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NITROGEN FIXATION BY A BANGLADESH DEEPWATER RICE-FIELD CALOTHRIX

by

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(M.Sc.(Ag.), Mymensingh)

A thesis submitted for the degree of
Doctor of Philosophy
in the University of Durham, England

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Department of Biological Sciences

February 1990



25 JUN 1991

This thesis is entirely my own work and has not previously been submitted for any other degree.

dedicated to the poor man behind the plough

ABSTRACT

In order to study the influence on blue-green algal nitrogenase activity of environmental variables in deepwater rice-fields (DWR), a laboratory study was planned on a DWR isolate of Calothrix (D764). The variables chosen were light, oxygen, combined nitrogen, phosphorus and iron. As availability of P is likely to play an especially important role for growth and nitrogen fixation in DWR, studies on phosphatase activity of the isolate were also included.

The method used for measuring nitrogenase activity was acetylene reduction assay (ARA). In order to convert nitrogenase activity to nitrogen fixation, the conversion ratio of $N_2 : C_2H_2$ reduced was determined by comparing the total amount of N fixed with total C_2H_2 reduced. The ratio was 1 : 4.1 and 1 : 5.2 at 85 and 10 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, respectively.

Changes in nitrogenase activity in batch culture were studied in relation to growth characteristics. Maximum activity (10.5 $\text{nmol } C_2H_4 \text{ mg d. wt}^{-1} \text{ min}^{-1}$) was observed after two days of growth. During this period, juvenile trichomes (hence maximum heterocyst frequency) were abundant and cyanophycin granules were absent; chl a, phycobiliprotein and algal N decreased. It is suggested that the juvenile filament is the most active nitrogen-fixer during the growth of the alga.

The response of nitrogenase to changes in light flux (down- or upshift) was rapid. The alga showed a marked drop in nitrogenase activity in the dark, but subsequent changes were slow, with detectable activity after 24 h. Higher nitrogenase activity was observed when the dark grown alga was re-illuminated, than the maximum activity found under continuous illumination.

Nitrogen fixation and heterocyst differentiation were suppressed when 10 mg l^{-1} $\text{NH}_4\text{-N}$ was added to a batch culture. Fe-deficient cultures had lower nitrogenase activity and N content than Fe-sufficient cultures. Fe-deficiency led to the development of a series of new heterocysts apical to the basal ones. Addition of Fe to Fe-deficient cultures led to a marked increase in nitrogenase activity and loss of the degenerated basal heterocysts.

The alga was capable of using a number of organic P substrates as the sole source of phosphorus and showed both cell-bound phosphomono- and phosphodiesterase activities. In batch culture, phosphatase activity was detected when cellular P content dropped to 0.98%. A brief study on the influence of the environmental factors on cell-bound phosphatase activities of the alga has been included.

A brief comparison in nitrogenase activity of a UK field Rivularia population and bacterised laboratory isolate Rivularia D403 was made and probable behaviour of algae in DWR is discussed.

ABBREVIATIONS

ARA	acetylene reduction assay (acetylene reducing activity)
PAR	photosynthetically active radiation (400-700 nm)
PMEase	phosphomonoesterase
PDEase	phosphodiesterase
nm	nanometre
μm	micrometre
cm	centimetre
m	metre
ha	hectare
μg	microgramme
mg	milligramme
g	gramme
kg	kilogramme
μl	microlitre
ml	millilitre
l	litre
s	second
min	minute
h	hour
d	day
nmol	nanomole
μM	micromolar
mM	millimolar
M	molar
d. wt	dry weight
chl <u>a</u>	chlorophyll <u>a</u>
N	nitrogen
P	phosphorus
Fe	iron
AMeP	2-amino-2-methyl-1-propanol
CAPS	(3-[cyclohexylamino]-1-propanesulfonic acid)
DMG	3,3-dimethyl-glutaric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
TES	(N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid)
EDTA	ethylenediaminetetra-acetic acid
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
ρNPP	ρ -nitrophenyl phosphate
bis- ρNPP	bis(ρ -nitrophenyl phosphate)
$^{\circ}\text{C}$	degree Celsius
n	number of replicates

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1 INTRODUCTION

1 General introduction

Bangladesh, a land of 144000 km² and situated between 20°35' to 26°75' N and 88°03' to 92°75' E, stretches from near the foot-hills of the Himalayas to the Bay of Bengal. The economy is predominantly agricultural and about 75% of the total population of over 100 million is engaged in it. The total cropped area is about 12.9 x 10⁶ ha. Deepwater rice is grown in nearly 2 x 10⁶ ha of land, which is about 12.2% of the total cropped area (Hoque et al., 1982). Its importance is evident from the fact that there is no other crop which can be grown in these areas during the monsoon season and produce a stable yield year after year with little or no addition of fertilizer. Brammer (1976, 1983) and Rother et al. (1988) attributed part of this fertility to the presence of blue-green algae.

It is well established that some blue-green algae fix atmospheric nitrogen and that part of this nitrogen can eventually be incorporated into the rice plant (Tirol et al., 1982; Watanabe and Ventura, 1982; Mian and Stewart, 1985). A number of studies on nitrogen fixation in Bangladesh deepwater rice-fields have been reported (Rother et al., 1988; Rother and Whitton, 1989), but relatively little is known about the influence of environmental variables on nitrogen fixation of axenic cultures in the laboratory. The present study was therefore planned to study the influence of environmental variables on nitrogen fixation and phosphatase activity of an axenic Bangladesh deepwater rice-field Calothrix in the laboratory.

1.2 Deepwater rice

Deepwater rice is variously called broadcast aman, low-land aman and floating rice. There are hundreds of cultivars and these are perhaps

physiological races of Oryza sativa L. The distinguishing character of this group of rice is that the internode, leaf sheath and blade can elongate by increasing cell length with rising floodwater, produce nodal roots and tiller, form knees and when uprooted, float on the water surface.

Deepwater rice is grown on nearly 2×10^6 ha of land which is about 12.2% of the total cropped area (Hoque et al., 1982). The bulk of deepwater rice is grown in the intermediate flooded zone where water depths are generally 0.91 to 1.83 m (Allison, 1975). Total elongation of the stem can be 2.5 to 25 cm d^{-1} (Choudhury and Zaman, 1970). Zaman et al. (1975) showed that a linear increase of culm length occurs despite the fluctuation of flood levels.

It is believed that the stable yield of deepwater rice is maintained by an annual deposit of alluvium from the seasonal flood and by biological activity in the floodwater itself (Brammer, 1983; Whitton et al., 1988c). Particular attention has been focussed on the nitrogen-fixing ability of many blue-green algae (Martinez and Catling 1982; Rother et al., 1988).

1.3 Environmental features of deepwater rice-field

The environment of deepwater rice-field is complex and heterogeneous because of the abrupt change from arid conditions in the early plant growth stages to a period of prolonged flooding lasting several months. During this period a wide aquatic flora develops and makes the environment more complex (Whitton et al., 1988b).

1.31 Soil

Deepwater rice soils with a single crop each year pass through three distinct phases: a drying phase from harvest to the commencement of the wet season, an alternately wet and dry phase in the initial stages of plant growth and a deeply flooded phase of up to 6 months. Where additional

crops are grown before or after deepwater rice, the cultural operations of these crops further influence the soil. Between harvest and the beginning of the wet season, cracking and drying can have marked effects on the physical conditions of the soil and thus on subsequent tillage operations. After the start of the wet season, low moisture status can increase phosphate fixation, while under fluctuating moisture status, nitrification proceeds more rapidly, producing a build up of nitrates, which, if not leached out by rain, are available to deepwater rice plants.

With the arrival of floodwater, numerous chemical, physical and microbiological changes occur. Soil submergence increases the availability of N, P, silicate, Fe, Mn and Mo, but can harm rice by reducing the availability of S, Cu and Zn. An increase in pH of acid soils benefits rice by depressing the toxicity of Al, Mn, Fe, CO₂ and organic acids; by increasing the availability of P, silicate and Mo; and by favouring microbial processes that release nutrients. A decrease in pH of alkaline soils favours rice by increasing the availability of P, Ca, Fe, Mn, Cu and Zn (Ponnamperuma, 1978).

Although the chemical changes that take place in flooded soil are generally beneficial to deepwater rice plants, they may come too late for maximum yields. Much of the deepwater rice plant growth takes place before flood arrival (Rother and Whitton, 1988) and this period may determine the success of the crop.

1.32 Flooding

In Bangladesh, flooding typically occurs from late June to early November. There are usually two distinct flood peaks, the first in late July and the second in mid September (Whitton *et al.*, 1988a). In deepwater rice areas of Bangladesh in 1979 - 80, the maximum water depth was 1 - 2 m in 56% of the fields, 2 - 3 m in 28% of the fields and > 3 m in

only 2% of the fields (Catling *et al.*, 1983). The maximum water depth at one location may vary 50 - 100 cm between years (Catling *et al.*, 1988). In large areas of deepwater rice flooding patterns are very heterogeneous, with deep pockets in the centre of the bils, that may flood 1.5 - 2.0 m deeper than the surrounding areas.

1.33 Temperature

As most of the time there is about 1 - 3 m of standing water in deepwater rice-fields the temperature range is very narrow. The temperature in the upper part of the water column at two locations in Bangladesh during July - October ranged between 29 - 35°C, although slightly lower temperatures were sometimes recorded near the bottom (Whitton *et al.*, 1988a). Vertical mixing is normally brought about by a cooling of the upper water layers to temperatures below those of deep layers. Though the fastest heating during the day takes place at the surface, night time cooling generally reduces the surface temperature no lower than the deeper water.

1.34 Light

During the monsoon season, the cloud cover in the sky varied widely and is the key factor influencing the total PAR^{which} reaches the rice-field. The mean values for PAR during May - August 1983 measured at hourly periods between 1000 - 1400 h fell within a narrow range, with 95% of values between 1000 - 3000 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$. The highest spot values recorded with the 2- π sensor were approximately 3400 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, but with the cosine sensor about 2200 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (Whitton *et al.*, 1988a). Daily PAR maxima at Sonargaon in Bangladesh in 1986 ranged from 310 - 2173 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, but 89% of the days had maxima greater than 1500 and only 2% less than 50 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$. Daily totals for light flux

ranged from 4.50 - 46.1 mol_{photon} m⁻² d⁻¹, with 10% of days less than 15 mol photon m⁻² d⁻¹ (Whitton and Rother, 1988b). The factors affecting any particular microhabitat include irradiance reaching the top of the deepwater rice leaf canopy, time of day and the extent of surface and under water plant biomass. In a 12 h measurement of light flux at 10 cm below of the surface water inside a deepwater rice field, light flux reached a maximum between 1200 - 1300, but the total light flux was much lower in October than June presumably due to greater rice leaf canopy and much greater under water biomass (Whitton et al., 1988a). PAR maxima inside the deepwater rice fields were less than 250 μmol photon m⁻² s⁻¹ for 10% of the days at 5 cm above the water surface and 62% of the days at -10 cm (Whitton and Rother, 1988b).

1.35 Dissolved oxygen

The first surge of floodwater arriving in the field and flash-flood water has high oxygen levels and wind increases dissolved oxygen (Puckridge et al., 1988). The concentration of dissolved oxygen varied markedly according to the location, time of day, position in the water column and whether the field was planted or fallow. Spot survey measurements in a range of habitats between 1400 - 1600, showed a trend for high pH values associated with high O₂ values. O₂ in two locations in Bangladesh was sometimes supersaturated in the upper part of the water column (Whitton and Rother, 1988a). The oxygen concentrations in floodwater of deepwater rice-fields exhibited diel changes and decreased with depth (Whitton and Rother, 1988a; Setter et al., 1987; Heckman, 1979), but there was no marked changes at 10 cm from the bottom. Concentrations of oxygen at the water surface significantly decreased between 1800 - 0600; presumably this was due partly to respiration by the submerged shoots and other organisms in the floodwater (Setter et al., 1987).

Cropping systems greatly affect O₂ regimes. The presence of the lower parts of aus rice left after harvest, or of rotting jute, both led to high O₂ demand and eventual release of nutrients. From September onwards the bottom water of deepwater rice-fields was found to be anoxic perhaps due to the decomposition of the remains of the aus crop mixed with deepwater rice crop (Whitton *et al.*, 1988a).

The high oxygen in the water column was contributed mainly by mass transport of gases from atmosphere through the gaseous film on the surface of the rice plants (Raskin and Kende, 1983; Whitton and Rother, 1988b), but standing crops of macrophytes and algae also contributed to the increased oxygen in the water by photosynthesis (Whitton *et al.*, 1988d). Dissolved oxygen concentration below 0.125 mol m⁻³ around plant tissues have been reported to affect metabolism adversely (Armstrong, 1979). Energy generation for nutrient uptake depends on respiration via the O₂ supply either from the floodwater or from gas spaces from the shoots to the roots.

1.36 Nutrients in flood water of rice-fields in Bangladesh

Flood water in Bangladesh is moderately soft (Whitton *et al.*, 1988a). Na, Mg and Ca are the elements in flood water likely to vary widely depending on the relative contributions from the sources of river water and partly drainage from rainwater in local catchment. N and P are also fairly low, but provide a "mesotrophic" environment for algae. There is a relatively low N : P ratio and most of the filtrable P is in the form of organic compounds. Most minor elements are either below or just above detectable limits and K, silica, Cl and SO₄⁻ are low (Whitton *et al.*, 1988a).

1.37 Algal flora

Studies on algae associated with deepwater rice in Bangladesh have been reported by Catling *et al.*, 1981 and Whitton *et al.*, 1988b. These surveys revealed a rich algal flora in deepwater rice-fields and adjacent areas. In deepwater rice-fields, algae were found to grow on soil prior to flooding, in the water column as free floating flocs and associated with deepwater rice plants or other aquatic macrophytes. After the recession of floodwater some algae were found to grow on the moist soil. Algae were usually much more abundant on isolated plants or tillers at the edge of a field next to a channel, or on submerged macrophytes in fallow areas than inside rice-fields, possibly because of reduced light and grazing pressure from snails and other microorganisms.

In the deepwater rice-fields of Bangladesh, Aulosira fertilissima and Scytonema mirabile, were widespread and equally successful on soil in the period prior to the arrival of floodwater and floating on the surface of the water during the flood (Whitton *et al.*, 1988b). Gloeotrichia natans, Tolypothrix penicillata, Aulosira fertilissima, Anabaena spp., Cylindrospermum majus, Nostoc spp., Scytonema mirabile, Tolypothrix penicillata and Aphanothece stagnina were the floating forms found in the deepwater rice-fields. Algae which occur commonly as epiphytes or associated with rice and other hydrophytes are G. pisum, G. natans, Anabaena spp., Nostoc, Scytonema mirabile, Aphanothece stagnina, Lyngbya aestuarii, Lyngbya spp., Oscillatoria spp., Phormidium spp., Microchaete, Oedogonium, Bulbochaete, Coleochaete, Chaetophora and Gomphonema. Among benthic forms during flooding period are species of Chara and Nitella. On the deepwater rice culms, Anabaena occurred most commonly, followed by Gloeotrichia, Oscillatoria, Chroococcus, Nostoc, Lyngbya and Microchaete in decreasing order (Martinez and Catling, 1982). Calothrix spp. and Nostoc were frequent on rotting sheaths and Nostoc sometimes formed colonies

inside the air cavities of older sheaths (Whitton *et al.*, 1988b). The algae Aulosira fertilissima, Nostoc, Tolypothrix byssoidea, T. penicillata, Oscillatoria, Porphyrosiphon notarissii were successful in the post-flood period. Among planktonic species Anabaena and Synechococcus were also observed in deepwater rice-fields in Bangladesh.

Different species of Gloeotrichia and a number of green algae e.g. Aphanochaete repens, Bulbochaete spinosa, Chaetophora sp., Coleochaete sp. and Draparnaldiosis indica have been shown to form long hairs in deepwater rice-fields of Bangladesh (Whitton *et al.*, 1988b).

Sixty-five taxa of diatoms were recorded belonging to 17 genera at two locations of Bangladesh. Navicula confervacea was the predominant diatom during the later part of the season. Periodicity of algae in deepwater rice-fields was also observed by (Catling *et al.*, 1981; Whitton *et al.*, 1988b) being abundant from July to the end of September and declined in number and variety with flood recession, except for the diatoms, which were possibly more tolerant to anoxic conditions.

1.4 Nitrogen fixation by blue-green algae in rice-field

N₂-fixing blue-green algae grow abundantly in tropical and subtropical regions and are particularly common in rice-fields (Watanabe and Yamamoto 1971; Whitton *et al.*, 1988b). De (1939) provided evidence, whilst working on blue-green algae of (present day) Bangladesh, that these organisms are the main agents for N₂-fixation in the rice field soil and an enormous volume of material on this subject has been published from various countries (e.g. Watanabe *et al.*, 1951; Singh, 1961; Venkataraman, 1972; Roger and Kulasooriya, 1980; Roger and Watanabe, 1982; Rother *et al.*, 1988; Rother and Whitton, 1989). The probable importance of blue-green algae in the nitrogen economy of Bangladesh rice-fields has been stressed by several authors (Brammer, 1976, 1983; Catling *et al.*, 1981; Martinez and Catling,

1982; Bhuiya et al., 1984; Rother et al., 1988; Rother and Whitton, 1989). Maintenance of natural nitrogen fertility of rice-fields has been explained by blue-green algal N₂-fixation, its slow mineralisation and accumulation in soil (De and Sulaiman, 1950; Hirano, 1958; Subrahmanyam et al., 1965; Watanabe, 1965; App et al., 1980; Tirol et al., 1982; Mian and Stewart, 1985).

Under a relatively low light intensity (800 lux) a N₂-fixation rate of 10 - 20 kg N ha⁻¹ crop⁻¹ (extrapolating nitrogen fixation activity of 5.1 μmol N plant⁻¹ h⁻¹ based on two determinations at heading and maturing stages of plant, ARA technique) has been observed in deepwater rice-fields mainly due to epiphytic Nostoc, Anabaena, Calothrix and Gloeotrichia (Kulasooriya et al., 1981a) and this value corresponds closely to that found by App et al. (1980). The blue-green algae attached to submerged weeds also play a positive role in nitrogen cycling by fixing 2 kg N ha⁻¹ crop⁻¹ under rice cultivation and 4 kg N ha⁻¹ crop⁻¹ under fallow land (Kulasooriya et al., 1981b). Most detailed surveys and quantifications of nitrogen fixation by blue-green algal communities in deepwater rice-fields of Bangladesh have been reported by Rother et al. (1988); Whitton et al., (1988c) and Rother and Whitton (1989). Nitrogen fixation in the pre-flood period at five locations in Bangladesh in 1983 was 1.0 - 10.2 kg ha⁻¹ depending on the blue-green algal cover in the fields (Rother and Whitton, 1989). Nitrogen fixation inside deepwater rice-fields during the flood season of 1983 at Manikganj and Sonargaon was 4.39 and 4.42 kg ha⁻¹, respectively (Rother et al., 1988). Nitrogen fixation in the post-flood period at Manikganj was 2.15 kg ha⁻¹ due to the development of Aulosira fertilissima and Scytonema mirabile mats, whereas at Sonargaon it was negligible. Total nitrogen fixation at Manikganj and Sonargaon in 1983 was 16.7 and 8.2 kg ha⁻¹ respectively. The difference in nitrogen fixation at those two locations was mainly due to greater algal diversity and

abundance at the former site. In low land rice-fields, variable N_2 -fixation rates have been found and a contribution in the range of 10 - 30 kg N ha⁻¹ crop⁻¹ appears to be typical (Agarwal, 1979; Venkataraman, 1981; Roger and Watanabe, 1982; Roger et al., 1986; Rother et al., 1988). The presence of blue-green algae can lead to a 10 - 15% increase in grain yield in the total absence of chemical fertilizer (Agarwal, 1979; Roger and Watanabe, 1982).

Although lot of work has been carried out on nitrogen fixation in rice-fields, little is known about the availability of algal nitrogen to the rice plants. Watanabe and Ventura (1982) reported a ¹⁵N study in a deepwater rice plot where about 15% of the total nitrogen in deepwater rice had been supplied by blue-green algal N_2 -fixation. However, Rother and Whitton (1989) speculated that much of the nitrogen fixed by algae may not be available to deepwater rice plants, because, during the pre-flood period, algal mats in Bangladesh deepwater rice-fields increased steadily and there was little evidence of grazing and lysis by which algal nitrogen might be made available to crop plants. Algae which were less successful during the flood might be recycled quickly and their nitrogen becoming available to plants. Algae which were successful during the flood period may not be recycled until flood water receded when deepwater rice had been harvested and the soil was ploughed for the next crop.

N_2 -fixation in rice-fields can be influenced by a variety of factors, the cumulative effect of which determines the ultimate nitrogen gain (Sethunathan et al., 1981). The most important are likely to be light intensity and nitrogenous compounds in the environments. The rate of nitrogen fixation is usually light dependent (Fogg, 1974; Roger and Reynaud, 1979). Deficiency of light limits N_2 -fixation e.g. highest activity was recorded when the plant canopy gave the lowest cover (Watanabe et al., 1978a; Boddey and Ahmad, 1981) and during cloudy weather, higher

activity in unplanted fields than in planted fields (Yoshida and Ancajas, 1973). In Bangladesh deepwater rice-fields the highest nitrogenase activity was recorded in the early afternoon when the light intensity is maximum (Aziz and Whitton, 1988; Rother et al., 1988). Asymmetric curves of ARA with a maximum either in the morning (with a low decreasing activity in the afternoon) or in the afternoon, recorded by Alimagno and Yoshida (1977), have been explained by Roger and Reynaud (1979) as the inhibitory effect of high light intensity and optimal light intensity in the afternoon, respectively.

In the presence of nitrogen fertilizer, blue-green algal N₂-fixing activity is inhibited or at least affected (Roger and Kulasoorya, 1980). Alimagno and Yoshida (1977) estimated about 18 - 33 kg N ha⁻¹ crop⁻¹ is fixed in the unfertilized lowland rice soil compared to 2.3 - 5.7 kg N ha⁻¹ crop⁻¹ in fertilized fields. In contrast, it has been estimated that the efficiency of N₂-fixation does not seem to have been affected even when algae were applied in combination with 75 kg N ha⁻¹ chemical fertilizer and the highest increase in grain yield was obtained in the third season, suggesting that the benefit accumulated over the years (Mudholkar et al., 1973). It has been estimated that about 14% additional energy yield (in terms of nitrogen contribution in the range of 20 - 30 kg ha⁻¹) could be obtained by blue-green algal complementation with nitrogen fertilizer (Venkataraman, 1981). After broadcast N fertilization, N in the floodwater returns to the original concentration within a few days (Fillery and De Datta, 1986; Fillery et al., 1986). Therefore blue-green algal inhibition by broadcast N fertilizer might be most indirect. Broadcasting N fertilizer favours the growth of green algae more than the blue-green algae early in the crop cycle (Roger et al., 1980; Fillery et al., 1986) which permits the establishment of grazers that initially cause the disappearance of green algae and might further inhibit blue-green algal

growth, even when the N concentration in the flood water has decreased to a level insufficient to inhibit blue-green algae either directly or indirectly through competition with green algae. On the other hand, deep placement of N fertilizer did not suppress the growth of N₂-fixing blue-green algae and did not affect the photodependent nitrogen fixation on the surface soil (Roger *et al.*, 1980). Seasonal variation in N₂-fixing activity has been attributed to the succession of blue-green algae (Watanabe *et al.*, 1978a), shading by the plant canopy (Yoshida and Ancajas, 1973; Boddey and Ahmad, 1981), or a predominant effect of light intensity in relation to both season and plant canopy (Roger and Reynaud, 1979; Rother *et al.*, 1988). In the wet season N₂-fixing activity can decrease to 20% (3 kg N ha⁻¹ crop⁻¹) in the flooded rice-field compared to the dry season (15 kg N ha⁻¹ crop⁻¹) (Yoshida and Ancajas, 1973). Two peaks have been observed by Watanabe *et al.*, (1978b), one at the early stage of rice cultivation and another after harvesting, in both dry and wet seasons, which the authors considered the effect of light. However, peak N₂-fixing activity may occur at any time during the cultivation cycle (Roger and Reynaud, 1979).

A considerable increase of N₂-fixation by blue-green algae in the presence of the rice crop due to the increased CO₂ supply, has been documented in a laboratory study (De and Sulaiman, 1950). Little or no information is available on the effect of changes of pH (with the change of CO₂) and O₂ in the rice-field on N₂-fixing activity by blue-green algae. Rarely, temperature could be a limiting factor for blue-green algal N₂-fixation, particularly in deepwater rice-fields which are well temperature buffered (Whitton *et al.*, 1988a), but in dryland, under weak plant cover, a high temperature in the middle of the day may inhibit blue-green algal N₂-fixation (Roger and Reynaud, 1979).

1.5 Influence of environmental factors on nitrogen fixation in blue-green algae

1.5.1 Light

The rate of nitrogen fixation by blue-green algae is usually light dependent and has a close relationship with photosynthesis (Fogg, 1974; Bothe, 1982). The electrons and ATP for N₂-fixation are generated via photosynthesis (Lex and Stewart, 1973) and both the rate and duration of nitrogenase activity in the dark are affected by the rate of photosynthesis and assimilation during the light period (Dugdale and Dugdale, 1962; Horne and Fogg, 1970; Stewart *et al.*, 1975; Fay, 1976). Lex and Stewart (1973) demonstrated that in cells with high carbohydrate reserves, DCMU did not affect ARA, but upon depletion of these reserves, inhibition progressively appeared. ATP can be supplied to nitrogenase either by phosphorylation or by terminal respiration, but in carbon starved *Anabaena cylindrica*, it is the reductant rather than ATP which limits the rate of ARA (Lex and Stewart, 1973; Donze *et al.*, 1974). Upon carbon starvation, the formation of reductant becomes progressively more dependent on light and in severely starved cells almost all reductant can be generated via photosystem I (Donze *et al.*, 1974).

Axenic strains of *Anabaena* showed different saturating light intensities for ARA, some at about 5 klux (Chen, 1983a) while others at about 15 klux (Antarikanonda and Lorenzen, 1982). In the field condition also the saturating light intensity for N₂-fixation is variable, e.g. 2000 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for a thermal *Calothrix* (Wickstorm, 1980), 900 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for *Nostoc muscorum* (Coxson and Kershaw, 1983) and ca 5 klux for *Gloeotrichia* in a rice-field (Roger and Watanabe, 1982). N₂-fixing response of light grown alga to darkness is variable, e.g. ca 4% of the light ARA after 60 min dark incubation by *G. echinulata* (Stewart *et al.*, 1967); 10 - 25% of light ARA after 60 min dark incubation by *G.*

echinulata in culture (Chang and Blauw, 1980) and about 50% loss of ARA after 100 min dark incubation by intertidal lagoon blue-green algae (Potts and Whitton, 1977); 14% of light ARA after 60 min incubation by a deepwater rice-field C. pisum (Aziz and Whitton, 1988). Rapid increase in nitrogenase activity of dark placed heterocystous blue-green algae on reillumination have been reported by many authors (Ramos et al., 1985; Ohmori, 1984; Chen, 1986; Khamees et al., 1987; Aziz and Whitton, 1988). The development of nitrogen fixation capacity was reported to be dependent upon de novo protein synthesis.

1.52 Oxygen

Nitrogen fixation is very sensitive to oxygen (Robson and Postgate, 1980, Pienkos et al., 1983). Blue-green algae are the only organisms that have solved the problem of protecting nitrogenase from simultaneous photosynthetic O₂ evolution. Most blue-green algae that grow diazotrophically elaborate the specialized cell known as the heterocyst. A special function in binding O₂ was suggested for four unique glycolipids detected in the laminated layer of the heterocyst envelope (Lambein and Wolk, 1973). Algae deficient in heterocyst envelope glycolipids had little or no nitrogenase activity when assayed under aerobic conditions (Haury and Wolk, 1978).

High oxygen tensions inhibit ARA of different species of blue-green algae (Stewart and Pearson, 1970; Brownlee and Murphy, 1983; Pienkos et al. 1983). The optimal nitrogenase activity occurs at low pO₂. Oxygen treatment acted directly on the intracellular nitrogenase and did not affect photophosphorylation or oxygen evolution of Anabaena spp. strain CA and 1F (Pienkos et al., 1983). Sensitivity of ARA to O₂ varies upon the physiological conditions of the algae. Preincubation of A. cylindrica under anoxic conditions raises the sensitivity towards oxygen (Ohmori and

Hattori, 1979; Mackey and Smith, 1983);^{it} was suggested that under lowered O₂ algae partially lost the ability to protect nitrogenase from oxygen. A switch on-and-off mechanism of nitrogenase depending on the external O₂ tension was reported in a mutant of Anabaena sp. strain CA (Gotto et al., 1979). On the other hand continuous inactivation of nitrogenase by O₂, hydrolysis and resynthesis during the growth of Anabaena spp. under aerobic growth has been suggested by Bone, 1971 and Murry et al., 1983a.

Among intact microorganisms, O₂ inactivation appears to be at least partly reversible (Stewart et al., 1975; Lambert and Smith, 1980; Pienkos et al., 1983). The reversibility was reported to be linked to intracellular O₂ protection mechanisms, including a hydrogenase-coupled Knallgas (O₂ respiration) reaction (Bothe et al., 1978), superoxide dismutase and catalase (Mackey and Smith, 1983) and by diverting reductive energy from photosynthesis to nitrogen-fixing processes (Paerl, 1978).

In water bodies with abundant algae, there is usually a diel cycle of dissolved oxygen, sometimes reaching supersaturation in the afternoon. Under these conditions, some algae have got an adaptive mechanism to combat oxygen supersaturation. Anabaena spp. reduce direct competition between nitrogen fixation and carbon fixation for light generated reductant by optimising carbon fixation during the late morning and nitrogen fixation during afternoon hours (Paerl, 1979).

1.53 Combined nitrogen

The effect of combined nitrogen on nitrogenase activity and biosynthesis has been examined in many nitrogen fixing organisms. It is often found that the addition of combined nitrogen suppresses nitrogenase biosynthesis rather than the activity of the existing enzyme (Stewart et al., 1968; Brill, 1975; Reich et al., 1986). The negative effect of ammonium on nitrogenase synthesis seems to be dependent on its

assimilation. Stewart and Rowell (1975) first demonstrated for Anabaena cylindrica that inactivation of glutamine synthetase by MSX allows full expression of nitrogenase activity (and heterocyst development) in blue-green algae regardless of the presence of ammonium. Since then, this has been verified with various strains by using not only MSX, but other GS inhibitors, such as 5-hydroxylysine (Ladha et al., 1978) or phosphinotricine (Lea et al., 1984). Although some workers have favoured the idea of glutamine synthetase having a direct role in nitrogenase regulation (Ownby, 1977; Singh et al., 1983a), this contention has been superseded by that of other products of ammonium assimilation being the actual effectors. Some authors, however still claim a key role for ammonium per se as the inhibitor of nitrogenase and heterocyst formation (Singh et al., 1983b; Turpin et al., 1984). Mackerras and Smith (1986) have shown that nitrogenase activity of Anabaena cylindrica was repressed upon addition of ammonium salts after preincubation in the presence of MSX sufficiently to inhibit totally glutamine synthetase and provided evidence that ammonia itself is a primary regulator of nitrogenase levels in A. cylindrica.

Inhibitors of ammonium assimilation also abolish the nitrogenase repression induced by nitrate, indicating that the nitrate effect is indirect and due to the ammonium generated in nitrate reduction. Anabaena 33047 cells treated with MSX and suspended in nitrate medium take up and reduce nitrate and N_2 simultaneously at high rates, without any apparent competition for assimilatory power, releasing large amounts of ammonium to the medium (Ramos and Guerrero, 1983). Thus several workers have found that there is no immediate effect of combined nitrogen on nitrogenase activity (Ohmori and Hattori, 1972; Stewart et al., 1975; Murry et al., 1983).

The response of nitrogenase activity of blue-green algae on addition of combined nitrogen depends on the metabolic state (e.g. C : N ratio). Thus, in N-starved filaments in Anabaena, the inhibitory effects of ammonium on nitrogenase synthesis can be absent or delayed until the cells recover from nitrogen starvation (Ramos et al., 1985). Nitrogenase development in Anabaena cells maintained in the absence of N₂ (Ar : CO₂ atmosphere) for 14 - 16 h is not sensitive to ammonium inhibition (Murry and Benemann, 1979; Murry et al., 1983b; Ramos et al., 1985). Cells from different stages of growth (Murry and Benemann, 1979; Murry et al., 1983b; Kumar and Kumar, 1984) or exposed to different light intensities (Ohmori and Hattori, 1974) also show a differential behaviour with regard to the response of nitrogenase to ammonium. It was found that the noticeable inhibitory effect is due to the interruption of reductants and/or ATP supply (Ohmori and Hattori, 1974, 1978; Upchurch and Mortenson, 1980; Murry et al., 1983b). However on long-term treatment the inhibitory effect is the result of inhibition of heterocyst differentiation and dilution of the enzymes due to growth (Stewart et al., 1975; Ramos and Guerrero, 1983).

The model of effect of combined nitrogen on nitrogen fixation of the blue-green algae appears to differ according to the organism tested and source of combined nitrogen used (Bothe, 1982). For instance, addition of ammonia to Anabaena cylindrica abolished its nitrogenase activity within 48 h, whereas this effect was only gradual when the organism was grown with nitrate as nitrogen source (Stewart et al., 1975; Bothe and Eisbrenner, 1977). In contrast to A. cylindrica Bottomley et al. (1979) found that addition of 10 mM KNO₃ or NH₄NO₃ to Anabaena sp. CA completely repressed nitrogenase activity while the same concentration of NH₄Cl reduced the activity by about 70%. Chen (1983b) found that the nitrogenase activity of three Anabaena strains isolated from rice-fields in Taiwan showed

different responses to the addition of combined nitrogen. For example, addition of 1 mM nitrite or ammonia completely repressed the activity of the two strains while the third one maintained the activity at 25% of the control.

In heterocystous blue-green algae detectable nitrogenase activity is usually concomitant with the appearance of fully differentiated heterocysts (Bradley and Carr, 1976). Combined nitrogen inhibits heterocyst differentiation in many strains. There are also differences between the strains in their response to the effect of combined nitrogen on heterocyst differentiation. Ogawa and Carr (1969) noticed that Anabaena varibilis still had few heterocysts at $28 \text{ mg l}^{-1} \text{ NO}_3\text{-N}$ while the same concentration of $\text{NH}_4\text{-N}$ stopped heterocyst formation completely.

Thomas and David (1971) found that heterocyst formation was totally inhibited when nitrate was supplied in batch culture, but not in continuous culture at high dilution rate. In contrast to other heterocystous blue-green algae, heterocyst differentiation in Anabaena sp. CA is much more strongly inhibited by nitrate than ammonia (Bottomley et al., 1979). Singh and Viswanathan (1972) found that 0.5 mM of either NaNO_3 , NH_4Cl or urea completely repressed heterocyst formation by Camptylonema lahorensis while 20 mM KNO_3 is required to achieve the same effect; the experiment was carried out on solid medium.

1.54 Phosphorus

Among nutritional factors, phosphorus limitation is of prime importance in regulating nitrogen fixation potential in different water bodies (Stewart and Alexander, 1971; Healey, 1972). Nitrogenase activities of natural populations of blue-green algae were found to vary markedly and rapidly, depending on the levels of phosphorus which were available for their metabolism (Stewart et al., 1970; Huber, 1986; Horne and Commins, 1987). Stewart and Alexander (1971) found that the addition of inorganic

phosphorus or phosphorus-containing detergent at very low concentration ($5 \mu\text{g l}^{-1}$) to phosphorus starved algae increased the nitrogenase activity by 81 - 169%. The same authors found the saturation level of phosphorus for nitrogenase activity to be very low ($<50 \mu\text{g l}^{-1} \text{P}$). Bone (1971) found that there is only little effect on the nitrogenase activity of Anabaena flos-aquae when the phosphate concentration was increased from 0.022 - 0.066 mM. In lakes and reservoirs, increased phosphorus loading from applied fertilizer, municipal sewage and industrial discharges have increased the dominance of planktonic heterocystous blue-green algae Anabaena, Gloeotrichia, Aphanizomenon and Nostoc (Fogg, 1969; Granhall and Lundgren, 1975). Phosphate fertilization of lakes or P enrichment in enclosures have been reported to increase the nitrogen fixation activity of natural populations of blue-green algae (Liao, 1977; Lundgren, 1978; Lean et al., 1978; Wurtsbaugh et al., 1985; Istvanovics et al., 1986). Stewart (1964) found that addition of about $9 \text{ mg l}^{-1} \text{PO}_4\text{-P}$ to natural sea water increased nitrogen fixation of a natural population of blue-green algae (Calothrix scopulorum and Nostoc entophyllum) by 17% and 12%, respectively. When samples of Rhizosolenia were enriched with orthophosphate 0.5 h prior assaying for AR, activity was doubled by adding 0.5 - 50 μM phosphate (Mague et al., 1974). Capone and Taylor (1977) could not find any stimulation of ARA by adding 2 μM PO_4 even in P-deficient sea water.

1.55 Iron

Iron demands for both synthesis and function of nitrogenase are appreciable, being second only to phosphorus. High rates of N_2 -fixation increase the need for soluble iron. An increased iron demand under nitrogen-fixing conditions has been reported for Nostoc muscorum (Carnahan and Castle, 1958; Eyster, 1972) and for Anabaena flos-aquae (Murphy et al., 1976). Goldman and Cartner (1965) reported that iron is implicated in

limiting phytoplankton growth in ultra oligotrophic lake Tahoe, California. Iron has been shown to limit N_2 -fixation by spring-early summer blooms of Aphanizomenon and by the autumn bloom of Anabaena in Clear Lake (Horne 1974, 1975). Very small additions of unchelated iron produced large increases in nitrogenase activity, but not in photosynthesis or pigment synthesis (Horne, 1978; Reuter, 1988). Wurtsbaugh and Horne (1983) found the ARA by the algae of the Clear lake was stimulated as much as 500% above control levels by iron additions in laboratory and in situ. C fixation and chl a were also significantly stimulated by iron addition, but less rapidly and to a lesser extent than N_2 fixation. A similar enhancement of nitrogen fixation rate on addition of iron was also reported in Lake Titicaca (Wurtsbaugh et al., 1985).

1.6 Effects of enviromental conditions on the morphology of blue-green algae

1.61 Nitrogen

Nitrogen deficiency results in the yellowing of the vegetative cells due to an overall reduction in the nitrogen containing pigments i.e. phycocyanin and chlorophyll (Fogg et al., 1973). Allen and Smith (1969) reported a levelling off protein synthesis in Anacystis nidulans in response of nitrogen deficiency, the requirements for cellular nitrogen being provided by phycocyanin which disappeared from the cells. These authors also reported simultaneous build up of polysaccharides. Further investigations into the effects of nitrogen deficiency by Neilson et al. (1971) on Anabaena species and De Vasconcelos and Fay (1974) on A. cylindrica confirmed earlier findings, although a gradual disappearance of cyanophycin granules. Restoring the nitrogen levels, by the addition of ammonia, resulted in algal cultures rapidly regaining their pigmentation consequent upon the onset of protein synthesis and the rapid synthesis of

cyanophycin.

In the case of tapered heterocystous blue-green algae, several workers have reported the repression of heterocyst differentiation in the presence of combined nitrogen, but also loss of typical polarity (Fay *et al.*, 1968; Kirkby, 1975; Wyatt *et al.*, 1973). However, combined nitrogen can suppress heterocyst formation, but leave polarity in several tapered blue-green algae (Sinclair and Whitton, 1977a; Rai *et al.*, 1978).

1.62 Phosphorus

Phosphorus is a component of lipids, carbohydrates and nucleic acids in addition to its involvement in energy transfer reactions.

Consequently, any conditions which limit its supply would be expected to have marked effects on the morphology and physiology of the algae.

The morphological variability of the blue-green algae as a response to the environmental conditions is well known and, among these, the Rivulariaceae show a particularly marked response (Livingstone and Whitton, 1983). The concentration of cellular phosphorus appears to be a key factor influencing morphology in this family (Sinclair and Whitton, 1977b). The morphological changes occurring in the strain *Calothrix parietina* D550 have been described (Livingstone and Whitton, 1983; Wood *et al.*, 1986). Under P-rich conditions, the apical region of the trichome continuously gives rise to hormogonia, but with increasing P-deficiency a multicellular colourless hair is formed instead (Livingstone and Whitton, 1983).

In a detailed study of phosphorus deficiency in *Plectonema boryanum*, Jensen and Sicko (1974) reported the development of abnormally long and short cells, suggesting some disturbance of cell division. Upon the restoration of the phosphate to normal levels, they observed a rapid accumulation of polyphosphate referred to as the "polyphosphate overplus". Healey (1973) reported that in *Anabaena flos-aquae* there was a greater

initial rate of phosphate uptake under phosphorus deficiency than in phosphorus sufficient conditions.

The yellowing of the vegetative cells, as observed by Stewart and Alexander (1971), was also noted in Anacystis nidulans by Ihlenfeldt and Gibson (1975), who related a fall in phycocyanin levels due to P-deficiency. Healey (1973) reported that the chlorophyll levels in Anabaena flos-aquae dropped considerably under reduced phosphorus conditions.

1.63 Iron

Iron is involved in photosynthesis (Hardie et al., 1983), nitrogen fixation (Eady and Smith, 1979), nitrogen assimilation (Verstreate et al., 1980) and hence any conditions which limits its availability have marked affect on the physiology and morphology of the algae. Fe-deficiency has been reported to the reduction of chlorophyll a and phycocyanin content of blue-green algae (Price, 1968; Öquist, 1971; Hardie et al., 1983).

Calothrix viguieri D253 produced hairs under Fe-deficient conditions in freshwater media and the sheaths had a much deeper brown colouration than other cultures (Whitton et al., 1973; Sinclair and Whitton, 1975b). The latter authors also showed that six out of 16 strains of Rivulariaceae produced hairs in response to Fe-deficiency. Heterocyst frequency increased and hairs and intra-thylakoidal vacuoles developed in Calothrix parietina D184 under Fe-deficiency (Douglas et al., 1986). A functional significance has been suggested for hairs formed in response to P-deficiency, but not so for those in response to Fe-deficiency (Whitton, 1988). However, the possibility has been raised that hair formation under Fe-deficiency might perhaps result from an interaction in some cell process leading to P-deficiency in spite of the presence of a high environmental concentration of P (Douglas et al., 1986; Whitton, 1988).

The addition of Fe to Fe-deficient cultures can lead to a quite rapid response; hairs were shed and hormogonia released in various Rivulariaceae strains from both field and laboratory (Sinclair and Whitton, 1977b). When Fe was restored to Fe-deficient Agmenellum quadruplicatum, the intracellular pigment levels increased rapidly and growth resumed (Hardie et al., 1983).

1.7 Aims

The aims of the research project were:

- a) To quantify the influence of environmental factors on nitrogen fixation activity of a deepwater rice-field Calothrix in the laboratory.
- b) To study the phosphatase activity of this isolate as P plays an important role in nitrogen fixation and growth of algae.

Because of the problem in conducting nitrogen fixation studies in deepwater rice-fields in Bangladesh, both field and laboratory, experiments were planned to determine the influence of environmental factors on another species, Rivularia sp. in the UK to understand the nitrogen fixation activity of Calothrix in the field.

2 MATERIALS AND METHODS

2.1 Computing

Three computing systems were used during the study. Routine calculations were performed on a Research Machines Nimbus XN16 using the MULTIPLAN spreadsheet program (Microsoft). Statistical analyses were carried out using SPSSX running on an AMDAHL 470/v8 mainframe operating under the Michigan Terminal System. Graphical output was carried out using the UNIRAS suite of software, incorporating UNIGRAPH and UNIEDIT, running on a SUN minicomputer operating under UNIX.

2.2 Laboratory procedures

2.21 Mass determination

Mass was measured on a Oertling R51 balance to five decimal places. All references to weight refer to mass.

2.22 Temperature

Temperature was measured by a thermometer. In the field temperature of the incubation vessel was measured at 15-min interval and the values integrated to mean temperature experienced by the alga.

2.23 Light

Light flux was measured using a SKP 210 PAR cosine corrected sensor (Skye Instruments Ltd, Wales) and expressed as $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ PAR. Light attenuation was achieved by wrapping the flasks with different grades of neutral density filters and placing at different places in the growth tank.

In the field measurements of light flux were taken at 15-min intervals throughout each survey and the values integrated to calculate the mean light experienced by the alga.

2.24 pH

For laboratory work pH was measured by WTW E50 (Wissenschaftliche-Technische Werkstätten) electrode and EIL pH meter (model 7050). The probe was calibrated with a BDH standard buffer solution immediately before a reading was taken.

2.25 Dissolved oxygen

Dissolved oxygen was measured using a WTW (Wissenschaftliche-Technische Werkstätten) meter with O₂ probe EOT 190. Atmospheric pressure was determined using a barometer and the meter was adjusted accordingly. The O₂ electrode was calibrated before taking a reading. The results were expressed as % saturation.

2.26 Colorimetric analysis

All large volume i.e. more than 330 μ l colorimetric analyses were carried out using a Shimadzu Digital Double-Beam Spectrophotometer (model UV-150-2). Glass cuvettes of 1 - 10 cm path length were used for all readings in the visible and infra-red.

The MCC Plate Reader was used for a large percentage of colorimetric analysis on phosphatase activity. Assays using *p*NPP and bis-*p*NPP as substrates, Absorbance Programme 1 and Filter Code 1 (405 nm) were used.

2.3 Standard culture techniques

2.31 General

To avoid contamination all isolation and subculturing work was carried

out in a Microflow Pathfinder laminar flow cabinet conforming to B.S. 5295 class 1. Aseptic techniques were used throughout and working surfaces were swabbed with alcohol before and after use. If a microscope was needed during the procedure a Nikon Stereoscopic Zoom microscope (model SMZ-2) was used inside the laminar flow cabinet.

2.32 Culture vessel and glassware

Experiments in batch culture were carried out in 100-ml Erlenmeyer flask. The flasks were plugged with siliconerubber stoppers (Sanko Plastics Co., Japan). Pre-sterilized plastic petri dishes (Sterilin, England) were used for solid media.

2.33 Cleaning glassware and utensils

The flasks were cleaned by scrubbing to remove all algal materials and soaked in 2% Decon (Decon 90, Decon Laboratories Ltd. Hove), for 30 min. They were then rinsed six times with hot tap water and six times with distilled water. The universal bottles used for field experiments were washed in 10% H_2SO_4 and washed six times with distilled water. Silicon rubber stoppers (Sanko Plastics Co., Japan) used for culture flasks were soaked in 2% Decon rinsing in hot water and then rinsed six times in distilled water before use.

2.34 Sterilization

Medium, pipette tips (wrapped with aluminium foil and plugged with cotton wool) and other glasswares were sterilized by autoclaving at 121°C for 20 min. Wire loops were pre-sterilized in a Bunsen flame, isolation needles were dipped in alcohol and flamed very briefly. Organic phosphorus compounds, sucrose, fructose, chloramphenicol and DCMU were sterilized by filter sterilization.

2.35 Media

2.351 Chu 10D

The water chemistry of rice-fields of Bangladesh from where the Calothrix D764 was isolated was taken into consideration when developing media. The water chemistry of the rice-field from where the alga was isolated has been reported by Whitton et al. (1988a). Of the available media, Chu 10D (Sinclair & Whitton, 1977a) was most suited to the water chemistry in the field. As the laboratory study was planned mainly to investigate the nitrogen fixation study, a nitrogen free version of the Chu 10D was used for all experiments (basal medium), unless stated otherwise. This was done by substituting $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ for $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$. When Rivularia D403 was grown in Chu 10D-N the alga did not form large colonies. As Ca and Mg content in the pond water was very high (Hudson et al., 1971), so the Ca and Mg content of Chu 10D-N was raised to 50.0 and 25.0 mg l^{-1} , respectively. The composition of the Chu 10D-N medium and the composition of each element are shown in Tables 2.1; 2.2. All chemicals used in the medium were of Analar grade and obtained from British Drug House Ltd (BDH), Poole, England, except HEPES which was supplied by Sigma Chemical Co., USA. Iron and EDTA was used in a single solution. Stock solutions were stored in a refrigerator. Medium was made up freshly as required using distilled water.

To prepare 1 l of medium, ca 500 ml of distilled water was taken in a beaker. For buffering 0.6 g HEPES was added. pH dropped to ca 5.0 and this was adjusted to 7.0 by adding ca 1.40 ml 1.0 M NaOH. This buffered aliquot was transferred into 1 l volumetric flasks and stock solutions were then added and final volume was made up with distilled water. The medium in the culture flasks was sterilized immediately, stored in the dark and used within 24 h after autoclaving. 1% agar (w/v) medium was also autoclaved, cooled to ca 50°C poured into presterilized Petri dishes

aseptically, solidified and stored in the refrigerator.

Thus, the liquid medium used in this study is not an absolute inorganic medium, but with two organic compounds, the EDTA as a chelating agent and HEPES as a buffer. Some morphological study was done with reduced P, - Fe, + NH₄-N and + NO₃-N in basal medium. In case of reduced concentration of P had the effect of reducing the K concentration which was compensated for by adding KCl to the medium. NH₄-N and NO₃-N were added as NH₄Cl and NaNO₃, respectively. In case of - Fe medium, one tenth of Na₂-EDTA was added.

Table 2.1 Composition of mineral salts in Chu 10D-N growth medium.

salt	mg l ⁻¹	μM
MgSO ₄ .7H ₂ O	25.0	101.40
NaHCO ₃	15.85	188.60
CaCl ₂ .2H ₂ O	35.84	243.70
FeCl ₃ .6H ₂ O	2.42	8.97
Na ₂ -EDTA.2H ₂ O	3.18	8.97
KH ₂ PO ₄	7.8	57.15
H ₃ BO ₃	0.72	115.60
MnCl ₂ .2H ₂ O	0.045	2.28
Na ₂ MoO ₄ .2H ₂ O	0.007	0.02
ZnSO ₄ .7H ₂ O	0.056	0.19
CuSO ₄ .5H ₂ O	0.02	0.07
CoSO ₄ .7H ₂ O	0.01	0.03
NiSO ₄ .7H ₂ O	0.009	0.03
NaOH	<u>ca</u> 60.00	1500.00
HEPES	600.00	2517.00

Table 2.2 Elemental composition of Chu 10D-N medium.

element	mg l ⁻¹	μM
P	1.78	57.45
S	3.25	101.70
Cl	17.26	532.00
Na	39.0	167.00
K	2.24	102.13
Ca	9.78	243.70
Mg	2.47	101.40
Mn	0.012	2.28
Fe	0.50	8.97
Ni	0.002	0.03
Co	0.002	0.03
Cu	0.005	0.07
Zn	0.013	0.19
B	0.125	11.15
Mo	0.0028	0.02

2.352 Phosphatase assay medium

This was a further modification of Chu 10D-N medium, where N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) was removed, KH_2PO_4 was replaced with KCl and the iron was halved.

Table 2.3 Composition of mineral salts in phosphatase assay medium.

salt	phosphatase assay medium	
	mg l ⁻¹	μM
MgSO ₄ .7H ₂ O	25.0000	101.40
NaHCO ₃	15.8500	188.60
CaCl ₂ .2H ₂ O	35.8300	243.70
FeCl ₃ .6H ₂ O	1.2125	4.45
Na ₂ EDTA.2H ₂ O	1.6685	4.45
KCl	4.2784	57.38
H ₃ BO ₃	0.7150	11.56
MnCl ₂ .4H ₂ O	0.4525	2.28
Na ₂ MoO ₂ .2H ₂ O	0.0067	0.02
ZnSO ₄ .7H ₂ O	0.0555	0.19
CuSO ₄ .5H ₂ O	0.0197	0.07
CoSO ₄ .7H ₂ O	0.0105	0.03
NiSO ₄ .7H ₂ O	0.0380	0.03
NaOH	-	-
HEPES	-	-

Table 2.4 Elemental composition of phosphatase assay medium.

element	mg l ⁻¹	μM
S	3.25	101.70
Cl	18.81	532.00
Na	39.23	167.00
K	2.24	57.38
Ca	9.78	243.70
Mg	2.47	101.40
Mn	0.012	2.28
Fe	0.25	4.48
Ni	0.002	0.03
Co	0.002	0.03
Cu	0.005	0.07
Zn	0.013	0.19
B	0.125	11.15
Mo	0.0028	0.02

2.36 Maintenance and subculturing

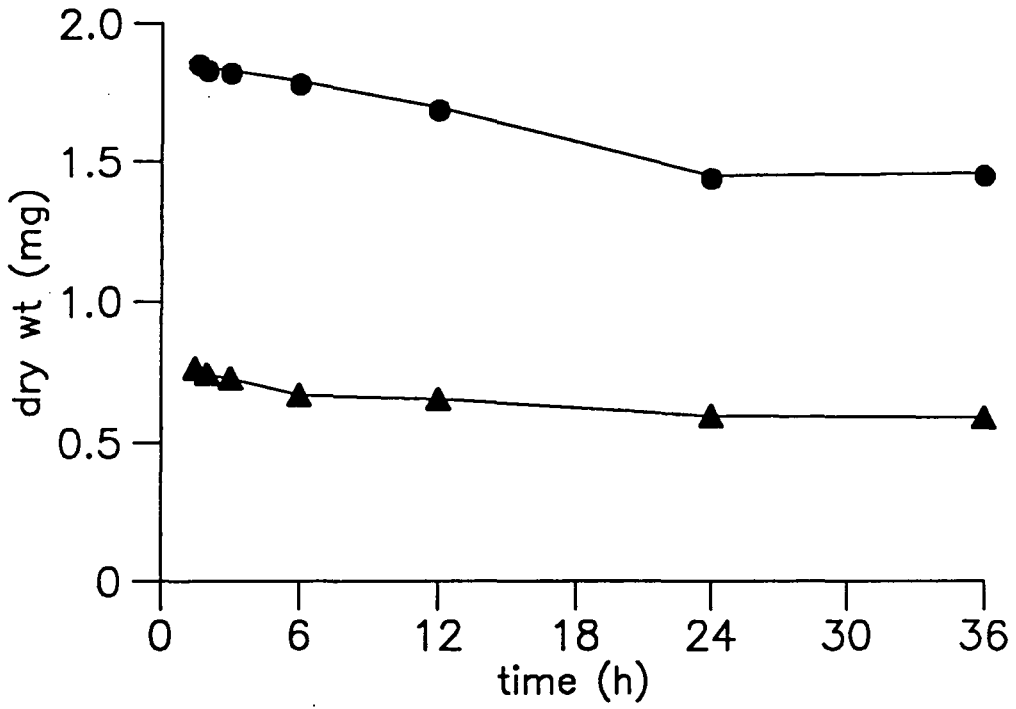
Stock cultures were maintained in 50-ml liquid medium incubated standing in the 32°C growth room under continuous light (ca 40 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$). Subcultures to fresh medium were made after 6 - 8 weeks. Stocks for experimental purposes were maintained in the shaker tank under continuous light of 85 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. Although the flasks were kept in a 32°C tank, the temperature of the culture was 31°C.

Subculturing for stock cultures was done by picking up a small aliquot of the alga with a sterilized wire loop and inoculating to fresh medium aseptically using a laminar flow cabinet (Microflow Pathfinder). Subcultures for experiments were done by inoculating a standard amount of homogenised algal material. The alga was pooled by automatic pipette fitted with sterile plastic tip. The algal mass with the parent medium was then homogenised by taking it in plastic syringe and passing through 50, 25 and 16 mm sterile needles (Gillette Surgical Ltd, U.K.) till a homogeneous algal suspension was attained. A known volume of algal aliquot was pipetted out, centrifuged and washed twice with distilled water and dried at 105°C for 2.0 h and transferred to desiccator and dry wt determined. From this dry weight the final dry weight of the alga was estimated using a drying curve (Fig. 2.1). A calculated volume of algal aliquot was pipetted to each flask having 10.0 mg d. wt l^{-1} .

2.37 Incubation

Experiments were carried out in batch culture under continuous light. In the growth room illumination was provided by white fluorescent tubes above the alga. All N_2 -fixation experiments were carried out in the thermostatically controlled tanks illuminated from below by warm white fluorescent tubes. Dark condition was achieved by wrapping flasks with two layers of aluminium foil and one layer of black polythene. The flasks

Fig. 2.1 Influence of drying period on changes in dry weight of Calothrix D764.



were randomized at 12 h intervals. Flasks in the tank were shaken at 60 oscillation min^{-1} . N_2 -fixation experiments were conducted in the Erlenmeyer flasks with 128.0 ± 1.0 ml gas volume with silicon rubber stopper. The gas phase of flasks with 25 ml medium was therefore about 103 ml.

2.38 Purification and test of purity

Small pieces of a young vigorously growing culture were placed onto the middle of the agar plate of the Chu 10D-N medium. Plates were then incubated in a growth room at 32°C at a photon flux density of 30 - 40 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. After 2 - 5 d there was usually a zone of hormogonia around the inocula, some sufficiently well separated for individuals to be picked off. Clean hormogonia were located using a stereobinocular microscope and a single hormogonium, together with a block of surrounding agar was transferred onto a fresh agar plate. When the hormogonium developed into a colony was transferred in to liquid medium. Flasks were incubated in the growth room at 32°C . After 6 - 9 d algal growth was quite visible. The cultures were then tested following the test media described by Hoshaw and Rosowski (1973) and also by examining materials by phase contrast microscopy (Nikon Fluophot type 109).

2.4 Algal cultures

The algae used in this study were obtained from the Durham Culture Collection. Calothrix D764 was isolated from a deepwater rice-field in Bangladesh and made axenic by picking single hormogonium. The strain Rivularia D403 was isolated from a calcareous pond Croft Kettle, near Darlington UK and has been in Durham Culture Collection for few years.

2.5 Microscopy and photomicrography

Algal cultures were examined routinely using a Nikon Fluophot fitted with a 35-mm camera. Small samples of algal mat were mounted on microscope slide in a drop of medium in which they had been growing thus preventing any osmotic effects. As the filaments were densely interwoven, they were gently teased apart with fine dissecting needles. This allowed individual filaments to be examined in order to assess their morphological characteristics.

2.6 Scoring morphological characters

2.61 Trichome dimensions in cultured alga

In order to quantify changes in trichome morphology under different conditions of growth a number of measurements were taken during the period of incubation from a random sample of 20 trichomes. The characters selected are:

- a) Basal diameter - This was determined the basal vegetative cell of the trichome.
- b) Heterocyst width and length - These were the maximum dimension of basal heterocyst, when more than one heterocyst was present at the base of the trichome, measurements were taken that from most recently formed.
- c) Apical diameter - The diameter of the most apical vegetative cell was taken.

2.62 Heterocyst character and heterocyst frequency

Heterocyst may develop a number of locations along a trichome, brief description of which are given below:

- a) Basal heterocyst - A single heterocyst in a basal terminal position.

- b) Secondary basal heterocyst - A heterocyst developed immediately to the original one.

Heterocyst frequency was studied in different conditions of growth and during growth and ARA. Heterocyst frequency is defined as the number of heterocyst per total number of cells and is expressed as a percentage. Heterocyst frequency at particular stage of trichome growth was studied by counting the total number of cells per intact trichome, while during growth and ARA studies, heterocyst frequency was counted in sonicated material using haemocytometer (improved Neubauer ruling 0.20 mm depth) as follows:

An aliquot of algal sample was transferred to a universal bottle and preserved in 2.5% gluteraldehyde, 1% paraformaldehyde and 0.05 M pipes at pH 7.0. The alga was sonicated (Soniprep 150 MSE) at 26 μ m amplitude for 3 - 4 min (during which vial was kept on ice). The material was then observed under microscope to check whether one celled condition was achieved. A few 2 - 3 celled fragments were always present. When this sample was sonicated again, fragmentation of individual cells occurred, resulting in a false increase in number of cells, so cells were counted without further sonication. About 0.05 ml of suspension was pipetted to each chamber. Only cells with distinct polar nodule and a thick walled were considered as heterocysts.

2.63 Cell inclusions

Cyanophycin granules were usually identified without staining by their characteristic refractive appearance. To confirm the presence of granules in the young trichomes they were stained with Schneider's acetocarmine (saturated solution of carmine in 45% acetic acid). Two drops of acetocarmine were placed over a drop of dense algal suspension and kept for 5 min for post vital staining. After placing coverslips cyanophycin granules were examined at high magnification (x 1000).

Polyphosphate granules were identified by the staining method of Ebel *et al.* (1958). Samples were taken in snap-cap vials were soaked for 15 min in 10% (w/v) $\text{Pb}(\text{NO}_3)_2$ in 0.1 N HNO_3 . The treated material was washed thoroughly (5 times) with distilled water and treated with 10% $(\text{NH}_4)_2\text{S}$ for 30 s and again rinsed thoroughly. Polyphosphate granules were stained dark brown to black.

2.7 Estimation of yield

2.71 Dry weight

For dry weight the alga was centrifuged at 5000 x g for 10 min, washed twice with distilled water and the pellet was collected in a preweighed crucible and oven dried at 105°C for 24 h. The dried sample was then placed in a desiccator, cooled to room temperature and reweighed using a Mettler H 51 balance.

2.72 Extraction and estimation of pigments

Pigment analyses were based on OD measurements, obtained using a Shimadzu digital double beam spectrophotometer (UV-150-02), using aqueous extracts and 90% methanol extracts. Chl *a* was estimated by following the procedure based on the recommendation of Marker *et al.* (1980). Extraction was done with 10 or 25 ml 90% hot (70°C) methanol for 15 min. Extracts were then cooled down, kept out of direct light, filtered through GF/C filters and absorbance was measured at 665 and 750 nm using a Shimadzu digital double beam spectrophotometer (uv-150-02). Absorbance was again measured at 665 and 750 nm after acidification with 0.1 M HCl (final concentration 10^{-3} M HCl) for 60 min and was calculated from the following equation:

$$\mu\text{g Chl } a = (A_b - A_a) \times \frac{R}{R - 1} \times K \times \frac{V}{L}$$

Where A_b = extract absorbance at 665 nm before acidification deducting

absorbance at 750 nm

A_a = extract absorbance at 665 nm after acidification deducting the absorbance at 750 nm

R = acid ratio (A_b/A_a)

K = 1000 x the reciprocal of the specific absorbance coefficient (SAC) of chl a at 665 nm in 90% methanol

V = volume of solvent used to extract chl a in ml

L = light path of the cuvette used in cm

Marker et al. (1980) recommended the specific absorbance coefficient of chl a in 90% methanol is 77. Therefore,

$$\mu\text{g chl } \underline{a} = 12.99 \cdot (A_b - A_a) \times \frac{R}{R - 1} \times \frac{V}{L}$$

Growth rate have been expressed in terms of relative growth constant or specific growth constant (K') (Fogg, 1975)

$$\frac{\log_{10}N_t - \log_{10} N_0}{t}$$

Where t = time in days, from the time of incubation

N_t = biomass after t days

N_0 = biomass at zero time

Maximum growth rate is defined as the maximum growth rate under light saturation at a specified temperature. The mean generation time or doubling time (G) has been calculated from specific growth constant K' :

$$G = \frac{0.301}{K} \text{ day}$$

Phycocyanin and allophycocyanin were estimated by following the procedure based on Bennett & Bogorad (1973). Extraction was done in distilled water (pH adjusted to 7.0) and sonicated (Soniprep 150 MSE) at 26 μm amplitude for 10 min during which vial was kept on ice. The algal extract was then centrifuged at 10,000 x g for 15 min, washed, sonicated

and centrifuged again unless and until phycobiliproteins were fully extracted. Absorption of algal phycobiliprotein extract was taken at 624 nm and 652 nm. Phycobiliproteins were calculated from the equation:

$$\text{Phycocyanin} = \frac{A_{624} - 0.474 (A_{652})}{5.34}$$

$$\text{Allophycocyanin} = \frac{A_{652} - 0.208 (A_{624})}{5.09}$$

where A_{624} = absorption at 624 nm

A_{652} = absorption at 652 nm

2.73 Yield estimation from a single sample

To obtain dry weight, chlorophyll a and phycobiliproteins from a single sample, the culture was homogenised to give a uniform suspension from which accurate aliquots could be taken.

A washed algal pellet was prepared as in section 2.71 and 30.0 ml of distilled water was added and homogenised by passing through different grades of needles. Aliquot for dry weight estimations was transferred directly into predried crucibles and dried as section 2.71. Material for chlorophyll a estimation was filtered through GF/C glass fibre paper and stored in the freezer until chlorophyll a analysis. Material for phycobiliprotein estimation was centrifuged at 5000 x g for 10 min and algal pellet was collected in a universal bottle with distilled water (pH adjusted to 7.0) for phycobiliprotein estimation as section 2.72.

2.8 Biological assays

2.81 Acetylene reduction assay

2.811 Laboratory

N_2 -fixation was studied using the acetylene reduction technique (Hardy et al., 1973). The alga was always kept and maintained in the parent

medium and flask, so that it did not experience a major shift in environmental variables. A shift of light during C_2H_2 injection and equilibration could not be overcome because of the ARA technique itself.

The silicon-rubber bung was replaced by suba seal immediately prior to gassing. Fifteen ml of C_2H_2 was injected into each flask and was then shaken slightly before equilibration of pressure by another needle. After equilibration, flasks were incubated in the experimental conditions. A control, with the autoclaved medium only, was used to estimate C_2H_4 contamination.

After incubation with C_2H_2 for 30 or 60 min the gas mixture was collected by 1 ml disposable plastic syringe and stuck into a rubber mat. Analysis of the gas mixture was made by injecting into the Varian Aerograph 1400 gas chromatograph equipped with hydrogen flame ionization detector. N_2 was used as a carrier gas at a rate of 45 ml min^{-1} through a 3.0 mm x 1.8 m column packed with Poropak R (Waters Associates Inc., USA). Other operating conditions were: detecting temperature, 150°C , column temperature 80°C ; air and hydrogen flow rates were 300 and 30 ml min^{-1} , respectively. C_2H_4 and C_2H_2 (as a check of accuracy of method) peaks were recorded from chart recorder, identified by their retention time. Acetylene was supplied by British Oxygen Co., and ethylene (99.8%) by BDH laboratory gas service. The gas chromatograph was calibrated each day of use, with high purity of ethylene standards. The results of nitrogenase activity (ARA) were expressed on the basis of dry weight as well as chl *a*.

2.812 Field

Rivularia colonies were collected from the submerged dead woods and kept in a plastic bucket with pond water. Colonies were then selected as small colonies (1 - 2 mm diameter) or large (4 - 5 mm diameter). Three large and 8 - 10 small colonies were used for ARA experiments in each universal

bottle (28 ml volume) with 2.0 ml of pond water. The different light fluxes on the incubation vessel were obtained by wrapping with neutral density filters and darkness was achieved by covering with aluminium foil. After incubation, the vessels were sealed with rubber lined perforated screw caps (W. Freeman & Co., Barnsley, UK). Three ml of C_2H_2 gas was injected with a plastic syringe (Beckton-Dickinson Co., Ireland). After gassing, the incubation vessel was shaken lightly and the gas pressure was equilibrated by another needle. The colonies were placed face upwards in the vessel and the vessels were put on a shallow wire rack laid on the pond water near the edge. The racks were shaken gently at 30 min interval. C_2H_2 gas was carried to the field in foot ball bladders with the adaptor temporarily removed. In all cases incubation was done for 2 - 3 h because of the low fixation rate found in this Rivularia population. The mid-time of incubation was considered as the time of nitrogenase activity.

At the end of the incubation, the gas mixture was collected in 5 ml evacuated blood collecting tubes (Venoject, Terumo Europe N.V., Belgium). Samples were analysed in Varian Aerograph Series 1400 (Section 2.811). After evacuation of the gas mixture, the incubation vessels plus alga were kept in the ice box for transfer to the laboratory where they were then stored in the deep freezer until chl a analysis. On the day of chl a analysis, the sample was thawed and the alga was filtered through GF/C filters. Chl a was determined as (Section 2.72). The result of nitrogenase activity (ARA) was expressed as $nmol C_2H_4 \mu g chl \underline{a}^{-1} min^{-1}$.

2.82 Phosphatase assay

Alga was separated from the growth medium by centrifugation at 5000 x g for 10 min. The supernatant was decanted and made up to the volume with MilliQ water. The centrifuged medium was passed through GF/C filters and regarded as extracellular phosphatase fraction. The algal pellet was

washed twice and resuspended in assay medium (Section 2.352). This allowed for the dilution effect in the assay. The volume required to resuspend the algal pellet would vary depending upon the concentration of alga required in the assay. The alga was homogenised by passing through different grades of needles and finally sonicated (Soniprep 150 MSE) at 26 μm amplitude for 2 min. During sonication the algal homogenate was cooled with an ice jacket. The algal suspension was examined under the light microscope for cellular damage.

For cell-bound and extracellular phosphatase activity a 30 μl algal sample was pipetted via a titertek 8-channel pipettman (EFLAB, Finland) into a 96 microwell plate (no. 96F, Inter Med, Nunc, Denmark). All the microwell plates had lids (no. 96L, Inter Med, NUNC, Denmark), which reduced contamination, prevented evaporation and acted as an insulator against heat-loss. 90 μl of standard buffer was pipetted into the microwells i.e. glycine-NaOH, pH 10.3 (50 mM final concentration). The plate was then incubated at 32°C for 20 min. Then 180 μl of 0.25 mM p NPP or 0.5 mM bis- p NPP (7.75 mg l^{-1} P final concentration) was pipetted out into the microwells. A T = 0 min reading was taken and subsequent readings at 10 min intervals for 30 min. There was no termination of phosphatase activity. Subsequently a time course was plotted from the readings, a value for phosphatase activity was taken from the linear part of the constructed graph. A calibration curve using p -nitrophenol (p NP) at pH 10.3 was constructed between 0.002 - 0.2 μmol . Activity was expressed as $\mu\text{mol } p\text{NP mg d. wt}^{-1} \text{ h}^{-1}$.

For determining the pH optima duplicate buffers were used for each pH unit to compensate for any inhibition of phosphatase activity by the buffers used (Table 2.5). The buffers were 50 mM (final concentration), which was chosen as a suitable concentration for buffering physiological media (Dawson *et al.*, 1986). PMEase activity was monitored using p NPP.

For assaying PMEase 20 μl of alga and 70 μl of buffer were used. This was incubated at 32°C for 30 min. After the incubation period 140 μl of $p\text{NPP}$ substrate (pre-incubated at 32°C) was added. The assay ran for 20 min and was terminated by the addition of 100 μl of 4.95 M NaOH, resulting in a final pH of ca 12.8 ± 0.1 . At each pH a T = 0 line was set up by the addition of NaOH before $p\text{NPP}$, which compensated for any optical variation.

A different protocol was used to assay for PDEase activity, because the addition of 100 μl of 4.95 M NaOH resulted in bis- $p\text{NPP}$ hydrolysis. So for PDEase activity 30 μl of alga; 90 μl of buffer and 180 μl of substrate were used. The assay was terminated with 30 μl of 0.33 M NaOH, resulting in a final pH of 12.3. For each of the above procedures calibration curves were set up at the requisite pH values using $p\text{NP}$ between 0.0002 - 0.2 μM .

Table 2.5 Buffers used to investigate the effect of pH on phosphatase activity. Two buffers were used at each pH value (A, B)

pH	buffer	set	buffering capacity	pKa at 20°C
3.0	DMG-NaOH	A	3.2-7.6	3.66 and 6.20
3.0	glycine-HCL	B	2.2-3.6	2.35 and 9.60
4.0	DMG-NaOH	A	3.2-7.6	3.66 and 6.20
4.0	succinic acid	A	3.8-6.0	4.18 and 5.60
5.0	DMG-NaOH	A	3.2-7.6	3.66 and 6.20
5.0	succinic acid-NaOH	B	3.8-6.0	4.18 and 5.60
6.0	DMG-NaOH	A	3.2-7.6	3.66 and 6.20
6.0	succinic acid-NaOH	B	3.8-6.0	4.18 and 5.60
7.0	DMG-NaOH	A	3.2-7.6	3.66 and 6.20
7.0	HEPES-NaOH	B	6.8-8.2	7.50
8.0	TES-NaOH	A	6.8-8.2	7.50
8.0	HEPES-NaOH	B	6.8-8.2	7.50
9.0	AMeP-NaOH	A	9.0-10.5	7.69
9.0	glycine-NaOH	B	8.6-10.6	2.35 and 9.60
10.0	AMeP-NaOH	A	9.0-10.5	9.69
10.0	glycine-NaOH	B	8.6-10.6	2.35 and 9.60
10.3	AMeP-NaOH	A	9.0-10.5	9.69
10.3	glycine-NaOH	B	8.6-10.6	2.35 and 9.60
11.0	CAPS-NaOH	A	9.8-11.1	10.40
11.0	Na ₂ CO ₃ -NaHCO ₃	B	9.2-10.8	10.33

2.9 Chemical analysis

2.91 Ammonium nitrogen

Analysis of ammonium nitrogen was made according to Solorzano (1969). Ammonia reacts with phenol and hypochlorite in an alkaline solution to form indophenol blue; the reaction is catalysed by nitroprusside. The resulting absorbance is proportional to the ammonia present and is measured spectrophotometrically at 640 nm.

Reagents:

1. Phenol- alcohol solution: 10.0 g phenol was dissolved in 100.0 ml 95% ethyl alcohol.
2. Na-nitroprusside: 0.5% solution in MilliQ water.
3. Alkaline solution: 100.0 g trisodium citrate and 5.0 g NaOH were dissolve in 500.0 ml MilliQ water.
4. Na-hypochlorite solution: 1.5 N solution.
5. Oxidizing solution: 100.0 ml of alkaline solution and 25 ml hypochlorite solution were mixed together.

Procedure:

To a 25.0 ml sample or ~~sample~~ diluted to 25.0 ml, 2.0 ml phenol, 2.0 ml Na nitroprusside, and 5.0 ml oxidising solution were added. Solution was mixed thoroughly after each addition and left for 1 h for colour development and absorbance was taken at 640 nm.

A calibration curve was constructed within the range 0 - 250 $\mu\text{g NH}_4\text{-N}$ and the absorbance value for the sample was calculated.

2.92 P fractions in medium

Analysis of the phosphorus fractions in the medium, termed "filtrable reactive phosphorus" and "filtrable total phosphorus" (organic and orthophosphate") were made according to Eisenreich et al. (1975). In a suitably acidified solution sodium molybdate and potassium antimony

tartarate react with orthophosphate to form molybdophosphoric acid which is then reduced to the intensely coloured molybdenum blue complex by ascorbic acid and determined spectrophotometrically at 882 nm wavelength.

Stock solutions:

1. H_2SO_4 -antimony: 53.3 ml of concentrated H_2SO_4 was mixed with 500 ml MilliQ water and cooled; 0.748 g of $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6 \cdot \frac{1}{2}\text{H}_2\text{O}$ was dissolved in H_2SO_4 solution and diluted to 1 l.
2. Molybdate: 10.839 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ was dissolved in about 500 ml MilliQ water and diluted to 1 l.
3. Digestion Acid: 100.0 ml concentrated H_2SO_4 was mixed with 500 ml MilliQ water, cooled and diluted to 1 l.

Reagents:

1. Digestion Reagent: 6.0 g $\text{K}_2\text{S}_2\text{O}_8$ was dissolved in about 80 ml MilliQ water containing 10.0 ml digestion acid and diluted to 100.0 ml.
2. Mixed reagent I. 25.0 ml each of the H_2SO_4 -antimony and molybdate stock solutions were mixed with 0.2 g ascorbic acid and diluted to 100.0 ml.
3. Mixed reagent II. 25.0 ml each of the H_2SO_4 -antimony and molybdate stock solutions was mixed with 10.0 ml of digestion reagent and 0.2 g ascorbic acid and diluted to 100.0 ml.

Procedure:

I. Filtrable reactive phosphate

A suitable aliquot of sample was diluted to 25 ml with MilliQ water in a 125-ml conical flask. Then 5.0 ml of mixed reagent II was added and mixed thoroughly.

II. Filtrable total phosphate

A suitable aliquot of sample was diluted to 25 ml with MilliQ water in a 125-ml conical flask. 5 ml digestion reagent was added to the sample.

The flask top was covered with aluminium foil and autoclaved at 121°C for 30 min. The sample was cooled to room temperature and 5.0 ml of mixed reagent I was added.

Calibration curves were prepared by treating two series of phosphate standards, as for filtrable reactive phosphate and filtrable total phosphate.

2.93 Algal N

Algal N was determined according to Yoshida *et al.* (1976).

Determination of algal N depends on the conversion of bound N into an ammonium salts when the sample is heated with concentrated H₂SO₄ and a catalyst; ammonia is then liberated from the salt with alkali and estimated titrimetrically after distillation.

Reagents:

1. Catalyst mixture- 5.0 g K₂SO₄ , 1.0 g CuSO₄.5H₂O and 0.1 g Se metal were ground to a fine powder in a pestle and mortar.
2. Concentrated H₂SO₄
3. Boric acid, 1% w/v in MilliQ water, plus 10 ml mixed indicator per l.
4. Mixed indicator : 0.3 g bromocresol green and 0.2 g methyl red dissolved in 400 ml 90% ethanol.
5. NaOH 40% (w/v)
6. HCl M/70.

Procedure:

The alga was digested by adding 20 mg catalyst mixture and 1 ml concentrated H₂SO₄ placing on a heater. The digest was cooled to room temperature and diluted to 25 ml by MilliQ water, stored in acid washed snap cap vial. 10 ml sample was distilled in presence of 3 ml 40% NaOH, and the distillate was collected in boric acid and indicator mixture. The

boric acid was titrated against M/70 HCl using micro burette. The accuracy of the method was checked by standard $(\text{NH}_4)_2\text{SO}_4$ solution following the above procedure and titrating against M/70 HCl.

2.94 Extracellular N

The alga was separated from the medium by centrifugation (Section 2.71). The medium was then analysed for total N by kjeldahl digestion following distillation and subsequent titration (Section 2.93). Flasks with medium only were also incubated in the tank along with the algal culture to check any contamination.

2.95 Algal P

Algal P was determined from the H_2SO_4 algal digest. 10.0 ml algal digest was transferred to snap cap vial and 5.0 ml MilliQ water was added and then neutralized to pH 7.0 by 6.0 N NaOH. The volume of the digest was made 25.0 ml by adding MilliQ water and phosphate was determined as filtrable total phosphate (Section 2.92).

3 MORPHOLOGY AND GROWTH OF CALOTHRIX D764

3.1 Morphology

3.11 Introduction

The environmental features of deepwater rice-fields in Bangladesh are documented quite well (Section 1.3). In order to study the influence of deepwater rice-field environments related to nitrogen fixation in the laboratory, an axenic strain of Calothrix from deepwater rice-field of Bangladesh was used (Section 2.4). This strain is a representative of the Rivulariaceae, the predominant blue-green algal taxon in deepwater rice-fields; it differs morphologically from another strain studied by Aziz and Whitton (1987) isolated from the same environment. The influence of light, nitrogen sources, phosphorus and iron on the morphology and physiology of the strain was carried out in order to plan for subsequent nitrogen fixation experiments.

3.111 Morphological changes in batch culture

The morphological study was carried out in batch culture in standard medium. Overall morphological changes occurring during the incubation period are describing using the stages of development proposed by Aziz and Whitton (1987): hormogonium, juvenile trichome, developing and mature trichome.

The hormogonia were short trichomes consisting of a variable number of cells, usually 6 - 12, but as high as 30. Gas vacuoles were not observed in the hormogonium (nor elsewhere in this strain). The hormogonia had cyanophycin granules, but polyphosphate granules were rarely visible with the light microscope. A chain of cells with a fully differentiated heterocyst and the same number of cells as the parent hormogonium was regarded as a juvenile trichome. The time period for heterocyst

differentiation after release of the hormogonium ranged from 12 - 24 h. In the juvenile trichome there were abundant polyphosphate granules but no visible cyanophycin granules.

A trichome with repeatedly dividing cells, but before the formation of a hormogonium at its apex, was regarded as a developing trichome. In the mature trichome cyanophycin granules started to increase, but polyphosphate granules started to decrease. As there was only one heterocyst in a trichome, the heterocyst frequency at later stages of development of the alga decreased. In the juvenile trichome it was 10 - 12%, while in the mature trichome the heterocyst frequency ranged from 2 - 3%. Heterocysts were 5.0 to 7.5 μm in length and usually spherical to slightly conical in shape. In the juvenile trichome the heterocyst was pale, but as the trichome increased in length the heterocyst became blue-green. The basal cells were constricted between the cells. Hormogonium liberation was associated with the formation of a bi-concave necridial cell. After liberation the hormogonia aggregated together to form a rope-like structure in the shaker tank. In standing culture they float to the surface and formed a surface mat and there was little tendency to form ropes. The sheath was hyaline, gradually becoming thicker as the alga became older. In old cultures the cells at the base of the trichome became quadrate to cylindrical similar to those of an Anabaena. Some of the trichomes showed bulging, which was presumably due to in situ germination of the hormogonia trapped within the thick sheath. Divisions of terminal cells of trichomes created pressure on the surrounding sheath. Ultimately the growing apex came out by bursting the sheath as a single false branch. Several hormogonia germinated in this way producing several false branches. Cells in the median region of the developing trichome might divide more than the terminal cells resulting in the formation of loops. This happened due to the ability of the growing trichome to slide through the thick sheath,

presumably due to the constriction of the sheath around the cross walls.

Table 3.1 Trichome length (μm) and granulation (% of cell profile) of different stages of development of Calothrix D764 in batch culture.

developmental stage	trichome length	granulation	
		cyanophycin	polyphosphate
1 hormogonium	33	10	-
2 juvenile trichome	40	-	30
3 developing trichome	210	20	30
4 mature trichome	280	30	20

Table 3.2 Dimensions of morphological characters (μm) and granulation (% cell profile) of Calothrix D764 in standard medium.

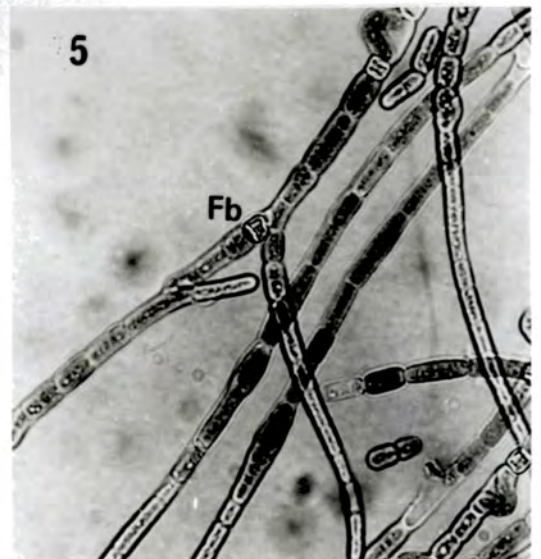
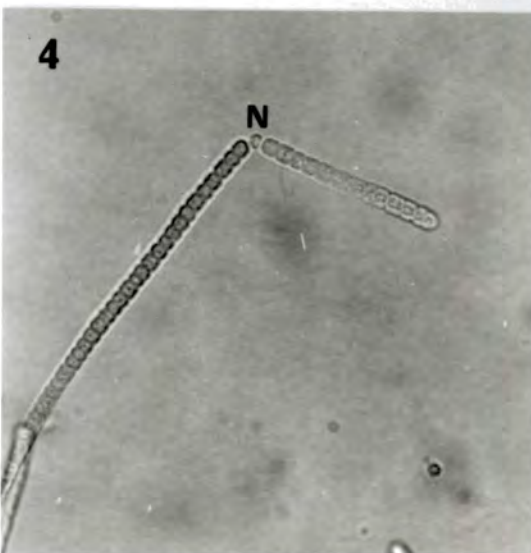
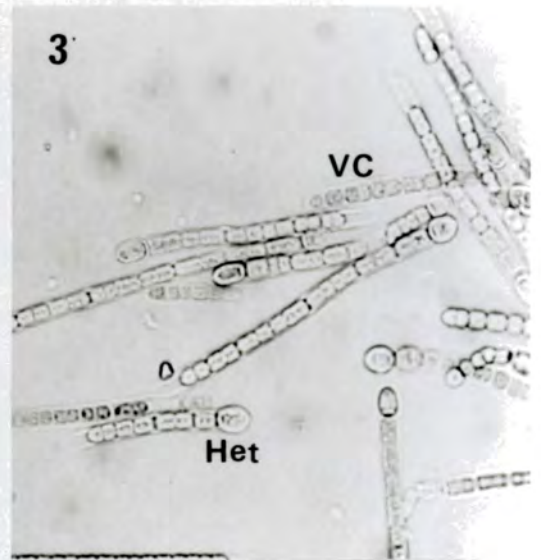
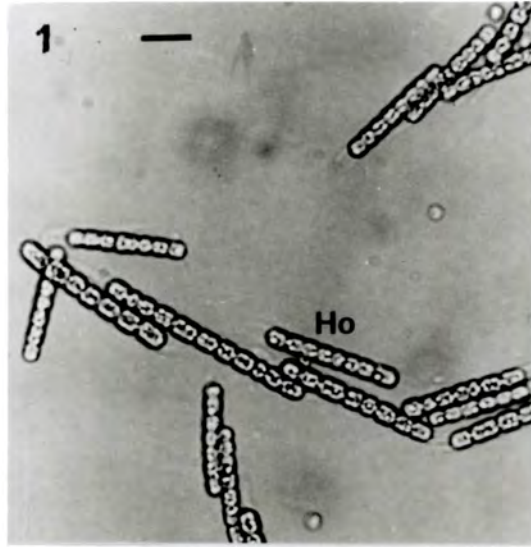
day	trichome length	basal cell		apical cell		cyanophycin		polyphosphate	
		length	width	length	width	basal	apical	basal	apical
0	110	6.0	4.0	4.0	3.0	10	-	20	10
2	145	6.5	4.0	5.0	3.0	20	10	20	20
4	240	7.0	5.0	6.0	3.0	20	10	20	20
6	270	7.0	5.0	7.0	4.0	30	10	10	10
8	310	7.5	5.0	7.0	4.0	30	20	10	-
10	450	7.5	5.0	7.0	4.5	30	20	-	-
12	480	7.5	5.0	7.5	4.5	30	20	-	-

Fig. 3.1 Photomicrographs of Calothrix D764 grown in standard medium taken at different stages from batch culture.

Vegetative cell (VC), hormogonium (Ho), necridium (N), heterocyst (Het), false branch (Fb)

Scale bar = 10 μ m

- 1) hormogonia.
- 2) aggregation of hormogonia and developing trichomes forming a rope.
- 3) developing trichome .
- 4) liberation of hormogonium by forming necridium.
- 5) development of false branch in old culture.



3.112 Influence of nitrogen sources on morphology

The influence of nitrogen sources on morphology of this alga was chosen because combined nitrogen has been reported to affect the morphology and nitrogen fixation of blue-green algae (Section 1.53).

After 3 - 4 d from transfer of trichomes from - N medium to 10 mg l^{-1} NH_4-N or 70 mg l^{-1} NO_3-N , they lost heterocysts and polarity. The width of the trichome increased and the colour became dark blue-green. The trichomes comprised several groups of cells, with 6 - 10 cells in each group and deeply constricted between the groups. Possibly these groups of cells originated from the same parent cell. The breakdown of the trichome took place at these constrictions. Some of the cells in the trichomes were lysed. The sheath was thick and loose.

On transfer of the undifferentiated trichomes to - N medium, they became yellowish-green and heterocyst differentiation started within 10 - 12 h. As the trichomes grew by division of the cells the sheath swelled further, giving rise to a zig-zag shape of the trichome. The trichome emerged by perforation of the sheath. In this stage the trichome showed branching like Scytonema.

3.113 Influence of P on morphology

Phosphorus has been reported to affect the trichome morphology and nitrogen fixation of the Rivulariaceae (Section 1.54). Therefore an experiment was planned to find out the changes in morphology of the alga under increasing phosphorus deficiency.

The alga was subcultured to 0.1 mg l^{-1} P and the reduced level of K concentration was compensated by the addition of KCl to the standard medium. The first change in P-limited trichomes was the rapid loss of polyphosphate granules. Hormogonia production ceased rapidly. Cyanophycin granules became abundant. With increasing P-deficiency the

Fig. 3.2 Photomicrographs of Calothrix D764 (1, 2) in standard medium with $10 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$ and (3, 4) transfer of trichomes from the same medium to standard medium .

Sheath (S), false branch (Fb).

Scale = $10 \mu\text{m}$

- 1) loss of heterocyst from the trichome.
- 2) loss of polarity and development of groups of cells along the trichome.
- 3) development of trichome in the parent sheath.
- 4) development of false branch.



colour of the trichome turned to olive-green. Along the trichome intercalary heterocysts developed. The trichomes became narrower and more elongated than those in standard medium. Cross walls were poorly developed.

Addition of P to P-limited cultures led to rapid formation of polyphosphate granules, particularly in the basal areas while cyanophycin granules decreased slightly. Hormogonia were released within 24 h. Consequently, as growth resumed, cyanophycin granules continued to decrease in the trichome.

3.114 Influence of Fe on morphology

Iron was also tested for morphological study because of its importance in nitrogen fixation (Section 1.55). The medium used here was different from the standard medium; modifications include the omission of Fe, reduction of EDTA (10% of the standard medium) and 10 mg l^{-1} P to avoid interactions between Fe- and P- limitation.

The colour of the trichomes in - Fe medium turned from blue-green to pale. A marked effect was the formation of new heterocysts apical to the original one. There was less production of hormogonia. Cyanophycin granules decreased more rapidly in the trichome until they became absent, whereas polyphosphate granules decreased more slowly. Occasional death of cells along the trichome was also observed. These trichomes were shorter than those in standard medium.

Addition of Fe to Fe-limited cultures resulted in a marked change in morphology. Vegetative cells returned to their normal colour although a few trichomes in the centre of masses of the alga were slower than the rest. The colour of the heterocyst turned to blue-green. Hormogonia were released after 24 h following addition of Fe. After 48 h most of the collapsed basal heterocysts were lost. Cyanophycin granules reappeared in

the trichome, whilst the polyphosphate granules remained unchanged.

Table 3.3 Dimensions of morphological characters (μm) and granulation (% cell profile) of Calothrix D764 in low P medium.

day	trichome length	basal cell		apical cell		cyanophycin		polyphosphate	
		length	width	length	width	basal	apical	basal	apical
0	115	6.0	4.0	5.0	3.0	20	10	20	10
2	140	6.0	4.0	5.0	3.0	30	10	10	-
4	250	7.0	4.0	6.0	3.0	30	10	10	-
6	370	7.5	5.0	7.5	3.0	40	10	-	-
8	450	7.5	5.0	7.5	2.5	50	20	-	-
10	550	7.5	5.0	8.0	3.0	50	20	-	-
12	600	8.0	5.0	9.0	3.0	50	20	-	-

Table 3.4 Dimensions of morphological characters (μm) and granulation (% cell profile) of Calothrix D764 after addition (day 12) of P to P- limited cultures.

day	trichome length	basal cell		apical cell		cyanophycin		polyphosphate	
		length	width	length	width	basal	apical	basal	apical
0	600	8.0	5.0	9.0	3.0	50	20	-	-
1	600	8.0	5.0	9.0	3.0	50	20	30	10
2	570	8.0	5.0	8.0	3.0	50	30	30	10
3	500	8.0	5.0	8.0	5.0	40	20	10	10

Table 3.5 Dimensions of morphological characters (μm) and granulation (% cell profile) of Calothrix D764 in - Fe medium.

day	trichome length	basal cell		apical cell		cyanophycin		polyphosphate	
		length	width	length	width	basal	apical	basal	apical
0	98	6.0	4.0	4.0	3.0	30	10	40	10
2	130	6.0	4.0	4.0	3.0	20	10	40	20
4	210	6.5	4.0	5.0	3.0	20	-	40	30
6	255	6.5	4.5	5.0	4.0	-	-	30	20
8	275	7.0	5.0	5.5	4.0	-	-	30	10
10	290	7.0	5.5	5.5	4.0	-	-	30	10
12	320	7.0	5.5	5.5	4.0	-	-	30	10

Table 3.6 Dimensions of morphological characters (μm) and granulation (% cell profile) of Calothrix D764 after addition (day 12) of Fe to - Fe culture.

day	trichome length	basal cell		apical cell		cyanophycin		polyphosphate	
		length	width	length	width	basal	apical	basal	apical
0	320	7.0	5.5	5.5	4.0	-	-	30	10
1	300	7.0	5.0	6.5	4.0	-	-	30	10
2	325	7.0	5.0	6.5	4.0	10	-	30	10
3	350	7.0	5.0	6.0	4.0	20	10	30	10

3.115 Hair formation

Different species of the Rivulariaceae have been found to form long hairs in deepwater rice-fields (Section 1.37). Limitations of P- and Fe- often led to the formation of hairs in different members of the Rivulariaceae (Section 1.6). However, under laboratory conditions Calothrix D764 did not form hairs in response to P- or Fe- limitation. It seemed possible that one of several factors might have caused this. High concentrations of Na or EDTA or the well buffered medium, might be inhibitory to hair formation. Thus the alga was subcultured to modified standard medium with 1.0 mg Na l^{-1} , one-tenth of normal HEPES, - EDTA or one-tenth of normal EDTA. The culture was placed in the shaker tank at 10 and $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ and also in the light - dark (12 : 12 h) growth room at 25 and $10 \text{ photon m}^{-2} \text{ s}^{-1}$. The trichomes were much more elongated than those in the standard medium but no hair was formed.

3.2 Growth

3.21 Introduction

The deepwater rice-field environment is very heterogeneous (Section 1.3). In particular, the amount of light reaching the algae is highly variable, so the growth of these algae inside or on the periphery of a rice-field or in adjacent fallow areas may vary widely. Experiments on Calothrix D764 were therefore planned with the following objectives:

- 1) to determine the growth rate at different values of light flux;
- 2) to determine the changes in growth parameters at different values of light flux;
- 3) to determine whether the alga grow photoheterotrophically or heterotrophically.

3.22 Influence of light flux on growth rate

The influence of light flux on growth rate was studied in standard conditions (apart from light flux) in the shaker tank (Section 2.37). The inoculum was grown in its respective experimental light flux. The alga showed approximately exponential growth from about 1 to 3 d of growth. However, in the calculation of growth constant (K') 1 to 2 and 2 to 3 d have been considered and summarized in Table 3.7. At $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$, and considering the changes in yield from 1 to 2 d, the generation time of the alga was 13 h.

Table.3.7 Influence of light flux on the growth constant and doubling time of Calothrix D764. (Inoculum 3-d old; continuous light; continuous shaking)

light flux $\mu\text{mol photon m}^{-2} \text{s}^{-1}$	time period (d)	growth constant (K')			doubling time (h)		
		d. wt	chl <u>a</u>	N	d. wt	chl <u>a</u>	N
10	1 - 2	0.221	0.268	0.229	32.7	26.9	31.5
	2 - 3	0.204	0.254	0.253	35.4	28.4	28.6
25	1 - 2	0.389	0.398	0.380	18.6	18.2	19.0
	2 - 3	0.289	0.293	0.313	24.9	24.6	23.1
50	1 - 2	0.479	0.573	0.508	15.1	12.6	14.2
	2 - 3	0.269	0.282	0.309	26.8	25.6	23.3
85	1 - 2	0.542	0.537	0.533	13.3	13.5	13.6
	2 - 3	0.328	0.388	0.432	22.0	18.6	16.7
140	1 - 2	0.475	0.459	0.467	15.2	15.7	15.5
	2 - 3	0.339	0.416	0.425	21.3	17.4	17.0

3.23 Influence of light flux on the growth parameters

Experiments were planned to test the influence of light flux on Calothrix D764. Growth parameters included dry wt, chl a, cellular N and phycobiliproteins. The results are shown in Figs 3.3 - 3.6. Differences

Fig. 3.3 Changes in growth parameters of *Calothrix* D764 in batch culture at $10 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. (Inoculum 3-d old; continuous light; 32°C)

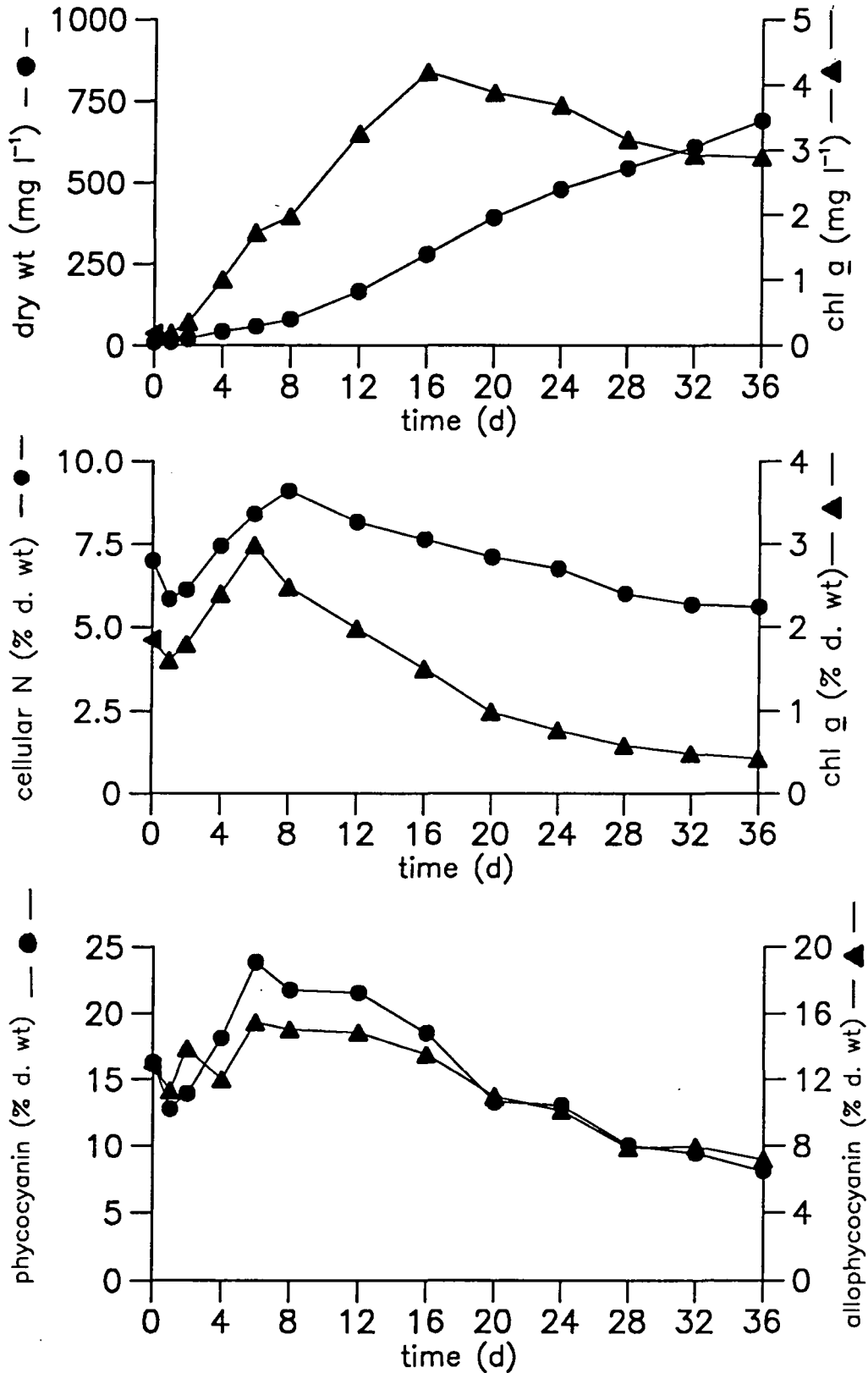


Fig. 3.4 Changes in growth parameters of *Calothrix* D764 in batch culture at $25 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. (Inoculum 3-d old; continuous light; 32°C)

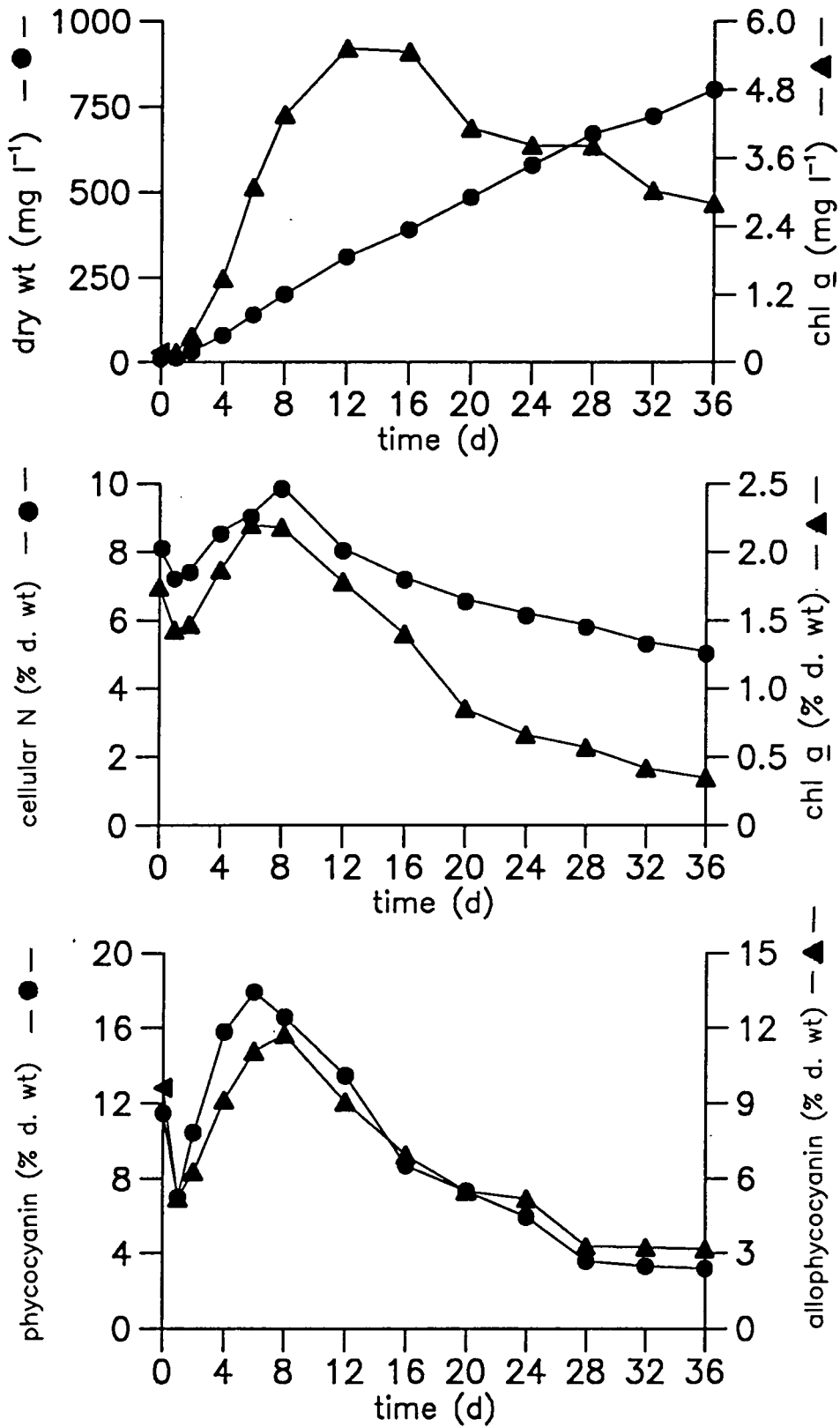


Fig. 3.5 Changes in growth parameters of *Calothrix* D764 in batch culture at $50 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. (Inoculum 3-d old; continuous light; 32°C)

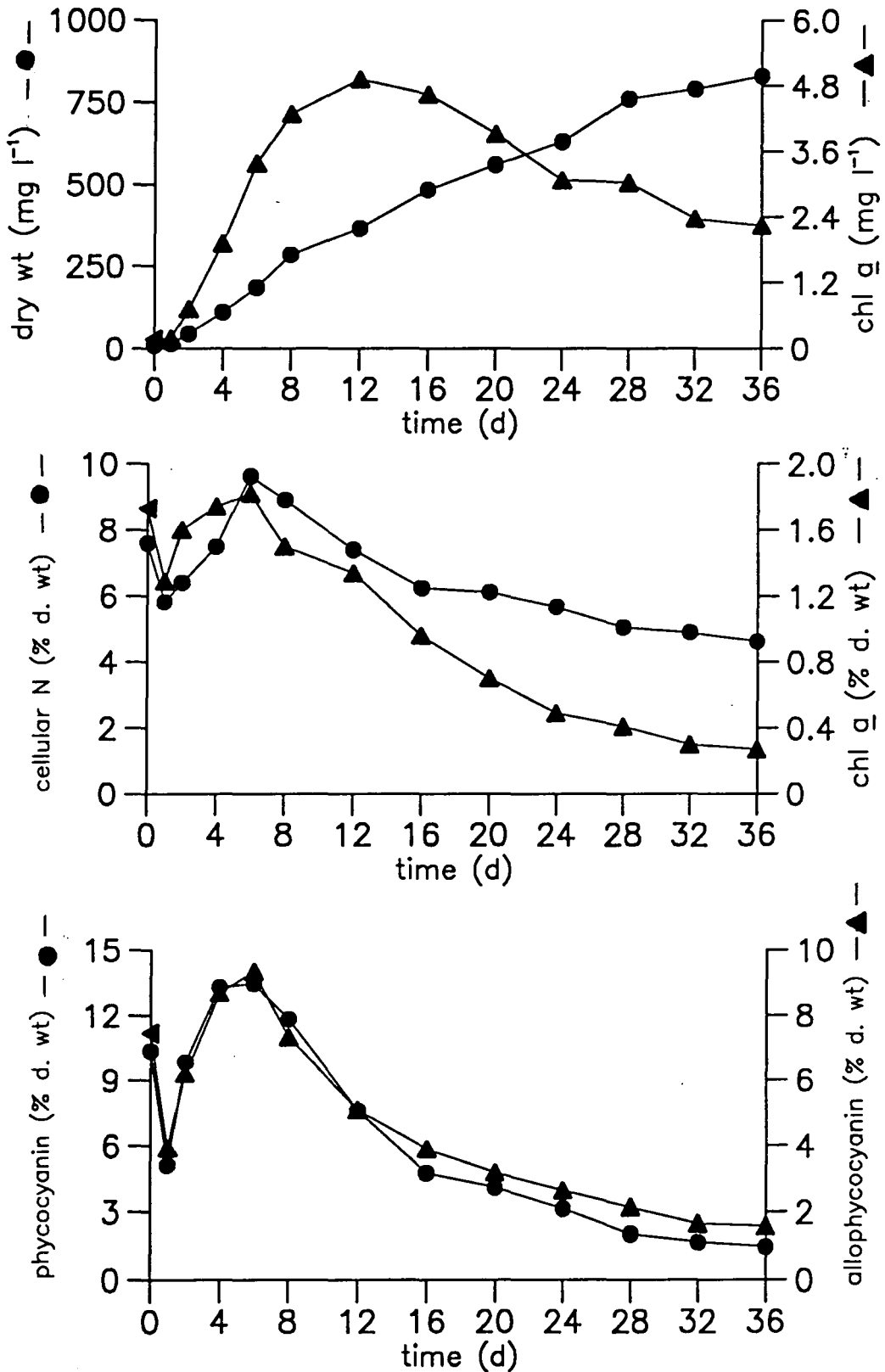
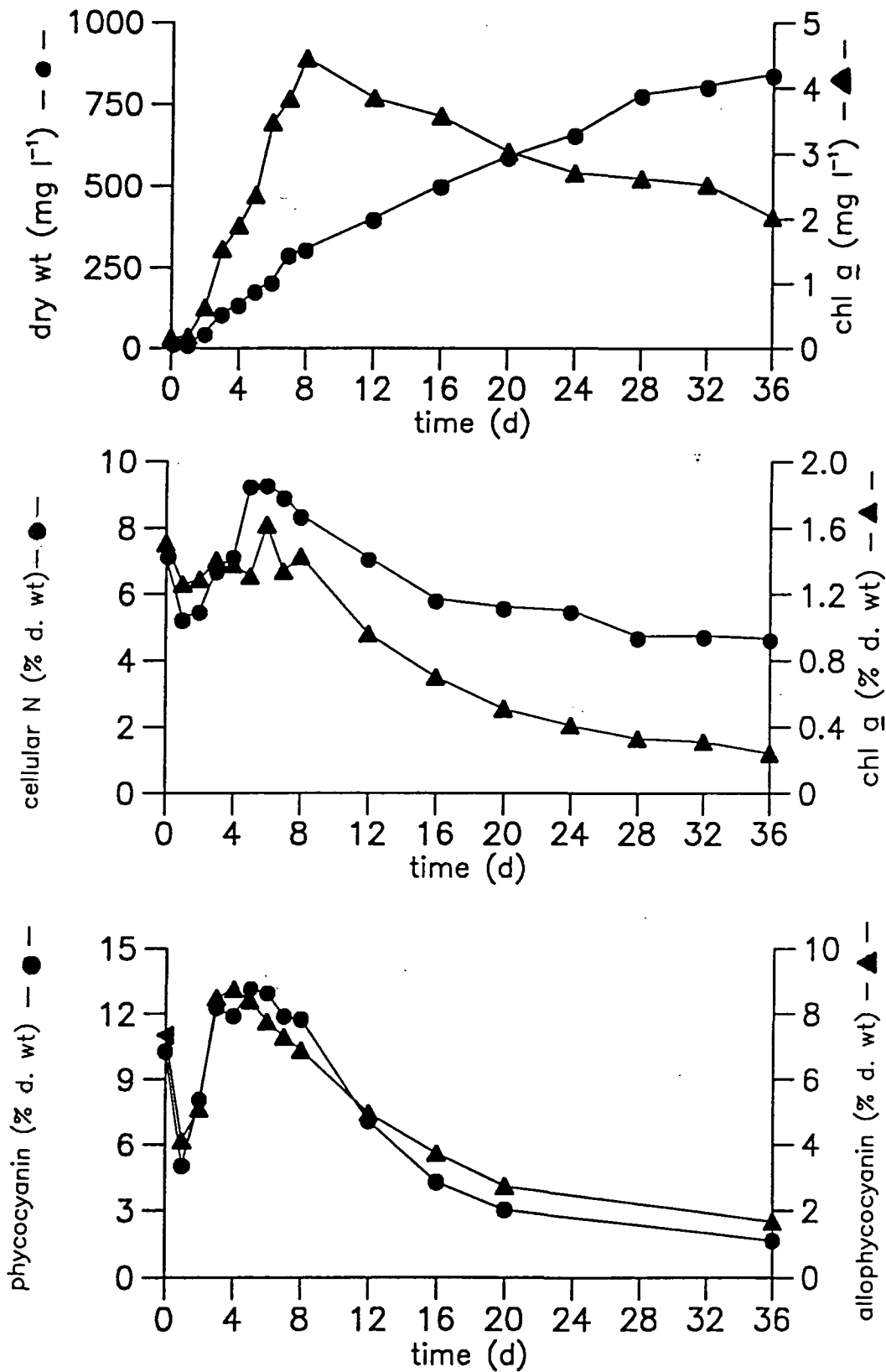


Fig. 3.6 Changes in growth parameters of *Calothrix* D764 in batch culture at $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$. (Inoculum 3-d old; continuous light; 32°C)



in dry weight and chl a were markedly observed during the early days of experiments. However, at the end of the growth experiments the dry weight of the alga grown at 25, 50 or 85 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ was the same. At low light flux the alga had higher contents of chl a and phycobiliproteins compared to the cultures at higher light flux. At all light fluxes, cellular N, chl a and phycobiliproteins dropped initially and increased gradually reaching a maximum between 6 - 8 d and decreased gradually.

3.24 Photoheterotrophic growth

During the later growth stages of the deepwater rice plant the amount of light reaching algae is low due to the increased above- and under-water biomass. At the same time a lot of under-water leaves of the rice plant start to decompose which might release organic substrates to the environment. An experiment was therefore carried out to elucidate the effects of sugars on the growth of Calothrix D764 at a relatively low light flux ($12 \mu\text{mol photon m}^{-2} \text{s}^{-1}$). The inoculum was prepared by subculturing the alga twice at $12 \mu\text{mol m}^{-2} \text{s}^{-1}$. The alga was subcultured with or without sugar (0.01 M) in the presence or absence of nitrogen sources ($10 \text{ mg l}^{-1} \text{ N}$). Yield was recorded after 8 d and the results are presented in Table 3.8.

Table 3.8 Influence of sugars and nitrogen sources on the yield of Calothrix D764. (Inoculum 3-d old; $12 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$; continuous light; $n = 4$)

treatment	yield (mg d. wt l^{-1})
standard medium	97.76 ± 11.92
,, + $\text{NO}_3\text{-N}$	124.72 ± 12.99
,, + $\text{NH}_4\text{-N}$	175.36 ± 13.36
,, + fructose	130.32 ± 13.29
,, + sucrose	118.32 ± 15.91
,, + $\text{NO}_3\text{-N}$ + fructose	185.12 ± 3.06
,, + $\text{NO}_3\text{-N}$ + sucrose	169.68 ± 13.58
,, + $\text{NH}_4\text{-N}$ + fructose	251.22 ± 25.82
,, + $\text{NH}_4\text{-N}$ + sucrose	235.30 ± 7.02

Fructose in the presence of $\text{NH}_4\text{-N}$ supported the highest yield. An experiment was therefore planned to see whether the alga can grow in the presence of the photosynthetic inhibitor DCMU ($1 \times 10^{-5} \text{ M}$). The alga was subcultured in the presence of DCMU, fructose and combined nitrogen in standard medium. The culture was killed within a few days. A further experiment was conducted to determine whether the solvent (ethyl alcohol) or DCMU killed the alga. The alga was grown in the presence of equal amount of ethanol to that added with DCMU; the culture grew normally.

3.25 Heterotrophic growth

In the above experiment, the alga was able to utilize the sugars effectively in the presence of nitrogen sources at low light flux. An experiment was planned to see whether the alga can grow in the dark in the presence of sugars and combined nitrogen. The culture was grown at 85

$\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ and subcultured to the standard medium with 10 mg l^{-1} $\text{NH}_4\text{-N}$ and fructose (0.01 M) and wrapped with two layers of aluminium foil and black polythene and kept in a dark box. The alga failed to grow heterotrophically, but remained viable after 3 months in the dark. The culture was pale. When the culture was placed in light it resumed growth.

4 NITROGENASE ACTIVITY (ARA) BY CALOTHRIX D764

4.1 Introduction

Nitrogen fixation by blue-green algae in deepwater rice-fields is associated with a complex set of environmental factors (Section 1.5). The results in Chapter 3 show that the morphology and growth of Calothrix D764 are affected markedly by changes in the environmental variables suggesting that these may affect nitrogenase activity. Experiments were therefore planned to quantify this. The influence of oxygen was also included, because it often changes markedly with time in the deepwater rice-fields (Section 1.35) and might affect nitrogenase activity.

4.2 Comparison of molar N_2 : C_2H_2 reduction ratio

The methods used for measuring nitrogenase activity were the acetylene reduction technique (Section 2.81) and total N determination (Section 2.94). The major disadvantage of the acetylene reduction technique is the conversion ratio from acetylene reduced to nitrogen fixed. Although the theoretical ratio is 1 : 3, it has been reported to shift widely due to the production of varying quantities of hydrogen (Peterson and Burris, 1976; Rother et al., 1988). An attempt was made to find out the molar ratio of N_2 : C_2H_2 reduced by determining the total N fixed and C_2H_2 reduced by the alga in batch culture at two values of light flux. A molar ratio of 1 : 4.1 fitted well during 36 d of growth at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (Fig. 4.3). On the other hand a ratio of 1 : 5.2 was found during 44 d of growth at $10 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (Fig. 4.4). For short periods of change in nitrogenase activity, ^{the} acetylene reduction technique was used, but for longer periods (> 24 h), both acetylene reduction and nitrogen determination were used.

4.3 Growth and nitrogenase activity

An experiment was carried out to quantify the changes in nitrogenase activity during batch culture in relationship to growth parameters. The alga was subcultured twice at 3-d intervals at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ before starting the experiment. The various changes are shown in Figs 4.1 - 4.3. Nitrogenase activity was measured daily up to day 8 and maximum activity was found after two days growth. This was followed by a decrease in nitrogenase activity (per unit d. wt), but with activity still detectable after growth for 36 d. A further experiment was also planned at a relatively low flux, $10 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ to compare nitrogenase activity values with the culture obtained at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (Fig. 4.4). Maximum nitrogenase activity was observed after three days of growth. Maximum nitrogenase activity was about 36% of the maximum nitrogenase activity recorded on day 2 with ^{the} culture grown at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. This was followed by a gradual decrease in activity, but the decrease in activity was much slower than that observed with the culture at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$.

4.4 Light

4.4.1 Introduction

In deepwater rice-fields, the amount of light that reaches and penetrates the water column varies widely depending on the season, above- and under-water plant biomass, cloud cover etc. Studies on nitrogenase activity were therefore planned with the following objectives:

- 1) to quantify the magnitude and rate of response in nitrogenase activity to changes in the light flux;
- 2) to quantify the influence of different periods of dark pretreatment upon nitrogenase activity after transfer of the culture to light;
- 3) to quantify nitrogenase activity in the dark.

Fig. 4.1 Changes in growth parameters of *Calothrix* D764 in batch culture.

(Inoculum 3-d old; $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$; continuous light; 32°C)

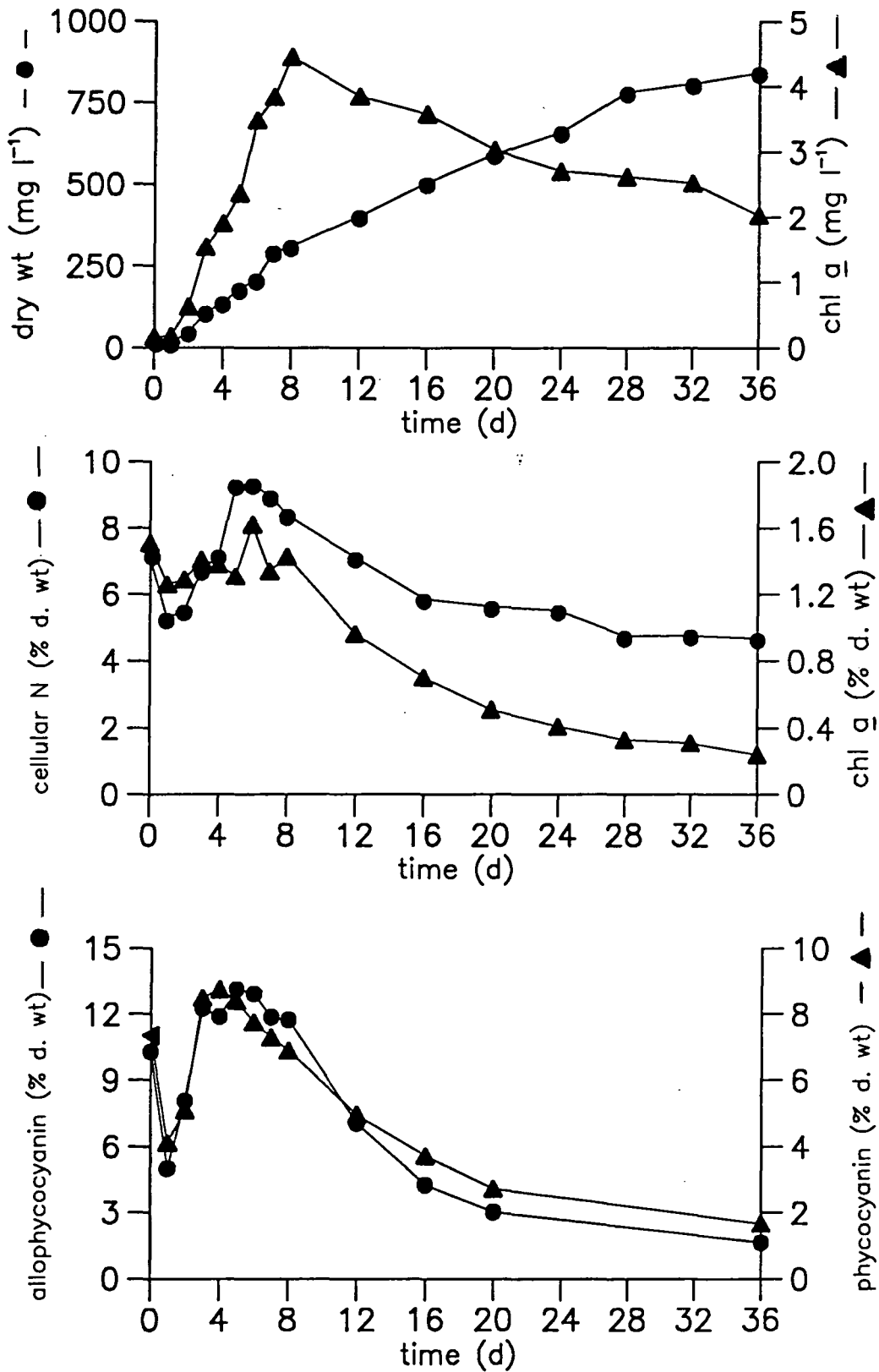


Fig. 4.2 Changes in growth parameters and nitrogenase activity of *Calothrix* D764 in batch culture. (Inoculum 3-d old; $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$; continuous light; 32°C)

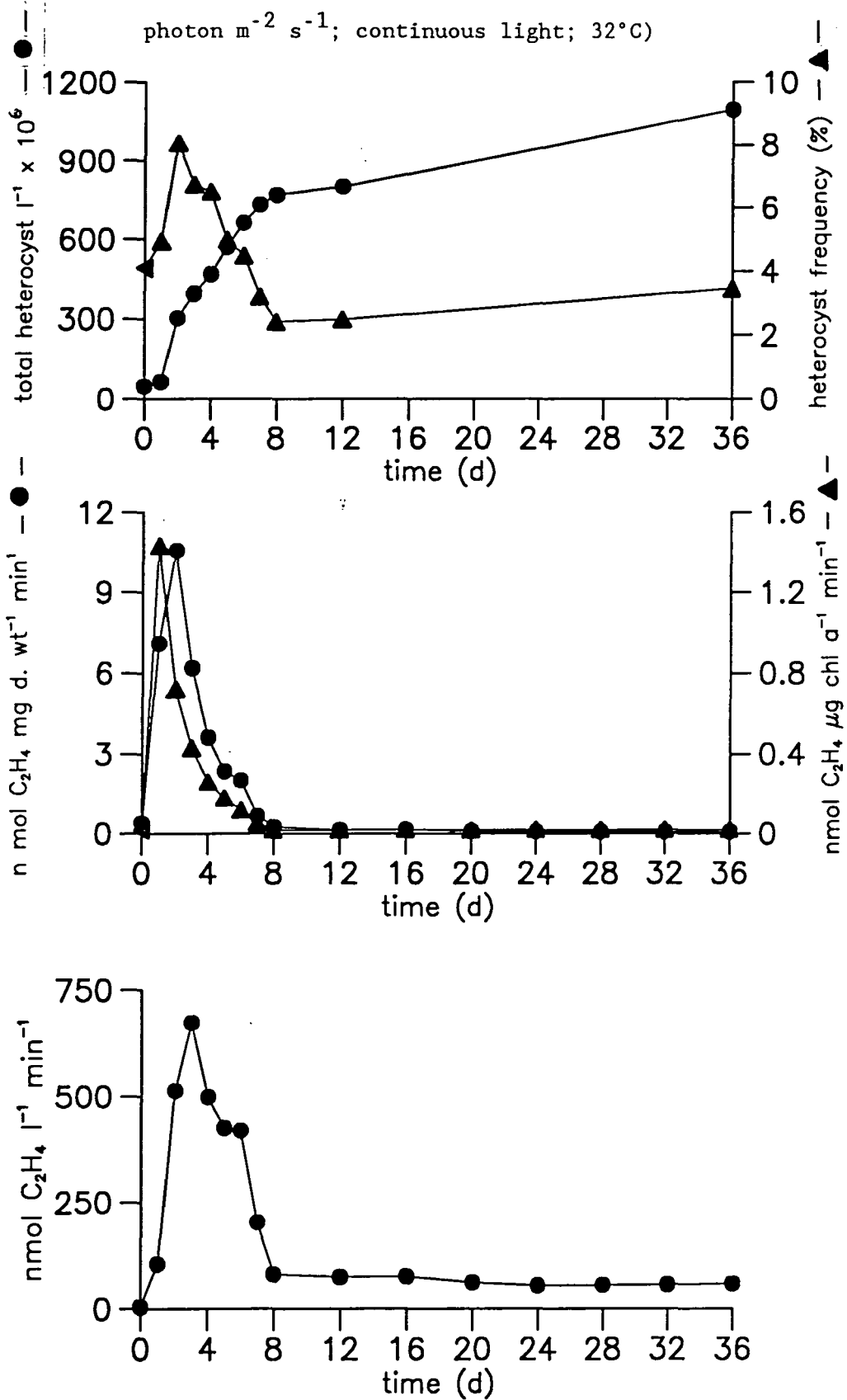


Fig. 4.3 Changes in nitrogen content as shown by direct measurement and indirectly from nitrogenase activity values (using $N_2 : C_2H_2$ reduced ratio of 1 : 4.1) of *Calothrix* D764 in batch culture. (Inoculum 3-d old; $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$; continuous light; 32°C)

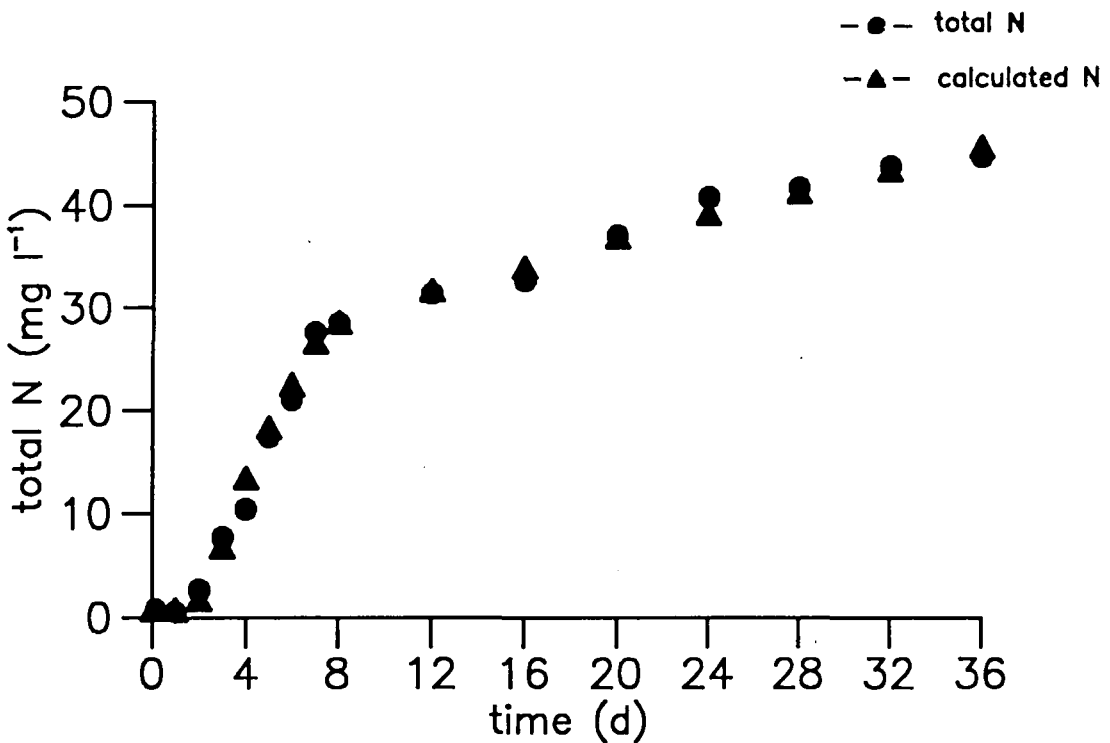
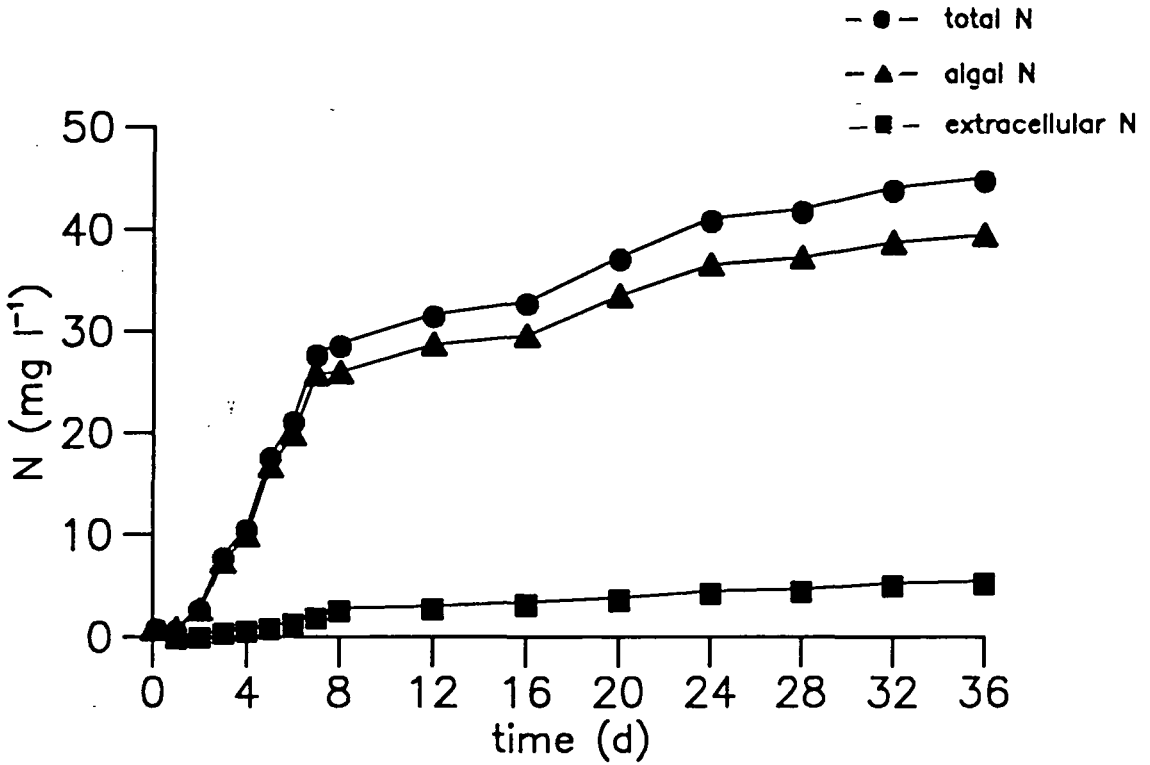
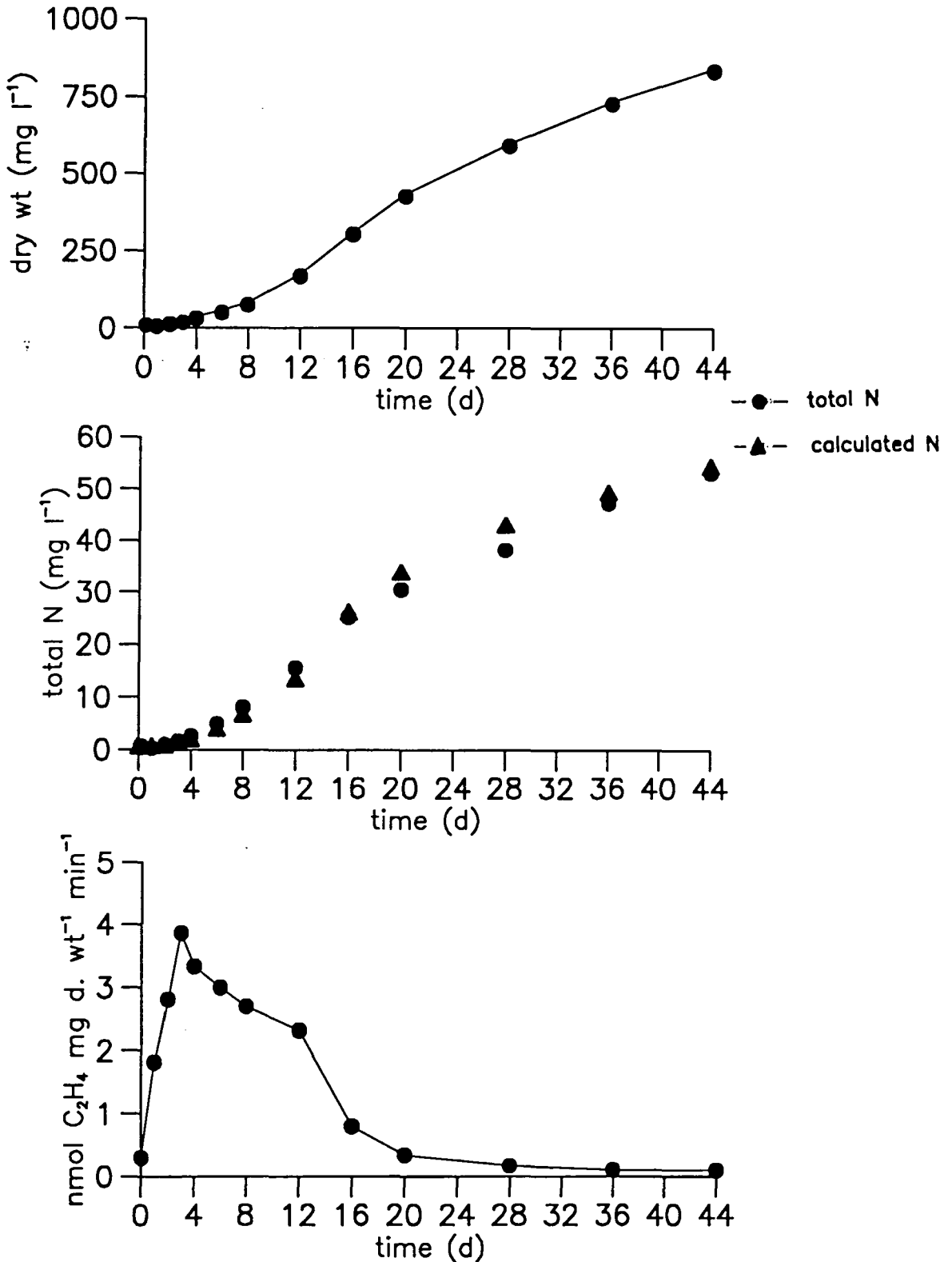


Fig. 4.4 Changes in dry weight and nitrogen content as shown by direct measurement and indirectly from nitrogenase activity values (using $N_2 : C_2H_2$ reduced ratio 1 :5.2) of *Calothrix* D764 in batch culture. (Inoculum 3-d old; $10 \mu\text{mol photon m}^{-2} \text{s}^{-1}$; continuous light; 32°C)



4.42 Influence of light flux on nitrogenase activity

The changes in light flux during the monsoon season in Bangladesh are very rapid. The variations in light flux may be several-fold in a short span of time (Section 1.34). The minimum time period for the determination of nitrogenase activity by the ARA technique is 30 min. Therefore the term rapid changes in nitrogenase activity in the text refers to a period of 30 min at least. A preliminary experiment was carried out to observe the influence of reduced light flux on nitrogenase activity of the alga for a period of 1 h. The alga was grown at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for 36 h and then incubated at lower values of light flux. The time delay incurred during the wrapping with neutral density filters, C_2H_2 injection etc of the flasks was about 50 s. A transfer to lower values of light flux or dark, brought about a marked reduction in nitrogenase activity during the first hour of incubation (Table 4.1).

Table 4.1 Influence on nitrogenase activity ($\text{nmol C}_2\text{H}_4 \text{ mg d. wt}^{-1} \text{ min}^{-1}$) of downshift in light flux from $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ to various lower light values for a period of one hour.

light flux to which shifted	% of original light flux	nitrogenase activity as % control value
50	59	88
25	29	78
10	12	35
dark	0	16

In the above experiment it is clear that the response of nitrogenase activity to a lower values of light flux is rapid. An experiment was carried out to monitor the changes in nitrogenase activity by placing the

culture from $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ to different lower values of light flux for a period of 12 h. The alga was grown at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for 36 h, then incubated at different values of light flux and nitrogenase activity was measured. The alga responded rapidly to the lower light flux apart from a drop at the intermediate light flux after 8 - 10 h (Fig. 4.5). An experiment was planned to study the speed of response to an upshift in light flux. In this case, the alga was grown at $10 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for 4 d. Growth (as d. wt) at this stage was similar to that obtained after 36 h of growth at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ in the previous experiment. When the alga was transferred to higher light flux, the response in nitrogenase activity was fast and appeared to level off after 2.0 - 2.5 h (Fig. 4.6).

4.43 Influence of pretreatment in dark on nitrogenase activity after transfer to light

Occasionally during the periods of monsoon rain, the algae in deepwater rice-fields are subjected to dark or near dark conditions for longer than one night. This longer period in the dark might have some ecological implications due to its influence on nitrogen fixation. Therefore experiments were planned to observe the influence of dark incubation for different periods on nitrogenase activity in the subsequent light period. In these cases, the alga was grown at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for 36 h and then incubated in the dark for 12, 24 and 36 h. Immediately before transfer to the dark the flasks were first wrapped with neutral density filters according to the light flux to be used in the subsequent light phase; this ensured that the alga would not be exposed to higher values of light flux during C_2H_2 gassing, equilibration etc. The flasks were then wrapped with aluminium foil and black polythene. After incubation in the dark, the culture was exposed to different values of light flux and

Fig. 4.5 Influence of downshift in light flux on nitrogenase activity of *Calothrix* D764. (Alga was grown at $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 36 h and then placed at different values of light flux and dark for 12 h)

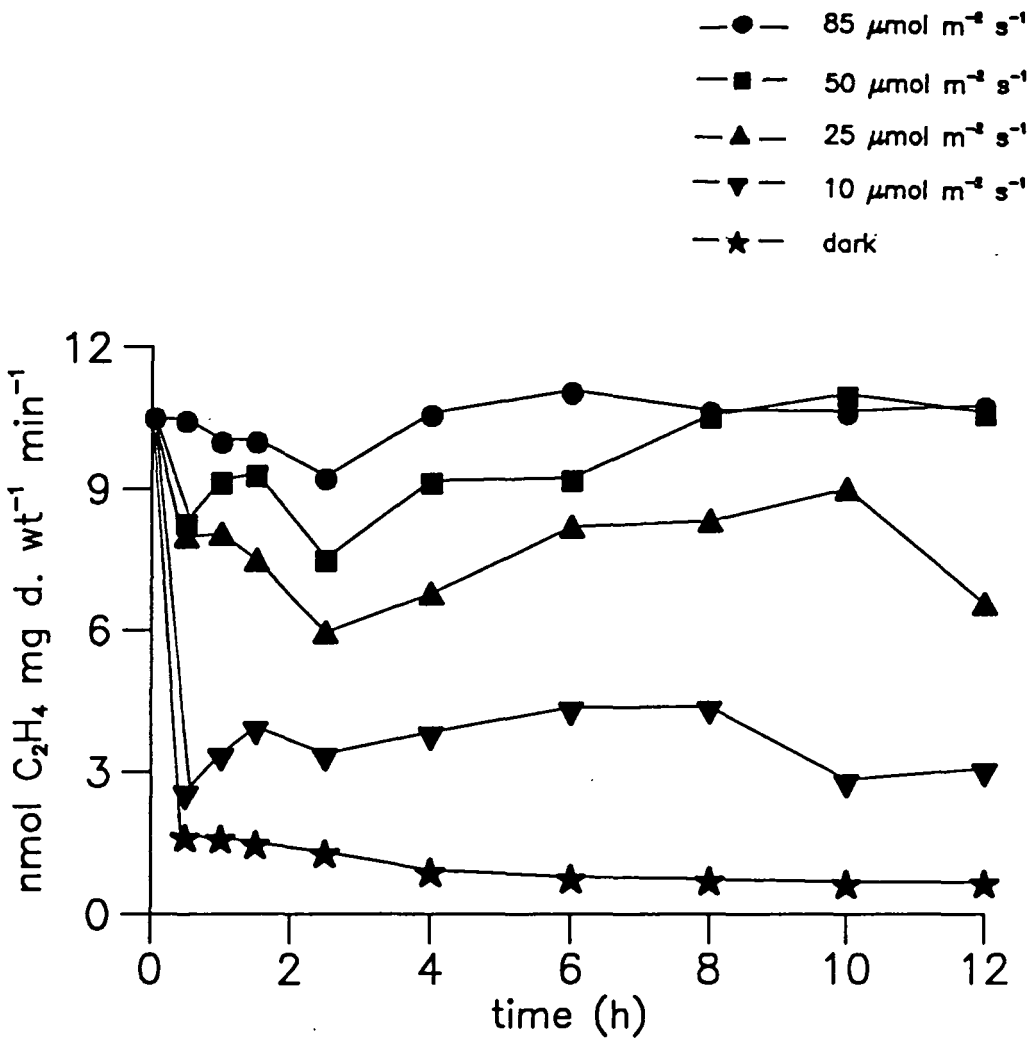
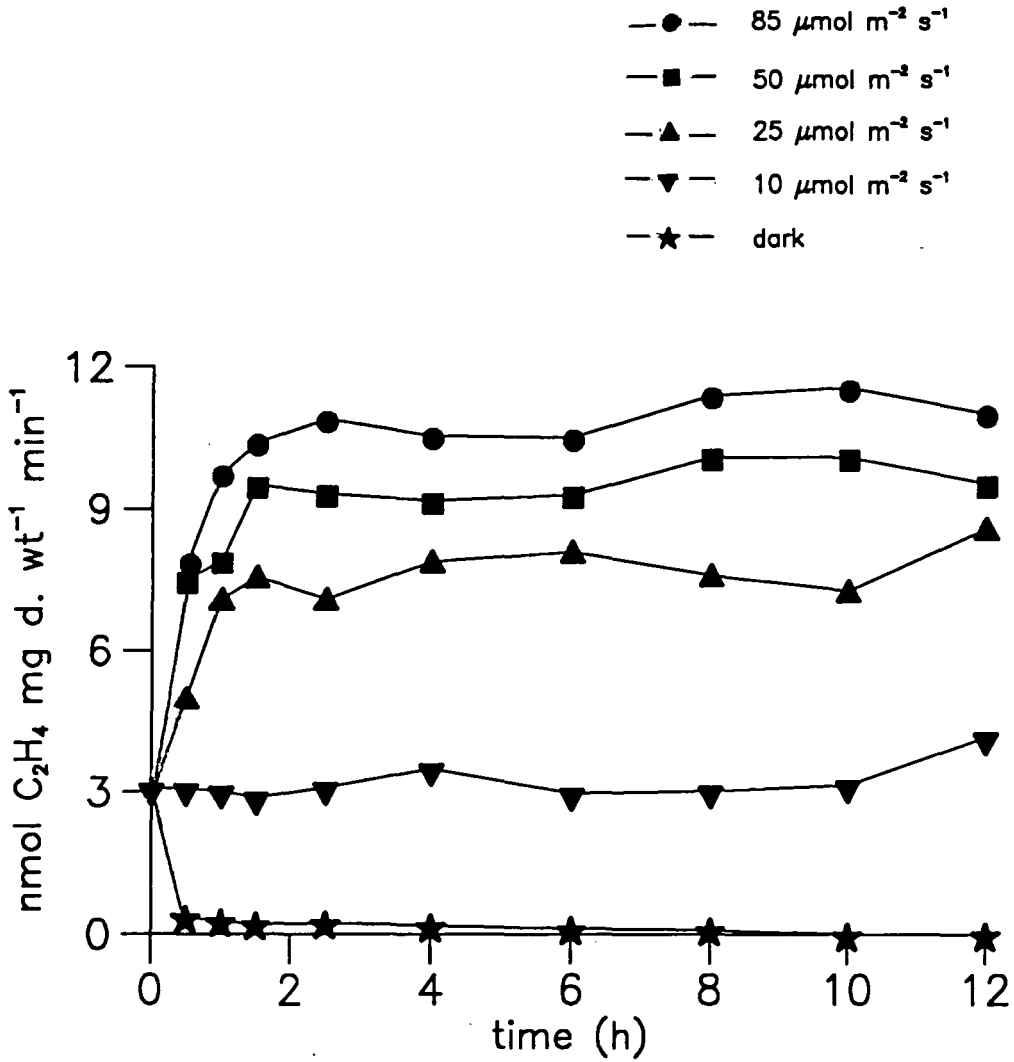


Fig. 4.6 Influence of upshift in light flux on nitrogenase activity of *Calothrix* D764. (Alga was grown at $10 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 4 d then placed at different higher values of light flux and dark for 12 h)



nitrogenase activity was measured. The influence of prior incubation in the dark on nitrogenase activity in the subsequent light phase is shown in Fig. 4.7. The resumption in nitrogenase activity was faster when the culture was placed in the dark for shorter period than the longer periods. Specific activity dropped markedly within 12 h of light treatment, after reaching maximum 4 - 6 h of re-illumination.

Pretreatment in the dark resulted in higher nitrogenase activity than was obtained during the growth under continuous light (Figs 4.2; 4.7). An experiment was therefore planned to see whether the loss in nitrogenase activity during the period of dark incubation is compensated for upon reillumination of the culture. As in the earlier experiment, the alga was grown at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for 36 h after which the flasks were divided into two series, continuous light and dark. The culture was placed in the dark for 12 h the approximate night time dark period. After 12 h of dark the alga was re-exposed to initial light conditions and nitrogenase activity was measured at regular intervals. The nitrogenase activity was also measured during growth under continuous light; a comparison is presented in Fig. 4.8. Transfer of the dark treated alga to the initial light led to much higher nitrogenase activity per unit dry weight, with an increase of about 60% in the subsequent light phase compared with continuous light. A similar experiment was also conducted on an 8-d old culture to see whether there is a similar response. The dark-treated alga also showed much higher nitrogenase activity in subsequent light compared with the continuous illuminated alga (Fig. 4.9). When total acetylene reduced was calculated for a period of 12 h in the dark followed by 48 h illumination, there was a 20% increase in activity compared with continuous illuminated alga during the same period. The total amount of nitrogen fixed by reilluminated alga was 11% higher than continuously illuminated alga (Table 4.2).

Fig. 4.7 Influence of pretreatment in dark for different periods of time on nitrogenase activity, upon re-illumination by *Calothrix* D764. (36-h grown culture at $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ was incubated in dark for a) 12 h, b) 24 h, c) 36 h; then exposed to different values of light flux for 12 h)

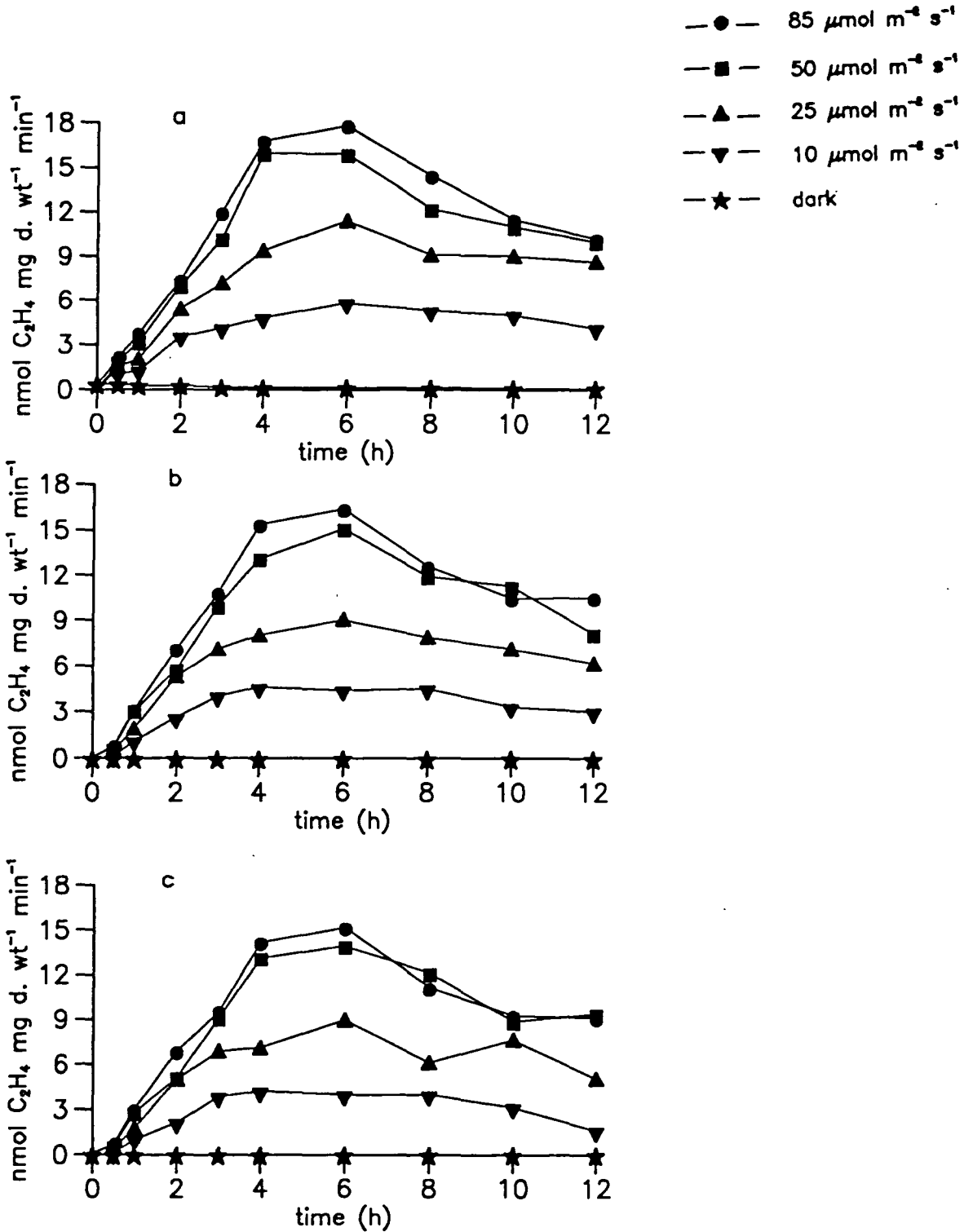


Fig. 4.8 Influence of pretreatment in dark for 12 h on nitrogenase activity upon re-illumination by 36-h old *Calothrix* D764. (Alga was grown for 36 h at $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ then divided into two series; one continuous light; another in dark for 12 h followed by re-illumination to original light flux)

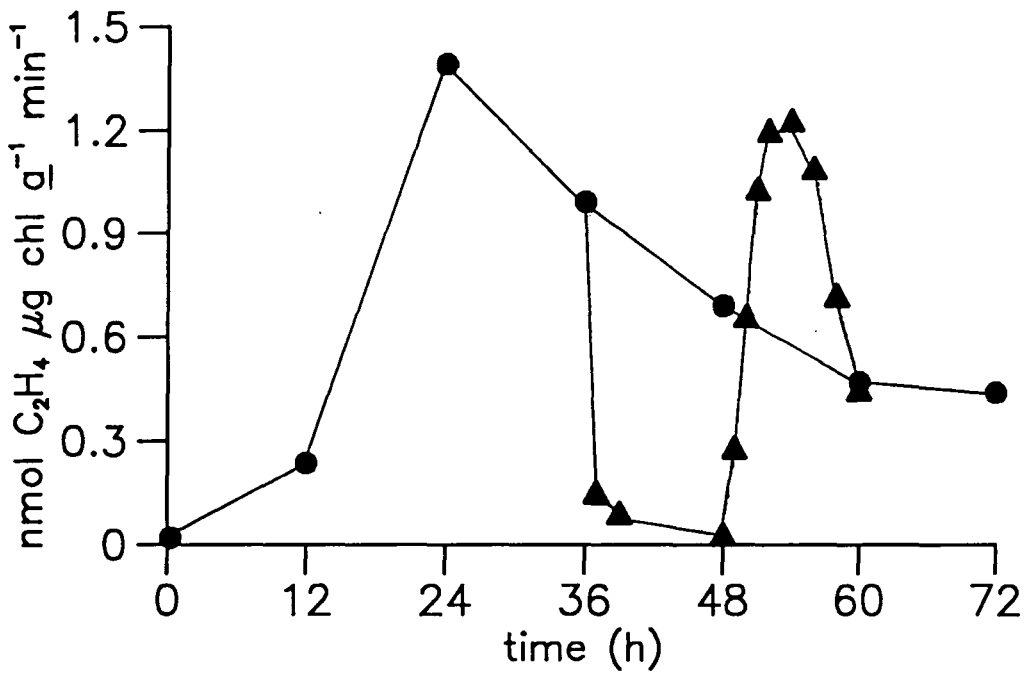
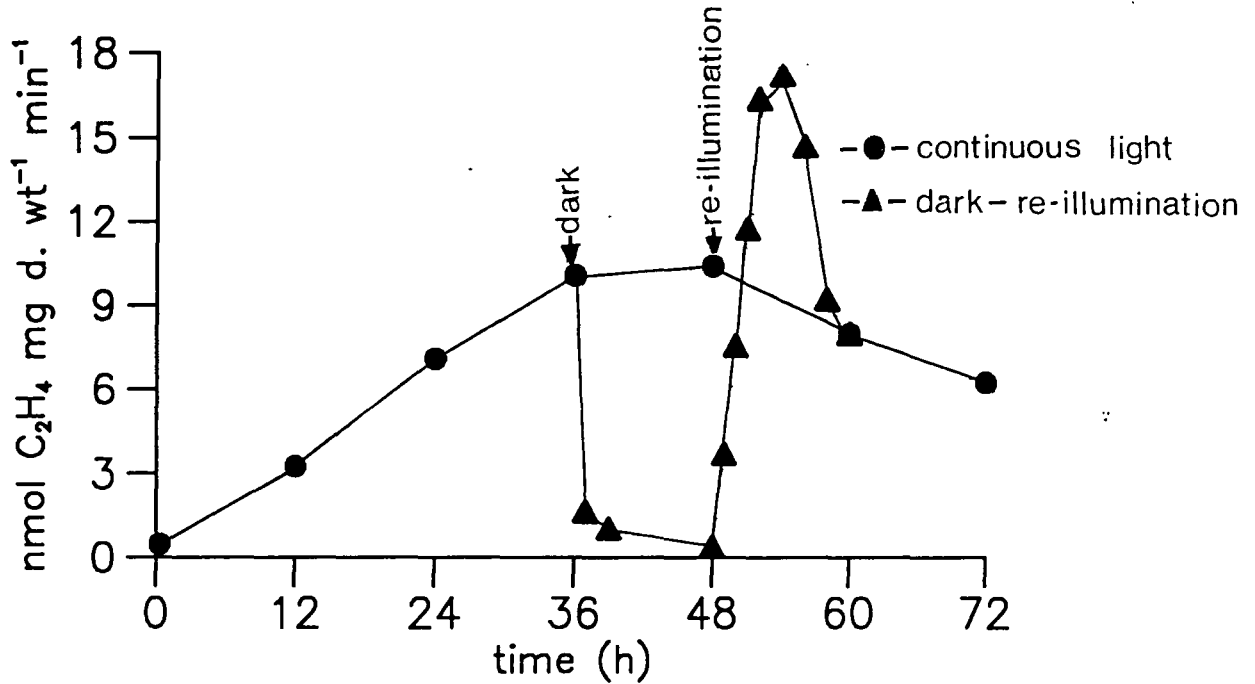


Fig. 4.9 Influence of pretreatment in dark for 12 h on nitrogenase activity upon re-illumination by 8-d old *Calothrix* D764. (Alga was grown for 8 d at $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ then divided into two series; one continuous light; another in dark for 12 h followed by re-illumination to original light flux)

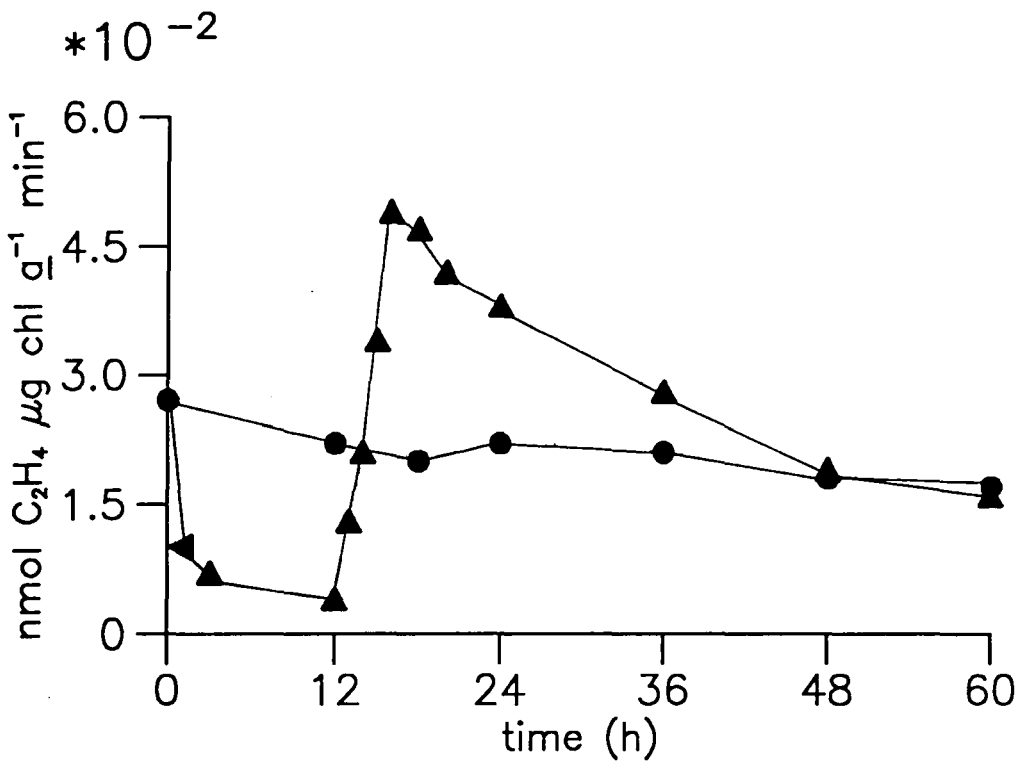
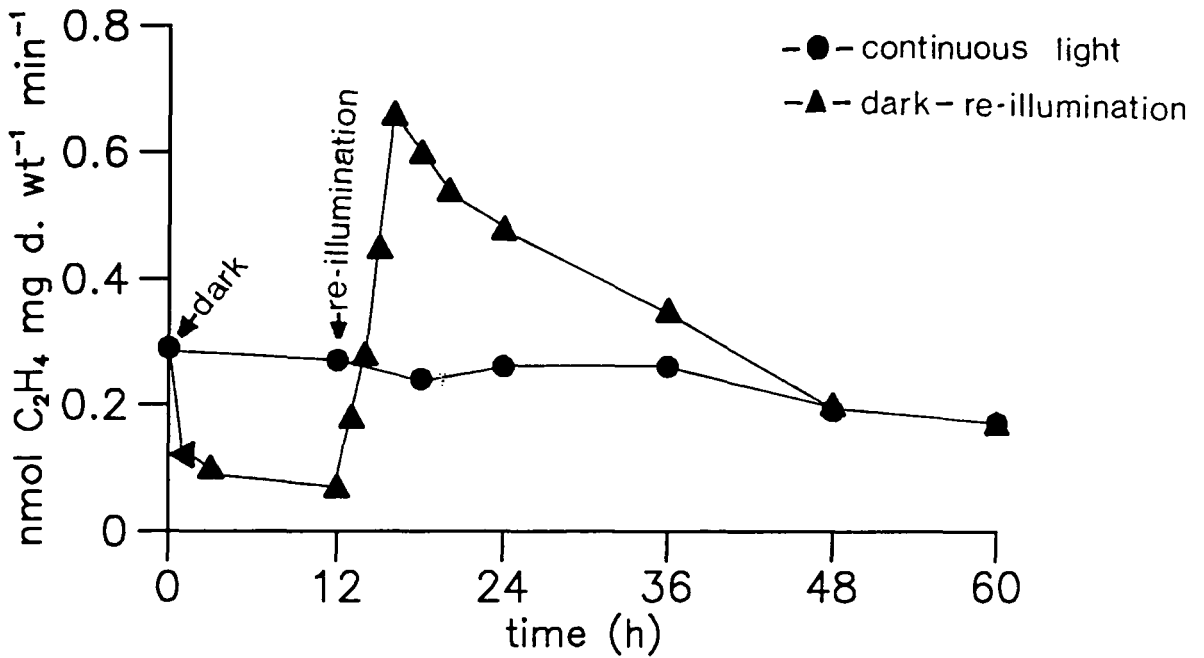


Table 4.2 Influence of light quantity on Calothrix D764 for a period of 60 h on nitrogen content. (Alga was grown at $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 8 d then divided into two sets; one kept at that light flux for 60 h; another placed in 12 h dark followed by 48 h re-illumination to original light flux; culture was harvested and analysed for cellular and extracellular N)

light conditions	cellular N (mg l^{-1})	extracellular N (mg l^{-1})	total N (mg l^{-1})
continuous light	1.76	0.22	1.98
12 h dark followed by re-illumination	1.56	0.65	2.21

Table 4.3 Changes in dry weight of Calothrix D764 in dark for varying periods of time. (Alga was grown for 36 h then placed in the dark for varying periods; $n = 4$)

time (h)	0	12	24	36
d. wt (mg l^{-1})	42.0	36.9	37.3	37.1
	\pm	\pm	\pm	\pm
	3.16	2.40	1.60	1.60

In the previous sections, it has been shown that the nitrogenase activity in the dark declined sharply and on reillumination nitrogenase activity increased rapidly. This may be associated with a number of changes at the cellular level. An experiment was planned to stop the synthesis of nitrogenase of the alga in the light period which was

previously incubated in the dark. The alga was grown for 36 h at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. The culture was placed in the dark for 12 h after which reilluminated in the presence or absence of chloramphenicol, a protein synthesis inhibitor (50 mg l^{-1}). There was no increase in nitrogenase activity with chloramphenicol treated alga (Fig. 4.10) presumably due to inhibition of nitrogenase synthesis.

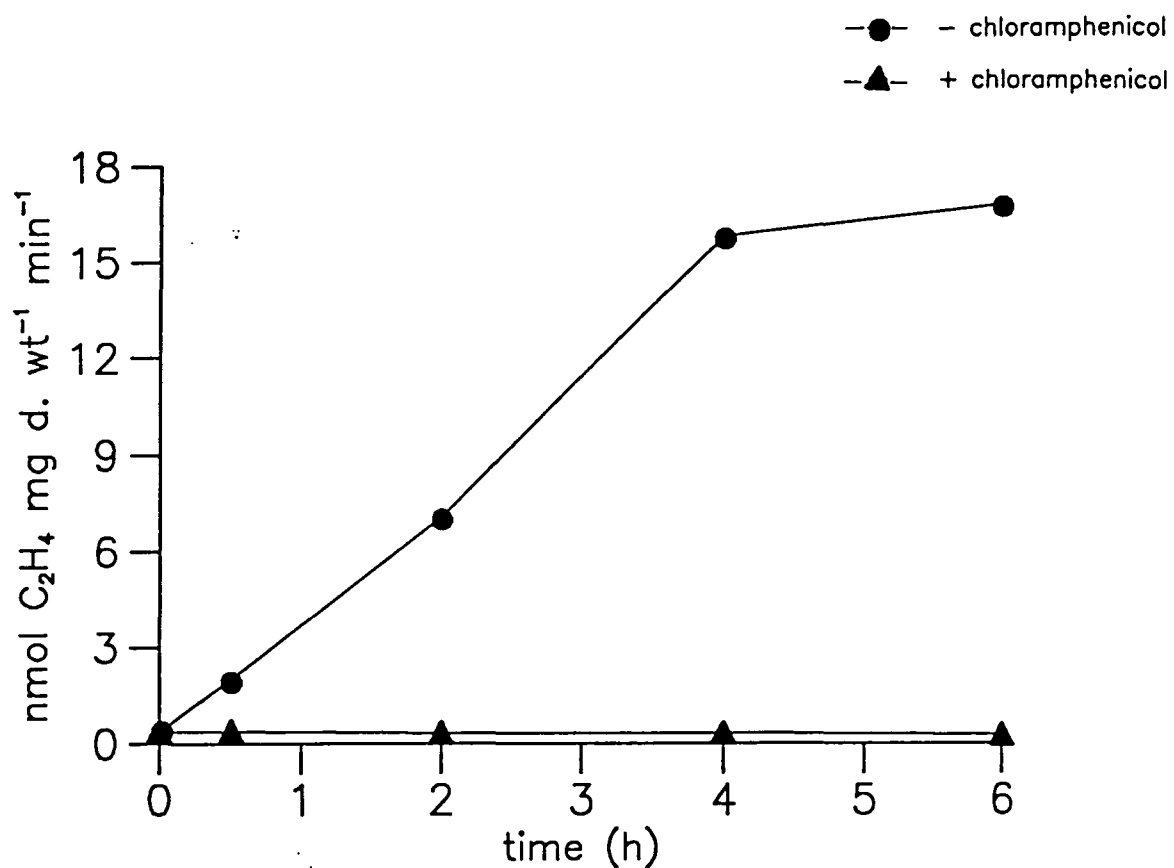
4.45 Nitrogenase activity in the dark

In the previous section, it has been shown that within an hour of transfer to dark, nitrogenase activity declined to about 16% of the light control. An experiment was carried out to quantify the nitrogenase activity in the dark. The alga was grown at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for 36 h and then incubated in the dark. Nitrogenase activity declined rapidly in the dark, dropping to 5% after 6 h (Table 4.4). Detectable nitrogenase activity was however still found after 24 h of dark incubation. At the end of the experiment the alga was tested for possible bacterial contamination and found to be axenic.

Table 4.4 Changes in nitrogenase activity ($\text{nmol C}_2\text{H}_4 \text{ mg d. wt}^{-1} \text{ min}^{-1}$) of Calothrix D764 in dark for varying periods of time. (Alga was grown at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for 36 h then placed in dark and nitrogenase activity was measured; $n = 4$)

time (h)	0	0.5	1.0	1.5	2.5	4.0	6.0	12.0	24.0	36.0
nitrogenase activity	10.33	1.78	1.64	1.54	1.38	0.69	0.52	0.37	0.12	n.d
	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm	
	0.98	0.06	0.15	0.07	0.02	0.08	0.09	0.07	0.03	

Fig. 4.10 Influence of chloramphenicol (final concentration 50 mg l^{-1}) on nitrogenase activity of *Calothrix* D764 in light. (Alga was grown for 36 h at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ and placed in the dark for 12 h then exposed to the original light in the presence or absence of chloramphenicol)



4.5 Oxygen

4.51 Introduction

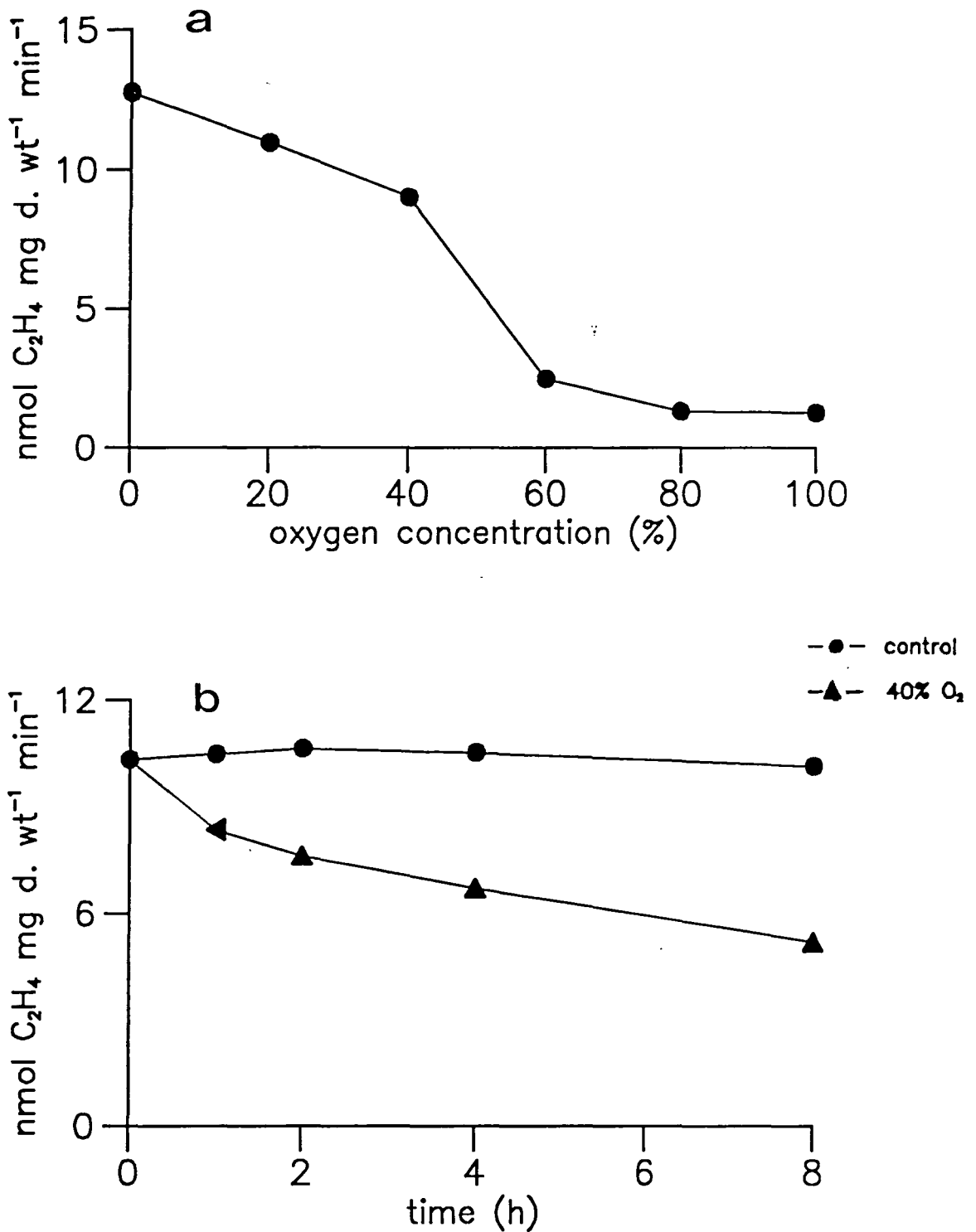
The dissolved oxygen concentration in ^{the} water column of deepwater rice-fields and adjacent fallow areas in Bangladesh rises to 200% supersaturation in the early afternoon. On the other hand during the later part of the season, the water column becomes anoxic (Section 1.35). A limited laboratory study was therefore included on the influence of oxygen on nitrogenase activity of Calothrix D764 in the laboratory.

4.52 Influence of higher oxygen concentrations on nitrogenase activity

The alga was grown for 36 h at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. The culture was flushed with either O_2 or N_2 and the required volume was withdrawn to inject different concentrations of O_2 , 0.03% CO_2 and 10% C_2H_2 . DCMU (10 μM) was added to prevent any photosynthetic O_2 evolution during the assay. Assay for nitrogenase activity was carried out for 30 min. At the end of the experiment, dissolved O_2 concentrations resulting from different treatments were 95% saturation at $p\text{O}_2 = 0.2 \text{ atm}$, 155% saturation at $p\text{O}_2 = 0.4 \text{ atm}$, 240% saturation at $p\text{O}_2 = 0.6 \text{ atm}$, 330% saturation at $p\text{O}_2 = 0.8 \text{ atm}$, and 350% saturation at $p\text{O}_2 = 1.0 \text{ atm}$. Anoxic conditions led to a 22% increase and 40% O_2 led to a 18% decrease in nitrogenase activity than the rate with 20% O_2 (Fig. 4.11a). Nitrogenase activity of the alga dropped sharply above 40% O_2 in the gas phase.

An experiment was planned to follow nitrogenase activity after incubation at ^{an} elevated concentration of oxygen (40% O_2), to see whether the alga can adapt at a higher O_2 concentration. The alga was grown for 36 h and flushed with N_2 and ^{the} required volume was withdrawn and O_2 was injected to obtain a 40% O_2 and 0.03% CO_2 mixture. After each 30 min the culture was flushed with the same gas mixture. There was a gradual decrease in nitrogenase activity with time (Fig. 4.11b). At the end of the experiment

Fig. 4.11 Influence of oxygen on nitrogenase activity of *Calothrix* D764. (36-h grown cultures at $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ were a) assayed with different concentrations of oxygen; b) incubated with 40% oxygen and then assayed for nitrogenase activity)



the dissolved O₂ concentration was 188% saturation. After 8 h of incubation at 40% O₂, nitrogenase activity declined to about 50% compared to the control.

4.6 Combined nitrogen

4.61 Introduction

The concentration of ammonium nitrogen in rice-fields may be quite high after the application of nitrogenous fertilizer (Section 1.4). This high concentration of ammonium nitrogen might have an effect on nitrogen fixation by the blue-green algae. Besides, the loss of heterocysts in this alga in the presence of nitrogen sources (Section 2.112) indicates that combined nitrogen might inhibit nitrogen fixation. Experiments were therefore planned to determine the influence of combined nitrogen on nitrogenase activity of this alga.

4.62 Influence of combined nitrogen on nitrogenase activity

An experiment was planned to determine the effect of combined nitrogen on nitrogenase activity. The alga was grown at 85 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ for 36 h and then 10 mg l^{-1} NH₄-N was added to the flasks. Nitrogenase activity declined to 83 and 55% compared to the control after 2.0 and 4.0 h of incubation.

Under natural conditions, the algae are subjected to a light - dark cycle. Thus, experiments were planned to see the effect of two levels of NH₄-N on nitrogenase activity of the alga in light and dark. Ammonium concentration in the medium after each nitrogenase activity study was also determined. Nitrogenase activity declined rapidly at 10 mg l^{-1} NH₄-N, whereas the inhibitory effect was slower on addition of 1.0 mg l^{-1} NH₄-N (Figs. 4.12 ; 4.13). Progressive inhibition of nitrogenase activity was

Fig. 4.12 Influence of ammonium nitrogen ($1.0 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$) on nitrogenase activity of *Calothrix* D764. (Alga was grown at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for 36 h then ammonium nitrogen was added)

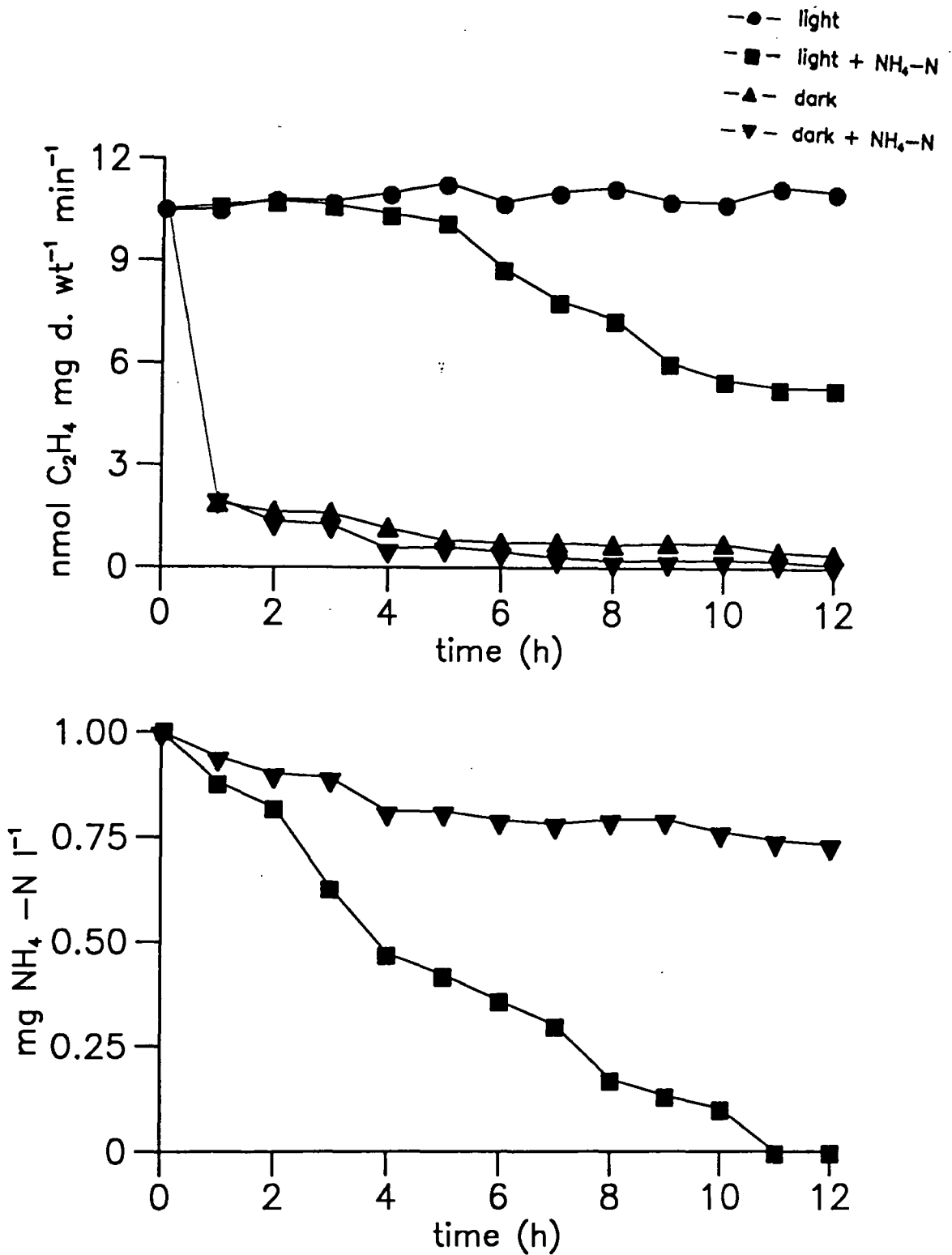
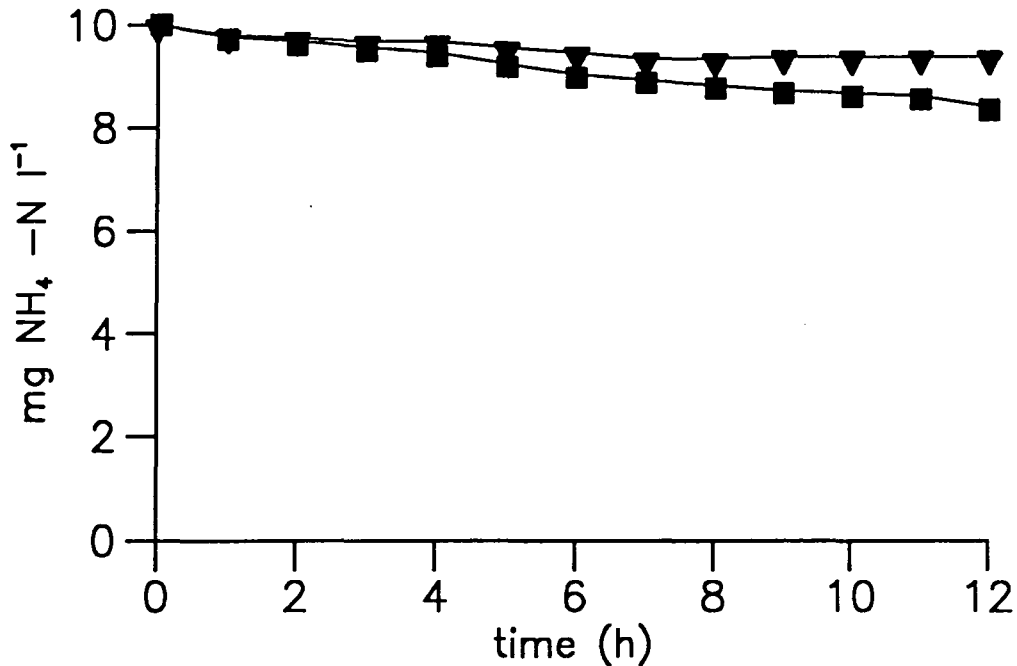
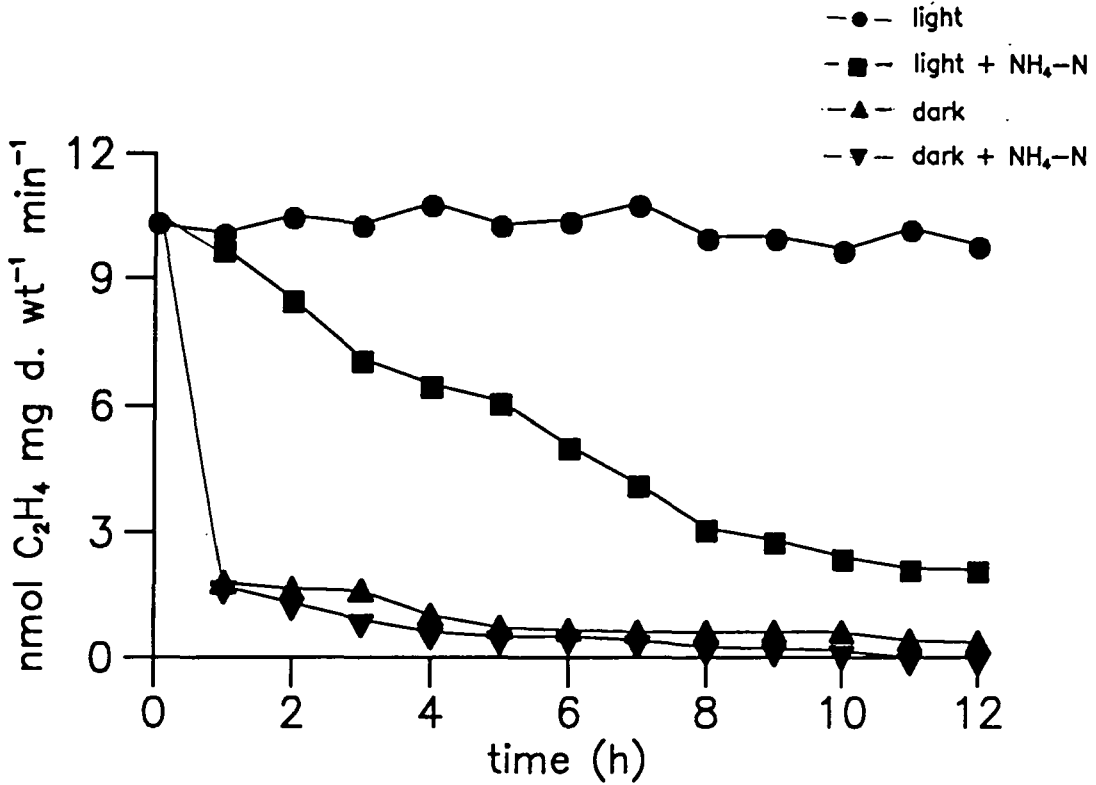


Fig. 4.13 Influence of ammonium nitrogen ($10.0 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$) on nitrogenase activity of *Calothrix* D764. (Alga was grown at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for 36 h then ammonium nitrogen was added)



noticed both in light and dark, but inhibition was more pronounced in the former.

4.7 Phosphorus

4.71 Introduction

In deepwater rice-fields in Bangladesh phosphorus levels are quite low and variable (Section 1.36). The low phosphorus in the environment might affect the nitrogen fixation of the algae. Therefore, an experiment was planned with Calothrix D764 to follow the changes in nitrogenase activity on addition of P to P-deficient cultures.

4.72 Influence of P addition to P-deficient culture on nitrogenase activity

The alga was grown in standard medium for 12 d to make the culture P deficient; and P (1.78 mg l^{-1}) was then added. The alga showed much higher nitrogenase activity on addition of P compared with the control and reached a maximum at day 2 and then started to decline (Fig. 4.14).

4.8 Iron

4.81 Introduction

The formation of secondary heterocysts of Calothrix D764 in response to Fe-limitation suggests this change may reflect a change in ability to fix nitrogen (Section 2.114). Therefore an experiment was planned to see the changes in nitrogenase activity and growth parameters of the alga in Fe-deficient conditions and also to follow the changes on addition of Fe to Fe-deficient culture.

4.82 Influence of Fe addition on nitrogenase activity

The culture was grown in standard medium apart from the omission of Fe and the reduction of EDTA. Before iron addition nitrogenase activity increased gradually to reach a maximum on day 2 and then decreased slowly. Iron addition on day 12 led to a rapid increase (detectable within 1 h) in nitrogenase activity, which reached a maximum within 24 h and then started to decline (Fig. 4.16).

4.73 Influence of protein synthesis inhibitor

It was not clear whether the rapid increase in activity was due to activation of the process by Fe or to synthesis of certain proteins. Therefore the influence of an inhibitor (chloramphenicol, 50 mg l⁻¹) of protein synthesis was tested on nitrogenase activity. A Fe-deficient culture was prepared as before and chloramphenicol and Fe were added to one series and Fe to another series. Chloramphenicol prevented any increase in nitrogenase activity, presumably by inhibiting the synthesis of nitrogenase (Fig. 4.17).

Fig. 4.14 Influence of P addition on nitrogenase activity by P-deficient culture of *Calothrix* D764. (Alga was grown in standard medium for 12 d then 1.78 mg l⁻¹ P was added)

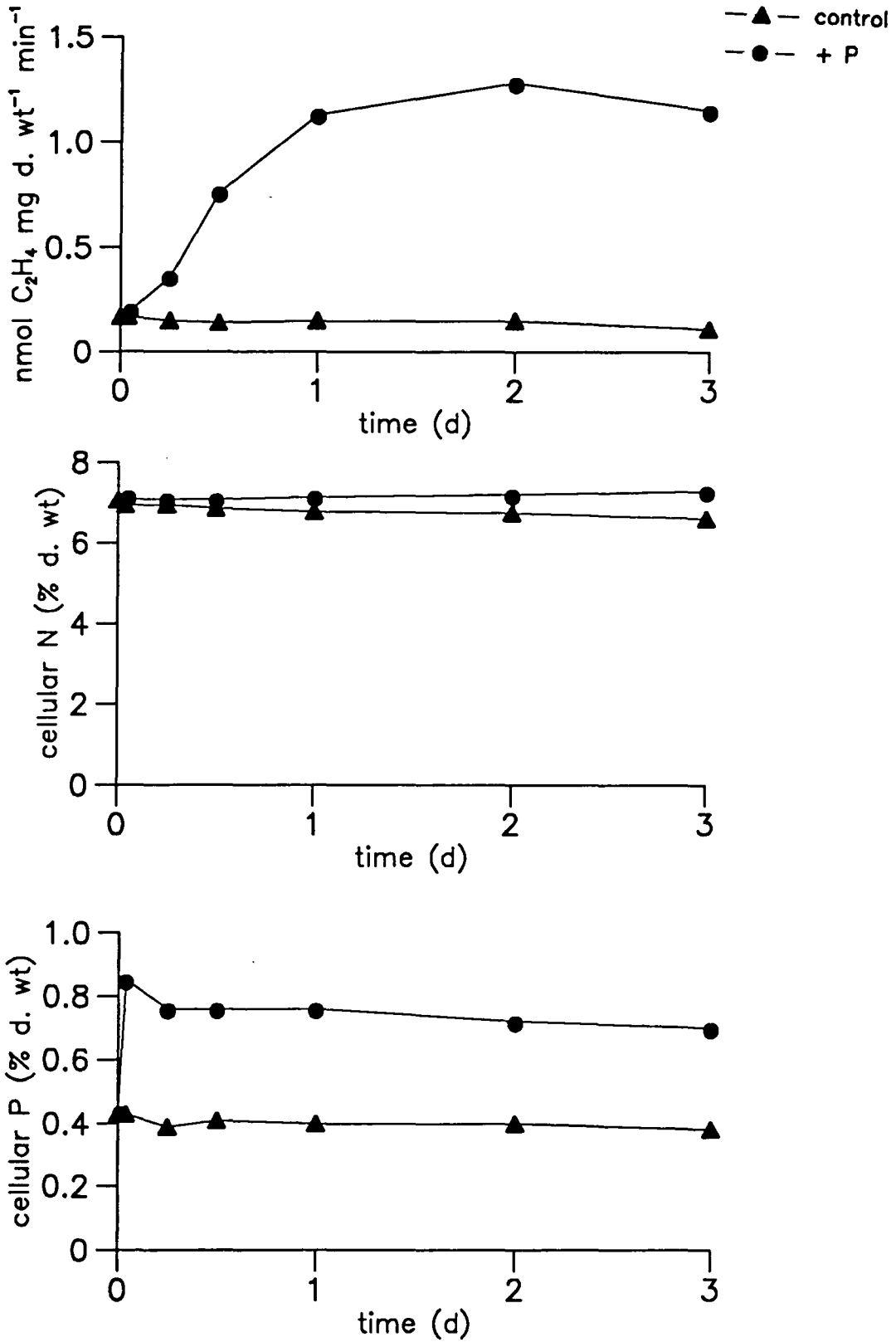


Fig. 4.15 Influence of Fe addition on dry weight and cellular N by Fe-deficient culture of *Calothrix* D764. (Alga was grown in - Fe medium and Fe (0.5 mg l^{-1}) was added on day 12)

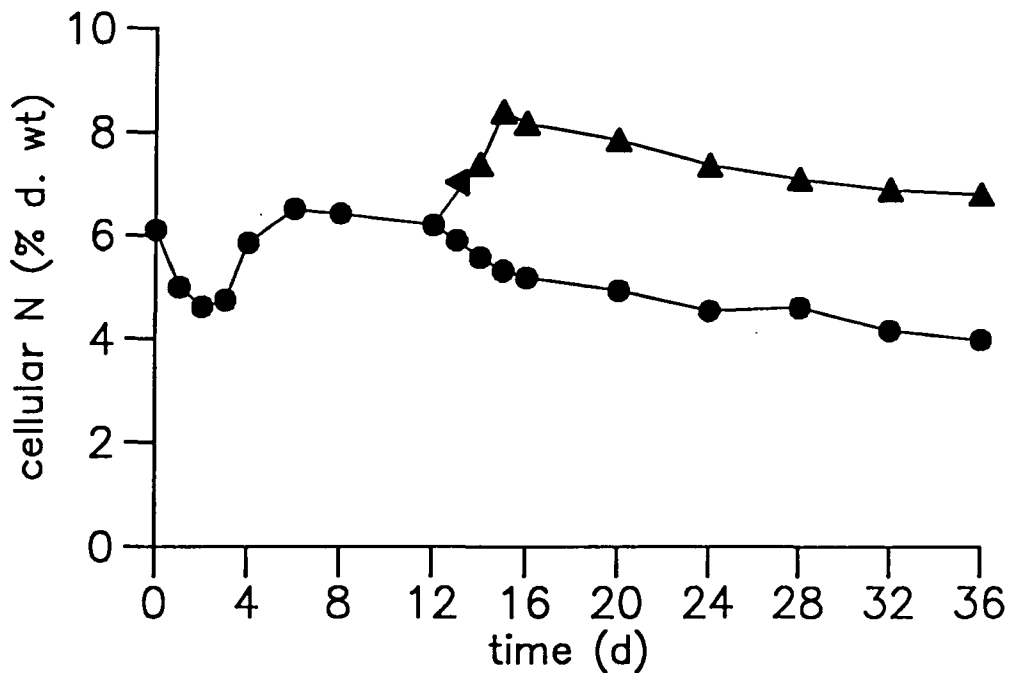
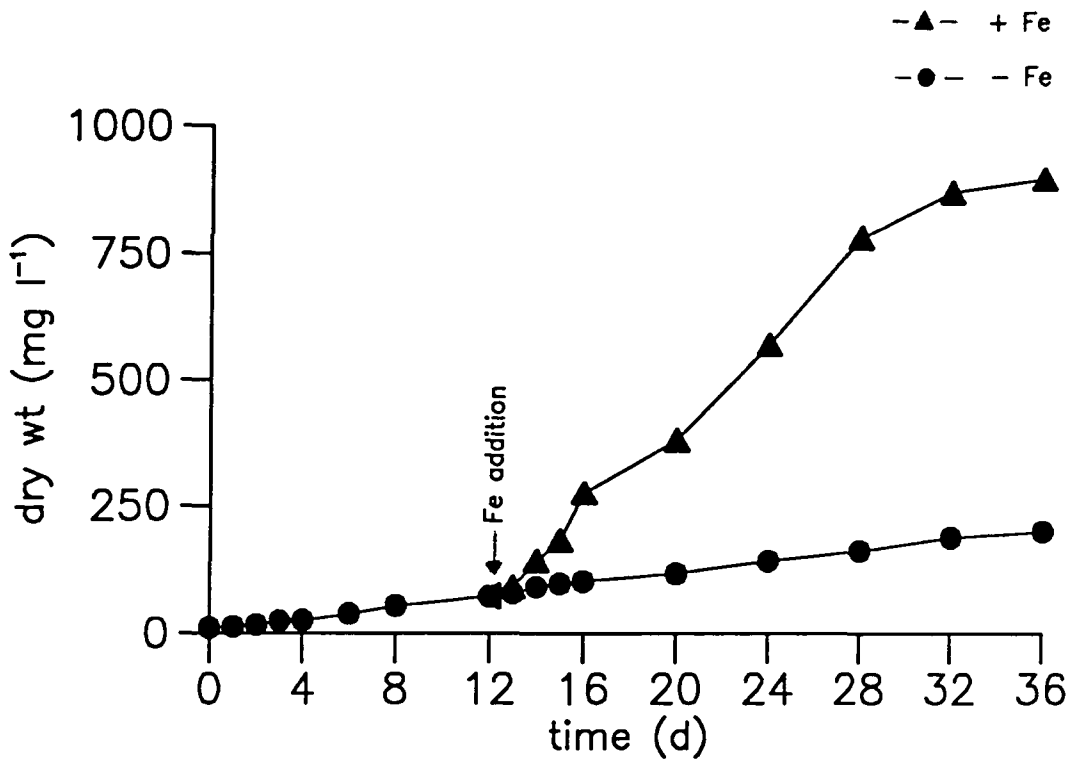


Fig. 4.16 Influence of Fe addition on heterocyst frequency and nitrogenase activity by Fe-deficient culture of *Calothrix* D764. (Alga was grown in - Fe medium and Fe (0.5 mg l^{-1}) was added on day 12)

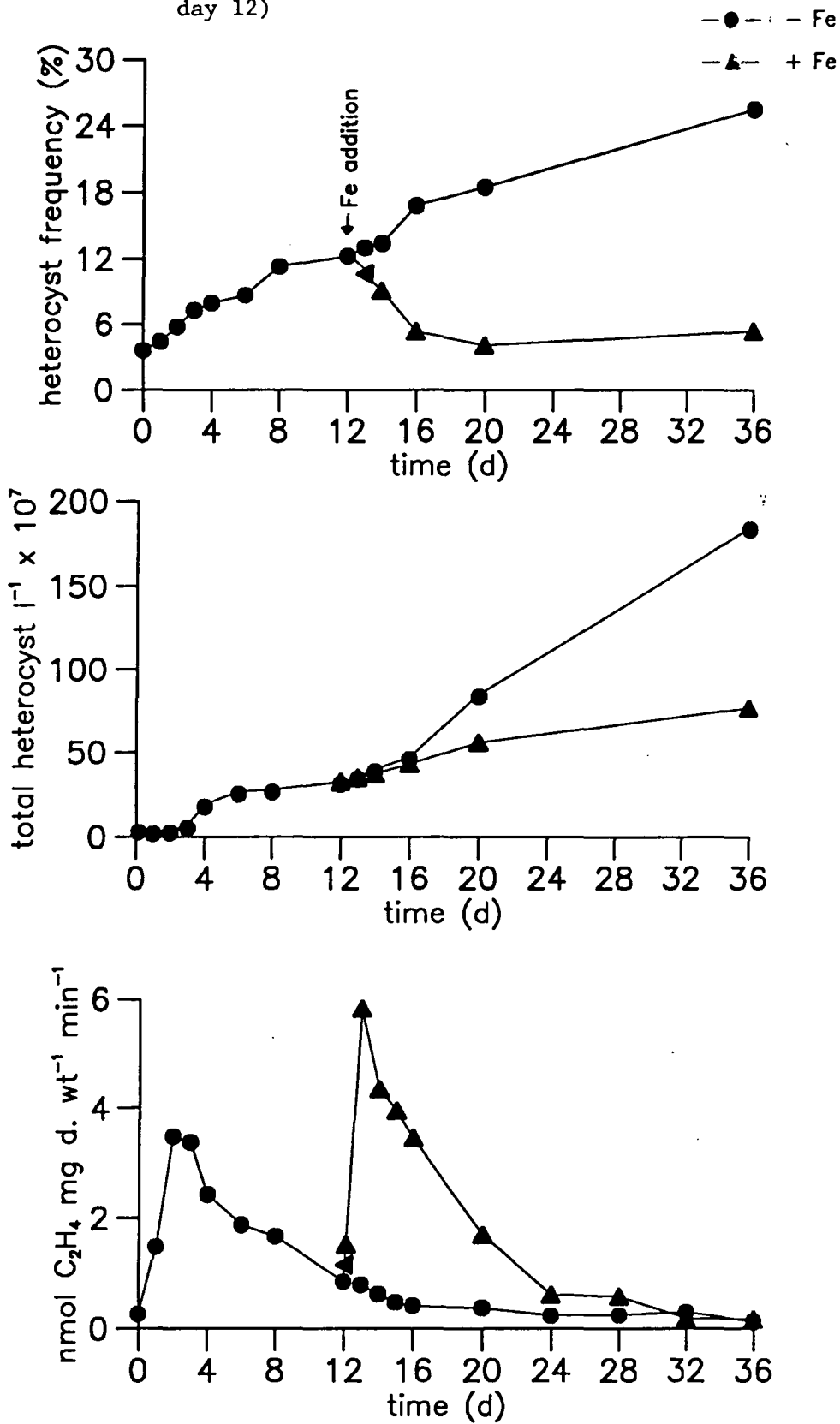
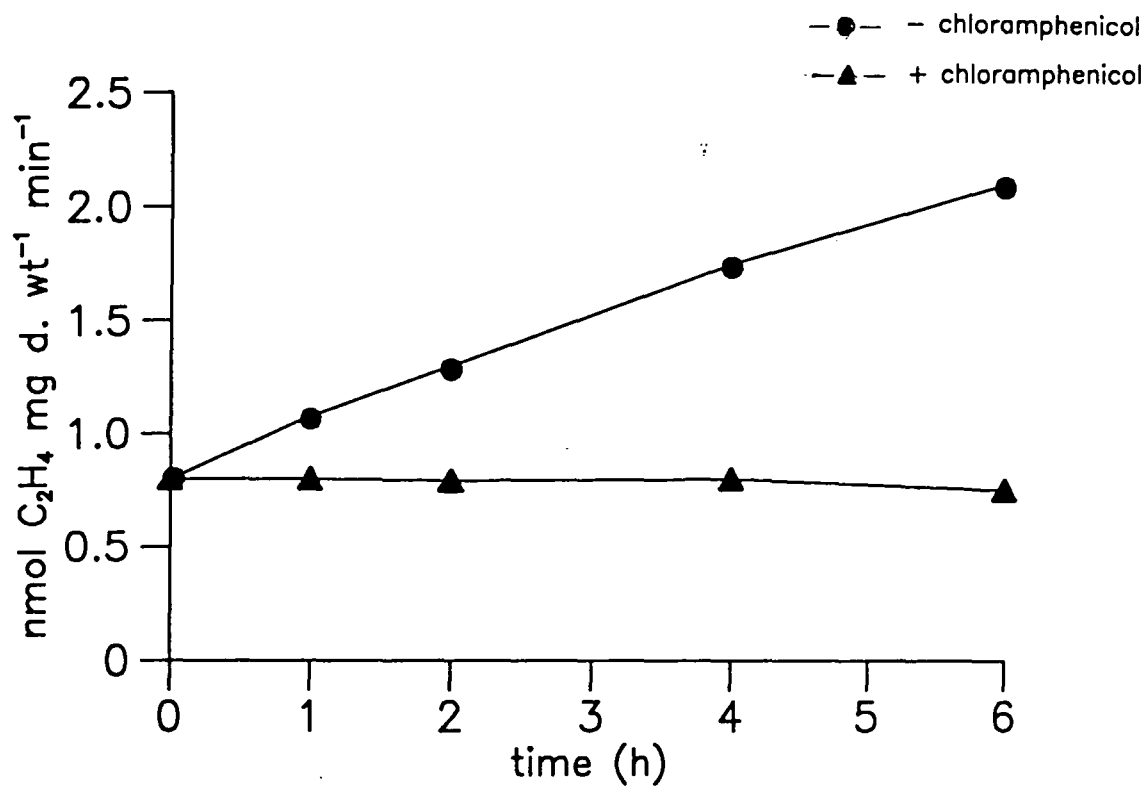


Fig. 4.17 Influence of chloramphenicol (final concentration 50 mg l^{-1}) and Fe (0.5 mg l^{-1}) addition on nitrogenase activity by Fe-deficient culture of *Calothrix* D764. (Alga was grown in - Fe medium for 12 d and then Fe or Fe plus chloramphenicol was added)



5 NITROGENASE ACTIVITY (ARA) BY RIVULARIA

5.1 Nitrogenase activity of field Rivularia

5.11 Introduction

In Chapter 4 nitrogen fixation (nitrogenase activity and total N) studies were carried out on the deepwater rice-field isolate Calothrix D764 in the laboratory. As it was impossible to conduct parallel studies on algae in deepwater rice-fields to compare laboratory results with field, combined field and laboratory experiments were planned with another member of the Rivulariaceae in the UK. The aim was to compare the response of nitrogenase activity of field populations of Rivularia and a laboratory isolate made from the field population with changes in light flux, the key environmental factor affecting nitrogen fixation in heterocystous blue-green algae.

5.111 Chemical environment of the field

Field experiments on Rivularia colonies were carried out in a pond, Croft Kettle near Darlington (grid reference NZ 282108). Water enters the pond from subterranean springs draining the limestone. Average pH value is 7.6. Water is highly calcareous, Mg and Ca content reaching values of 164 and 510 mg l⁻¹ (Hudson et al., 1971). Phosphorus status of the water is about 15 µg l⁻¹; mostly in the form of organic phosphate (D. Livingstone and B. A. Whitton, unpublished data). Ecological details of the pond have been described by Wheeler and Whitton (1971).

Rivularia was found to grow on submerged dead woods as distinct hemispherical colonies. The size of the colonies varied widely, reaching 5 - 6 mm across. Colonies were bright green. The alga formed multicellular long hairs which came out from the colonies. Calcite deposition was observed in the colonies.

5.112 Influence of light flux on nitrogenase activity

Nitrogenase activity of the alga varied widely depending on the age in batch culture (Section 4.3). A preliminary experiment was therefore planned to compare nitrogenase activity of small (1 - 2 mm diameter) and large (4 - 5 mm) Rivularia colonies in the field. The experiment was carried out at 1000 - 1200 and 1400 - 1600 on four different dates in 1987. The amount of light reaching the colonies varied widely depending on the position of the colonies in the water column and shading from surrounding plants. Care was therefore taken to collect colonies from well illuminated places in order to minimize the influence of light flux on nitrogenase activity. After collection the colonies were kept in a plastic bucket with pond water.

Dark condition was achieved by covering with two layers of aluminium foil. The colonies were incubated for 2 h for nitrogenase activity in universal bottles. The results are presented in Table 5.1. Small colonies showed higher nitrogenase activity per unit chlorophyll a than large colonies in all eight comparisons made in four dates, but the difference was significant only on 9 August during 1400 - 1600. Out of eight determinations of nitrogenase activity, seven were available for dark, of which small colonies showed higher activity in five determinations than large colonies. There was no significant difference in activity between small and large colonies in the dark. The ratio of light : dark in nitrogenase activity of small colonies on different dates ranged from 1 : 0.13 to 1 : 0.52 with a mean of 1 : 0.24, while for large colonies the range of the ratio was from 1 : 0.08 to 1 : 0.42 with a mean of 1 : 0.26 (Table 5.1).

Table 5.1 In situ nitrogenase activity ($\text{nmol C}_2\text{H}_4 \mu\text{g chl a}^{-1} \text{min}^{-1} \times 10^{-3}$) of small and large colonies of Rivularia incubated at full sunlight and in darkness at two different times on various dates in 1987. (Mean light flux $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ experienced by the alga is the mean of eight readings taken 15-min intervals; n = 6)

a) nitrogenase activity measured during 1000 - 1200

date	mean light flux ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	small colonies (1 - 2 mm)		large colonies (4 - 5 mm)	
		light	dark	light	dark
9 Aug.	523 ± 198	24.9 ± 11.7	-	13.7 ± 7.0	-
25 Aug.	254 ± 47	17.4 ± 3.0	9.1 ± 1.3	15.3 ± 4.4	5.4 ± 2.6
3 Sep.	493 ± 296	12.7 ± 3.0	2.1 ± 0.4	8.3 ± 2.1	2.0 ± 0.7
26 Sep.	583 ± 364	12.9 ± 2.9	3.5 ± 1.0	9.8 ± 2.7	3.2 ± 0.9

b) nitrogenase activity measured during 1400 - 1600

9 Aug.	679 ± 450	32.0 ± 6.0	4.2 ± 3.0	13.4 ± 6.2	2.3 ± 0.8
25 Aug.	227 ± 38	17.5 ± 1.1	5.3 ± 0.8	16.0 ± 5.4	5.5 ± 3.2
3 Sep.	120 ± 78	15.7 ± 2.0	2.6 ± 0.2	10.9 ± 1.5	0.9 ± 0.2
26 Sep.	333 ± 237	11.6 ± 1.8	3.0 ± 0.9	7.1 ± 1.9	3.0 ± 0.7

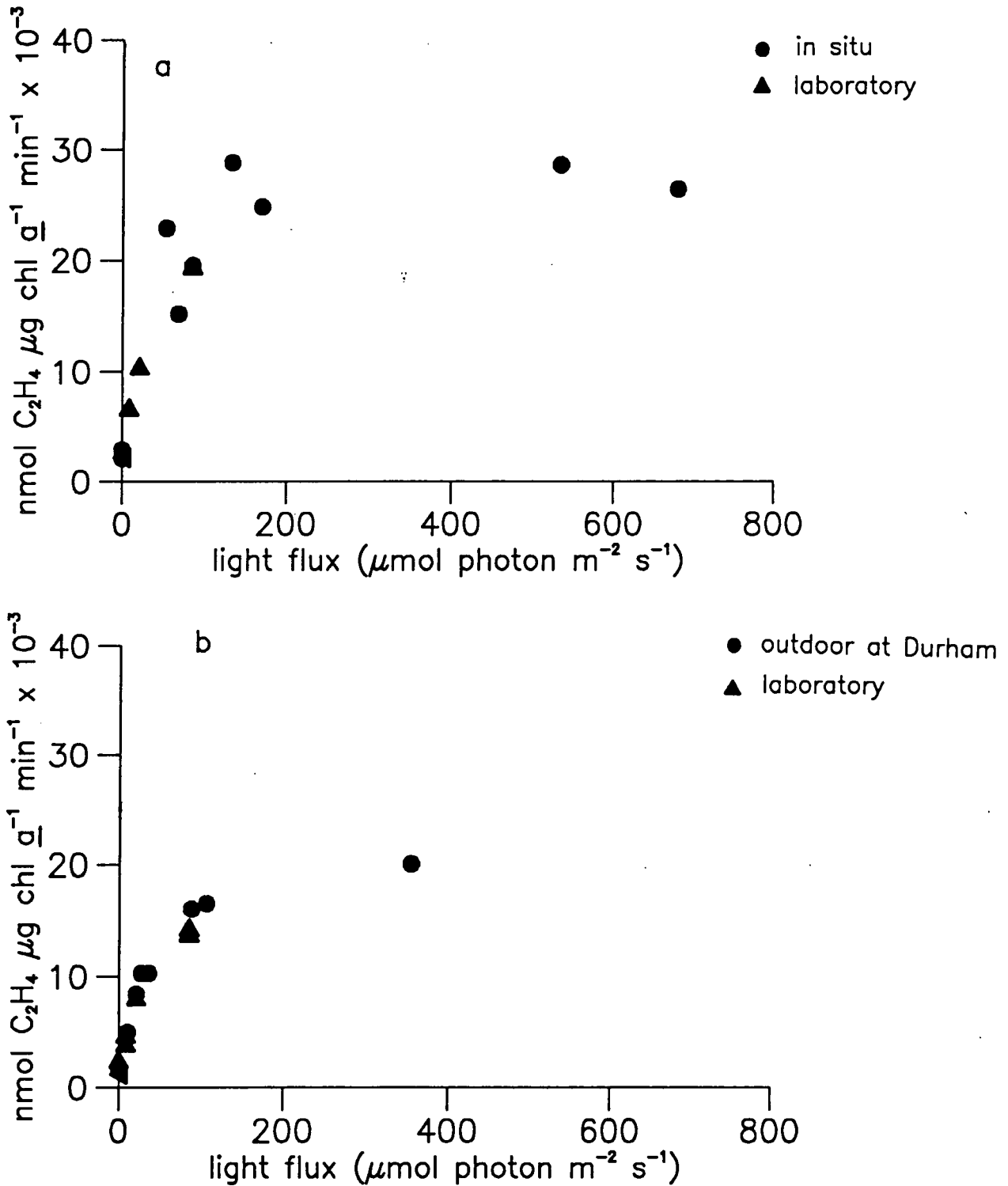
Light flux in the field often varies markedly even for a short period of time (Table 5.1). An experiment was therefore planned to compare the influence of mean light flux on nitrogenase activity of colonies in the field with nitrogenase activity in the laboratory under a constant light flux. Small colonies of Rivularia were chosen for subsequent comparison with the laboratory culture results of Rivularia D403 (Section 5.2) which formed small colonies. In the laboratory it is extremely difficult to achieve the high values of light flux to which the algae are exposed in the field. Therefore nitrogenase activity of the alga in the laboratory was carried out at ^{the} lower end of the light flux available in the field. Different values of light flux were obtained by wrapping with neutral density filters and dark conditions by wrapping with aluminium foil. Nitrogenase activity of the colonies was carried out in the field between 1000 - 1200 at a mean light flux of $680 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ at 15°C . The colonies were brought to the laboratory which took about 40 min. During this transit period the colonies were kept at low light flux. The colonies were incubated for 2 h in the laboratory at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ at 15°C before conducting nitrogenase activity study. Nitrogenase activity was measured at different values of light flux in the laboratory. Nitrogenase activity of the alga declined both in the field and laboratory when incubated at a light flux below $106 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (Fig. 5.1a).

In the previous experiment, the time period between the field and laboratory experiments might have influenced nitrogenase activity. Therefore an experiment was planned to compare nitrogenase activity of the colonies simultaneously in the outdoor at Durham and in the laboratory. Small colonies were collected from the field as ⁱⁿ the previous experiment. Nitrogenase activity was measured simultaneously outdoors at the Science site of Durham University and in the laboratory. In this case the alga

Fig. 5.1 Influence of mean light flux on nitrogenase activity of field *Rivularia* colonies.

a) nitrogenase activity in situ and in Durham;

b) nitrogenase activity in the outdoor at Durham and in laboratory.



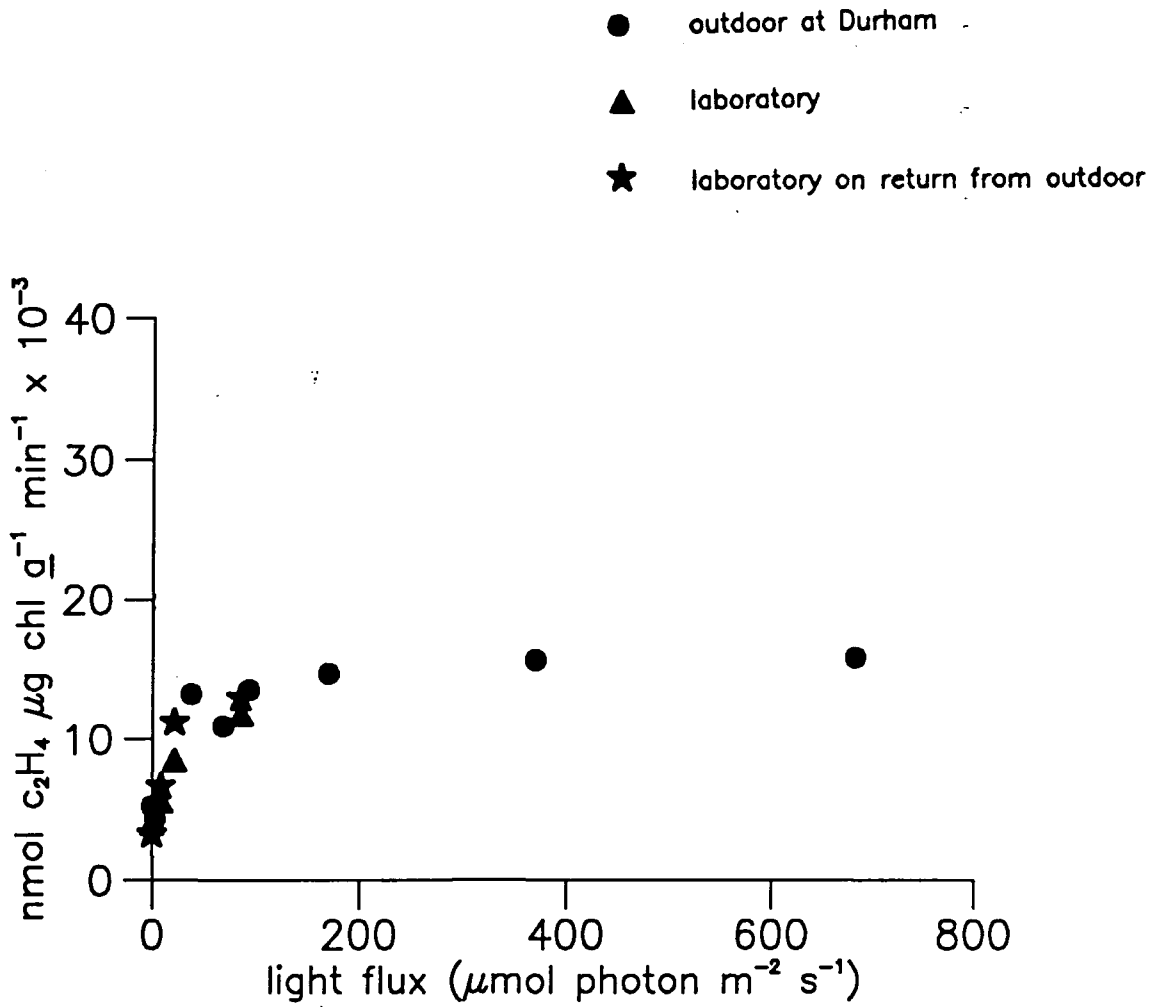
had lower values of nitrogenase activity than the previous experiment. Nitrogenase activity decreased when incubated below $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (Fig. 5.1b).

5.2 Nitrogenase activity of Rivularia D403

5.21 Influence of light flux on nitrogenase activity

In Section 5.112 the influence of light flux on nitrogenase activity of field Rivularia colonies has been tested both in the field and laboratory. It was planned to quantify the changes in nitrogenase activity with changing light flux of a bacterised Rivularia D403 culture (Section 2.4) in order to compare the results with those of field alga (Section 5.112). The alga was grown at $48 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ at 25°C for 30 d. The culture was then adapted to 15°C at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ for 3 d to simulate the temperature of the field. Nitrogenase activity was measured in the laboratory at different values of light flux. The culture was then taken outdoors; nitrogenase activity was carried out at different values of light flux, after which the culture brought back to the laboratory and nitrogenase activity at different values of light flux was measured. Nitrogenase activity of the alga reduced when incubated below $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (Fig. 5.2).

Fig. 5.2 Influence of mean light flux on nitrogenase activity of Rivularia D403 in the outdoor at Durham and in the laboratory.



6 PHOSPHATASE ACTIVITY OF CALOTHRIX D764

6.1 Introduction

Nitrogen fixation of Calothrix D764 is affected markedly by the availability of phosphorus (Section 4.62). As different species of blue-green algae have been reported to utilize organic phosphates for their growth and nitrogen fixation (Section 1.54), a limited study on phosphatase activity of Calothrix D764 was undertaken. The specific objectives were:

- 1) to determine the ability of the alga to utilize a range of organic phosphate substrates;
- 2) to quantify the influence of environmental variables on phosphatase activity.

6.2 Growth on different P substrates

A preliminary experiment was planned to determine the ability of Calothrix D764 to utilize different organic phosphates for growth. Eight different organic phosphate substrates were tested. One control (- P) and another with inorganic P were also included for comparison. All substrates were sterilized by filtration and added aseptically to Chu 10D-N-P medium. The alga was grown in standard growth conditions (Section 2.37) and yield was determined after 16 d. The alga showed moderate growth (compared with orthophosphate) with glucose-6-phosphate, bis- ρ NPP, ρ NPP, MNP and β -glycerophosphate, but could not grow with ATP or phytic acid or DNA (Table 6.1).

Table 6.1 Influence of different P sources (1.78 mg l^{-1} P) on yield of Calothrix D764 after 16 d of growth in batch culture. (Inoculum 10 mg l^{-1} dry wt; $85 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$; $n = 4$)

P source	yield (mg d. wt l^{-1})	
	\bar{x}	SD
- P	78.4	10.38
potassium dihydrogen orthophosphate	493.4	33.68
<i>p</i> -nitrophenyl phosphate	345.0	31.65
2-methoxy-4-(2'-nitrovinyl)-phenyl phosphate	197.2	30.86
glucose-6-phosphate	445.4	19.27
sodium- β -glycerophosphate	293.2	9.13
adenosine triphosphate	60.8	9.05
bis-(<i>p</i> -nitrophenyl) phosphate	300.0	10.05
deoxyribonucleic acid	88.2	4.03
phytic acid	95.1	5.83

6.3 Growth and phosphatase activity

In the previous experiment the alga was able to utilise both phosphomono- and phosphodiester substrates as a source of phosphorus. A further experiment was carried out to monitor the changes in both cell-bound and extracellular phosphatase activities of the alga in batch culture. In addition to phosphatase activities cellular P content of the alga was also determined to relate the result with switch-on of phosphatase activity. The alga was subcultured twice at 3-d intervals to ensure that there was no detectable phosphatase activity. Changes in dry weight, cellular P content and phosphatase activities of the alga in batch culture are presented in Figs 6.1 - 6.2. Detectable cell-bound PMEase activity was observed only in one replicate out of four replicates by day 4 and the cellular P content of the alga was 0.98%. The alga showed both cell-bound PMEase and PDEase activity. There was only detectable extracellular PMEase activity, but no extracellular PDEase activity during growth in batch culture. Maximum rate (expressed as unit d. wt) of cell-bound PMEase activity was observed on day 12 after which it decreased steadily. The rate of cell-bound PDEase increased gradually up to day 7 and maintained almost the same level to day 28; after that the activity declined.

6.4 Influence of temperature

An experiment was carried out to test the influence of temperature on cell-bound and extracellular phosphatase activities of the alga. PMEase and PDEase were assayed using *p*NPP and bis-*p*NPP, respectively. In order to test the effect of temperature on phosphatase activity, aliquots of algal homogenate or medium were preincubated for 30 min at respective temperature. Deviations in pH from 10.3 due to temperature were compensated for by the addition of 1.0 M NaOH or HCl. Phosphatase

Fig. 6.1 Changes in dry weight and cellular P content of *Calothrix* D764 in batch culture. (Inoculum 3-d old; $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$; 32°C)

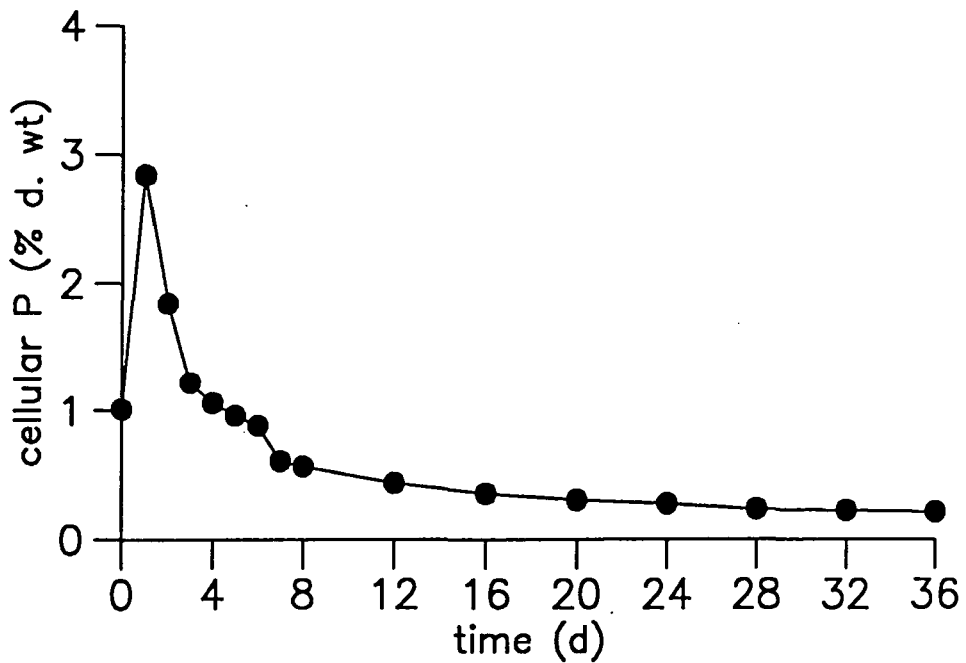
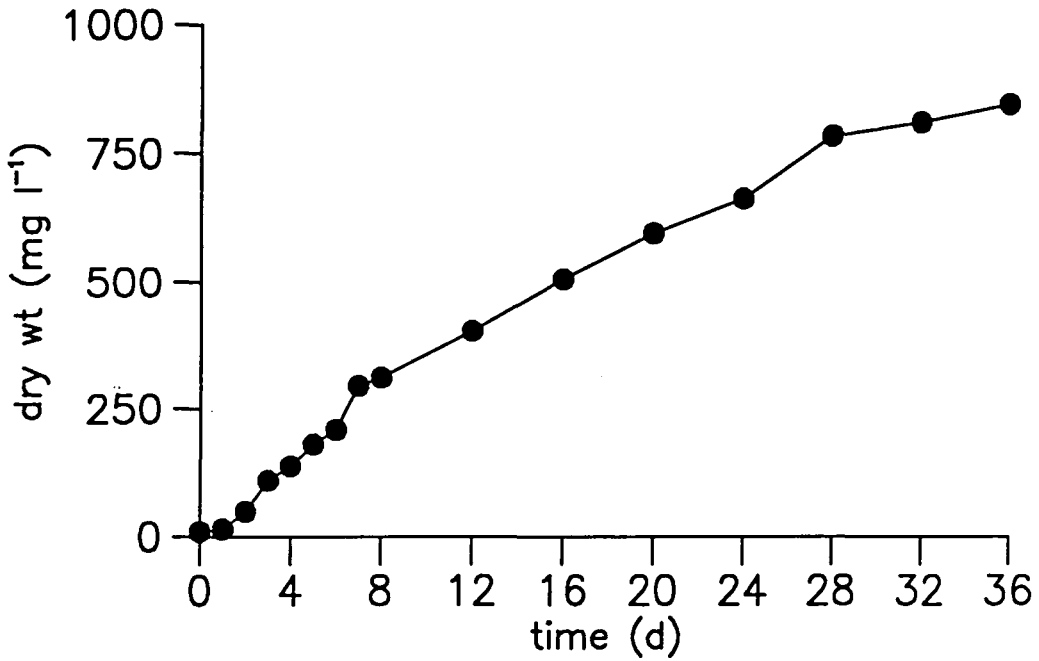
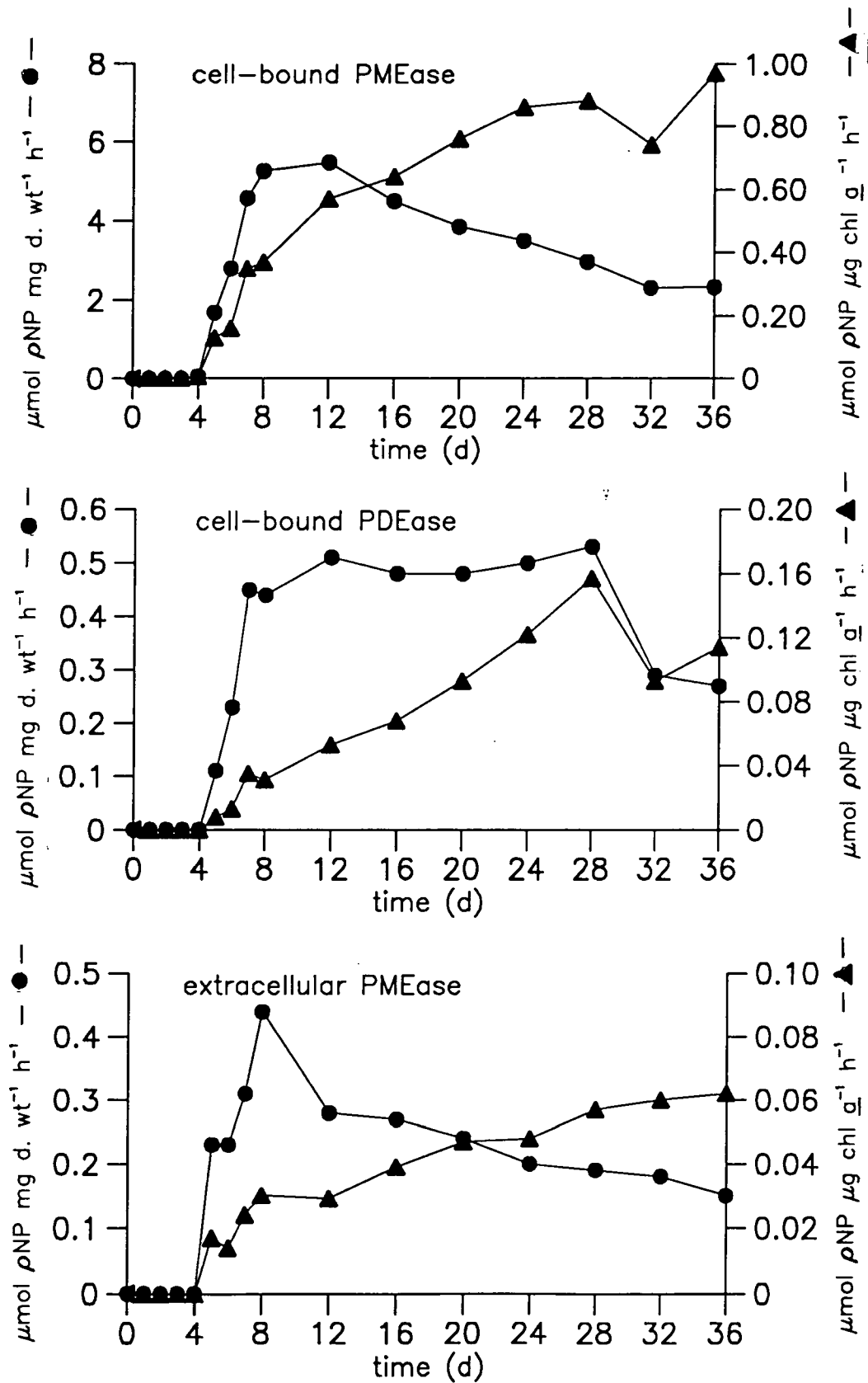


Fig. 6.2 Changes of phosphatase activities of *Calothrix* D764 in batch culture. (Inoculum 3-d old; $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$; 32°C)



activity was measured at 5°C intervals. Temperatures above 80°C could not be tested directly because of the spontaneous hydrolysis of ρ NPP.

Activity was terminated after 20 min of incubation. Temperature optimum for both cell-bound and extracellular PMEase was 35°C, while for cell-bound PDEase it was 40°C (Fig. 6.3).

6.5 Influence of pH

Experiment was also carried out to determine the influence of pH on phosphatase activities of the alga because the pH of water in deepwater rice-fields of Bangladesh and the adjacent fallow areas showed marked diel changes, which might affect the phosphatase activity of the alga. The influence of pH was tested on cell-bound PMEase and PDEase and on extracellular PMEase (there was no detectable extracellular PDEase of this alga in batch culture: Section 6.3). Both cell-bound and extracellular PMEase were assayed using ρ NPP and cell-bound PDEase by bis- ρ NPP (Section 2.82). The assay was carried out at 32°C. The influence of pH between 3.0 - 11.0 was tested using a range of buffers (Section 2.82). Two different buffer sets were tested at each pH value and both are plotted in Fig. 6.4. The optimum pH of both cell-bound and extracellular PMEase was 9.0, while for cell-bound PDEase it was 10.0.

6.6 Influence of ions

The elemental composition of water in deepwater rice-fields of Bangladesh depends primarily on the river water(s) flooding a particular area. The concentrations of Na, Mg and Ca vary widely in different areas of deepwater rice-fields in Bangladesh (Section 1.36). In addition to the effect of Na, Mg and Ca, other ions were also tested which have been reported to affect algal phosphatase activity. Cell-bound PMEase and PDEase were assayed using ρ NPP and bis- ρ NPP, respectively at pH 10.3

Fig. 6.3 Influence of temperature on phosphatase activities of *Calothrix* D764. (16-d old culture was used for experiment; $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$; 32°C)

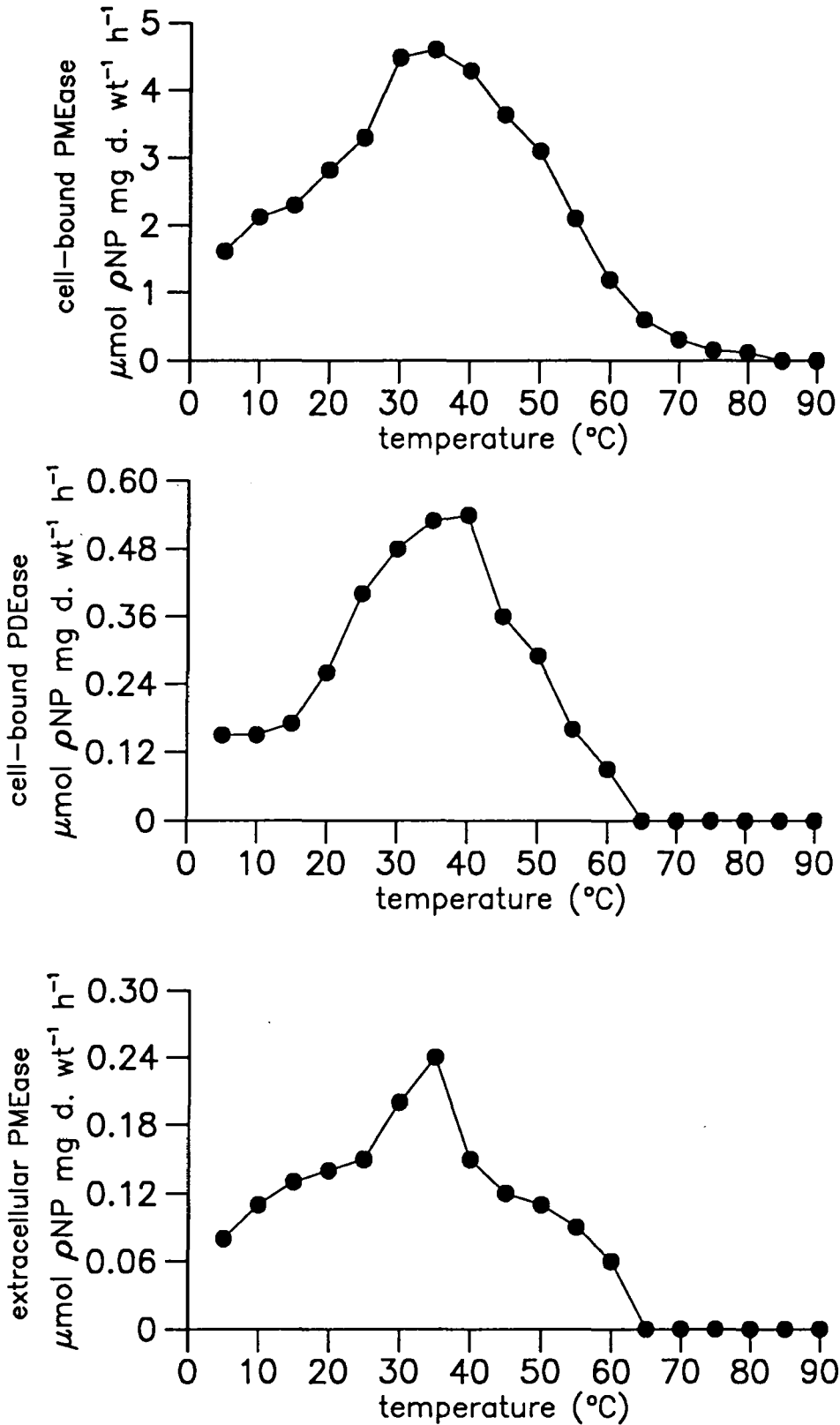
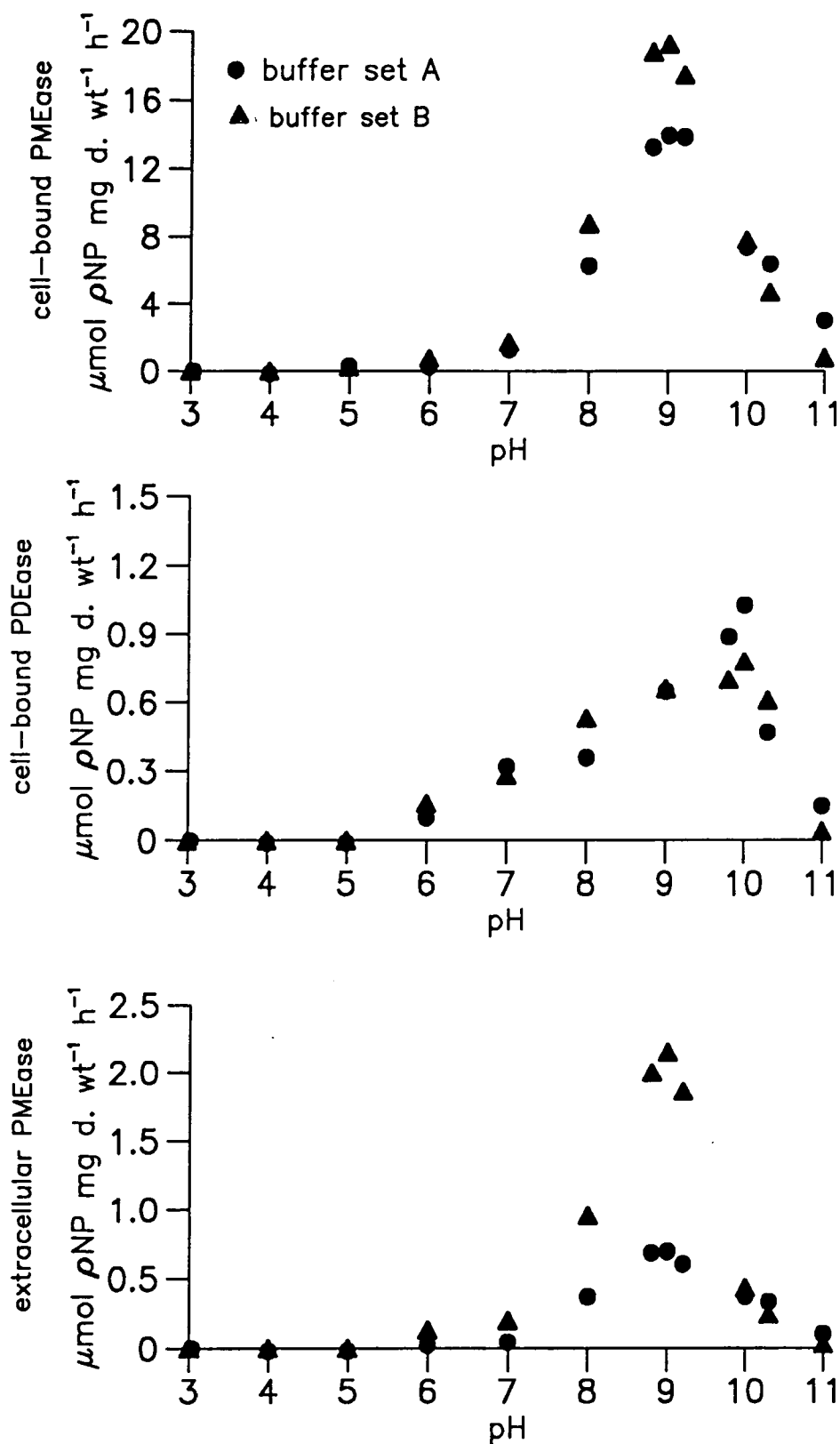
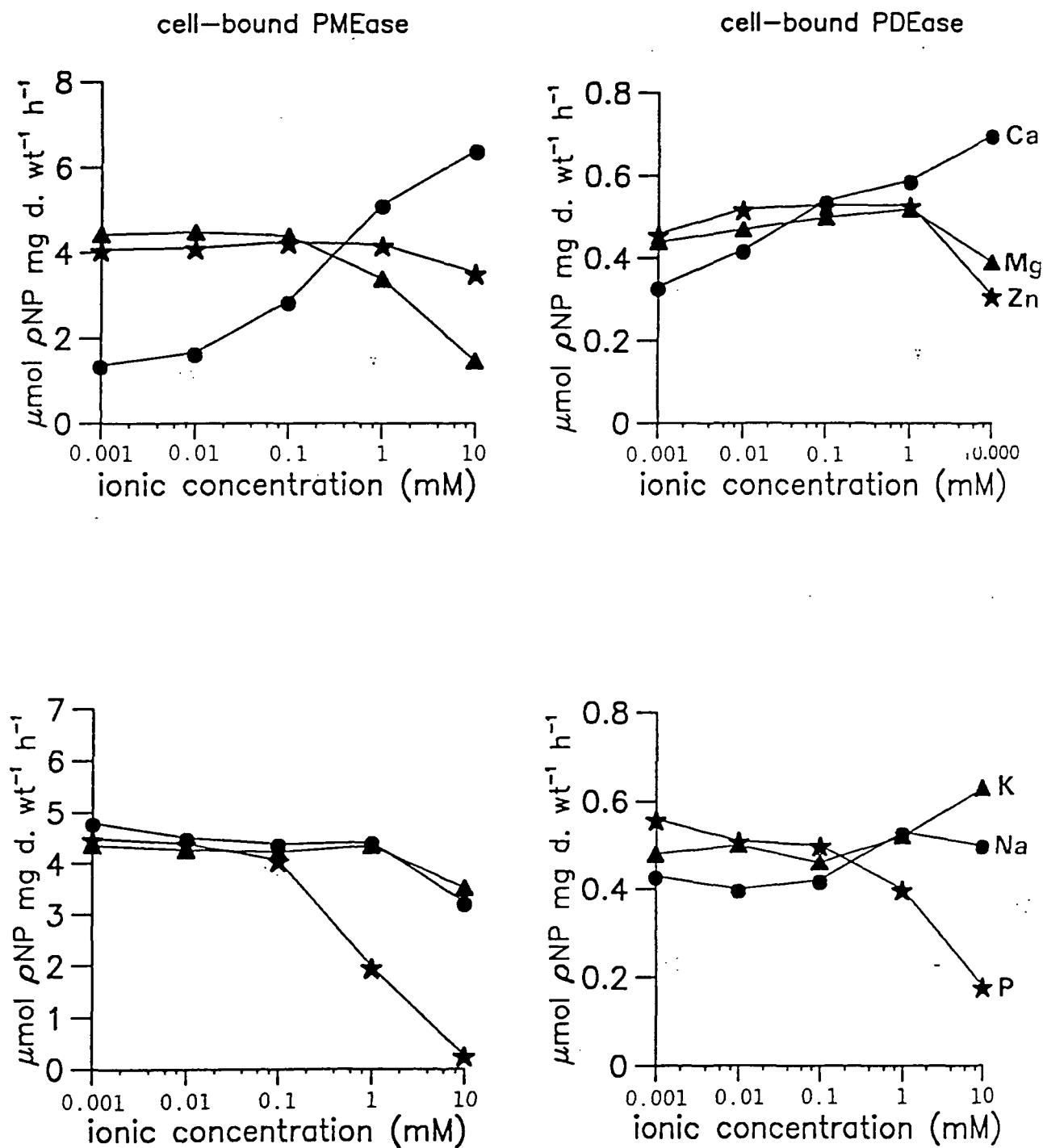


Fig. 6.4 Influence of pH (3 - 11) on phosphatase activities of *Calothrix* D764. (16-d old culture was used for experiment; $85 \mu\text{mol photon m}^{-2} \text{s}^{-1}$; 32°C)



(glycine-NaOH, 50 mM) and 32°C. The range of concentrations was 0.001 mM, 0.01 mM, 0.1 mM, 1 mM and 10 mM. The ions tested were Mg^{++} , Ca^{++} , Zn^{++} , Na^+ , K^+ and phosphate. Ions were added as the relevant chloride or sulphate, these anions having no reported effect on phosphatase activity. NaOH used for buffering glycine was replaced by KOH in the case of Na assay. Phosphate was added as the sodium salt. The response of cell-bound phosphatases is depicted in Fig. 6.5. Calcium had the greatest stimulatory effect. All other cations above 1.0 mM were inhibitory. Phosphate up to 0.1 mM had no significant inhibitory effect. 10 mM phosphate decreased the activity of PMEase and PDEase by 6 and 32%, respectively.

Fig. 6.5 Influence of ions on cell-bound phosphatases of *Calothrix* D764.
 (16-d old culture was used for experiment; 85 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$; 32°C)



7 DISCUSSION

7.1 Introduction

This study on nitrogenase activity has quantified the influence of environmental variables likely to be important in deepwater rice-fields on an axenic alga in the laboratory in batch culture. The isolate used was Calothrix D764, a representative of the predominant blue-green algal family, the Rivulariaceae, in deepwater rice-fields in Bangladesh. There were marked changes in nitrogenase activity with changes in environmental variables. The results of the key experiment on Calothrix D764 have been presented together in Fig. 7.1 for ease of comparison, although during the discussion the original reference from Chapter 4 has been mentioned.

7.2 Morphology of Calothrix D764

The morphology of different members of ^{the} Rivulariaceae has been reported to vary widely depending on the environmental conditions (Whitton, 1988), so it was planned first to determine this for the strain chosen.

Calothrix D764 is relatively narrow compared to other members of the Rivulariaceae (Livingstone and Whitton, 1983; Aziz and Whitton, 1987). It did not form hairs under P- or Fe- limitation, although many other species of Calothrix have been reported to form hairs under such conditions (Sinclair and Whitton, 1977b; Wood et al., 1986).

Combined nitrogen led to the complete loss of heterocysts and development of undifferentiated trichomes (Section 3.112). The loss of heterocysts in the presence of combined nitrogen is a common feature in heterocystous blue-green algae (Sinclair and Whitton, 1977a; Rai et al., 1978; Helber et al., 1988). Transfer of undifferentiated trichomes to - N medium led to initial yellowish colour, which turned blue-green after heterocyst differentiation. During the transition period the alga had to

pass through nitrogen starvation, presumably leading to the partial breakdown of phycobiliprotein and chl a, which in turn led to yellowish colour of the alga.

Increasing P-deficiency in Calothrix D764 led to rapid cessation of hormogonia production (Section 3.113). A similar response has been shown previously for Calothrix D550 (Livingstone and Whitton, 1983) and Calothrix D184 (Wood, 1984). The cessation of hormogonia production might be due to the decreased cellular P status of the alga. Another marked influence of P-deficiency was the increase in cyanophycin content of trichomes,^a similar increase was also reported by Sinclair (1977). Simon (1973) demonstrated that cyanophycin granules acted as a nitrogen reserve and accumulated in Anabaena cylindrica under conditions which prevented protein synthesis. On the basis of those observations it is probable that a factor leading to the accumulation of cyanophycin is the disturbance of protein synthesis as a consequence of the nutrient deficient condition.

The most pronounced influence of iron limitation on Calothrix D764 was the formation of a new heterocyst apical to the original basal one (Section 3.114), similar to that reported by Sinclair (1977); Wood (1984); Douglas et al. (1986) for various Calothrix strains. Increasing Fe-deficiency led to the repeated replacement of the heterocyst basal to it. Douglas et al. (1986) suggested that the successive formation of heterocysts may be associated with a switch to molecular components with reduced demand of Fe. The previous heterocyst presumably became non-functional, possibly by loss of the ability to synthesize nitrogenase.

7.3 Physiology, growth and cellular composition of Calothrix D764

Changes in cellular N, chl a, phycobiliprotein and nitrogenase activity of Calothrix D764 have been studied at different values of light flux in batch culture (Figs 3.3 - 3.6; 4.2; 4.4). (The changes in nitrogenase activity at different values of light flux will be discussed in Section 7.4). The most pronounced effect of light flux on the alga was the increase in chl a and phycobiliprotein contents at lower values of light flux. At $10 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ the alga had almost twice chl a and phycobiliprotein contents than at $85 \mu\text{mol photon}$ (Figs 3.3; 3.6). The increase in chl a and phycobiliprotein contents in blue-green algae at lower values of light flux than higher light flux was reported in previous research (Jones and Myers, 1965; Wyman and Fay, 1986).

The allophycocyanin content at different values of light flux was about 40% of the total phycobiliprotein (Figs 3.3 -3.6). Although allophycocyanin content of heterocystous blue-green algae at different values of light flux has not been reported in the literature, a similar high allophycocyanin content was reported in Synechococcus sp. 6312 when grown at a light flux of 2000 - 3000 lux at 25°C (Lemmasson et al., 1973). When Plectonema boryanum and P. calotrichoides were grown at 25°C at a light flux of 500 lux, the total phycocyanin content was 22.1 and 21.0% respectively (Khoja and Whitton, 1975).

For any particular light flux, higher chl a and phycobiliprotein contents per unit dry weight were correlated with cellular N (Figs 3.3 - 3.6). As N is a component of chl a and phycobiliprotein, changes in chl a and phycobiliprotein will themselves influence cellular N.

At all values of light flux chl a, phycobiliprotein and cellular N contents declined initially during growth in batch culture then increased gradually, reaching a maximum between 6 - 8 d of growth (Figs 3.3 - 3.6). The initial decrease in pigment content and cellular N might occur when the

alga was homogenised and subcultured to fresh medium, fragments of trichomes without heterocysts passed through nitrogen starvation before they differentiated heterocysts and fixed nitrogen.

The growth of the alga was enhanced slightly when presented with sugars (fructose and sucrose) in the growth medium compared to the control at $12 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, but when combined nitrogen and sugars were supplied the yield of the alga increased significantly (Table 3.8). This indicated that the photochemically generated energy and reductant at low light flux were used to a considerable extent for nitrogen fixation. A similar experiment in the dark in the presence of combined nitrogen and fructose showed that the viability of the alga increased compared with the control (Section 3.25).

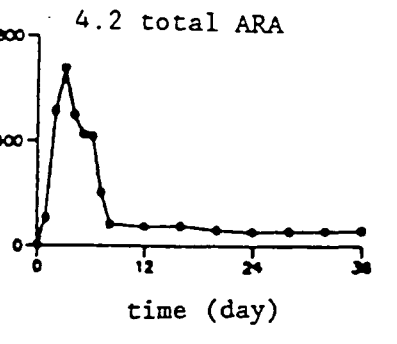
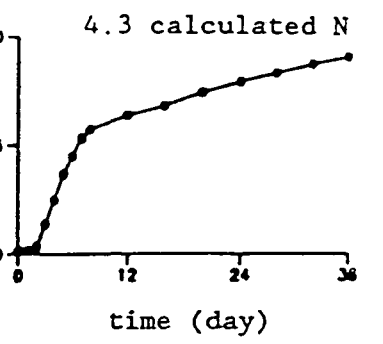
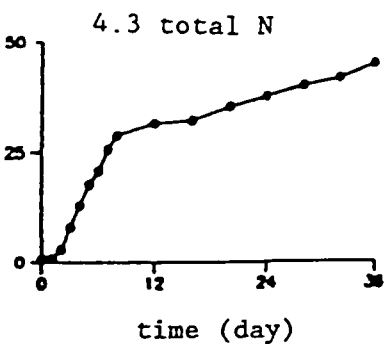
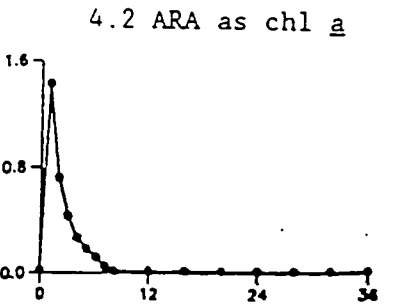
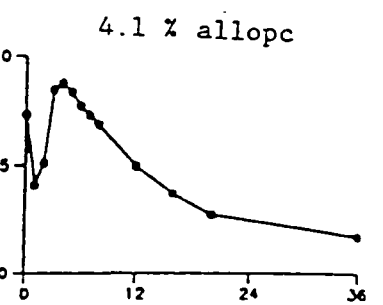
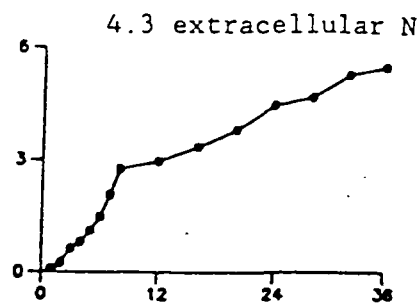
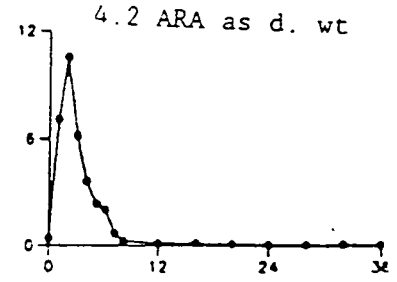
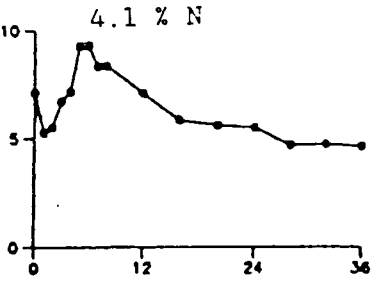
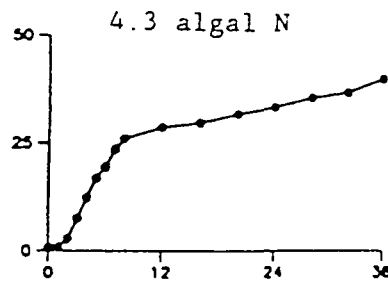
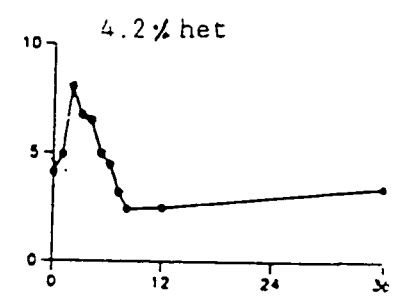
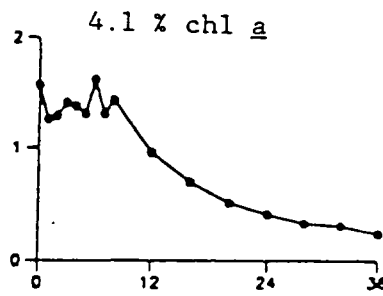
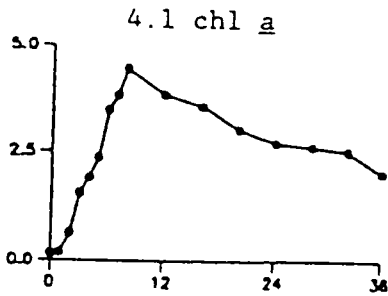
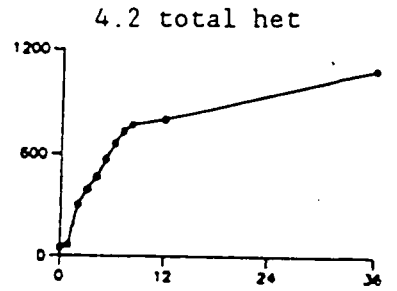
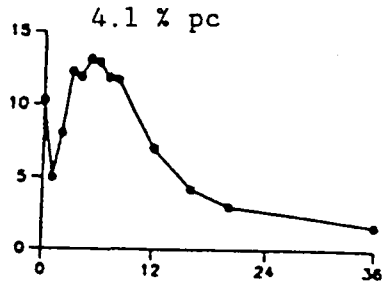
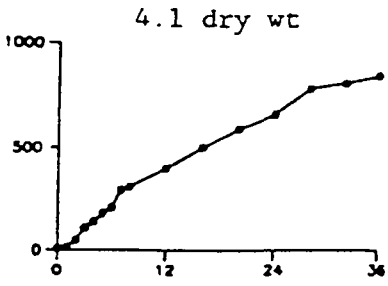
7.4 Nitrogenase activity of Calothrix D764

In order to relate nitrogenase activity with nitrogen fixation of the alga, experiments were planned to find out the molar ratio of $\text{N}_2 : \text{C}_2\text{H}_2$ reduced in batch culture at 10 and $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. The method used to calculate the molar ratio was to compare the total amount of N fixed with total C_2H_2 reduced by the alga. When the total (cellular and extracellular) N fixed was compared with C_2H_2 reduced during 44 d of growth in batch culture at $10 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ a molar ratio of 1 : 5.2 was obtained. Nitrogen fixation estimated using this ratio was underestimated during the early stages and overestimated during the later stages of growth (Fig. 4.4). On the other hand a molar ratio of 1 : 4.1 was obtained during 36 d of growth at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ (Fig. 4.3). Estimated nitrogen fixation using the ratio 1 : 4.1 was closer to the actual N fixed and there was no clear difference between growth stages as observed in the previous experiment. The regression equations between actual N and

Fig. 7.1 Changes in growth parameters and ARA of Calothrix D764 in batch culture.

The number preceding the text in the graph refers to the Fig. No. in Chapter 4. The three pairs of columns correspond to the three columns in the figure.

parameters	expressed as	parameters	expressed as	parameters	expressed as
dry wt	mg l ⁻¹	% pc	% dry wt	total het	l ⁻¹ x 10 ⁶
chl <u>a</u>	mg l ⁻¹	% chl <u>a</u>	% dry wt	% het	% total cell
algal N	mg l ⁻¹	% N	% dry wt	ARA dry wt	nmol C ₂ H ₄ mg d. wt -1 min ⁻¹
extracel- lular N	mg l ⁻¹	% allopc	% dry wt	ARA chl <u>a</u>	nmol C ₂ H ₄ μg chl <u>a</u> ⁻¹ min ⁻¹
total N	mg l ⁻¹	calcula- lated N	mg l ⁻¹	total ARA	nmol C ₂ H ₄ l ⁻¹ min ⁻¹



estimated N at 10 and 85 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ were: $y = -0.93 + 1.06x$, $r = 0.997$ and $y = -0.05 + 0.99x$, $r = 0.998$, respectively. The lower ratio at 85 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ compared with 10 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ showed that more efficient nitrogen fixation occurred at optimum growth conditions.

Most results for the molar ratio of $\text{N}_2 : \text{C}_2\text{H}_2$ reduced in the literature are from a particular growth stage in situ (Peterson and Burris, 1976; Graham et al., 1980) or from cultured algae (Rother et al., 1988). The ratio of the experiments discussed above may be compared with the mean values of 1 : 5.66 and 1 : 4.66 at a light flux of 160 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ during the exponential and later growth stages of six other axenic strains in the laboratory by ^{15}N and C_2H_2 reduction technique. The mean ratio of 1 : 4.4 reported by Peterson and Burris (1976) in situ in lakes, which has been used widely by other researchers is also closer to the present ratio obtained in the present experiments. The shift from the theoretical ratio for short-period incubation is presumably due to the evolution of H_2 by nitrogenase (Peterson and Burris, 1976; Bothe et al., 1978; Graham et al., 1980).

In order to relate nitrogenase activity with growth parameters, an experiment was planned at 85 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ in batch culture. Nitrogenase activity and growth parameters were measured at different growth stages. Maximum nitrogenase activity (per unit d. wt) occurred after two days growth (Fig. 4.2). During this period, juvenile trichomes were abundant, cyanophycin granules were lacking, phycocyanin and allophycocyanin declined to about 50%, chl a and cellular N to 80%. It is probable that high heterocyst frequency and high rate of ARA were a response to this decrease in N content. At this stage the alga was yellowish green. A decrease in chl a and phycobiliprotein has been reported to be the features of nitrogen deficiency in other blue-green algae (Allen and Smith, 1969; Fay, 1983; De Laura et al., 1987; Aziz and

Whitton, 1988). Besides absorption of light energy, phycocyanin and phycoerythrin have been shown to be the nitrogen reserve in some species of blue-green algae (Allen and Smith, 1969; Boussiba and Richmond; 1980; Wymann *et al.*, 1985; Carr, 1989). In this alga allophycocyanin also decreased concomitantly with phycocyanin, indicating that it might also serve as a storage of nitrogen.

Rapid division of the cells occurred in trichomes only after heterocyst differentiation and when normal level of pigmentation was restored. This suggests that nitrogen fixation occurred at a high rate at this stage to meet the nitrogen requirement for cell division (Jewell and Kulasooriya, 1970). As the high fixation rate continued, the cellular N of the alga gradually increased, reaching a maximum of 9.32% on day 6 (Fig. 4.1). However, total ARA started to decline after day 3 (Fig. 4.2) and abundant developing and mature trichomes occurred. The decrease in nitrogenase activity in batch culture may be related to a number of factors such as limitation of CO₂, nutrient elements or developmental stage of the alga. In this experiment FRP was exhausted from the medium by day 6 indicating that P-limitation was not involved in earlier days. Chl *a* and phycobiliprotein contents started to increase after day 1 and reached a maximum of 1.6 and 21.5%, respectively, on day 6. After day 6 cellular N, chl *a* and phycobiliprotein contents started to decrease gradually (Fig. 4.1) Hormogonia production ceased at day 8. Probably N₂-fixation at this stage was limited by nutrient deficiency. The increase in weight may be due largely to accumulation of carbohydrate. It appears from the above discussion that the juvenile trichome is the most active nitrogen fixer during the life of the alga.

Some comparison can be made between values of nitrogenase activity found in the present study and values reported in the literature for other blue-green algae. In vivo nitrogenase activity (ARA) in batch cultures, is

typically in the range 1 - 10 nmol C₂H₄ mg protein⁻¹ min⁻¹ (Stewart, 1973). Assuming that a blue-green algal cell during exponential phase consists 50% of dry weight as protein (Collyer and Fogg, 1955) and 1% as chl *a* (Fay, 1969), then the above value corresponds to 0.05 - 0.5 nmol C₂H₄ μg chl *a*⁻¹ min⁻¹ (= 0.5 - 5.0 nmol C₂H₄ mg d. wt⁻¹ min⁻¹). Accordingly the maximum rate obtained in the present study is higher. Several strains of blue-green algae isolated from rice-fields showed high rates of nitrogen fixation under optimal growth conditions, ranging from 0.45 to 1.0 nmol C₂H₄ mg chl *a*⁻¹ min⁻¹ (= 4.5 - 10.0 nmol C₂H₄ mg d. wt⁻¹ min⁻¹: Antarikanonda and Lorenzen 1982; Chen, 1983a). However in the present study *Calothrix* D764 showed higher ARA (10.0 - 11.0 nmol C₂H₄ mg d. wt⁻¹ min⁻¹) than reported for other members of Rivulariaceae in the laboratory e.g. two-day old unialgal culture of *Gloeotrichia* (0.81 nmol C₂H₄ mg d. wt⁻¹ min⁻¹ at 4000 lux: Finke and Seeley, 1978) or the axenic culture of *G. echinulata* (0.61 nmol C₂H₄ mg d. wt⁻¹ min⁻¹: Chang and Blauw, 1980), but comparable with the nitrogenase activity of *G. pisum* (13.0 nmol C₂H₄ mg d. wt⁻¹ min⁻¹ at 105 μmol photon m⁻² s⁻¹: Aziz and Whitton, 1988).

In order to relate the changes in nitrogenase activity with changes in light flux, experiments were planned by upshift and downshift in light flux. The alga showed a rapid response to the changes in the light flux. Transfer to the dark for 1.0 h reduced nitrogenase activity by 84% compared with parallel incubation in the light (Table 4.1). This percentage reduction in nitrogenase activity is consistent with the activity of *G. pisum* (Aziz and Whitton, 1988) though Finke and Seeley (1978) observed 50 to 66% reduction in activity within 1 - 2 h dark treatment. The large fall of nitrogenase activity in the dark may be because the alga did not accumulate a large amount of reserve carbohydrate during the light period. Lex and Stewart (1973) demonstrated that photosynthesis promotes N₂-fixation by producing carbon compounds, which act as reductant for

nitrogenase. Quick responses and decrease of nitrogenase activity due to a down-shift in light flux suggest that N_2 -fixation in the light is dependent upon reductant produced during recent photosynthesis (Peterson et al., 1977).

The other feature of nitrogenase activity studied was behaviour in the dark. Considerable nitrogenase activity was observed during a dark period of 12 h, although the organism could not grow in the dark under heterotrophic conditions (Section 3.25). Assuming a molar ratio for N_2 : C_2H_2 reduced in ^{the}dark of 1 : 5.2 (using the value obtained for growth at $10 \mu\text{mol photon m}^{-2} \text{s}^{-1}$), the amount of nitrogen fixed (as NH_3) during 12 h in the dark was 0.17 mg. Net loss of carbohydrate was therefore approximately 5.27 mg. If each molecule of glucose produces 6 ATP or 2 NADPH and fixation of one molecule of N_2 requires 15 ATP and 4 NADPH then by catabolizing 5.27 mg carbohydrate the alga can fix 0.22 mg N. Most of the energy generated by mobilizing polyglucoside was diverted to nitrogen fixation. Both the rate and duration of enzyme activity in the dark are affected by the rate of carbon assimilation during the light period (Lex and Stewart 1973; Fay, 1976; Ernst et al., 1984). Nitrogenase activity in the dark has also been attributed to the utilization of suitable organic substances (Fay 1976; Haury and Spiller, 1981). Dark nitrogenase activity by Calothrix D764 in a medium without any organic substrate indicated that this was not involved, but rather a pool of carbon compounds.

One of the most interesting properties of Calothrix D764 was higher nitrogenase activity when the dark incubated culture was reilluminated, than the activity found under continuous illumination (Figs. 4.1; 4.7). Aziz and Whitton (1988) found a similar type of response with an axenic strain of G. pisum in the laboratory. This parallels the high rates of phosphate accumulation found when phosphate deficient blue-green algae are presented with phosphate (Healey, 1982). Meyer et al. (1978) provided

evidence of extremely active biosynthesis of nitrogenase upon re-illumination of Rhodospseudomonas capsulata, a purple non-sulphur bacterium (photosynthetic and facultative anaerobe). In a medium free of combined nitrogen and carbon, they observed that nitrogenase synthesis proceeded at a rate such that within 3 - 4 h, the activity was equal to that found in light grown cultures. They also observed consistently higher activity under intermittent illumination and the retention of the ability to synthesize nitrogenase over a longer period of time under discontinuous illumination. In a young culture of Calothrix D764 it was observed that following re-illumination for 48 h, maximum nitrogenase activity occurred at 6 h, after which activity declined to the level obtained under continuous illumination (Fig. 4.8). In an 8-d old culture maximum nitrogenase activity occurred after 4 h of re-illumination and then decreased slowly and after 48 h it reached the levels obtained under continuous light (Fig. 4.9). The increase in nitrogen fixation during a period of 60 h by the reilluminated alga was 11%, while the increase in acetylene reduced was 20%, which is due presumably to shifting in $N_2 : C_2H_2$ reduced ratio during ^{the} light-dark-light transition period. The resumption in nitrogenase activity was stopped when chloramphenicol was added (Fig. 4.10), indicating that fresh biosynthesis of the proteins was involved for such increase.

Nitrogenase activity of blue-green algal communities in Bangladesh deepwater rice-fields showed peak activity in the early afternoon, when the dissolved oxygen concentration was at maximum (Rother *et al.*, 1988). It was therefore of interest to quantify the influence of elevated oxygen concentrations on nitrogenase activity of Calothrix D764 in batch culture. Although the alga was isolated from a Bangladesh deepwater rice-field nitrogenase activity declined rapidly when incubated at higher oxygen concentration (Fig. 4.11a). Peak nitrogenase activity in the early

afternoon of the algae was observed by Rother et al. (1988), presumably due to an increase in photosynthesis with increasing PAR which might have increased nitrogenase activity more than the inhibition by higher oxygen concentration. In the experiment discussed above the culture was not exposed to a higher light flux, so photosynthesis may not have increased enough to compensate for as a result inhibition by oxygen was noticed.

When the alga was incubated with oxygen enriched air for a period of 8 h nitrogenase activity decreased steadily (Fig. 4.11b). The alga could apparently not tolerate a higher concentration of oxygen, although other blue-green algae have been reported to do so (Paerl, 1978; Mackey and Smith, 1983).

In general when heterocystous blue-green algae are presented with combined nitrogen, nitrogenase activity decreases gradually and on prolonged incubation complete inhibition occurs (Murry and Benemann, 1979; Bothe and Eisbrenner, 1978; Ramos and Guerrero, 1983). It was therefore decided to monitor the rate of changes in nitrogenase activity of the strain chosen at two levels of ammonium nitrogen in batch culture. Fifty per cent inhibition was noticed after 6 h incubation with $10.0 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$, whereas it took 12 h with $1.0 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$ (Figs 4.12; 4.13). This reduction in activity may be compared with Anabaena cylindrica, where 50% inhibition was observed in 3 h when $1 \text{ mM NH}_4\text{-N}$ was added (Ohmori and Hattori, 1974). Complete loss of heterocysts and the development of undifferentiated trichomes were observed when the alga was transferred to medium with combined nitrogen (Section 3.112), indicated that nitrogen fixation was inhibited completely. In deepwater rice-fields, the concentration of combined nitrogen is generally low (Whitton et al., 1988a), which may not be sufficient to inhibit nitrogenase activity to a great extent, except when the farmers use a heavy dose of nitrogenous fertilizer.

Iron plays an especially important role for the growth of nitrogen fixing blue-green algae. It was therefore decided to quantify the influence of iron on nitrogenase activity of Calothrix D764 in batch culture. Maximum nitrogenase activity observed in Fe-deficient cultures was about one third compared with standard medium (Figs 4.2; 4.16). Addition of Fe at day 12 led to a rapid increase in nitrogenase activity detectable within an hour and reached a maximum at day 1 which was about seven times higher than the activity before addition of Fe (Fig 4.16). This increase in activity can be compared with Calothrix D253 where addition of 0.5 mg l^{-1} Fe to a Fe-limited culture led to nineteen times higher activity than the activity before addition of Fe (Mahasneh, 1988). A similar increase in nitrogenase activity in situ was also reported by Wurstbaugh and Horne (1983). The increase in activity may be explained by Fe addition leading to the synthesis of either nitrogenase or other Fe-containing molecules required in the fixation process.

7.5 Phosphatase activity of Calothrix D764

Dissolved organic phosphate constitutes more than half of the filterable total phosphorus in water of deepwater rice-fields in Bangladesh (Whitton et al., 1988a), so the ability of Calothrix D764 to utilise different organic P substrates was investigated, together with the influence of environmental factors on phosphatase activity. The alga showed marked PMEase and PDEase activity with increasing phosphorus deficiency in batch culture (Fig. 6.2). Possibly due to the presence of both PMEase and PDEase activity the alga was able to grow on different organic P substrates (Table 6.1), but this does not explain the failure to grow on DNA or ATP. High concentration of ATP has been reported to be toxic in three other Calothrix species (Grainger, 1989). It may be that ATP was also inhibitory for this strain. In Calothrix D764 the cellular P content at which detectable cellular

phosphatase activity developed was 0.98% (Fig. 6.1). At an approximately similar cellular P concentration, phosphatase activity was also detected in Calothrix parietina D550 (Livingstone et al., 1983).

The temperature optimum of PMEase in laboratory assay was almost the same as the day-time temperature prevalent in Bangladesh deepwater rice-fields (Whitton et al., 1988a), whereas for PDEase it was slightly higher (Fig. 6.3), suggesting that the temperature in deepwater rice-fields would not limit the phosphatase activity. The pH optimum for PMEase was at 9.0 while for cell-bound PDEase it was 10.0 (Fig. 6.4), quite similar to that observed for other blue-green algae by Healey (1973) and Healey and Hendzel (1979). The pH in the water column of deepwater rice-fields showed a diel cycle with a pH maximum in early afternoon (Whitton et al., 1988d). Maximum pH of deepwater rice-fields was about 10.0, a value at which laboratory activity for PMEase is about 40% of the maximum while for PDEase 100% activity. The environment remains at this pH for only a few hours each day and is generally around pH 7.0; at this pH PMEase is about 10% maximum activity and 30% maximum for PDEase.

Absence of calcium from the assay medium reduced PMEase and PDEase activity by 30 and 70%, respectively. The absence of other cations did not have any marked effect on phosphatase activity (Fig. 6.5). Inorganic phosphate (Pi) inhibited phosphatase activity of Calothrix D764, a common feature to all inducible phosphatase systems (Healey, 1973; Ingram et al., 1973; Doonam and Jensen, 1977; Grainger et al., 1989). Calcium had the greatest stimulatory effect on both cell-bound PMEase and PDEase activities, which matches the results for other blue-green algae (Glew and Heath, 1971; Healey, 1973; Doonan and Jensen, 1977; Grainger et al., 1989). The concentration of calcium reported for Bangladesh deepwater rice-fields ranged from 0.1 - 1.0 mM (Whitton et al., 1988a), which is sufficient to increase the phosphatase activity.

7.6 Nitrogenase activity of Rivularia in the field and laboratory

Nitrogenase activity of Calothrix D764 was quantified in the laboratory (Chapter 4), but it was impossible to go back to Bangladesh with Calothrix D764 to compare laboratory results with those in ^{the} field. Therefore to compare the nitrogenase activity of a laboratory isolate with ^afield population a limited study was carried on Rivularia in the UK. The laboratory isolate Rivularia D403 and field populations of Rivularia responded quite similarly in changing light flux. A 25% decrease in light flux from $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ resulted in a drop in nitrogenase activity to 72% by Rivularia D403 (Fig. 5.2), whereas in the case of field populations of Rivularia a decrease in 25% light flux from 85 or $106 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, led to a drop in nitrogenase activity by 59 and 62% respectively (Fig. 5.1b).

As nitrogenase activity of the laboratory isolate and field populations of Rivularia responded quite similarly to changes in light flux, it is probable that Calothrix species in rice-fields may behave similarly as Calothrix D764 in the laboratory. If this is the case then light flux has tremendous potential influence on the nitrogen input in the rice-field ecosystem, where light flux varies markedly in space and time (Whitton et al., 1988d).

The ability of Calothrix D764 to fix nitrogen at a high rate is of special ecological importance. This is because nitrogen is one of the limiting nutrient elements in rice-fields (Whitton et al., 1988a). Nitrogen fixation by the alga may play an important role in maintaining the nitrogen fertility of the rice-fields.

SUMMARY

1. Calothrix D764 was isolated from a deepwater rice-field in Bangladesh. Experiments were focussed mainly on the influence of environmental variables on nitrogen fixation in batch culture. As P plays a key role in nitrogen fixation and growth of blue-green algae studies on phosphatase activity were also included.
2. Calothrix D764 is relatively narrow ($\bar{x} = 4 - 5 \mu\text{M}$) compared to other Calothrix strains reported in literature. It did not form hairs in the laboratory under any environmental conditions.
3. The mean generation time of the alga at $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ was 13 h. The exponential phase of growth was about 2 d in batch culture.
4. The alga had higher chl a and phycobiliprotein contents at low values of light flux than at high light fluxes. At all values of light flux, chl a, cellular N and biliprotein contents dropped initially, then increased gradually, reaching a maximum between 6 - 8 d and then decreased gradually.
5. By comparing the total amount of nitrogen fixed with total amount of C_2H_2 reduced, the molar ratios of $\text{N}_2 : \text{C}_2\text{H}_2$ reduced obtained were 1 : 5.2 and 1 : 4.1 during growth at 10 and $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, respectively.
6. The alga showed maximum nitrogenase activity ($10.5 \text{ nmol C}_2\text{H}_4 \text{ mg d. wt}^{-1} \text{ min}^{-1}$ or $1.4 \text{ nmol C}_2\text{H}_4 \mu\text{g chl a}^{-1} \text{ min}^{-1}$) after two days of growth under continuous light flux of $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, with a concomitant drop in chl a, phycobiliprotein and cellular N.

7. The response of nitrogenase to changes in light flux (down- or upshift) was rapid (within 30 min). At all values for light flux, nitrogenase activity following transfer levelled off after about 2.5 h. Transfer to dark for 1 h from $85 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ light led to 84% reduction in activity. Subsequent changes in the dark were slow, with detectable activity after 24 h.
8. Nitrogenase activity was much higher upon illumination after a period of time in the dark than under continuous illumination at the same value of light flux.
9. The alga showed higher nitrogenase activity in anoxic condition, the activity decreased with increasing oxygen concentration. When the alga was incubated at higher oxygen (40%) concentration nitrogenase activity decreased gradually.
10. Addition of $10 \text{ mg l}^{-1} \text{ NH}_4\text{-N}$ to nitrogen fixing cultures led to a gradual decrease in nitrogenase activity both in light and dark. Prolonged incubation with combined nitrogen led to loss of heterocysts and trichome polarity. Transfer of undifferentiated trichomes to - N medium led to yellowish green colour, heterocyst differentiation and development of trichome polarity.
11. Fe-limited cultures showed decreased nitrogenase activity and N content, pale appearance, formation of a series of new heterocysts apical to the original heterocyst and absence of the cyanophycin granules compared to Fe-sufficient cultures. Addition of Fe to Fe-deficient cultures led to rapid increase in nitrogenase activity, colour restoration, loss of

degenerated basal heterocysts, appearance of cyanophycin granules and differentiation of hormogonia.

12. The alga was capable of utilizing a wide range of phosphorus substrates and showed marked both cell-bound PMEase and PDEase activity and released PMEase to the growth medium.

13. Phosphatase activity was detected when the P content dropped to 0.98%. The rate of activity of PMEase (expressed as d. wt) increased to 12 d, when the P content dropped to 0.43%, after which it decreased steadily but PDEase activity increased up to 12 d and maintained the level up to 28 d at that time the P level in the alga dropped to 0.23%.

14. Temperature optima for PMEase and PDEase were 35 and 40°C, respectively. The pH optimum for both cellular and extracellular PMEase was 9.0, but for PDEase it was 10.0.

15. Small colonies (1 - 2 mm diameter) of field Rivularia had higher nitrogenase activity than large (4 - 5 mm diameter) ones.

16. The field populations of Rivularia and the laboratory isolate Rivularia D403 responded quite similarly in changing light flux. In case of field populations a 25% reduction in light flux from 106 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ led to a drop in nitrogenase activity by 62%, whereas in laboratory isolate Rivularia D403 a 25% decrease in light flux from 85 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ resulted in a drop in nitrogenase activity by 72%.

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