accumulating strains grow in the most extreme hypersaline media. Since the upper salinity limit for the growth of sucrose accumulators is not below full-strength seawater, and since several cyanobacteria isolated from marine habitats have been shown to synthesize sucrose as their major organic osmoticum in response to salinity stress (Reed *et al.*, 1984 b; Warr *et al.*, 1984 a, b), we are forced to reject the hypothesis of Mackay *et al.* (1983) that such forms cannot be regarded as marine. The occurrence of euryhaline glucosylglycerol-accumulating freshwater isolates (Richardson *et al.*, 1983; see also present Fig. 5) also weakens the proposal that heteroside-accumulating forms are uniquely marine. Thus, while the upper salinity limit for growth appears to be directly related to the type of organic osmoticum, the lower salinity limit for growth, which will be independent of organic solute accumulation, will be determined by the particular ionic requirements of each individual species (see Rippka *et al.*, 1979). The findings of Mackay *et al.* (1983, 1984) do not wholly conflict with the present study, although our interpretation of results is at odds with their habitat-linked hypothesis. Thus, we conclude (1) that cyanobacteria fall into three rather broad (and overlapping) categories, characterized by the accumulation of disaccharides (least halotolerant), heterosides (intermediate halotolerant) or betaines (most halotolerant); (2) that no *absolute* correlation exists between organic solute accumulation profile and habitat; (3) that glucosylglycerol accumulation and growth in hypersaline media are not unique characteristics of marine cyanobacteria; and (4) that halotolerance may be linked to the compatibility of organic osmotica, rather than habitat.

*Acknowledgements.* We wish to thank Dr. R. Rippka of the Institut Pasteur for her assistance in the selection of suitable strains. This research was supported by the Agricultural & Food Research Council and the Royal Society. R.H.R. currently holds a Royal Society Research Fellowship.

## Literature cited

Ben Amotz, A. and M. Avron: Accumulation of metabolites by halotolerant algae and its industrial potential. A. Rev. Microbiol. *37,* 95–119 (1983)

Bisson, M. A. and G. O. Kirst: Osmotic adaptation in the marine alga *Griffithsia monilis* (Rhodophyceae): the role of ions and organic compounds. Aust. J. Pl. Physiol. *6,* 523–538 (1979)

Bisson, M. A. and G. O. Kirst: *Lamprothamnium,* a euryhaline charophyte. II. Time course of turgor regulation. J. exp. Bot. *31,* 1237–1244 (1980)

Blumwald, E., R. J. Mehlhorn and L. Packer: Studies of osmoregulation in salt adaptation of cyanobacteria with ESR spinprobe techniques. Proc. natn. Acad. Sci. USA. *80,* 2599–2602 (1983)

Blumwald, E. and E. Tel-Or: Osmoregulation and cell composition in salt-adaptation of *Nostoc muscorum.* Archs Microbiol. *132,* 168–172 (1982)

Borowitzka, L. J.: Solute accumulation and regulation of cell water activity. *In:* The physiology and biochemistry of drought resistance in plants, pp 97–130. Ed. by L. G. Paleg and D. Aspinall. London: Academic Press 1981

Borowitzka, L. J., S. Demmerle, M. A. Mackay and R. S. Norton: Carbon-13 nuclear magnetic resonance study of osmoregulation in a blue-green alga. Science, N. Y. *210,* 650–651 (1980)

Bouillard, L. and D. Le Rudulier: Nitrogen fixation under osmotic stress: enhancement of nitrogenase biosynthesis in *Klebsiella pneumoniae* by glycine betaine. Physiologie vég. *21,* 447–457 (1983)

Brown, A. D.: Microbial water stress. Bact. Rev. *40,* 803–846 (1976)

Brown, A. D.: Halophilic prokaryotes. *In:* Encyclopedia of plant physiology, Vol. 12C. pp 137–162. Ed. by O. L. Lange, P. S. Nobel, C. B. Osmond and H. Ziegler. Berlin: Springer-Verlag 1983

Brown A. D. and J. R. Simpson: Water relations of sugar-tolerant yeasts: the role of intracellular polyols. J. gen. Microbiol. *72,* 589–591 (1972)

Brown, L. M. and J. A. Hellebust: The contribution of organic solutes to osmotic balance in some green and eustigmatophyte algae. J. Phycol. *16,* 265–270 (1980)

Carr, N. G. and B. A. Whitton (Eds.): The biology of Cyanobacteria, 688 pp. Oxford: Blackwell 1982

Craigie, J. S.: Storage products. *In:* Algal physiology and biochemistry, pp 206–235. Ed. by W. D. P. Stewart. Oxford: Blackwell 1974

Craigie, J. S. and J. McLachlan: Glycerol as a photosynthetic product in *Dunaliella tertiolecta* Butcher. Can. J. Bot. *42,* 777–778 (1964)

Cram, W. J.: Negative feedback regulation of transport in cells. The maintenance of turgor, volume and nutrient supply. *In:* Encyclopedia of plant physiology, Vol. 2A. pp 284–326. Ed. by U. Luttge and M. G. Pitman. Berlin: Springer-Verlag 1976

Dickson, D. M., J. Davenport and R. G. Wyn Jones: Steady state osmotic adaptation in *Ulva lactuca.* Planta *150,* 158–165 (1980)

Erdmann, N.: Organic osmoregulatory solutes in blue-green algae. Z. PflPhysiol. *110,* 147–155 (1983)

Fogg, G. E., W. D. P. Stewart, P . Fay and A. E. Walsby: The blue-green algae, 459 pp. London: Academic Press 1973

Hellebust, J. A.: Osmoregulation. A. Rev. Pl. Physiol. *27,* 485–507 (1976)

Hinton, R. H., M. L. E. Burge and G. C. Hartman: Sucrose interference in the assay of enzymes and protein. Analyt. Biochem. *29,* 248–256 (1969)

Holligan, P. M. and E. A. Drew: Routine analysis by gas-liquid chromatography of soluble carbohydrates in extracts of plant tissues. II. Quantitative analysis of standard carbohydrates, and the separation and estimation of soluble sugars and polyols from a variety of plant tissues. New Phytol. *70,* 271–297 (1971)

Kauss, H.: Isofloridosid und Osmoregulation bei *Ochromonas malhamensis.* Z. PflPhysiol, *56,* 453–465 (1967)

Kauss, H.: α-Galaktosylglyzeride und Osmoregulation in Rotalgen. Z. PflPhysiol. *58,* 428–433 (1968)

Kauss, H.: Osmotic regulation in algae. Prog. Phytochem. *5,* 1–27 (1978)

Kirst, G. O.: The cell volume of the unicellular alga *Platymonas subcordiformis* Hazen.: effect of the salinity of the culture medium and of osmotic stresses. Z. PflPhysiol. *81,* 386–394 (1977)

Kirst, G. O. and M. A. Bisson: Regulation of turgor in marine algae: ions and low-molecular weight organic compounds. Aust. J. Pl. Physiol. *6,* 539–556 (1979)

Kremer, B. P.: Aspects of carbon metabolism in marine macroalgae. Oceanogr. mar. Biol. A. Rev. *19,* 41–94 (1981)

Mackay, M. A., R. S. Norton and L. J. Borowitzka: Marine blue-green algae have a unique osmoregulatory system. Mar. Biol. *73,* 301–307 (1983)

Mackay, M. A., R. S. Norton and L. J. Borowitzka: Organic osmoregulatory solutes in cyanobacteria. J. gen. Microbiol. *130,* 2177–2191 (1984)

Miller, D. M., J. H. Jones, J. H. Yopp, D. R. Tindall and W. E. Schmid: Ion metabolism in a halophilic blue-green alga, *Aphanothece halophytica.* Archs Microbiol. *111,* 145–149 (1976)

Mohammad, F. A. A., R. H. Reed and W. D. P. Stewart: The halophilic cyanobacterium *Synechocystis* DUN52 and its osmotic responses. Fedn eur. microbiol. Soc. (FEMS) Lett. *16,* 287–290 (1983)

Parsons, T. R.: Coulter counter for phytoplankton. *In:* Handbook of phycological methods, pp 345–358. Ed. by J. R. Stein. Cambridge: Cambridge University Press 1973

Pollard, A. and R. G. Wyn Jones: Enzyme activities in concentrated solutions of glycine betaine and other solutes. Planta *144,* 291–298 (1979)

Reed, R. H.: Taxonomic implications of osmoacclimation in *Cyanidium caldarium* (Tilden) Geitler. Phycologia *22,* 351–354 (1983 a)

Reed, R. H.: Measurement and osmotic significance of $\beta$-dimethylsulphoniopropionate in marine macroalgae. Mar. Biol. Lett. *4,* 173–181 (1983 b)

Reed, R. H.: Osmoacclimation in *Bangia atropurpurea* (Rhodophyta, Bangiales): the osmotic role of floridoside. Br. phycol. J. *20* (In press). (1985)

Reed, R. H., J. C. Collins and G. Russell: The effects of salinity upon galactosyl-glycerol content and concentration of the marine red alga *Porphyra purpurea* (Roth) C. Ag. J. exp. Bot. *31,* 1539–1554 (1980)

Reed, R. H., J. A. Chudek, R. Foster and W. D. P. Stewart: Osmotic adjustment in cyanobacteria from hypersaline environments. Archs Microbiol. *138,* 333–337 (1984 a)

Reed, R. H., D. L. Richardson and W. D. P. Stewart: $Na^+$ uptake and extrusion in the cyanobacterium *Synechocystis* PCC 6714 in response to hypersaline treatment: evidence for transient changes in membrane permeability. Biochim. biophys. Acta *814,* 347–355 (1985)

Reed, R. H., D. L. Richardson, S. R. C. Warr and W. D. P. Stewart: Carbohydrate accumulation and osmotic stress in cyanobacteria. J. gen. Microbiol. *130,* 1–4 (1984 b)

Reed, R. H. and W. D. P. Stewart: Physiological responses of *Rivularia atra* to salinity: osmotic adjustment in hyposaline media. New Phytol. *95,* 595–603 (1983)

Richardson, D. L., R. H. Reed and W. D. P. Stewart: *Synechocystis* PCC 6803: a euryhaline cyanobacterium. Fedn eur. microbiol. Soc. (FEMS) Lett. *18,* 99–102 (1983)

Rippka, R. and G. Cohen-Bazire: The Cyanobacteriales: a legitimate order based on the type strain *Cyanobacterium stanieri?* Annls Inst. Pasteur, Paris (Microbiol.) *134 B,* 21–36 (1983)

Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman and R. Y. Stanier: Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. gen. Microbiol. *111,* 1–61 (1979)

Setter, T. L. and H. Greenway. Growth and osmoregulation of *Chlorella emersonii* in NaCl and neutral osmotica. Aust. J. Pl. Physiol. *6,* 47–60 and (Corrigendum) 569–572 (1979)

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

Warr, S. R. C., R. H. Reed and W. D. P. Stewart: Physiological responses of *Nodularia harveyana* to osmotic stress. Mar. Biol. *79,* 21–26 (1984 a)

Warr, S. R. C., R. H. Reed and W. D. P. Stewart: Osmotic adjustment of cyanobacteria: the effects of NaCl, KCl, sucrose, and glycine betaine on glutamine synthetase activity in a marine and a halotolerant strain. J. gen. Microbiol. *130,* 2169–2175 (1984 b)

Warr, S. R. C., R. H. Reed and W. D. P. Stewart: Carbohydrate accumulation in osmotically stressed cyanobacteria (blue-green algae): interactions of temperature and salinity. New Phytol. (In press). (1985 b)

Wetherall, D. F.: Osmotic equilibration and growth of *Scenedesmus obliquus* in saline media. Physiologia Pl. *16,* 82–91 (1963)

Wyn Jones, R. G. and J. Gorham: Osmoregulation. *In:* Encyclopedia of plant physiology, Vol. 12C. pp 35–58. Ed. by O. L. Lange, P. S. Nobel, C. B. Osmond and H. Ziegler. Berlin: Springer-Verlag 1983

Yopp, J. H., D. M. Miller and D. R. Tindall: Regulation of intracellular water potential in the halophilic blue-green alga *Aphanothece halophytica* (Chroococcales). *In:* Energetics and structure of halophilic microorganisms, pp 619–624. Ed. by S. R. Caplan and M. Ginzburg. Amsterdam: Elsevier 1978

Date of final manuscript acceptance: May 2, 1985.
Communicated by J. Mauchline, Oban