Sulpate-limited growth in the N2-fixing unicellular cyanobacterium Gloeothece (Nageli) sp. PCC 6909.

Ortega-Calvo, J.J.; Stal, L.J.

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Sulphate-limited growth in the N₂-fixing unicellular cyanobacterium *Gloeothece* (Nägeli) sp. PCC 6909

**By Jose-Julio Ortega-Calvo† and Lucas J. Stal**

1Laboratory for Microbiology, University of Amsterdam, Nieuwe Achtergracht 127, NL-1018 WS Amsterdam, The Netherlands

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**SUMMARY**

The unicellular diazotrophic cyanobacterium *Gloeothece* PCC 6909 was grown in batch and continuous culture. It was found that the organism scavenged sulphate from the medium and incorporated it in the polysaccharide sheath. As a result batch cultures became sulphur-depleted whilst continuous cultures were sulphur-limited. This was observed regardless of the nitrogen source of the culture. Sulphur-depletion was the reason for the low nitrogenase activities usually observed in batch cultures of *Gloeothece*. Sulphate-amended cultures showed sustained high nitrogenase activity in the light. Sulphate uptake from the medium was light-dependent. Earlier work on N₂ fixation in *Gloeothece* is discussed in the light of possible sulphur limitation.

**Key words:** *Gloeothece*, cyanobacteria, N₂ fixation, sulphate, continuous culture.

**INTRODUCTION**

The unicellular cyanobacterium *Gloeothece* sp. is capable of diazotrophic growth under oxic conditions (Gallon, LaRue & Kurz, 1974; Kallas et al., 1983). The protection of the oxygen-sensitive nitrogenase in this organism has been the subject of a large number of investigations (see Gallon, 1992; Gallon & Stal, 1992 for reviews) but, in spite of considerable effort, no completely satisfactory explanation for aerobic N₂ fixation in this non-heterocystous cyanobacterium can be offered. One of the hypotheses used to explain diazotrophic growth in non-heterocystous cyanobacteria like *Gloeothece* sp. is the temporal separation of oxygenic photosynthesis and N₂ fixation (Gallon et al., 1974; Kallas et al., 1983). The latter would be confined to the night under natural conditions or to the dark period when grown artificially as batch cultures under alternating light-dark cycles (Mullineaux, Gallon & Chaplin, 1981). However, at least one cyanobacterium does not fit this hypothesis. N₂ fixation in the filamentous, non-heterocystous cyanobacterium *Trichodesmium* is strictly light-dependent (Capone et al., 1990). Moreover, all non-heterocystous diazotrophic cyanobacteria can grow at the expense of N₂ under continuous light. By using continuous cultures of *Gloeothece*, Ortega-Calvo & Stal (1991) found that this organism may also confine virtually all nitrogenase activity to the light period. They estimated a maximum specific growth rate of close to 0.02 h⁻¹ (doubling time 35 h) which clearly exceeded rates estimated from batch cultures (Gallon et al. 1988; Tease & Walker, 1987; Myint, 1991). Batch cultures turned yellow-green (bleached) because of degradation of phycobiliproteins, which is often the case when cultures are nitrogen-starved (Allen & Hutchison, 1980). However, Tease & Walker (1987) and Myint (1991) did not find any difference in growth rate between N₂-fixing or nitrate-grown batch cultures. However, sulphur limitation results in similar morphological and physiological changes as nitrogen limitation (Wanner et al., 1986), and during a systematic investigation of batch cultures of *Gloeothece* we recently found that these cultures were sulphur-limited (Stal, unpublished).

Many cyanobacteria produce a polysaccharide sheath. *Gloeothece* sp. PCC 6909 produces a complex sheath which surrounds up to 16 cells. The sheath is composed of different layers. The inner sheath encloses 1–2 cells whereas the outer sheath encloses cell groups (Tease et al., 1991). The sheath of *Gloeothece* is composed of complex heteropolysac-

* To whom correspondence should be addressed.
† Present address: Instituto de recursos Naturales y Agrobiología de Sevilla, CSIC, PO Box 1052, E-41080 Sevilla, Spain.
Pack, The Netherlands), using acetylene as internal standard (Ortega-Calvo & Stal, 1991). Sulphated polysaccharides are common in eukaryotic algae (Percival, 1979) but are extremely rare among prokaryotes. Nevertheless, as well as in Gloeothecae, sulphated polysaccharides have been found in Synechocystis (Panoff et al., 1988) and in the filamentous Phormidium sp. (Bar-Or & Shilo, 1987). The amount of sulphate that was found in the sheath of Gloeothecae was considerable. Tease et al. (1991) measured 18.6, 8.2 and 13.8% (wt/wt of polysaccharide) in the inner sheath, outer sheath and (extracellular) soluble polysaccharide, respectively. In this paper we show that the high sulphate content of the sheath of Gloeothecae may result in sulphur-limited growth and low activity of nitrogenase.

MATERIALS AND METHODS
Organism and growth conditions
Gloeotheca sp. PCC 6909 was obtained from the Pasteur Culture Collection (Paris, France). The organism was routinely grown in medium BG 11° which was devoid of any source of combined nitrogen (Rippka et al., 1979). For some experiments regular BG 11 was used which contained 7.5 mM nitrate as the nitrogen source. The culture was maintained in 300 ml Erlemeyer flasks containing 150 ml of growth medium. Stock cultures were grown on an orbital shaking platform at 20°C and at an incident light intensity of 16 μmol m⁻² s⁻¹, provided by Philips TLD 18W/33 fluorescent tubes. These cultures were transferred to fresh medium every 6 wk and were used as inoculum for continuous cultures. The continuous cultures were identical to those used previously (Ortega-Calvo & Stal, 1991) and their construction has been described in detail by Van Liere & Mur (1978). The culture vessel was a modified 21 Kluyver flask. The cultures were grown in nitrate-free medium at 20°C under either continuous light or an alternating light-dark cycle of 16–18 h. The cultures were aerated with 1 l min⁻¹ of air. The air was freed of traces of ammonia by passage through an acid trap (33 mM phosphoric acid). Batch culture experiments were carried out with steady-state samples of the diazotrophic continuous culture running at a dilution rate of D = 0.007 h⁻¹ and given continuous illumination. Further details are given in the Results section.

Nitrogenase activity
Nitrogenase activity was measured using the acetylene reduction assay, essentially as described by Ortega-Calvo & Stal (1991). Acetylene and ethylene were determined gas chromatographically (Chrompack, The Netherlands), using acetylene as internal standard (Stal, 1988).

Photosynthesis
Light response curves of photosynthesis were measured as described by Dubinsky et al. (1987). A custom-made oxygen cell with a volume of 12 ml was used. The cell was provided with a magnetic stirrer, a water jacket, and kept at 20°C. Oxygen was measured polarographically using a Clark type electrode (YSI, Ohio, model 5331). Polarization was at 0.75 V using a custom-made voltage source. Current was measured using a Keithley Picoammeter connected to a datalogger. Light was provided by a slide projector. Light intensity was varied by neutral density filters and by varying the distance of the projector to the oxygen cell. For each intensity, oxygen production was measured for 3 min.

Light measurements
Light was measured with a LiCor, Inc. (USA) model LI-185B Quantum/Radiometer/Photometer equipped with a LI-190SB quantum sensor. Mean photon flux density (PFD) in the culture was calculated according to Van Liere, Loogman & Mur (1978).

Analytical procedures
Cell carbohydrate was extracted from the culture after washing the cells in sulphate-free BG 11° medium. A sample (5 ml) of culture was treated with 5 ml of 1 M HCl for 15 min at 100°C. Subsequently the cells were centrifuged and the carbohydrate content of the supernatant was determined using the anthrone method (Trevelyan & Harrison, 1952), with glucose as reference. The supernatant was then transferred to sealed vials and maintained at 100°C for 48 h. Sulphate that was released from the sheath carbohydrate was determined using the turbidimetric-barium chloride assay (Tease et al., 1991). Extended hydrolysis (48 h) was necessary in order fully to recover sulphate from the sheath. Microscopic examinations showed that the initial, 15 min, acid treatment completely removed the sheath from the cells without destroying the cells. However, intracellular storage polyglucose was also hydrolyzed and accounted for part of the carbohydrate measured. Absorbance of the culture was measured at 720 nm. For chlorophyll a determination, cell pellets were extracted twice for 1 h in the dark and at room temperature, with an appropriate volume of methanol. The specific absorbance coefficient of 745 ml mg⁻¹ cm⁻¹ was used (MacKinney, 1941). Protein was determined on pellets after chlorophyll extraction, using Folin reagent (Lowry et al., 1951). The pellets were hydrolysed at 100°C for 1 h in 0.5 M NaOH.

In vivo spectra were run on an Aminco DW2000
spectrophotometer. Cell suspensions were made in 50%, glycerol which prevented sedimentation during the run. The spectra were used to calculate the ratio of phycocyanin (624 nm) to chlorophyll a (680 nm).

RESULTS

In Table 1, some properties of continuous cultures of Gloeothecae sp. PCC 6909 are shown. Gloeothecae sp. was grown either diazotrophically (nitrate-free medium, BG 11°) or with nitrate as the source of nitrogen. When the total daily doses of light of the culture was increased by changing from a 16–18 h light–dark regime to conditions of continuous light (D = 0.007 h⁻¹) the optical density of the culture did not change and the protein content of the cultures remained approximately constant (106 and 97 mg 1⁻¹ in the light–dark and continuous light cultures, respectively). From this observation we concluded that light was not the limiting factor in this culture. Furthermore, since there was no difference in optical density between the N₂-fixing and nitrate-grown culture at D = 0.007 h⁻¹, nitrogen was also unlikely to be the limiting factor. The fact that no difference in optical density and dry weight was seen between the nitrate-grown cultures at D = 0.007 and 0.015 h⁻¹ also showed that light was not the limiting factor to growth of these cultures. However, there was a large difference between the optical densities and dry weights of the N₂-fixing and nitrate-grown cultures at D = 0.015 h⁻¹. In addition, the N₂-fixing culture at D = 0.007 h⁻¹ showed a lower optical density and dry weight compared to that grown at D = 0.015 h⁻¹. Therefore, it appears that both light and nitrogen limit growth. In all cultures the concentration of sulphate in the growth medium was below the limit of detection. Sulphate was therefore considered as a possible growth-limiting factor. In order to investigate this possibility further, we carried out a number of experiments with samples of the culture grown at D = 0.007 h⁻¹ in nitrate-free medium and under continuous light.

The chemostat culture at steady state was switched to batch conditions but the other conditions (light and aeration) were kept the same. At the beginning of the experiment 0.3 mM sulphate was added to the culture (which corresponds to the normal concentration in BG 11 medium). The increase of culture protein content followed precisely the theoretical growth at 0.007 h⁻¹ (Fig. 1a). The sulphate that was added at day 0 disappeared from the medium within 2 d. This sulphate could be found associated with the sheath (Fig. 1b), which already contained a large amount of sulphate at the start of the experiment. The ratio of phycocyanin:chlorophyll a decreased, indicating an impairment of protein synthesis. The concentration of carbohydrate showed a dramatic increase (Fig. 1c), particularly when sulphate in the medium fell below the limit of detection. This dramatic increase in carbohydrate indicated severe unbalanced growth. In contrast, whilst sulphate was still detectable in the medium, the increase in concentration of carbohydrate was in balance with growth. If no sulphate was added to the culture at the beginning of the experiment it is evident that growth, if at all, was very much slower than 0.007 h⁻¹ (Fig. 1d). Under these conditions, the culture immediately accumulated large amounts of carbohydrate and bleached, as judged from the decrease in the phycocyanin:chlorophyll a ratio (Fig. 1f). The sheath contained a large, but constant amount of sulphate. When, after 7 d of batch cultivation, sulphate was added to the culture it was immediately taken up and associated with the sheath (Fig. 1e). The phycocyanin:chlorophyll a ratio started to increase and also the production of carbohydrate ceased. Growth (increase of protein) was not observed. However, recovery from starvation for sulphate for 7 d would presumably require a lag and, during this period, sulphate may have become unavailable for growth because it had been totally bound to the sheath.

In Figure 2 the photosynthetic characteristics of the sulphate-starved culture are shown as photosynthesis versus irradiance curves of samples from different points of time during the experiment. It can be seen that after 7 d of batch cultivation all photosynthetic parameters had decreased. In particular, the maximum rate of photosynthesis (Pmax) decreased to a very low level. Photosynthetic efficiency (α) also decreased, but to a lesser extent (Table 2). After addition of sulphate, Pmax and α were restored to their original values more rapidly then was the concentration of light-harvesting phycobiliproteins. It seems therefore unlikely that the original changes are caused by the decline in the phycobiliproteins. In addition, since mainly Pmax was inhibited, we concluded that the overall light-harvesting ca-

<table>
<thead>
<tr>
<th>Lighta Cycleb Dilution² Mediuma A730c Dry wt³</th>
<th>22 16–8 0.007 N 0.31 635</th>
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<tbody>
<tr>
<td>22 16–8 0.007 N 0.31 880</td>
<td></td>
</tr>
<tr>
<td>28 16–8 0.015 N 0.20 255</td>
<td></td>
</tr>
<tr>
<td>22 16–8 0.015 N 0.30 715</td>
<td></td>
</tr>
<tr>
<td>18 CL 0.007 N 0.29 730</td>
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<tr>
<td>18 CL 0.007 N 0.29 730</td>
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Table 1. Some properties of continuous cultures of Gloeothecae sp. PCC 6909

a Light is the photon flux density (PFD) in the culture and expressed as μE m⁻² s⁻¹.
b Cycle means continuous light.
c Dilution rate of the culture in h⁻¹.
d Medium is either BG 11 (+ N) or its nitrate-free variant (BG 11°; -N).
e A730 is the absorbance of the culture measured at 720 nm.
f Dry weight of the culture measured as mg 1⁻¹.

SLATE-LIMITED GROWTH IN GLOEO THECE
Figure 1. Effects of addition of sulphate to continuous and batch cultures of Gloeothece PCC 6909. (a–c) Chemostat culture grown in nitrate-free medium at $D = 0.07$ h$^{-1}$ and continuous light was switched to batch mode and given 0.3 mM sulphate. (a) Increase of protein in the culture (—-O--O). The solid line indicates the theoretical growth at $\mu = 0.07$ h$^{-1}$. (b) Pattern of sulphate in the culture. Sulphate concentration in the medium (—-△-△); sulphate associated with sheath (—-□-□). (c) Carbohydrate content of cells (—-∇-∇); ratio of phycocyanin to chlorophyll a (—+- + +). (d–f) Chemostat culture grown in nitrate-free medium at $D = 0.07$ h$^{-1}$ and continuous light was switched to batch mode and given 0.3 mM sulphate at day 7 (arrows). (d) Increase of protein in the culture (—-O--O). The solid line indicates the theoretical growth at $\mu = 0.07$ h$^{-1}$. (e) Pattern of sulphate in the culture. Sulphate concentration in the medium (—-△-△); sulphate associated with sheath (—-□-□). (f) Carbohydrate content of cells (—-∇-∇); ratio of phycocyanin (PC) to chlorophyll a (—+- + +).

Capacity was not affected by sulphate depletion. However, the rate of dark respiration showed a dramatic decline. Addition of sulphate resulted within 2 d in a total recovery of the photosynthetic capacity of the culture, and a marked increase in respiratory $O_2$ consumption.

In another experiment a sample of the continuous culture, grown at $D = 0.07$ h$^{-1}$ in nitrate-free medium and continuous light, was washed and resuspended in a double volume of either regular BG 11 medium or its nitrate-free variant. These suspensions were then incubated in aerated Kluvyer flasks either under low ($10 \mu$mol m$^{-2}$ s$^{-1}$) or high ($70 \mu$mol m$^{-2}$ s$^{-1}$) incident irradiation. Sulphate (dissolved and sheath-associated), carbohydrate and protein were measured in these cultures. The results of this experiment are depicted in Figures 3 and 4. In all cases sulphate was taken up from the medium and incorporated into the sheath. However, a great difference was observed between high and low light suspensions. At high irradiation ($70 \mu$mol m$^{-2}$ s$^{-1}$), sulphate was taken up from the medium within 3 (BG 11, Fig. 3a) or 4 (BG 11°, Fig. 3b) d. Under low light ($10 \mu$mol m$^{-2}$ s$^{-1}$) this took 11 d in either medium (Fig. 3c–d). The cultures grew under all conditions at a rate similar to that in the continuous culture from which they were taken (Fig. 4). Under high light, carbohydrate accumulation exceeded
growth when sulphate was depleted (Fig. 4a–b). It was also noted that carbohydrate accumulated faster and reached higher concentrations in the culture grown with nitrate (Fig. 4a). Under low light, carbohydrate accumulation lagged behind growth (Fig. 4c–d). Growth was slightly better in the presence of nitrate.

The photosynthetic characteristics of Gloeothecae showed remarkable changes after 3 d incubation (Table 2d–g) when compared to the original culture

### Table 2. Photosynthetic characteristics of Gloeothecae species PCC 6909 incubated under various conditions

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\alpha^b$</th>
<th>$P_{\text{max}}^c$</th>
<th>Dark respiration$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Continuous culture</td>
<td>0.18</td>
<td>2.76</td>
<td>0.85</td>
</tr>
<tr>
<td>B 7 d batch cultivation</td>
<td>0.13</td>
<td>0.47</td>
<td>0.208</td>
</tr>
<tr>
<td>C 7 d batch + sulphate</td>
<td>0.22</td>
<td>2.71</td>
<td>0.696</td>
</tr>
<tr>
<td>D 3 d batch, 70 $\mu$E, $NO_3^-$</td>
<td>0.32</td>
<td>1.92</td>
<td>2.149</td>
</tr>
<tr>
<td>E 3 d batch, 70 $\mu$E, $NO_3^-$</td>
<td>0.26</td>
<td>4.48</td>
<td>1.927</td>
</tr>
<tr>
<td>F 3 d batch, 10 $\mu$E, $NO_3^-$</td>
<td>0.14</td>
<td>1.78</td>
<td>1.714</td>
</tr>
<tr>
<td>G 3 d batch, 10 $\mu$E, $NO_3^-$</td>
<td>0.11</td>
<td>3.14</td>
<td>1.061</td>
</tr>
</tbody>
</table>

- $^a$ A; continuous culture grown in nitrate-free medium under continuous light at $D = 0.007$ h$^{-1}$. B; as A but after 7 d batch cultivation. C; as B 2 d after addition of 0.3 mM sulphate. D; as A but after 3 d batch cultivation in fresh BG 11° medium and at 70 $\mu$E m$^{-2}$ s$^{-1}$. E; as D but incubated in BG 11° medium. F; as D but incubated at 10 $\mu$E m$^{-2}$ s$^{-1}$. G; as F but incubated in BG 11° medium.
- $^b$ Photosynthetic efficiency ($\mu$mol O$_2$ mg$^{-1}$ protein h$^{-1}$).
- $^c$ Maximum rate of photosynthesis ($\mu$mol O$_2$ mg$^{-1}$ protein h$^{-1}$).
- $^d$ Dark respiration ($\mu$mol O$_2$ mg$^{-1}$ protein h$^{-1}$).

(Table 2a). $P_{\text{max}}$ was much lower in $N_2$-fixing cultures (Fig. 5). Highest photosynthetic rates were found in cultures incubated at high light intensity but the differences were not very great. Photosynthetic efficiency ($\alpha$) showed larger differences.
Figure 4. Changes in carbohydrate and protein content of a steady state sample of a chemostat culture of Gloeoeprce PCC 6909, grown at $D = 0.007 \, \text{h}^{-1}$ under continuous light and resuspended (a, c) in nitrate-containing medium (BG 11) or (b, d) in nitrate-free medium (BG 11*). Incubation was under 70 (a, b) or 10 (c, d) $\mu$mol m$^{-2}$ s$^{-1}$ light as a batch culture. Carbohydrate (g l$^{-1}$) (— ▽ — ▽ ); protein (g l$^{-1}$) (— ○ — ○ ).

Figure 5. Photosynthesis versus light curves of batch cultures of Gloeothece PCC 6909 incubated for 3 d at 70 (a) or 10 (b) $\mu$mol m$^{-2}$ s$^{-1}$ light in sulphate containing BG 11 or BG 11* medium. $\text{N}_2$-fixing culture (— △ — △ ); culture with nitrate as nitrogen source (— □ — □ ). (Table 2). Cultures incubated at high intensity had a value of $x$ more than twice that found in the light cultures. Photosynthetic efficiency was slightly higher in $\text{N}_2$-fixing cultures (Table 2). Dark respiration was also highest in cultures incubated at high light intensity and $\text{N}_2$-fixing cultures showed slightly higher respiration rates than nitrate cultures.

$\text{N}_2$ fixation (acetylene reduction) was measured in the cultures that were incubated in nitrate-free medium. Resuspension of the cells into fresh (sulphate-containing) medium resulted in the almost immediate induction of nitrogenase (Fig. 6). Under high light, nitrogenase increased dramatically, reaching a maximum after 5 d (Fig. 6a). Subsequently, nitrogenase activity dropped to a very low activity which is usually found in batch cultures (Stal, Myint & Ortega-Calvo, 1991). Under low light, nitrogenase was also clearly stimulated. However, maximum nitrogenase activity was lower and a cyclic behaviour was observed with a period of 3 d (Fig. 6b). Also, during the course of the experiment, nitrogenase activity did not drop to the very low level of the culture incubated under high light.
Despite considerable effort, the mechanisms by which *Gloeotheca* sp. is capable of protecting nitrogenase from inactivation by oxygen have not yet been completely elucidated (see Gallon 1992 and Gallon & Stal 1992 for reviews). Kallas *et al.* (1983) emphasized that a low level of irradiance is required for growth of *Gloeotheca*. This may seem obvious since high light intensities would result in a higher sp. This may seem obvious for growth of *Gloeotheca* sp. PCC 6909. A steady-state sample of the chemostat culture grown at $D = 0.007$ h$^{-1}$ under continuous light was resuspended in fresh nitrate-free medium and incubated under 70 (a) or 10 (b) µmol m$^{-2}$ s$^{-1}$ light as a batch culture.

**Figure 6.** Nitrogenase activity (acetylene reduction) in *Gloeotheca* PCC 6909. A steady-state sample of the chemostat culture grown at $D = 0.007$ h$^{-1}$ under continuous light was resuspended in fresh nitrate-free medium and incubated under 70 (a) or 10 (b) µmol m$^{-2}$ s$^{-1}$ light as a batch culture.

**DISCUSSION**

Despite considerable effort, the mechanisms by which *Gloeotheca* sp. is capable of protecting nitrogenase from inactivation by oxygen have not yet been completely elucidated (see Gallon 1992 and Gallon & Stal 1992 for reviews). Kallas *et al.* (1983) emphasized that a low level of irradiance is required for growth of *Gloeotheca*. This may seem obvious since high light intensities would result in a higher evolution of oxygen and consequently inhibit nitrogenase (Giani & Krumbein, 1986). However, this explanation is unlikely because, regardless of the source of nitrogen, good growth of *Gloeotheca* PCC 6909 occurs only under low light (Myint, 1991; Stal, unpublished observations). In batch culture the cells bleached as a result of the decomposition of phycobiliproteins. In cyanobacteria the degradation of phycobiliproteins is usually the result of the impairment of protein synthesis (Ownby, Shannahan & Hood, 1979), which is mostly reported as a consequence of nitrogen depletion (Allen & Smith, 1969; Allen & Hutchison, 1980; Duke, Cezeaux & Allen, 1989). However, sulphur starvation is also known to induce bleaching (Wanner *et al.*, 1986; Kramer & Schmidt, 1989). Because we showed that bleaching in batch cultures of *Gloeotheca* sp. PCC 6909 occurred both in diazotrophic and nitrate-grown cultures, we concluded that impairment of nitrogen fixation could not be the cause of bleaching and therefore that bleaching was probably unrelated to nitrogen depletion.

Tease & Walker (1987) and Tease *et al.* (1991) reported the occurrence of large amounts of sulphated polysaccharides in the sheath of *Gloeotheca*. Sulphated polysaccharides are common in eukaryotic algae but are rarely found in prokaryotes (Percival, 1979). The particular large amount of sulphate (17.5% of sheath dry weight) in the sheath and the large amounts of sheath that are produced by *Gloeotheca* suggested to us the possibility that sulphate depletion rather than nitrogen depletion may explain the observed degradation of phycobiliproteins. The results we have presented here clearly showed that sulphate-depleted cultures of *Gloeotheca* were (i) growing at very low rates (if at all), (ii) bleached, (iii) contained a large amount of sulphate in the sheath, (iv) showed very low maximum rates of photosynthesis and respiration, though affinit for light was much less affected, and (v) showed very low nitrogenase activities. We also showed that addition of sulphate to such cultures resulted in (i) growth, (ii) synthesis of phycobiliproteins, (iii) further incorporation of sulphate into the sheath, (iv) restoration of the maximum rate of photosynthesis and the rate of dark respiration, and (v) induced nitrogenase activity.

An important observation was the fact that sulphate incorporation into the sheath was light-dependent. We therefore believe that incorporation of sulphate into the sheath represents newly synthesized sulphated polysaccharides, as is the case in eukaryotic algae (Percival, 1979). It is very likely that this synthesis depends on light and photosynthetic CO$_2$ fixation. At low light intensity, sulphate is less rapidly taken up from the medium and presumably remains available for growth for a longer period of time. Under low light the culture will therefore not bleach, but light limitation allows only slow growth of the culture.

*Gloeotheca* sp. PCC 6909 is capable of diazotrophic growth under continuous illumination (Gallon *et al.*, 1974; Mullineaux *et al.*, 1981; Myint, 1991). However, when grown in batch culture under alternating light–dark cycles, nitrogenase activity in this organism was confined to periods of darkness (Mullineaux *et al.*, 1981; Stal & Krumbein, 1985; Myint, 1991). This pattern of $N_2$ fixation can be found in all non-heterocystous nitrogen-fixing cyanobacteria with the notable exception of the marine planktonic filamentous *Trichodesmium* (Gallon & Stal, 1992). Such diel variations of $N_2$ fixation have been interpreted as the temporal separation of the incompatible processes oxygenic photosynthesis and $N_2$ fixation (Kallas *et al.*, 1983). However, Ortega-Calvo & Stal (1991) cultivated *Gloeotheca* sp. PCC 6909 in a continuous culture and made two important observations. These were: (i) nitrogenase activity was totally or predominantly confined to the light period when imposing as 12–12 or 16–18 h light–dark cycle, respectively, (ii) the maximum growth rate
was close to 0.02 h⁻¹ and measured nitrogenase activity was proportional to this growth rate. The observed growth rate was several fold higher than that reported for batch cultures of the same organism (Tease & Walker, 1987; Gallon et al., 1988; Myint, 1991). Moreover, the rate of acetylene reduction measured in continuous culture was up to three orders of magnitude greater than that in batch cultures (Stal et al., 1991). At that time Ortega-Calvo & Stal (1991) assumed that their continuous cultures were light-limited. Taking into account the results presented here we believe that their assumption was not correct and that sulphate limitation seems more likely.

Addition of sulphate to the culture resulted in an almost immediate stimulation of nitrogenase activity (Fig. 6) which reached specific activities similar to those observed in continuous cultures (Ortega-Calvo & Stal, 1991). In continuous culture, sulphate is available for growth because of the continuous supply of sterile medium, whereas in batch culture sulphate will be depleted. Moreover, when sulphate is available, high nitrogenase activity may be observed in Gloeothecae sp. but the requirement for energy and reductant makes it dependent on light. Indeed, nitrogenase activity in sulphate-sufficient batch cultures grown under a light-dark regime was confined to the light period (Stal, preliminary results). On the other hand, light-dependent N₂ fixation reawakens the paradox of contemporaneous nitrogen fixation and oxygenic photosynthesis. At present, we cannot offer a good explanation for this, but it is certain that the mechanism that allows simultaneous N₂ fixation and photosynthesis in Gloeothecae sp. may be limited by the requirement for energy and reductant, which depends on the antennae of oxygen. Therefore, sufficient aeration of the cultures, preventing oxygen supersaturation, may be of paramount importance. In the experiments presented in this paper, batch cultures were aerated in the same manner as continuous cultures (Ortega-Calvo & Stal, 1991).

We have compared the photosynthetic parameters of sulphate-amended N₂-fixing and nitrate-grown cultures (Fig. 5; Table 2). Sulphate starvation resulted in a dramatic decrease of Pmax but z was less affected. Pmax depends either on the number of reaction centres or on the efficiency of photosynthetic electron transport or the CO₂-fixing enzyme RuBisCO, while z depends rather on the antennae of the reaction centres. Because the rate of dark respiration was dramatically decreased in the sulphate-starved culture, a change in electron transport rather than a change in the photosynthetic apparatus may be a more likely explanation of the effect of sulphate starvation on Pmax. Both z and Pmax are higher at high light intensity, implying that synthesis of the photosynthetic apparatus may be stimulated by light. However, increased photosynthetic capacity could not be translated into growth but rather resulted in accumulation of carbohydrate (Fig. 4a-b). Although the affinity (z) for light is similar in N₂-fixing cells and nitrate-grown cells, N₂-fixing cells show much lower maximum rates of photosynthesis than do nitrate-grown cells. The lower (net) rate of photosynthesis in the N₂-fixing culture consequently leads to much lower accumulation of carbohydrate in cultures incubated at 70 μmol m⁻² s⁻¹ (Fig. 4a-b). This is not the case in the light cultures. At 10 μmol m⁻² s⁻¹ incident light intensity, the actual rates of photosynthesis are not very different between nitrate-grown and N₂-fixing cultures. Thus, no difference in carbohydrate accumulation between these cultures was observed. It is not clear whether the differences in Pmax and carbohydrate accumulation in both cultures is the result of adaptation of the photosynthetic apparatus or of the balance between respiration (or oxygen uptake) and carbohydrate (or reductant) mobilization. Decreased photosynthetic oxygen production, or even net oxygen uptake in the light, has been observed in Gloeothecae sp. PCC 6909 (Myint, 1991) as well as in other non-heterocystous N₂-fixing cyanobacteria (Mitsui et al., 1986; Stal & Krumpen, 1987). In this respect the results of Maryan et al. (1986) who showed the importance of respiration for N₂ fixation in Gloeothecae sp., are important.

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