The physiological-ecology of the cyanobacterium Microcoleus

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The physiological-ecology of the cyanobacterium *Microcoleus*

by

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This thesis in entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.

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ABSTRACT

A study was carried out to determine how widespread N_2 fixation is in the cyanobacterium Microcoleus, both in the laboratory and in the field. The research was extended to compare the influence of environmental variables on both N_2 fixing and non-fixing strains of Microcoleus isolated from a range of habitats.

Since the morphology of Microcoleus strains is likely to influence their physiology, attempts were made to grow them in vitro in a form morphologically akin to that in the field i.e. with a communal sheath. Limited success was achieved with Microcoleus D634 on incubating in standard medium supplemented with 516 mM Na and 125 mM Ca (salinity of 30‰ as shown by hydrometer), where a thin, communal sheath was only found intermittently surrounding 2 - 3 trichomes.

All five strains were mixohaline, growing at salinities of 0.5 - 30 (40)‰ and surviving periods under euryhaline (30 - 40‰) and polyhaline (>40‰) conditions. The shorter the time of exposure the higher the salinity tolerated. Growth varied according to the ratio of Na^+ to Ca^{2+}, Na^+ to K^+ and Na^+ to Mg^{2+}.

Despite many changes in the nutrient status of the medium (Na^+, Ca^{2+}, Mg^{2+}, K^+) at varying PAR and temperature, under oxic and micro-oxic conditions, only one of the five strains (Microcoleus D778) fixed N_2 as shown by acetylene reduction activity (ARA) and growth in the absence of combined N.

No ARA was detected for Microcoleus mats at Gibraltar Point, over two diel cycles in August 1986; however, when ARA was measured at Church Island, Anglesey (from whence Pearson et al., (1979) isolated Microcoleus D778), over 6 diel periods between June and October, 1987, ARA was detectable at all times between 0.1 and 3.4 nmol C_2H_4 cm^{-2} h^{-1} using 4 h incubation periods. A different response in ARA was found on each occasion; generally, high activity (with > 70% in the dark) was found on sunny days and low, fairly constant ARA during cloudy, overcast days.

On incubating Microcoleus D778 in 86 mM Na (salinity of 5‰) at 20°C under 16:8 and 8:16 light (50 µmol photon m^{-2} s^{-1}):dark, the majority (72 and 92% respectively) of ARA occurred in the dark, whereas in 20:4 and 16:8 light:dark only 19 and 40% ARA occurred in the light. However, ARA over a 16:8 light:dark cycle varied with PAR, salinity and temperature. In addition, the optimum temperature for ARA varied according to pH and salinity. When DCMU was added to Microcoleus mats and to axenic cultures, ARA increased markedly; the precise value depending on PAR and preincubation conditions.

ABBREVIATIONS

g	gramme
mg	milligramme
µg	microgramme
l	litre
ml	millilitre
µl	microlitre
m	metre
cm	centimetre
mm	millimetre
µm	micrometre
nm	nanometre
M	molar
mM	millimolar
µM	micromolar
d	day
h	hour
min	minute
s	second
pO_2	partial pressure of O_2
MPa	Mega Pascal
‰	salinity
°C	degrees Celsius
PAR	photosynthetically active radiation
BST	British summer time
GMT	Grenwich mean time
log	logarithm
log_e	natural logarithm
mid. log	midpoint of logarithmic growth
µ	mean specific growth rate in days
K	mean exponential growth rate
t_d	mean doubling time in days
n	number of samples
x	mean

s.d.	standard deviation
CV	coefficient of variation
Q_1 Q_3	quartiles one and three
p 0.05	95% confidence limits
N	nitrogen
P	phosphorus
C	carbon
AAS	atomic absorption spectroscopy
ARA	acetylene reduction activity
C_2H_4	ethylene
C_2H_2	acetylene
v/v	volume to volume
Chl a	chlorophyll a
AMP	2-amino-2-methyl-1-propanol
CAPS	(3- Cyclohexylamino -1-propane sulphonic acid)
DCMU	(3-(3.4-Dichlorophenyl)-1,1-dimethyl urea)
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
EPPS	(N- 2-Hydroxyethyl -piperazine-N'- 3-propanesulphonic acid)
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid)
MES	(2- N-Mopholino ethanesulphonic acid)
PIPES	(Piperazine-N,N'-bis 2-ethanesulphonic acid)
TES	(N-tris hydroxymethyl methyl-2-aminoethane sulphonic acid. (hydroxyl,1bis(hydroxymethyl) ethylamino)ethane sulphonic acid)
C	cyanophycin
t	thylakoid
cm	cytoplasmic membrane
pl	peptidoglycan layer
om	outer membrane
S	sheath

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CONTENTS

page

page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Microcoleus is one of the most widespread genera of cyanobacteria in tropical and sub-tropical regions. It is prevalent in alkaline, calcareous marine and freshwater habitats exposed to intermittent flooding and desiccation. Despite the numerous accounts of its occurrence, comparatively little is known of its physiology or ecology, few strains having been obtained in clonal axenic culture.

1.1 Taxonomy of Microcoleus

The blue-green algae were traditionally assigned to the algae, consequently their classification was developed by phycologists working under the provision of the Botanical Code, the discriminatory properties, both generic and specific, being either structural or more rarely ecological. Geitler's (1932) flora which lists 22 species describes the genus as "filaments unbranched or sparsely branched, sheaths mostly colourless, more or less regularly cylindrical, not layered, often becoming diffluent when old. Trichomes very many inside the sheath, grouped thickly, often wound together. End cells mostly more or less cone shaped, seldom with a head".

According to the scheme put forward by Fritsch (1942), Microcoleus is classified as:

Phylum:	Cyanophyta (Smith, 1938)
Class:	Cyanophyceae (Sachs, 1874)
Order:	Nostocales (Geitler, 1925)
Family:	Oscillatoriaceae (Dumortier ex Keichner, 1898)
Genus:	Microcoleus (Gomont, 1892)

Most records are for one of two species: M. chthonoplastes from saline environments and with trichomes lacking a calyptra and M. vaginatus from non-saline environments and with many trichomes possessing a calyptra. The genus is not sharply delimited from several others, namely Schizothrix, Hydrocoleus and Sirocoleus (Geitler (1932; Bergey Manual, Vol. 3 (1989)). The situation is further complicated by the loss of a sheath in culture (Gupta, 1967; Rippka et al, 1979; Pearson et al 1979; Stal, pers. comm.) although fascicles of

trichomes have been reported within thin sheaths (Javor & Castenholz, 1981) particularly under unidirectional light (Pearson et al., 1979). Little characterisation of Microcoleus has come from work with cultures, apart from an account by Gupta (1967).

Recognition of the close relationship between the blue-green algae and the bacteria was implemented in the 7th and 8th addition of the Bergey Manual of Determinative Bacteriology. Stanier et al. (1978) proposed a new classification system for the cyanobacteria based on the bacteriological code and using biochemical, physiological, genetic and morphological characteristics of pure cultures.

1.2 Ecology of Microcoleus

Microcoleus occurs in a wide range of environments including mangrove swamps (Potts & Whitton, 1977), coastal pools (Potts & Whitton, 1979), salt-marshes (Carter, 1932), soils (Gupta, 1967), desert crusts (Brock, 1975) rice-fields (Al-Mousawi, 1984), sewage (Pentecost, 1984), old moss (Gymnostomum recurvirostrum) growths (Whitton, pers. comm.), relics of tufa mounds (Pentecost, 1984) and intertidal mud-flats (Golubic, 1973; Margulis et al, 1983) where the majority of work on this genus has been carried out.

1.2.1 Cyanobacterial mats

Assemblages of cyanobacteria associated with the trapping, binding and to a lesser extent cementing of carbonate sediments in marine coastal environments are called recent, cyanobacterial stromatolites or cyanobacterial mats (Golubic, 1973). They occur in a variety of environments such as hot springs (Castenholz, 1984), hypersaline lakes and sabkhas (Friedmann and Krumbein, 1985), freshwater lakes (Pentecost, 1984), salt marshes (Carpenter & Price, 1976; Whitney et al., 1975) as well as in upper littoral and intertidal flats (Bauld, 1984; Javor and Castenholz, 1981; 1984).

Microcoleus mats in arid carbonate-rich intertidal areas, such as Shark Bay, Australia (Davies, 1970) and the Arabian coast of the Arabian /Persian Gulf (Kendal & Skipwith, 1968; Golubic, 1973) are often layered, with the Microcoleus overlain by one or more layer of another species (e.g. Solar Lake - Jørgensen et al., 1983; coastal pools of the Sinai Peninsula - Potts, 1980). There are also many examples where Microcoleus is the dominant species of the mat (e.g. parts of the lagoon at Aldabra Atoll - Potts & Whitton, 1979);

intertidal mats on the Island of Mellum in the southern North Sea - L.J. Stal, pers. comm.).

At different sites Microcoleus may occur on quite different parts of the intertidal region, including extensive areas of the lower intertidal region where the community is always submerged by at least a film of water (Javor and Castenholz, 1984).

1.3 Morphology of Microcoleus

Cyanobacteria secrete a fibrillar or amorphous sheath around their cells. According to Desikachary (1959), "all blue-green algae secrete mucilage, but they differ in the amount of mucilage secreted, the consistency of the mucilage, whether it is homogeneous or lamellated and whether it is coloured or hyaline". The sheaths of Microcoleus are usually colourless; however, those of closely related genera such as Schizothrix may be yellow, brown, red, violet or blue. Sheaths are composed mainly of polysaccharides (Weckesser et al., 1988).

1.4 Physiology of Microcoleus

1.4.1 Motility

Filamentous cyanobacteria which move by gliding are common in intertidal areas (Malin & Walsby, 1985). Whale & Walsby (1984) found M. chthonoplastes from the upper intertidal mudflats of the Menai Straits to be positively phototactic but negatively geotactic and magnetotactic.

1.4.2 Desiccation

Microcoleus appears to be favoured by slightly saline and poorly drained sites subject to intermittent flooding and desiccation. M. chthonoplastes is the dominant form (together with Nostoc) on rice-field soils in the Iraqi marshes at the time of the year when the soil is starting to dry (Maulood et al., 1979). Microcoleus is also part of the cyanobacterial flora of deserts; however, Brock (1975) showed that a population of Microcoleus from a desert near Mud Lake, Idaho, was not especially adapted to grow and photosynthesise at low water potentials, although it showed considerable ability to withstand drought conditions. It was more sensitive to matric than osmotic reduction in

water potential. Growth was partially inhibited at a water potential of -0.7 MPa and completely at - 1.8 MPa. Photosynthesis was markedly inhibited at - 1.8 MPa and virtually ceased at -2.8 MPa (water potential of sea water).

1.4.3 Salinity

Salinity can vary widely in intertidal environments as a result of periodic flooding and the effect of precipitation and evaporation (Fogg et al., 1973). Salinity is most constant in the open oceans (31-33%), coastal seas (30-38%) and secondary seas (0-41%) and least in rock pools and wave spray zones of coasts (0-240%). Lagoons, tidal flats, salt marshes and mangrove estuaries are intermediate in the range of salinity fluctuation to which they are exposed.

M. chthonoplastes from saline waters in Algeria and Tunisia (Hof & Frémy, 1933) grew at low concentrations of sodium, but not at 2.0 M; the authors regarded it as halotolerant. However, Javor and Castenholz (1981) found an axenic isolate of M. chthonoplastes from a Baja California intertidal mat (Laguna Guerrero Negro, Mexico) to be quite tolerant of hypersaline conditions during 1 - 2 h experiments.

1.4.4 pH

Cyanobacteria are mainly associated with alkaline or circumneutral environments (Fay, 1983) and species have been known to flourish at a pH of up to 11.0 (Cifferi, 1983).

Revsbech et al., (1983) working on a Microcoleus mat in Solar Lake, Sinai, found pH to vary from 7.7 in the early morning to 9.6 in the early afternoon whilst the pH of Oscillatoria mats on the island of Mellum, Wadden Sea, fluctuated daily from 7.4 in the dark to 7.9 in the light (L.J. Stal, per. comm).

1.4.5 Temperature

Pentecost (1984) examined the relationship between temperature, solar radiation and photosynthesis (^{14}C) in temperate Microcoleus mats. The mats were found to be temperature limited for much of the year with an optimum for photosynthesis at 24 - 30°C. Wet mat temperatures did not exceed those of air by more than 4°C and was often at or slightly below air temperature. Dry mat temperatures were significantly higher than air temperatures.

A non-axenic strain of M. chthonoplastes isolated from USAR soil, India was found by Gupta (1967) to grow poorly at 27°C but vigorously at 32°C. At 38-40°C growth was stimulated initially but ceased almost entirely after a few days, the strain becoming chlorotic. Exposure to temperatures greater than 44°C for 24-h were lethal.

1.4.6 Oxygenic and anoxygenic photosynthesis

Microbial mats have a widespread occurrence in nutrient-depleted oligotrophic, marine waters but despite this they often exhibit remarkably high rates of primary production and biomass accumulation (Paerl et al., 1981; Whitton & Potts, 1982)

Jørgenson et al. (1983) reported that photosynthetic activity in a layered mat at Solar Lake reached a peak of 50 µmol O_2 cm^{-3} h^{-1} in the dense Microcoleus layer. Several workers have found variations in O_2 concentration over a diel period with levels during the day increasing up to five times that of supersaturation (Jorgensen et al., 1983; Revsbech & Ward, 1984; Stal, 1985; L.J. Stal pers.comm). Revsbech et al. (1983) reported O_2 concentrations during the night to be 0 µM, increasing in the light to reach a maximum of 1400 µM; no sulphide was detected during the day but in the dark concentrations rose to 50 µM.

Bauld (1984) measured primary production rates in Microcoleus and Lyngbya dominated mats in Shark Bay and the Spencer Gulf. Rates were generally in the range 100 - 300 mg C m^{-2} h^{-1}, but extremes of 17 - 613 mg C m^{-2} h^{-1} were measured. The latter were associated with prolonged drying and wetting of mats. Cohen (1984) reported that M. chthonoplastes (from Solar Lake) was capable of anoxygenic photosynthesis, yet even with 0.2 mM sulphide at pH 8.0 it only carried out oxygenic photosynthesis; rather than changing to anoxygenic photosynthesis it protected oxygenic photosynthesis from sulphide. Partial inhibition occurred at sulphide concentrations between 0.2 and 0.8 mM. Concomitantly, anoxygenic DCMU-insensitive photosynthesis was gradually induced and operated in addition to oxygenic photosynthesis. Sulphide toxicity to oxygenic photosynthesis was found to increase with increasing dark period before exposure to light, indicating a build up of an internal sulphide pool in the dark and its exclusion on exposure to the light. Cohen et al. (1986) further found that at high sulphide levels (100 µmol H_2S l^{-1}), oxygenic photosynthesis was relatively insignificant but at 70 µmol H_2S l^{-1} about 50% of photosynthesis was oxygenic. As the sulphide concentration decreased, there was a gradual transition to oxygenic photosynthesis. Jorgensen et

al., (1988) examined the potential for M. chthonoplaste to carry out oxygenic photosynthesis after prolonged burial below the photic zone. 20% loss of photosynthetic potential was lost with depth per annual layer down to a depth of 8- to 10- old layers (depth 14 mm). Below 30 mm, light dependent oxygen consumption increased with increasing light intensity.

1.4.7 Heterotrophy

A number of cyanobacteria are capable of photoheterotrophy and /or chemoteterotrophy (Rippka et al., 1972; Stanier, 1973; Huang & Chow, 1987; Pescheck, 1987). Paerl (1989) showed that maltose was able to support growth and N_2 fixation in M. chthonoplastes.

1.5 Dinitrogen fixation

Biological N2 fixation is the reduction of elemental nitrogen to ammonia catalyzed by the enzyme complex nitrogenase.

$$N_2 + 6H^+ + 6e^- \longrightarrow 2NH_3$$

Research into all aspects of N_2 fixation including its biochemistry, ecology, genetics and physiology has intensified over the last several decades, partly in response to the need for alternatives to synthetic fertilizers for agriculture.

1.5.1 Nitrogenase complex

Nitrogenase was isolated initially from Clostridium pasteurianum, an anaerobic bacterium common in soil. The enzyme has since been extracted from some 30 diazotrophs (Postgate, 1987), including bacteria and cyanbacteria and found to show a high degree of homology.

The nitrogen complex consists of two highly oxygen sensitive, iron-sulphur protein components, dinitrogenase and dinitrogenase reductase. The role of the dinitrogenase reductase is to accept electrons from ferredoxin and possibly flavodoxin during Fe deficiency (Bothe, 1982; Houchins, 1984), form a complex with MgATP and finally pass electrons onto dinitrogenase accompanied by hydrolysis of ATP to ADP. The precise ATP requirement is still uncertain; however, a minimum of 12 ATP is believed to be required for the reduction of one molecule of N_2 to two molecules of NH_3 (Guerrero & Lara, 1987).

Photophosphorylation is believed to be the major source of ATP in the light and respiration in the dark (Bothe, 1982; Houchins, 1984; Ohmori, 1984; Van Baalan, 1987).

1.5.2 Organisms expressing nitrogenase activity

Nitrogenase activity has only been confirmed in prokaryotic organisms (Gallon, 1988): chemoheterotrophs Azotobacteriaceae, Bacillaceae, Enterobacteriaceae and Spirillaceae; chemoautotrophs Xanthobacter autotrophicus, Methylococcus capsulatus); photoautotrophs Chlorobineae, Rhodospirilliaceae, cyanobacteria.

Numerous genera of cyanobacteria, including Anabaena, Calothrix, Chlorogloeopsis, Cylindrospermum, Fischerella, Hapalosiphon, Mastigocladus, Nostoc, Stigonema, Tolypothrix and Westiellopsis are able to fix N_2. They possess modified vegetative cells called heterocysts which provide the required reducing environment for nitrogenase activity. Reducing conditions are brought about by the absence of oxygen evolving photosynthesis, an active oxidative metabolism and the presence of an elaborate envelope which is believed to reduce the diffusion of oxygen into the heterocyst.

Reports have been published of N_2 fixation by the vegetative cells of heterocystous forms under microaerophilic conditions (Smith and Evans, 1970; van Gorkom and Donze). Rippka and Stanier (1978) working on Anabaena spiroides and Nostoc UTEX 1597 investigated this phenomenon under anaerobic conditions (inhibiting PSII using DCMU). Both strains fixed N2 even though heterocyst development was arrested at the pro-heterocyst stage; nitrogenase activity was not confined to the pro-heterocyst but was located in the vegetative cells. This phenomenon has also been found in Anabaena sp. ATCC 27892 (Gallon, 1980). In all cases N_2 fixation required low levels of oxygen. There have been several reports of nitrogenase activity in pure cultures of unicellular and filamentous, non-heterocystous cyanobacteria incubated oxically or micro-oxically including Stewart & Lex, 1970; Rippka et al., 1971; Kenyon et al., 1972; Rippka and Waterbury, 1977; Carpenter et al., 1978; Gallon, 1980; Potts, 1980; Stal & Krumbein, 1981; 1985; Huang and Chew, 1988. Strains of Gloeothece, Gloeocapsa, Synechoccocus, Myxosarcina, Xenococcus, Pleurocapsa, Oscillatoria, Lyngbya, Phormidium, Plectonema and Microcoleus have been found to carry out such activity.

Several hypotheses have been proposed to explain aerobic nitrogenase activity: a. growth under microaerophillic conditions (Postgate, 1987); b. aggregation of filaments into bundles as in Trichodesmium or formation of

glutinous colonies as in Derxia gummosa such that the partial pressure in certain regions would be low enough to enable nitrogenase activity (Postgate, 1987); c. secretion of large quantities of extracellular polysaccharide acting as a physical barrier to extensive oxygenation; d. respiratory protection as in Azotobacter vinelandii in which high respiratory rates remove O_2 and produce the necessary ATP required (Postgate, 1987); e. enzymic protection from superoxide dismutase and catalase (Gallon, 1980); f. removal of O_2 (and possible production of ATP) by H_2 (produced as a by-product of nitrogenase activity) in a Knallgas reaction catalysed by hydrogenase; g. separation of photosynthesis and nitrogenase activity in time, i.e. nitrogenase activity in young cultures when photosynthesis is still developing towards maximum oxygen production and ceasing at a later period when photosynthetic oxygen production is at a maximum (Postgate, 1987) as well as temporal separation of both activities to different phases of the cell cycle as in Synechococcus (Mitsui et al., 1986). Cultures of Oscillatoria and Gloeothece spp. grown under aerobic conditions in 12:12 light:dark fixed N_2 almost exclusively in the dark (Mullineaux et al., 1981; Stal & Heyer, 1987).

1.5.3 Nitrogenase activity in Microcoleus

Marine environments are believed to be low in biologically available nitrogen (Bautista and Paerl, 1985; Bohlool & Wiebe, 1978; Carpenter & Capone, 1983; Ryther & Dunston, 1971); however, concentrations of ammonium are generally much higher in sediments than in water columns (Howarth et al., 1988).

Potts and Whitton (1977) studied nitrogenase activity of Microcoleus dominant cyanobacterial mats in intertidal region of the lagoon of Aldabra Atoll. They found that one of the mats exhibited high nitrogenase activity whilst the other was negligible. The former was later recalculated by Whitton and Potts (1982) to be 0.41 nM C_2H_4 μg chl a^{-1} min^{-1}. Comparison of nitrogenase activity at three sites on Mellum island off the German coast (Stal et al., 1984) showed considerably higher values per unit area in two mat communities dominated by Microcoleus than in a more open community dominated by an Oscillatoria sp.. Circumstantial evidence suggested that most of the activity at the Microcoleus site was due to this cyanobacterium.

Paerl et al (1989) have studied diel N_2 fixation (acetylene reduction activity (ARA)) in an intertidal community consisting of a Microcoleus sp., Lyngbya aestuarii and an underlying layer of anaerobic bacteria on the

Shackleford Bank off the coast of North Carolina. On a cloudy, overcast day, ARA was fairly constant during the 24 h period, whereas on sunny days the majority of activity occurred in the dark.

Pearson et al. (1981), demonstrated nitrogenase activity in an axenic culture of an intertidal Microcoleus directly by the $^{15}N_2$ technique and indirectly by ARA; the organism was more sensitive to oxygen than heterocystous cyanobacteria. Nitrogenase activity was evident whether or not trichomes were aggregated into bundles (Pearson et al., 1979). Stal and Krumbein (1985) found only anaerobically induced nitrogenase activity in a non-axenic isolate of M. chthonoplastes from Mellum Isl., Germany.

1.5.4 Measurement of nitrogenase activity

The activity of nitrogenase can be determined directly or indirectly

a. directly - by measuring the fixation product ammonia and the further products of its assimilation i.e. measuring an increase in the total nitrogen when N_2 is the sole source of nitrogen. This process is time consuming, insufficiently sensitive and impractical in the field.

- using the stable, non-radioactive isotope ^{15}N present to the extent of 0.360 to 0.370 atoms of ^{15}N per 100 atoms of ^{14}N (Prokaryotes are believed to take up these two isotopse indiscriminately). This method is expensive and requires the use of a mass spectrometer.

b. Indirectly - based on the ability of nitrogenase to reduce acetylene to ethylene i.e. acetylene reduction activity (ARA). This indirect test was found to be more sensitive than the ^{15}N assay and has become widely used since due to its high sensitivity, relative low equipment cost, rapidity of use and range of use from pure enzymes to field samples.

1.6 Aims

Microcoleus has mainly been studied in intertidal microbial mats where what limited data there are suggest it may be one of the most active organisms within the mat. Since intertidal sand and mudflats are believed to be environments low in biologically available nitrogen the ability of certain strains of Microcoleus to fix N_2 is of great ecological importance.

The aims of the project were to locate Microcoleus mats from a range of environments, isolate strains, grow them in a form morphologically akin to that in the field (i.e. with a sheath) and to establish whether they were capable of fixing N_2. Thereafter to compare the effect of environmental variables on growth and nitrogenase activity in the laboratory and where feasible in the field.

CHAPTER 2

MATERIALS AND METHODS

2.1. Durham coding system: cyanobacterial cultures

All unicyanobacterial cultures within the Durham Culture Collection are allocated a unique three-digit number. It has no taxonomic significance but is a reference for use in a computer database. The database holds information concerning the details of sample collection and culture conditions.

2.2 Statistical analyses

'Classical' statistical techniques assume that:

1. data follow a normal distribution
2. variance of the sample must be independent of the mean
3. components of the variance should be additive

A sample may be considered normally distributed if $\pm$ 1 S.D. acccounts for 68% of all observations and $\pm$ 2 S.D. 96% (Statistical Research Laboratories, 1976). The variance and the mean tend to increase together; thus the second condition is never fully fulfilled (Elliott, 1977). All laboratory data (n = 5) was normally distributed and therefore subjected to parametric analyses with 95% confidence limits. The coefficient of variation for all laboratory assays was < 4%.

Transformation can normalise the frequency distribution of counts, eliminate the dependence of variance on the mean and ensure that the components of the variance are additive. The majority of statistical methods applied with the normal distribution can also be applied to transformed data. A wide range of transformations can be obtained from Taylor's Power Law (Taylor, 1961; 1971). No simple transformation could be found to normalise the field data, thus non-parametric methods were employed. Non-parametric methods do not require the assumption of parametric methods and are particularly suitable for small samples from contiguous distributions (Elliott, 1977). There is also the added practical advantage that non-parametric statistics are usually simpler mathematically compared to 'normal tests' on transformed data.

Non-parametric tests are sometimes criticised because they do not utilize all the information provided by the sample. When the assumption of a parametric test is valid, this is more powerful than any other in rejecting

the null hypothesis (Ho) when Ho is false. In addition, many non-parametric tests are about as efficient as their parametric equivalents when all the conditions for parametric tests are fulfilled. When these conditions are not fulfilled, the non-parametric test is usually more powerful than its parametric equivalent.

Non-parametric analyses use the median rather than the mean. The median is the value which splits a distribution in half i.e. there should be as many observed values greater than the median as there are less. The Mann-Whitney non-parametric test was selected for its high power efficiency and suitability for small samples.

Mann-Whitney U test

This test is the non-parametric alternative to the t-test. The power efficiency of the U-test is never less than 86%, is between 90 and 96% for normal data and may be much higher than the efficiency of 'methods' with non-normal data (Siegel 1956; Wilcoxon & Wilcox, 1964).

The null-hypothesis (Ho) is that two independent random samples are drawn from populations having the same parent distribution and the same medians. The Ho does not specify the form of the parent distribution, but simply assumes that it is the same for both samples.

Statistical analyses were carried out using MINITABS and graphics using HARVARD.

2.3 Common procedures

2.3.1 Mass determination

Mass was measured on an Oertling R51 five place balance if accuracy was essential. Routine measurements were made on a top-pan balance (Sartorius type 1474). All references to weight refer to mass.

2.3.2 pH

An Ingold combination electrode (type E SP/SK) and an EIL pH meter (model 7050) were routinely used for measuring liquid pH. All probes were calibrated with BDH standard buffer solutions (pH 4.0, 7.0 and 9.2), prepared in

distilled water and distilled water supplemented with NaCl to a salinity of 5%., immediately before a reading was taken.

2.3.3 Redox

The redox probe (Phillips pw9411 fitted with an Orion combination electrode, 91-06) was calibrated against a Zobell solution (0.003 M $K_4Fe(CN)_6.3H_2O$ and 0.003 M $K_4Fe(CN)6$ in 0.1 M KCl solution (Zobell, 1946) giving a potential of + 252 mV for 3 M KCl at 20 C. Brown (1933) showed 12 electrodes to agree to within 0.5 mV when calibrated in this solution. In Zobell solution only one reaction, the ferro-ferricyanide reaction occurs therefore a second buffer needs to be used. Readings were compared in a saturated solution of quinhydrone prepared in pH 4.00, 7.02 and 9.00. The difference in readings between a set of buffer saturated solution of quinhydrone must correspond to the pH difference x 58.1 mV (at 20 C) (Ingold, 1966; Potts and Whitton 1976). The values are illustrated in Table 2.1

Table 2.1 Calibration of a platinum electrode using quinhydrone saturated buffers (Ingold, 1966)

pH of buffer	potential (mV) of a Pt-Ag/$AgCl_3$ KCl (3M) electrode
7.02	+ 77
4.00	+ 25
9.20	- 51

2.3.4 Salinity

Salinity was determined using a hand refractometer (S/Mill, Atago, Japan) of specific gravity 1.000 to 1.070 and salinity range of 1 - 100%.. Readings were compared with hydrometers (Gallenkamp, UK; British standard a718 series 550) of specific gravity 0.90 - 0.095, 0.095 - 1.00 & 1.00 -1.05. The temperature was recorded and salinity determined from the salinity tables (APHA-STANDARD METHODS For the examination of water and wastewater, AWWA-WPCF, 1980).

2.3.5 Light

A Macam (Macam photometrics Ltd, Scotland) light meter (model Q101 and a Skye (Skye Instruments, Llandrindod Wells, Powys) meter (model SKP 2200) fitted with a PAR sensor head (model SKP 210) were used for measuring

photosynthetically active radiation (PAR) as photon flux density in mmol photon m^{-2} s^{-1}. Each sensor contained a PAR adaptor with a sharp cut off at the near infra-red and ultra-violet range. The shape of the detector response was such that the quantum efficiency was nearly flat from the 400 - 700 nm. The sensors were also cosine corrected to provide a 180° field of view.

Due to the size of the sensor heads, they could not be inserted into the incubation vessels. The Skye sensor was only 20 mm in diameter, therefore a flat section of glass (25 mm^2) was cut from a universal bottle and an Erlenmeyer flask (from the side and base) and placed over the sensor in an attempt to compare light incident on the flask with that transmitted into the flask (Table 2.3).

Laboratory light was provided by Warm, white (Phillips, UK) and daylight (Phillips, UK) 40 Watt fluorescent tubes. Light quality and quantity was altered by means of Lee filters (Lee filters Ltd, England) as outlined in Table 2.3. Light intensity was compared using the above mentioned Macam and Skye light meters (Table 2.4).

Table 2.2 % transmission and transmitted wavelength of Lee filters

Colour	Code No.	% Transmission	Transmitted wavelength (nm)
yellow	101	80.00	500-800
primary red	106	9.32	600-800
dark green	124	29.71	430-555 675-800
medium blue	132	8.27	355-560 685-800

Table 2.3 Comparison of light flux (PAR) from warm, white and daylight tubes incident on incubation vessels with those inside; measurements using Skye meter.

Filter	% light intensity			
	Incident	Flask side	Flask base	Universal bottle
Warm, white tubes				
None	100.0	100.0	94.0	89.0
Primary red	16.0	16.0	14.1	14.1
Medium blue	10.0	7.5	7.5	7.5
Dark green	15.0	12.5	12.5	12.5
Yellow	69.0	64.9	64.9	58.0
Neutral density (1)	44.0	44.0	41.4	39.6
Neutral density (2)	10.0	10.0	9.4	8.9
Daylight tubes				
None	106.1	89.9	97.1	98.3
Primary red	18.8	16.9	17.4	17.4
Medium blue	10.9	9.1	10.6	9.7
Dark green	16.2	13.6	14.6	14.9
Yellow	72.9	65.6	70.4	69.0
Neutral density (1)	49.0	42.8	44.9	45.8
Neutral density (2)	11.5	10.6	10.9	10.5

Table 2.4 **Comparison of light intensity values using the Macam and Skye meters.**

Filter	Macam meter		Skye meter	
	μmol photon $m^{-2} s^{-1}$	% light	μmol photon $m^{-2} s^{-1}$	% light
Warm, white tubes				
None	44.0	100.0	32.9	100.0
Primary red	6.0	14.0	5.0	15.2
Medium blue	5.5	13.0	3.4	10.3
Dark green	7.5	17.0	5.2	15.0
Yellow	27.5	63.0	24.1	73.2
Neutral density (1)	21.0	48.0	15.1	46.0
Neutral density (2)	6.0	14.0	3.4	10.3
Daylight tubes				
None	100.0	100.0	72.9	100.0
Primary red	15.0	15.0	12.0	16.5
Medium blue	11.0	11.0	7.1	9.7
Dark green	16.0	16.0	11.0	15.1
Yellow	78.0	78.0	56.2	56.2
Neutral density (1)	47.0	47.0	31.0	42.5
Neutral density (2)	10.0	10.0	7.7	10.6
Natural daylight				
None	1006.1	100.0	739.5	100.0
Primary red	150.9	15.0	116.7	15.9
Medium blue	100.6	10.0	80.7	11.0
Dark green	191.1	19.0	88.1	12.0
Yellow	794.7	79.0	587.2	81.0
Neutral density (1)	472.9	47.0	322.7	100.0
Neutral density (2)	101.6	10.1	74.1	10.1

2.3.6 Temperature

Temperature in the laboratory and in the field were measured using 60°C mercury thermometers (Baird & Tatlock) calibrated against a WTW (model FC91) portable conductivity/temperature meter.

A Skye data logger (model SDL 600) was used to record temperature fluctuation during light:dark cycles. Temperatures in these thermostatically controlled areas were found to be restricted to $\pm$ 0.5°c.
Experiments on the effect of temperature were carried out in a temperature gradient over the range 16 - 48 $\pm$ 0.5°C. The gradient was comprised of a

heated aluminium block thermostatically maintained at a steady temperature (± 0.5). Light was provided from above by warm, white fluorescent tubes at an intensity of 190 ± 6 and at 50 ± 4 μmol photons using neutral density filter. Temperatures lower than 16°C were attained by placing a shallow glass dish on a relevant section of the gradient and adjusting the temperature by adding dried ice. ARA was compared in cultures maintained at 20°C directly on the aluminium block and indirectly by means of the above described 'bath'. There was no significant difference ($p<0.05$).

2.3.7 Absorption

All colourimetric analyses were carried out using a Shimadzu Digital Double Beam Spectrophotometer (model UV-150-2).

2.4 Standard Culture Techniques

2.4.1 General

To avoid contamination all isolation and subculturing work was carried out in a microflow pathfinder laminar flow cabinet conforming to BS 5295 class 1. Aseptic technique was used throughout and working surfaces were swabbed with ethanol 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.

2.4.2 Preparation of glassware and utensils

Glassware, plastics, silicon rubber-bungs (Sanko Plastics Company, Japan), Suba-seals (W. Freeman & Co. Barnsley, U.K.) and metal caps were washed in 2% Decon 90 (Decon Laboratories Ltd, Hove), a phosphate-free detergent and rinsed thoroughly in distilled water with a final rinse in Milli-Q water. Glassware used in $-N$ experiments were soaked in 10% H_2SO_4 and rinsed as above. The teflon plungers of the hand homogenisers were flamed several times in ethanol and allowed to cool in sterile Milli-Q water. Wire loops were sterilised in a bunsen flame.

2.4.3 Sterilization

Culture media and utensils were sterilized by autoclaving at 121°C (105 Pa) for 20 min. Sediment extract was prepared by autoclaving (100°C for 30 min) on three consecutive days to destroy spores (Wilkinson, 1977). Media containing sea water were pasteurized for 60 min at 105°C to reduce precipitation. Solutions with components destabilized by heat (ammonium

chloride, sodium sulphide, sulphite and thiosulphite as well as all organics) were filter sterilized by filtration through pre-sterilized 0.22 µm millipore cellulose acetate filter enclosed in a Swinnex filter holder.

2.4.4 Media

A literature search was carried out to compare media available for brackish and marine environments. None were considered suitable for the isolation and culture of Microcoleus on the basis of buffering capacity, chelation, salinity and micro-nutrient concentrations. A new medium was devised (medium GD_{10}) as outlined in Table 2.5 based broadly on medium ASN_{111} (Rippka et al., 1979) The sub-script refers to the salinity of the medium which was altered by adjusting the concentration of NaCl.

Table 2.5 Composition of medium GD_{10}-N in mM and mg l^{-1}

Salt	Concentration (mM)	mg l^{-1}
NaCl	172.0	10000.0
$MgSO_4 7H_2O$	10.0	1202.8
$MgCl_2 6H_2O$	10.0	950.0
KCl	10.0	2375.0
$CaCl_2 2H_2O$	10.0	1108.7
$K_2HPO_4 3H_2O$	1.20×10^{-1}	2.00
Na_2EDTA	7.55×10^{-3}	2.54
$FeCl_3 3H_2O$	1.96×10^{-2}	1.94
H_3BO_3	4.46×10^{-2}	2.86×10^{-6}
$Na_2MoO_4 2H_2O$	2.52×10^{-2}	5.04×10^{-6}
$MnCl_2 4H_2O$	1.43×10^{-2}	1.80×10^{-6}
$ZnSO_4 7H_2O$	6.19×10^{-3}	1.11×10^{-6}
$CoCl_2 2H_2O$	2.77×10^{-3}	0.49×10^{-6}
$CuSO_4 6H_2O$	4.95×10^{-3}	0.79×10^{-6}
$NiSO_4 7H_2O$	3.10×10^{-3}	0.48×10^{-6}
HEPES	5.0	1.20×10^{-3}
pH	8.0	
Salinity (‰)	10	

Buffer

Since the concentration of nutrients in the medium was to be altered considerably during the course of this work a buffer was considered necessary for adequate pH control (± 0.1). HEPES was chosen because of its favourable

pKa of 7.55, the negligible metal binding capacity reported by Good et al., (1966) and the success of this buffer in supporting a range of cyanobacteria in this laboratory.

Growth of Microcoleus D634, D761 and D778 was compared in HEPES at 2.5 - 20.0 mM and found to be non-toxic i.e. it did not inhibit growth. HEPES was thereafter used at 5.0 mM, the lowest concentration at which a steady pH of 8.0 could be maintained over a range of variations in the medium.

To compare ARA over a range of pH several buffers were employed (Table 2.6).

Table 2.6 The pKa (25 C), pH range and concentration (mM) of buffers used for comparing ARA.

Buffer	pKa	pH range	concentration
PIPES	6.8	6.1 - 7.5	10
HEPES	7.5	6.8 - 8.2	10
TES	7.5	6.8 - 8.2	10
EPPS	8.0	7.3 - 8.7	10
Glycine	9.6	9.0 - 10.0	10
AMP	9.7	9.0 - 10.4	10
CAPS	10.4	9.7 - 11.1	10

Chelator

Iron precipitates as a phospate salt in the absence of a chelating agent (Stein, 1973; Fogg, 1979). However, EDTA (ethylenediaminetetraacetic acid) has a high affinity for ferric iron (Jackson & Morgan, 1978) thus rendering iron available to the cell in a soluble form. Fe:EDTA was used in a ratio of 1:1.

Nitrogen

Nitrogen was added to the medium mainly in the form of NH_4-N (NH_4Cl) but also as NO_3-N (Na_2NO_3) at 0.5 mM. Unless specified to the contrary medium GD_5+N refers to the addition of NH_4-N. When no combined nitrogen was incorporated the medium was referred to as GD_5-N.

Erdschreiber medium

Modifications were made to the original Erdschreiber medium (Erdschreiber, 1927) as outlined in Table 2.7.

Table 2.7 Modification of Erdschreiber medium.

Constituent	Modifications (volume in ml)		
Seawater	1000	500	250
Distilled water	-	500	750
Vitamins	+	+	+
Sediment extract	+/-	+/-	+/-
Cyanobacterial extract	+/-	+/-	+/-
NH_4-N (0.7 mM)	+/-	+/-	+/-
PO_4-P (0.06 mM)	+/-	+/-	+/-
$CaCl_2.2H_2O$	+	+	+
HEPES	+	+	+
pH	7.8	7.8	7.8

Sea water was collected off the coast of Lindisfarne, N. E. England and the Menai Straits, N. Wales. Care was taken not to disturb the sea bed during collection. The water was returned to the laboratory in a polypropylene container within a maximum of 6-h after collection to be filtered through a Whatman number 1 filter paper and subsequently stored at 4°C in the dark. Sediment extract was obtained by steaming the upper 5 mm of sediment in twice the volume of Milli-Q water at 100°C for 60 min. on each of three consecutive days. After cooling, the extract was filtered through a Whatman number 1 filter and stored in the dark at 4°C for a maximum of 8 weeks. Cyanobacterial extract was obtained by extracting Microcoleus dominant mats from the

underlying sediment and steaming it for 60 min. at 100°C in twice the volume of Milli-Q water. Filtration and storage was as above.
Vitamin B_1 and B_{12} were used at a final concentration of 200 and 20 $\mu g\ l^{-1}$ respectively. They were filter sterilized and added to the medium after autoclaving.

Stock solutions

Stock solutions of the salts employed were prepared in Milli-Q water and stored at 4°C in the dark for a maximum of three months. All chemicals, unless otherwise specified in Table 2.9 were AnalaR grade.

Table 2.8 Suppliers of chemicals other than BDH, AnalaR grade.

Chemical	Supplier
agar	Difco, UK
peptone	Difco, UK
Nutrient agar	Oxoid, UK
Tryptone	Oxoid, UK
Yeast extract	Oxoid, UK
HEPES	Sigma
PIPES	Sigma
TES	Sigma
EPPS	Sigma
AMP	Sigma
CAPS	Sigma
Glycine	BDH, UK
DCMU	Sigma

Experimental media

The standard medium was almost invariably the control for experiments investigating nutrient variations. All variations to the 'normal' media were made after autoclaving at less than or equal to 2% v/v. Changes in salinity were noted.

Since cultures at mid. log grew attached to glass-liquid interfaces the medium could be decanted without dislodging the organism. When the pH, salinity or nutrient status of the medium needed changing cultures were rinsed gently three times in fresh, sterile medium (maintained in a water bath at the

required temperature) before the final test medium was added using a 50-ml sterile syringe. The process required 6 min. for 10 samples.

Experiments

Experiments to determine the effect of environmental variables on growth and ARA

Unless specified to the contrary all inocula were prepared from cultures at mid-log and all experiments examining the effect of environmental variables on growth were carried out at mid. log. Microcoleus D634, D761. D781 and D782 were cultured at 32.0 ± 0.5 C in 14:10 light (50 ± 3 μmol photon m^{-2} s^{-1}):dark whereas Microcoleus D778 was cultured at 20.0 ± 0.5 C, either in continuous light or in 16:8 light (50 ± 3 μmol photon m^{-2} s^{-1}): dark.

All experiments comparing the effect of these variables on ARA were carried out during late lag phase or early log growth when ARA was highest.

To determine the influence of nutrient concentrations on growth

In order to determine the effect of nutrient concentrations on growth, cultures were grown to mid-log in medium GD at the required salinity (5 or 10‰) and cultures harvested for chl a analysis on the required days.

To determine the influence of temperature on growth

In order to determine the influence of temperature on growth cultures were removed from their incubations areas and transferred to a temperature gradient (2.3.6) for a pre-incubation period of 1-h, the end of which time was considered t = 0 and samples harvested for chlorophyll analysis 24, 48 or 72 h later. Chl a concentrations were compared with those maintained under 'normal' growth conditions to assess any differences arising from the experiment.

If no growth was found at the end of the incubation period but commenced on re-incubating under normal culture conditions, strains was termed as having survived.

To determine the influence of salinity on growth

In order to determine the influence of salinity on growth, cultures were removed from the incubation vessel and the medium changed as above before

returning samples to the incubation area and pre-incubating for 1-h, the end of which time was considered t = 0. Growth was compared 24, 48 or 72-h later.

Survival was tested as above.

To determine the influence of pH on growth

Experimental methods as above. Details of the buffers used are given in Table 2.6 and section 7.8.

2.4.5 Incubation area

All experiments were carried out without shaking although flasks were disturbed approximately every 4-h (during the light period) when they were moved in an attempt to compensate for the uneven light source. A summary of the incubation areas available is given in Table 2.10

Table 2.10 Temperature and light (PAR) in incubation facilities.

Temperature (°C)	PAR (μmol photon m^{-2} s^{-1}) source	intensity	direction	light:dark
20	Warm, white	30 ± 4	above	variable
20	Warm, white	50 ± 3	below	variable
20	Warm, white	100 ± 5	above	variable
20	Daylight	100 ± 4	above	variable
25	Warm, white	50 ± 4	above	24:0
32	Warm,	50 ± 3	above	14:10

2.4.6 Culture vessels

Isolation was carried out in 8-ml serum bottles, 29-ml universal bottles, 100 ml Erlenmeyer flasks and 50-ml plastic Petridishes. Acetylene reduction assays were carried out in 29-ml universal bottles and 100-ml Erlenmeyer flasks containing 5 and 25-ml of media respectively.

Stock cultures were maintained in 100-ml Erlenmeyer flasks containing 25-ml of medium.

2.4.7 Oxic and micro-oxic environments

Oxic

Oxic conditions refer to cultures grown in Erlenmeyer flasks or universal bottles in atmospheric oxygen without shaking. The solubility of oxygen in

water decreases as both the temperature and chloride concentration of the water increases (Standard Methods for the Examination of Waste Water, APHA-AWWA-WPCF (1980) pp. 392 - 393).

Experiments were carried out under ambient oxygen, both in the presence and absence of DCMU.

Micro-oxic

Micro-oxic conditions -DCMU refer to the removal of air by argon and allowing the atmosphere to re-equilibriate before re-introducing 10% v/v air, giving and O_2 concentration c. 2% that under normal atmospheric conditions.

Anoxic

Anoxic condition refer to incubations in an atmosphere of argon in the presence of DCMU.

Experiments under anoxic and micro-oxic conditions were carried out in 29-ml universal bottles and 100-ml Erlenmeyer flasks. The liquid phase (5-ml and 25-ml respectively) and gas phase was de-aerated with argon (BOC, industrial grade) for 5 min and 2 min in universal bottles and 10 min and 4 min in Erlenmeyer flasks. Using silicon tubes and polypropylene Y connectors to which 0.63 mm needles (B-D, Ireland) were fitted, the gas stream could be split enabling four bottles to be de-aerated simultaneously. A sterile Swinex filter holder (Millipore) containing a 0.22μm nitrocellulose acetate filter was initially fitted to this apparatus to sterilise the argon however, this was found to be unsatisfactory due to the resulting reduction in flow rate and consequent lengthening of the de-aeration period.

DCMU (3-(3.4-Dichlorophenyl)-1,1-dimethyl urea)

DCMU was dissolved in ethanol and diluted to aqueous solution with sterile distilled water giving a final concentration of less than 0.03% as recommended by Hirose (1987). DCMU was compared over a range of concentrations ($1x10^{-6}$, $5x10^{-6}$, $1x10^{-5}$, $5x10^{-5}$, $1x10^{-4}$ and $5x10^{-4}$) to be used finally in laboratory experiments at a concentration of $1x10^{-5}$ M (lower concentrations did not fully inhibit PSII). Mats were submerged in Milli-Q water containing $1x10^{-5}$ DCMU for 5 min and the mats left for 15 min to allow for penetration of DCMU through the sediment and sheath into the cells.

Carbonate

Carbonate, as Na_2CO_3 (0.1 mM) was added to medium GD under micro-oxic conditions since in exchanging air for argon CO_2 was removed. Carbonate was only added under oxic conditions when ARA was compared under oxic and micro-oxic conditions.

Reducing solutions

Sodium sulphide, sulphite and thiosulphite solutions were prepared using warm, sterile Milli-Q water in sterile universal bottles immediately before use. The Milli-Q water had been autoclaved as 14-ml aliquots in 28-ml universal bottles. Two 14-ml portions were pooled on removing the bottles from the autoclave at 80°C, to be sealed with as little air as possible and maintained at 30°C for a maximum of 30 min. before use. All media with sulphide employed HEPES at 20 mM.

2.4.8 Inoculating and Harvesting

Homogeneous inoculum

A homogeneous inoculum was prepared by crushing the cyanobacterium in a hand homogenizer (1500 mm x 20 mm) of precision made glass (Jencons) with a teflon plunger. After this initial shearing, the material was passed successively through 60 ml plastic syringes (B-D) fitted with 1.1 and 0.63 mm needles (microlance, B-D). No cell lysis occurred using this method.

Harvesting

Cultures did not grow homogeneously throughout the medium therefore it was impractical to extract an aliquot for biomass determination. Material adhering to the wall of the flask was dislodged using a glass rod fitted with a silicon tip; the whole content of the flask was then assayed.

Stock cultures

Stock cultures were maintained in 25-ml of medium GD_5 +N in 100-ml Erlenmeyer flasks.

2.4.9 Isolation

Cyanobacterial material was teased from the sediments in one of three ways: (1) applying lens tissue, (ii) applying coverslip of microscope slide (iii) gently scraping the surface with a needle. Material identified

microscopically as Microcoleus was transferred to liquid medium, onto agar, into modified Winogradsky columns or embedded in calcium alginate beads. Numerous variations were made on medium GD (Table 2.11) for the isolation of strains under a light:dark regime, at two light intensities (30 ± 2 and 50 ± 2 mmol photon m^{-2} s^{-1}) and at temperatures of 15.0 and 20.0 ± 0.5°C for temperate strains and at 25.0 and 32.0 ± 0.5°C for sub-tropical strains. Samples were incubated for up to 16 weeks.

Table 2.11 Changes in the nutrient status of medium GD for the isolation of Microcoleus strains.

Cation	Concentration (mM)					
Na^+	40	86	172	255	340	510
K^+	10	25	50			
Ca^{2+}	10	25	50	125		
Mg^+	10	25	50			

Solid and semi-solid environment

0.1, 0.5, 0.75, 1.0, 2.0 & 3.0% agar were used with and without purified sand. Samples were incubated in or on the agar, in which case cooled agar (45°C) was poured over the material forming a 0.5-1.0 mm layer.

Modified Winogradsky columns were prepared by coring (using sawn-off plastic syringes 21 mm in diameter) 3 cm deep cores and transferring them to universal bottles where they were incubated both aerobically and anaerobically (de-gasing with oxygen-free nitrogen), the latter in the presence and absence of sulphide (0.05, 0.10 and 0.25 mM) over a range of temperatures and light intensity. The sediment was overlain with sea water, distilled water and medium buffered at pH 7.8. Incubations involving sulphide were carried out at pH 7.0 and 7.8 employing HEPES at 20.0 mM.

Calcium alginate beads were also used to try and isolate Microcoleus. Sodium alginate (Sigma) was autoclaved in Milli-Q water and allowed to cool at room temperature. Cyanobacterial mat (including microscopically identified Microcoleus) dislodged from the underlying sediment was thoroughly mixed with the alginate solution. Calcium alginate beads were prepared by dropping the mixture via a syringe (B-D, Ireland) fitted with a 0.63 mm needle into a solution of 0.1 M $CaCl_2$. The beads, (4.0% sodium alginate) about 2 mm in

diameter, were allowed to harden for 30 min. in the $CaCl_2$ solution. They were washed thrice in the relevant medium.

Since these beads could be transferred to a salinities of up to 35‰. without disintegrating (at 20.0 to 32.0 ± 0.5 C) they were used as a means of changing the salinity of the environment. Beads containing cultures (maximum of 50 beads 25 ml^{-1}) were washed several times in the new media before finally transferring to that medium for the required period.

Visking dialysis tubing (Medicell International Ltd, London) 6.3 mm wide and 20 mm in length was boiled for 10 min in 10 mM EDTA before rinsing thoroughly several times in sterile distilled water. Serial dilution of field samples as well as enriched cultures in a range of media were injected into the tubes using 10 ml sterile syringes (B-D, Ireland), sealed and transferred to the relevant medium. This technique was also used for changing the salinity of the surrounding medium, the samples being washed several times in the new medium before finally incubating in the given medium.

2.4.10 Purity of strains

Several methods were attempted to render strains axenic including physical manipulation, sonication (Soniprep 150, MSE, UK) centrifugation and antibiotics. Young, three day-old cultures were employed for all purification methods.

Centrifugation

Media from three day old cultures were discarded and replaced with fresh, sterile media. The cultures were washed thrice in this way before homogenising (2.4.8) and centrifuging in Eppendorf (Sarstedt, England) tubes at 10000 r.p.m. for 1 min in a bench top centrifuge. The whole process was repeated three times with cultures tested for purity at all stages. Cultures were thereafter grown for three days before the process was repeated.

Sonication

Cultures were washed as above before sonicating. The process was repeated thrice and the cultures tested for purity at each stage.

Cultures cleaned by centrifugation and sonication were transferred into 10 and 25 ml volumes of liquid media. In addition concentrated samples as well

as serial dilutions were prepared and streaked or sprayed onto agar (0.8 and 1.0%).

Spray technique

The spray technique was developed by Wiedman et al. (1964). A suspension of Microcoleus was injected through a 0.5 mm diameter needle (BD, Ireland) onto the tip of a 1.0 mm diameter needle (BD, Ireland) in the path of sterile, compressed air to be atomized onto a suitably positioned agar (1%) plate.

Taxis

Half the streaked and sprayed plates were covered by a thin layer of agar (0.5, 0.8 or 1.0%) in an attempt to use the gliding ability of the organism to separate it from the bacteria.

Cultures were also placed at the centre of an agar (0.8 and 1.0%) plate and the whole apparatus darkened by foil, but split at the junction of the two halves and covered by Nescofilm to allow gaseous diffusion. Four windows were cut in the surface of the foil and covered by red, green and neutral density filters to alter the quality and quantity of light incident on the plates.

Antibiotics

Microcoleus cultures from both early and mid. log were used to streak a variety of bacterial test plates. These were incubated at 25.0°C and 32.0 ± 0.5°C and examined after 24-h, 48-h, 1, 2 and 3 weeks. A lawn of bacteria was prepared from each colony type on each bacterial plate. These bacterial lawns were grown for 2 - 4 h in the dark at 25.0 and 32.0 ± 0.5°C before testing with Oxoid Multidiscs (Code. 30-1H, 30-12L, 30-1N) over a period of 24-h. Stock solutions of antibiotics giving rise to clear zones were freshly prepared and both early log and mid log. cultures incubated with one or more antibiotics over 2, 4, 6, 12 and 24-h at final concentrations of 100, 500, 1000, 1500 and 2000 $\mu g\ l^{-1}$. Cultures were then washed several times in fresh, sterile medium before incubating at 32.0 C under 30 ± 3 and 50 ± 3 μmol photon $m^{-2}\ s^{-1}$.

Testing for purity

Stock cultures of axenic strains were tested for contamination prior to and after each subculturing and on preparing an experimental inoculum. Strains were examined microscopically and if no contamination was apparent, further tests were carried out. A sample was homogenized and then streaked onto a four types of media as outlined in Stein (1979). Plates were incubated

in the dark for 4 weeks at 15, 20, 25 and 30°C, during which period plates were examined by eye, with the use of a compound microscope and under oil immersion.

2.5 Atomic Absorption

Atomic absorption studies were carried out on all the media variations employed to determine if any precipitation occurred after autoclaving and on allowing the flasks to stand.

All analyses were performed on a Perkin-Elmer (PE) model 5000 Atomic Absorption Spectrophotometer with a PE model 5000 Automatic Burner Control Unit. Low level determinations were performed on the above fitted with a graphite furnace (PE model HGA 500) and autosampler (PE model AS 40) replacing the burner unit.

Determinations were performed using an air-acetylene flame. Analyses of potassium, magnesium and calcium required pretreatment; for K and addition of 15000 mg l^{-1} NaCl to a 4 ml aliquot sample (final concentration equal to 10% v/v) to suppress ionization interference (Perkin-Elmer CO., 1982) and for Mg and Ca, an addition of 7% $LaCl_3$ (final concentration of 0.51% v/v) to suppress chemical interference from Al, P, Si, Ti and Zi (Perkin-Elmer Co., 1982).

All metals were above the detection limit by a minimal factor of 10.

2.6 Microscopy

2.6.1 Light microscopy

Strains were examined using a Nikon fluorophot microscope, type 109, fitted with a Nikon micrometer eyepiece. Light micrographs were taken using a Nikon-M-350 automatic exposure camera. Kodak Ektachrome Tungsten Professional film was used for colour slides and Kodak Technical Pan 2415 for black and white.

Field observations were determined using a Cook MacArthur field microscope (Vickers Instruments Ltd, England).

2.6.2 Electron microscopy

The fixative for routine fixation (pH 7.0) was: 2.5% (v/v) glutaraldehyde, 2.5% (v/v) formaldehyde and 0.05 M PIPES.
Material was incubated overnight at room temperature, followed by a rinse in Milli-Q water and an overnight soak in PIPES (pH 7.0). A 3-5 min. period in a 0.05 M HEPES buffer completed the fixation.

Fixed material was washed twice in Milli-Q water (15 min. each) before staining with 1% osmium tetroxide for 1 h at room temperature. Stained material was washed in Milli-Q water before passing through a de-hydration series consisting of two 10 min. changes in 10%, 20%, 50%, 75% and 100% ethanol. Material was soaked in 1:1 alcohol:Spurr resin (Spurr, 1969) for 24 h before being polymerized at 70°C in fresh Spurr resin. Ultrathin sections were cut using glass knives on an LKB Ultratome 111 Ultra-microtone and collected on acid etched (4% HCl for 1 min.) copper apalladium grids. Sections were then stained following the recommendation of Reynolds (1963) for 20 min. in uranyl acetate, rinsed with distilled water and counterstained in lead citrate for 20 min. The grids were washed in distilled water before being dried and examined under a Phillips SM 400 transmission electron microscope. Kodak electron microscopy film used for all electron micrographs.

2.7 Biomass determination

2.7.1 Chlorophyll a and phaeopigments

Laboratory material

Cultures were filtered through a Whatman 2.5cm GF/C filter and extracted in 5 or 20 ml of 90% methanol for 15 min. at 70°C in the dark. Samples were thereafter re-filtered and made up to 5 or 25 ml respectively in volumetric flasks. Absorbance was read at 665 and 750 nm against 90% methanol before acidifying with HCl to a final concentration of 0.01 mM. Samples were re-read after one hour against an acidified 90% methanol blank.

Natural populations

Microcoleus mats were removed from the incubation vessel immediately after collecting the gas sample. Cores were sectioned into layers approximately 2 mm thick and placed on blotting paper until no leakage of water was evident. They were then transferred to clean blotting paper and incubated in the dark at 2-5 C (in a cool box on a bed of dried ice). Samples were returned to the laboratory within a maximum of 30-h, to be frozen at -20 C for a maximum of 7

days. Neither drying, transporting nor freezing the mats as described above was found to significantly (p 0.05) alter the chl a or phaeopigment levels. Microcoleus was only found in the upper 2 mm with the majority in the upper 1 mm, therefore only the upper 2 mm was tested and the bulk of the sediment beneath discarded to aid drying of the cores and to minimise any interference which may have arisen from any organic compounds present. Samples were extracted twice in 25 ml of 90% methanol at 70.0 C for 15 min. A third extraction was always carried out to ensure all the chlorophyll had been extracted. Samples were acidified as described earlier. Absorbances were re-read after 1-h (there was no significant difference (p 0.05) in pigment levels 1 - 20-h after acidifying). HCl at final concentrations of 0.05, 0.01 and 0.025 mM were compared but no significant difference (p 0.005) was found therefore 0.01 mM HCl was used. Where samples are likely to contain high levels of phaeophytin (e.g. in the field), Marker et al. (1980) suggests that neutralisation after acidification is required for accurate estimates of phaeophytin. However, neutralisation did not alter the level of phaeopigment (which were subsequently found to be low in the field samples examined) significantly (p 0.05). Chl a was determined from equation 1 and phaeopigments from equation 2.

$$c = \frac{(Ab - Aa)\ R \quad K. \quad V}{R-1 \qquad L} \qquad (1)$$

$$P = \frac{(Ab - R\ (Ab-Aa)).\ K.\ R.\ 0.973.\ V}{R-1 \qquad\qquad\qquad\qquad L} \qquad (2)$$

where

Ab = reading at 665 nm minus that at 750 nm before acidification

Aa = reading at 665 nm minus that at 750 nm after acidification

R = the maximum acid ratio for samples without pigments: (Ab/Aa)

K = 1000 x the reciprocal of the specific absorption coefficient of chl a at 665 nm. Taking this value for chlorophyll in methanol as 77 then K = 12.99

V = volume of extract

L = path length

c = concentration of chlorophyll a in $\mu g\ l^{-1}/cm^{-2}$

P = concentration of phaeopigments in $\mu g\ l^{-1}/cm^{-2}$

2.8 Studies on natural populations

2.8.1 Collection of *Microcoleus* mat

To isolate strains

The bottom half of a plastic petri-dish (Sterilin, England) was used as a corer to remove a section of sediment approximately 1 cm deep. The core was then inverted and maintained in the open petridish to be returned to the laboratory.

To examine nitrogenase activity

An area of mat sufficient to provide the required experimental inoculum was located by eye and *Microcoleus* confirmed microscopically. As there was only a limited quantity of mat available and in order to minimise any environmental damage as little an area as possible was disturbed (20 cm by 20 cm was randomly sampled for each experiment). Cores 1 cm in depth were removed using 'sawn off' plastic syringes of surface area 4.72 cm^2. Cores were placed on plastic petridishes and the upper 5.0 $\pm$ 1.0 mm cut with a razor blade and inserted into universal bottles by means of forceps. Handling of the cores was kept to a minimum; no physical contact was made with the mat.

For submerging in seawater

An area of mat approximately 40 cm x 20 cm was dug (using a garden spade) to a depth of about 3 cm. The mat was transferred to a deep, black polythene covered metal tray and freshly collected sea water poured over it. The tray gently rocked to mimic the action of the water covering the 'real' mats. During the light period, light intensity was kept as close to that incident on the 'real' mats by means of neutral density filters.

For laboratory experiments

Mats returned for experimentation in the laboratory were collected as above and were in transit for a maximum of 6-h before returning to the greenhouse.

For storage of *Microcoleus* mats

Mats were maintained in the greenhouse under reduced daylight (c. 50%) at 17 $\pm$ 3°C for a maximum of 4 days. During this time samples were watered four times daily in the morning, afternoon, evening and night with sterile distilled water. To keep the mats 'just moist' the quantity of water varied from c. 2 - 3 l^{-1} m^{-2} day^{-1} depending on temperature, light intensity and

humidity. Certain experiments required the mats to be pre-treated in different ways in which case details are given with the experiment.

Prior to experimenting a section of mat was cut with a knife and transferred with minimal contact to a perspex container 20 cm x 20 cm x 5 cm such that the mat fitted exactly. When required liquid (sea water, distilled water or media) was gently poured from the side to minimise any disturbance. 40 ml of liquid was added per container giving a liquid depth of c. 1 cm. This height did not vary even over an incubation period of 6-h unless dry ice was added in which case details of changes are given in individual experiments.

2.8.2 Physical variables

The Skye light meter was employed in the field to measure light both in the air and in water (useful range up to 2 m, -25°C to +75°C and at all humidities).

Prior to tidal submergence a light probe was positioned on the mats and secured in position with rocks (they did not cast a shadow over the probe). Light readings were taken every 5 min. except when it was found to be highly variable (e.g. cloudy with sunny spells) when reading were taken continuously. Two light probes were compared on all occasions.

Air and water temperatures were recorded using mercury thermometers (2.36). Temperatures underwater were recorded by attaching these thermometers to a portable wooden pole.

pH determinations were carried out on mats returned to the laboratory. The Microcoleus mats were separated by cutting with a scalpal and teased apart with two needles. These samples were tested for pH immediately and after air drying at 20 and 50°C. The samples were mixed with distilled water in a ratio of 1 - 5 and stirred for 15 min. The pH was read using a WTW meter (pH-91) fitted with an Ingold combination electrode.

2.9 Acetylene reduction activity (ARA)

2.9.1 Laboratory technique

To minimise any disturbance to the cultures the whole content of the flask was used for the assay. Unless specified to the contrary replicates of 5 (n = 5) are used for all experiments.

Vessels were removed from the incubation area for 2, 3, 5, 8 and 10 min respectively when n = 5, 10, 20, 30 and 40 and placed in a water bath at the same temperature whilst the bungs were exchanged for suba-seals. Light levels were unavoidably reduced for this period. During dark 'test' periods, the low light provided by torch did not register on the light meter. Samples were returned to the incubation area for 10 min. before acetylene was added.

ARA was compared using 1 - 25% C_2H_2 v/v (n = 4) over 1-h and 2-h incubation periods in samples containing 800.31 $\pm$ 30.66 mg chl $\underline{a}$ l^{-1}. ARA reached a plateau of activity between 8 and 18% C_2H_2 v/v (Fig 2.1). Since many other laboratories working on cyanobacteria use 10% acetylene this concentration was chosen for all future laboratory assays.

10 ml of air was removed from the incubation vessel and 10% C_2H_2 v/v added using a 20 ml syringe (B-D, Ireland). The gas phase was allowed to equilibriate to atmospheric and each vessel gently stirred to ensure a homogeneous gas phase.

ARA was also compared in 10% v/v C_2H_2 over 0.25 - 4.0-h incubation periods. Rates did not vary significantly over 0.75 - 3.0-h incubation periods (Fig. 2.2). A 1-h incubation period was chosen for future experimentation.

Before collecting C_2H_4, incubation vessels were gently swirled to ensure a homogeneous gas sample and 1 ml of gas was collected using 1 ml disposable plastic syringes (Becton-Dickinson, Ireland) fitted with a 0.5 mm needle (B-D, Ireland). The tips were placed immediately in rubber stoppers (previously tested to ensure no ethylene release). New syringes could be stored for up to 12-h without any significant leakage. In practice syringes were injected into the gas chromatograph within 3-h of collection.

Due to the time required for de-aerating these cultures, they were removed from the incubation area for 20 min. (n = 12), during which time cultures were unavoidably subjected to a decrease in light intensity and temperature. For all experiments comparing ARA under oxic and micro-oxic conditions, samples were maintained under the same pre-experimental conditions.

Fig. 2.1 Influence of % C_2H_2 on ARA in Microcoleus D778 over 1 and 2-h incubation periods. 20°C, 50 umol photon m^{-2} s^{-1}

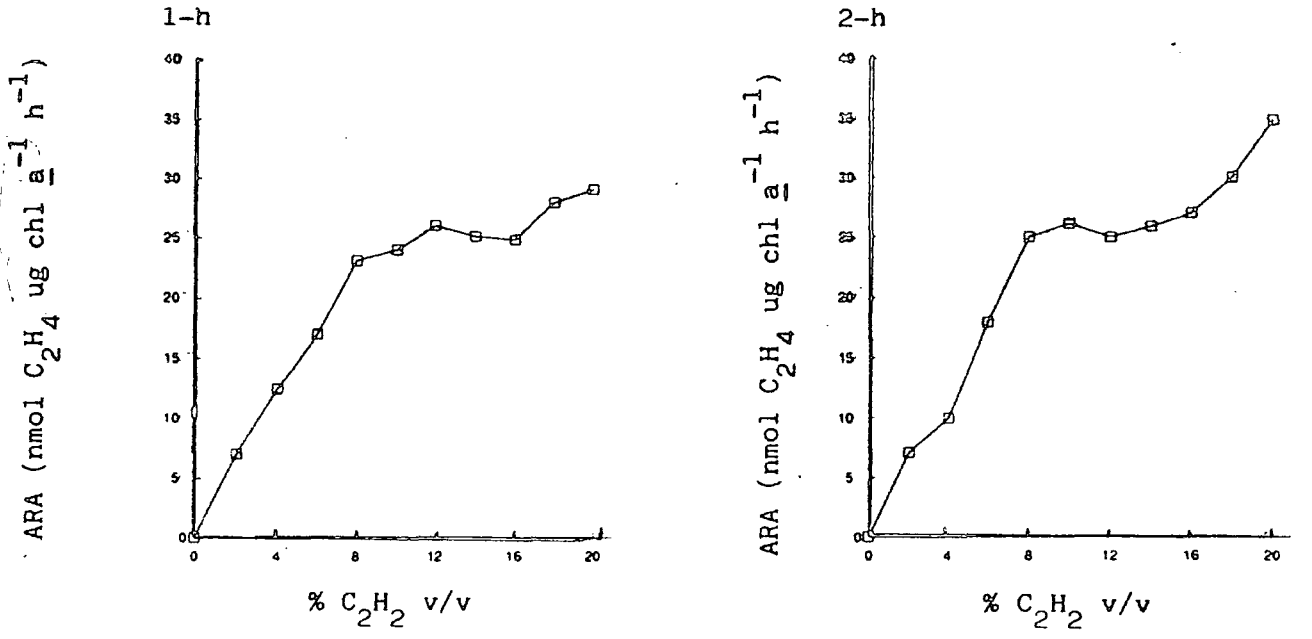
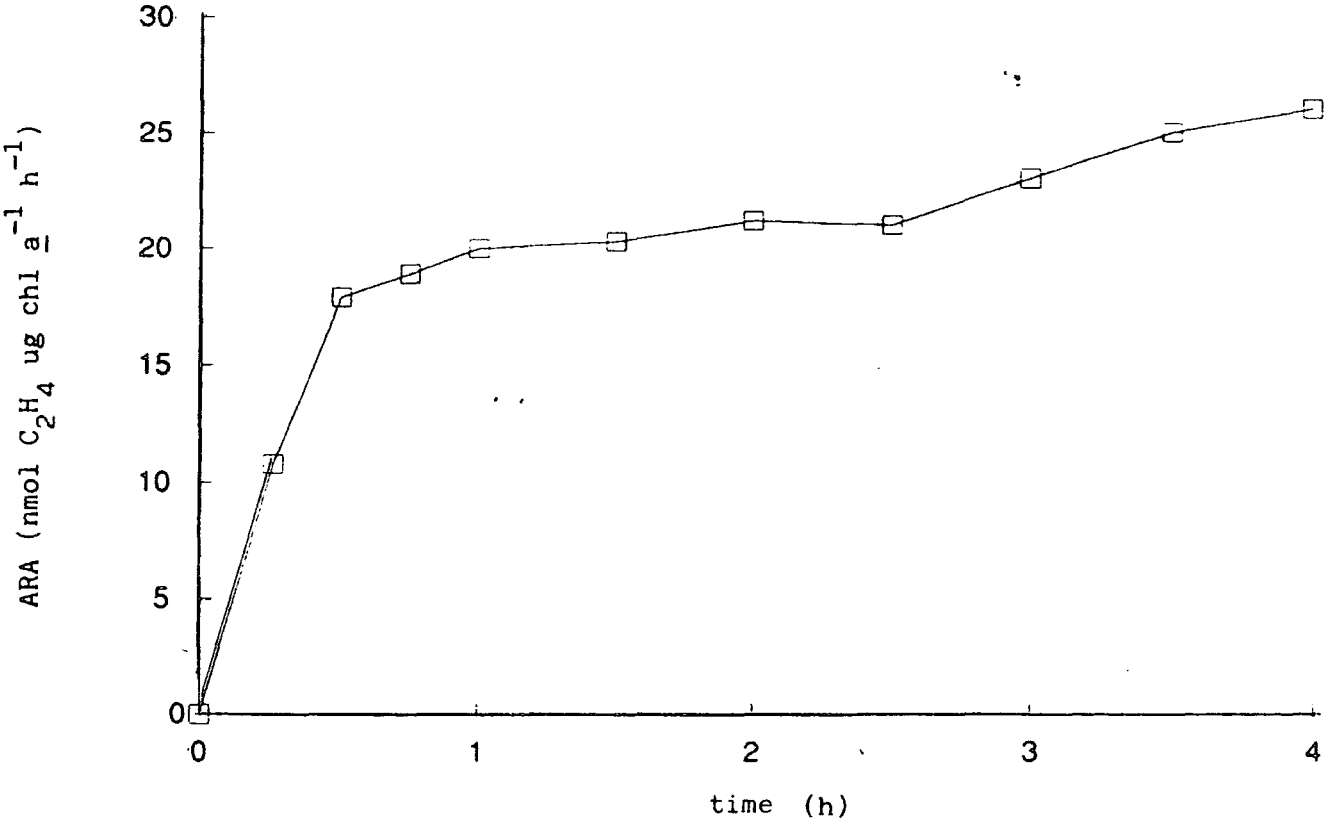


Fig. 2.2 Influence of incubation period on ARA in Microcoleus D778 using 10% C_2H_2 v/v. 20°C, 50 umol photon m^{-2} s^{-1}



Controls included:

Oxic incubation:	acetylene and medium
	cyanobacterium and medium
Micro-oxic conditions:	acetylene and medium
	cyanobacterium and medium
	acetylene, medium and DCMU
	cyanobacterium, medium and DCMU

Controls were incubated either in the light or in the dark depending on the experimental requirements. During a 24-h ARA experiment, controls were carried out twice in the dark and twice during the light period. No ethylene was detected in any of the controls.

2.9.2 Field technique

Cores 1 cm in depth were removed using 'sawn off' plastic syringes. ARA was examined on cores of different depth (mat + 1, 3, 6 and 9 mm) in the light, firstly on predominantly oxic cores and secondly on microoxic cores (as indicated by the presence of a black precipitate of FeS). No significant ($p<0.05$) increase in ARA was apparent with increasing depth of core either in the light or in the dark, therefore cores composed of the mat plus the underlying 3 mm or so of sediment were used for further experiments.

After collection the cores were placed on plastic petri dishes and the upper 3.0 + 1.0 mm cut with a razor blade. They were replaced on clean petri dishes and inserted into universal bottles using forceps. Cores were orientated such that the under surface was attached to the glass walls of the vessels approximately three-quarters of the way down the bottles (no shadow was cast on them from the caps) at an angle of 45 .

0 - 25% C_2H_2 v/v were compared (on Microcoleus mats returned to the laboratory) over a 2-h incubation period. (Controls containing equal volumes of argon were included to ensure any increase in ARA was not due to a reduction in the partial pressure of O_2). ARA increased steadily with increasing % C_2H_2; however, there was no significant difference in ARA between 8, 10 and 15% C_2H_4 v/v (Figs 2.3). ARA using 0 - 10 % C_2H_4 v/v were also compared between 0900 - 1300 on the 6.6.87, 20..7.87, 19.8.87, 4.8.87, 9.9.87

and 15.10.87 when the mats were at a different stage of development. There was no significant (p 0.05) difference in ARA at 6 - 10% C_2H_2 v/v (Fig. 2.6).

Acetylene was added using 5 ml syringed (B-D, Ireland)) fitted with 0.5 mm needles (microlance, B-D, Ireland).

ARA was compared during 1 (1800 - 1900), 2 (1800 - 2000) and 4 (1800 - 2200) h incubation periods and the rates found to differ significantly (p 0.05) between them; however, when ARA over four 1-h incubations (0600 - 0700, 0700 - 0800, 0800 - 0900, 0900 -1000) was compared with one 4-h incubation (0600 - 1000) there was no significant ($p<0.05$) difference. Differences in the former experiment may have arisen as a consequence of normal diel variations in ARA.

At the end of the incubation period universal bottles were shaken to ensure a homogeneous gas phase. Experiments were terminated by transferring 5 ml of the gas phase into a Venoject evacuated, glass, blood collecting tube (Terumo, Belgium) by means of a B-D (Ireland multiple sample needle composed of two needles able to simultaneously pierce both the evacuated tube and incubation vessel septum. Each septum was used a maximum of five times. Samples could be stored in the Venojects for up to 3 months (taking note of expiry date!) without any loss of gas. In practice, samples were analysed within 14 days. Venojects were periodically checked to ensure a complete vacuum.

Timing of each stage of the process is summarised in Table 2.13 where stage 1 refers to coring; stage 2 sectioning cores, inserting them into universal bottles and sealing these vessels; stage 3 removing air; stage 4 adding acetylene and equilibriating the pressure; stage 5 is the collection of gas. The median of 12 test periods is given taking into account variations arising due to e.g. a) dark period v light period b) climate, c) assistant.

Table 2.12 Time required for each stage of the acetylene reduction technique.

Stage	n	Median	Quartile 1	Quartile 3
1	12	2.2	2.0	2.6
2	12	8.1	4.7	8.4
3	12	2.0	1.8	2.3
4	12	3.0	2.8	3.3
5	12	2.2	2.0	2.6

Controls included Microcoleus mat alone and acetylene alone under the different light regimes tested.

ARA was expressed as living biomass (nmol C_2H_4 /mg chl a min^{-1}) and per surface area (nmol C_2H_4 cm^{-2} min^{-1}). No correlation was found between ARA and concentration of chl a per sample.

2.9.3 Mats returned to the laboratory

When required, mats were transferred from the greenhouse to the laboratory (5 min). Sections of mat were cut 15 cm x 30 cm and a depth of c. 2 cm, transferred to perspex containers 15 cm x 30 cm x 7 cm to be incubated under the required conditions.

When mats were submerged in liquid to a depth of 2 cm (Milli-Q or seawater) the temperature of the liquid remained constant by either adding dry ice or medium pre-heated to a given temperature according to the requirements of the experiment.

Fig. 2.3 Influence of % C_2H_2 v/v on ARA in Microcoleus mats returned to the laboratory (1.6.87) over a 2-h incubation period. 20°C, 50 umol photon m^{-2} s^{-1}

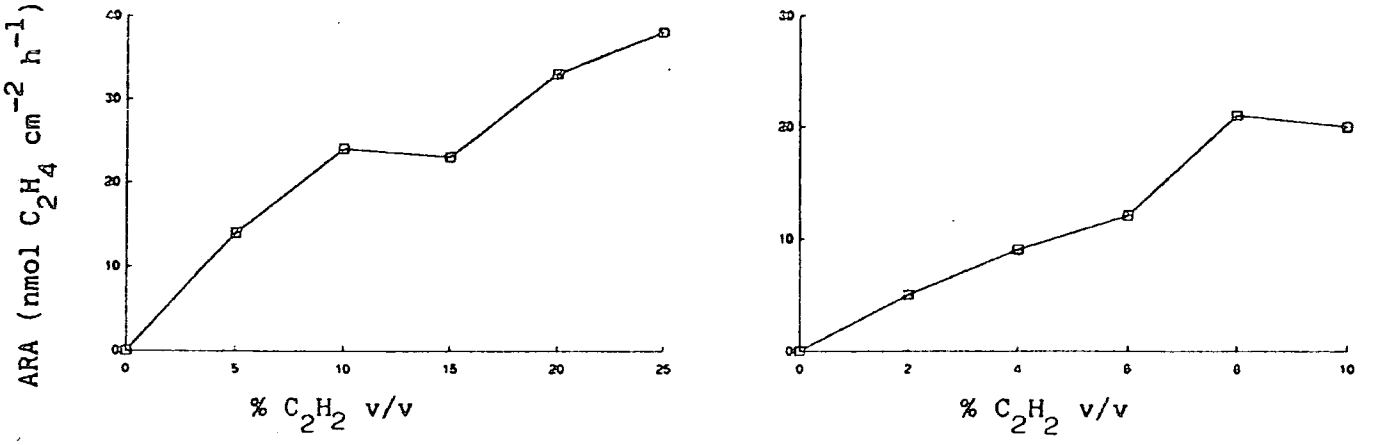
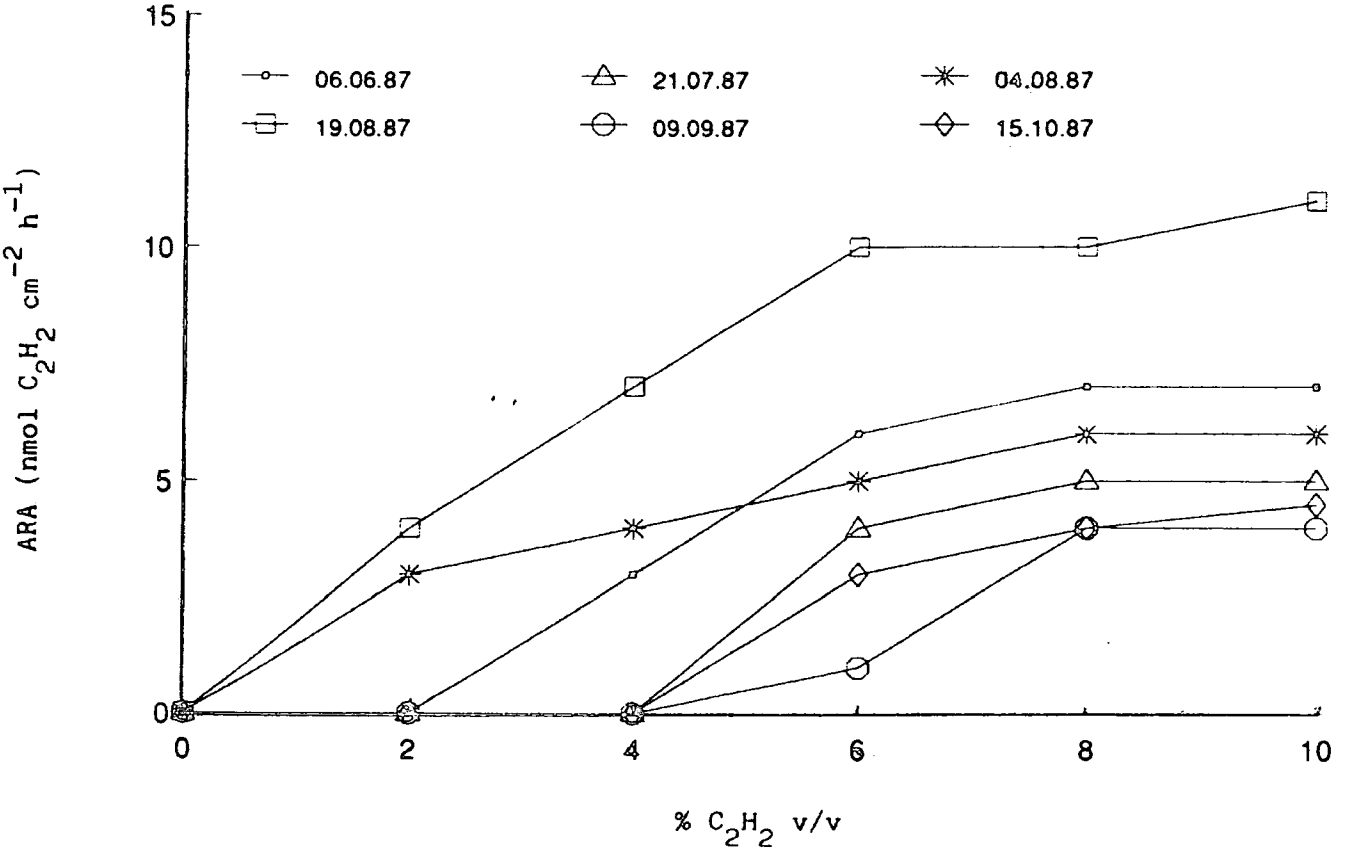


Fig. 2.4 Influence of % C_2H_4 v/v on ARA in Microcoleus mats in situ between 0900 and 1300



2.10.3 Use of Gas Chromatographs for the detection of ethylene

Samples were analysed on a Varian Aerograph (model 1400) gas chromatograph fitted with a flame ionization detector.

Column:	Stainless steel, 175 mm x 2 mm; Poropak R
Oven temperature:	Isothermal, 80°C.
Carrier gas:	N_2 at 45 ml l^{-1}
Other gases:	Air at 300 ml l^{-1}; Hydrogen at 30 ml l^{-1}
Septum type:	Silicon
Retention times:	Ethylene 1 min. 46 s; Acetylene 1 min. 55s

Samples were run against ethylene as well as ethylene and acetylene standards covering the working range.

CHAPTER 3

ENVIRONMENT FROM WHICH *MICROCOLEUS* STRAINS WERE ISOLATED

3.1 Introduction

Samples of soil or sediment were collected from a range of environments (Table 3.1).

Table 3.1 Details of sample collection.

Strain No.	Collected by	date	References	Environment	Country
D634	B.A.Whitton	21.09.79	Al-Mousawi & Whitton (1983)	Salt Marsh	Iraq
D761	B.A.Whitton	05.08.84	Mahasneh (1988)	Lagoon	Seychelle Isl.
D778	A.H.Pearson	xx.xx.78	Pearson *et al.*, (1979)	Intertidal sediment	Britain
D781	D.R.Griffiths	10.12.85	(pers. comm.)	Mangrove forest	Belize
D782	T.N.Tiwari	14.04.89	(pers. comm.)	Usar soil	India

3.2 *Microcoleus* strains

3.2.1 *Microcoleus* D634

Material was pooled from sediment ranging from wet to dry within an area of 2 m^2 and a depth of 3 mm from the southern marshes of Iraq (30° 50′ N, 47° E; Al-Mousawi and Whitton, 1983). The sample site lay near site D (Um al Schwalch) used in a study by Maulood *et al.*, (1981) on the algal ecology of the permanent marshes.

In the southern marshes of Iraq, rice is grown on land which is under water during the peak period of flood (March - April) from the Tigris and Euphrates but which requires earth dams to maintain the water level in the fields during the summer. Rice is planted in late spring and standing water typically remains until late August or September (Al-Mousawi and Whitton, 1983). The soils gradually dry out thereafter.

Immediately after harvesting the land is sometimes ploughed (Buringh, 1960) and may be planted with other crops e.g. wheat, barley or oats during the winter season (Al-Kaisi, 1976). The upper part of the soil consists of very fine loamy sand, silt and clay deposits by the river in flood. The soils are extremely calcareous with a calcium content of 172.3 mg g^{-1} ash weight (Al-Mousawi, 1984) and a moderate to high sodium levels of 172.0 mg g^{-1} ash weight (Al-Mousawi, 1984). Crystals of gypsum and sodium chloride were present in considerable amounts (Al-Kaisi, 1976).

The climate is arid, sub-tropical, characterised by a long, dry, summer with temperatures often exceeding 40°C during July and August. Rainfall of only 200 - 300 mm is restricted to autumn, winter and spring. There are occasional slight frosts during cold winters (General Establishment for Studies and Design, 1979). Maximum relative humidity during the summer lies between 46 - 48% and 70 - 80% in the winter. Evaporation is very high from a free water surface and irrigated land, often exceeding ten times the rainfall (General Establishment for Studies and Design, 1979).

3.2.2 *Microcoleus* D761

Aldabra Atoll is situated in the extreme south west corner of the Indian Ocean (9° 24'S, 46° 20'E). The land rim has a circumference of 96 km, varying in width from 0.25 and 5.0 km and enclosing a lagoon. Passes break the rim and connect the lagoon with the sea (Barnes *et al.*, 1971). The shallow nature of the lagoon (Farrow and Brander, 1971) does not allow tides to flow evenly inside and outside the atoll therefore the tidal range within the lagoon is very much reduced (Potts, 1977) and extreme low water is associated with neap tides rather than spring tides. The land is mainly flat and composed of coral limestone with guano-derived calcium phosphate in several areas (Potts, 1977). Mangroves form an almost continuous fringe around the coast of the lagoon and are the dominant vegetation between the tide marks (Potts, 1977). (The term "mangrove " refers to the assemblage of plants from five families with common ecological, morphological and physiological characters that allow them to live in tidal swamps (Rutzler & Feller, 1987). Tomlinson (1986) cited in Rutzler and Feller, 1987) defines the group of plants by five features: i. they are ecologically restricted to tidal swamps ii). the major element of the community frequently forms pure strands iii). the plants are morphologically adapted with aerial roots and viviparity iv). they are physiologically adapted for salt exclusion or salt excretion and v). they are taxanomically isolated from terrestrial relatives, at least at the generic level).

The atoll experiences two climatic seasons each six months long, the wet season or monsoon running from December to April and the dry season from May to November. Mean summer and winter temperatures are 32°C and 22°C respectively (Farrow, 1971).

3.2.3 *Microcoleus* D778

Microcoleus D778 was isolated from the upper-intertidal sand and mud flats on Church Island, Anglesey, Wales (53° 14'N, 4° 6'W).

The western shores of the Menai Strait are of the Precambrian, mainly of the Carboniferous system (Jones, 1968); superficial deposits are of boulder clay with low to medium base brown soil of good drainage. The shores of the Menai Straits bare no cliffs, the land sloping gently to the sea. Several small rivers run into the straits and since their gathering grounds are at no great height above sea level these rivers tend to be sluggish and consequently develop mudflats and salt marshes at their shallow estuaries.

Microcoleus mats were found in the upper inter-tidal mud, sand and shingle mats of Church island, above the *Fucus* zone (Fig. 3.1). Three sites were chosen for experimentation: site 1, 2 and 3 (Figs. 3.1, 3.2) c. 6 m below the wall surrounding the island and approximately 3 m away from each other. Sites 1 and 2 were submerged by spring tides 5.0 m whereas site 3 composed of *Salicornia* as well as *Microcoleus* mats was submerged by tides > 4.9 m.

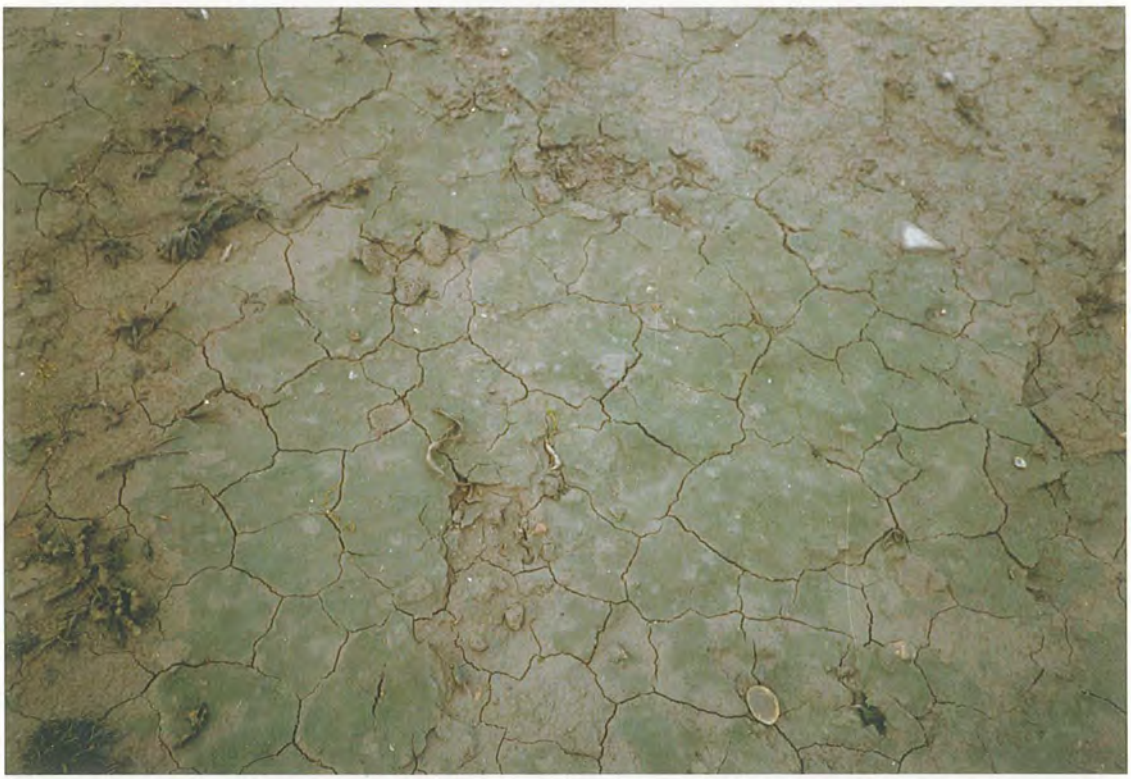
Table 3.2 Climatic data for Anglesey (1916 - 1950).

Month	Air temp. (°C) Holyhead 1921 - 1950	Mean rainfall (") Menai Straits 1916 - 1950	Mean sunshine (h) Menai Straits 1921 - 1950
Jan.	6.1	4.1	55
Feb.	5.8	2.7	75
March	6.8	2.4	125
April	8.4	2.1	174
May	10.7	2.5	219
June	13.3	2.5	222
July	15.1	2.8	185
Aug.	15.4	3.4	175
Sept.	14.3	3.6	139
Oct.	11.4	4.5	102
Nov.	8.6	4.2	60
Dec.	6.7	4.0	40

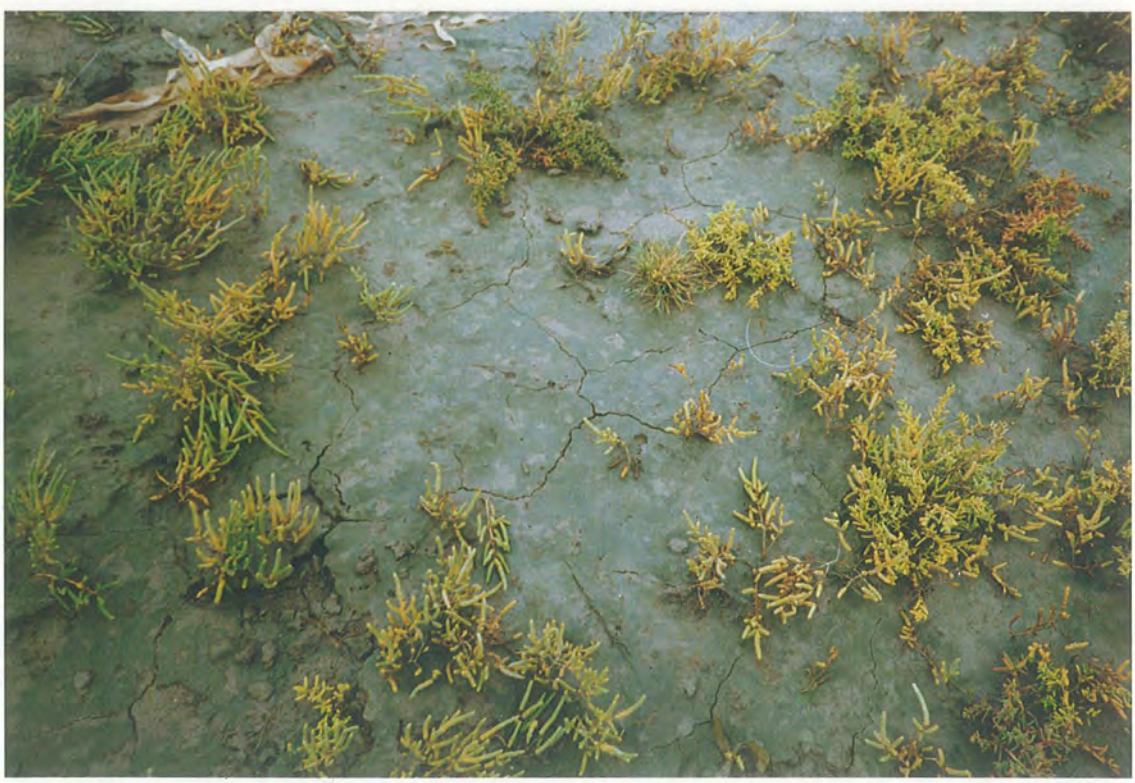
Fig. 3.1 Sites 1, 2 and 3, Church Island (14.8.87)

Fig. 3.2 Uneven distribution of *Microcoleus* mats at sites 1 and 3 of Church Island (14.8.88)

(a) Site 1 (⟼ = 10 cm)



(b) Site 3 *Microcoleus* distributed amongst *Salicornia* plants (⟼ = 10 cm)



3.2.4 Microcoleus D781

Material was collected by D.R.G. Griffith from partly dried out sediment in a mangrove swamp, San Pedro, Belize (17° 15'N, 88° 45' W) off the Caribbean coast of Central America.

In Belize the coast and inlands are closely connected with a 220 Km long barrier reef lying from just a few meters to 40 km offshore. Behind the reef and across the shallow coastal waters dotted with approximately 450 sand and mangrove cays, is the coastline fringed with mangroves and interrupted by numerous coastal lagoons and creek systems (Hain, 1987). Inland across the coastal flatlands lies the tropical lowland rain forest and to the west rise mountains of granite and limestone serving as the origin for a series of short, often estuarine, rivers.

Mangrove swamp communities dominate the world's tropical and sub-tropical coasts, paralleling the geographical distribution of coral reefs. Like reefs, mangrove swamps are environments formed by organisms thriving in the intertidal zone and enduring a wide range of salinities (Rutzler and Feller, 1987) although they are more tolerant of brackish than hyperhaline environments (Long and Mason, 1983). Mangroves are believed to be the principal source of nutrients enriching the coastal waters of Belize and together with the lagoons provide nursery and feeding areas for coastal fish species, function as sediment traps and act as a physical buffer against marine storms (Hain, 1987).

3.2.5 Microcoleus D782

Soil from a section of land previously used by the Agricultural Department of Benaras Hindu University (c. 60 Km west of Varavesi (25° 20' N; 83° 00' E) in N.E. India) but since left fallow for several years was collected by T.N. Tiwari (pers. comm). The sample was collected in April when the area appeared as a blue-green mat composed mainly of Microcoleus sp. but also Anabaena sp, Aphanothece sp., Nostoc sp. and Oscillatoria sp. Microcoleus was dominant from March to mid June. Limited chemical and physical data on the soil gave a pH of 7.9 and a relatively high calcium and sodium content (T.N. Tiwari pers. comm.) .

The climate in this region is characterised by three phases: cool, mainly dry winter from November to February; hot, dry season from March to early June; wet monsoon from the end of June to October. Mean monthly temperatures for Allahabad (100 km west of Varanasi) show the hottest months to precede the onset of rain in June, falling sharply during the monsoon and reaching a modest plateau in July, August and September. Temperatures fluctuate further

with the approach of winter when clear skies lead to a higher diurnal range (Johnson, 1979). The average annual rainfall is 1000 mm, only a small proportion of which falls outside the monsoon, usually as thundery showers in April. No frost has been recorded in the area (Johnson, 1979).

3.3 Survey for *Microcoleus* along a section of the English coastline

A literature survey was carried out to determine where along the British coast *Microcoleus* had previously been recorded. A survey was thereafter carried out along the upper intertidal areas and salt marshes of Lindisfarne and Budle Bay in Northumbria, the Tetney marshes and Donna Nook in Humberside, Gibraltar Point, Saltfleetby, Thedlethorpe dunes and Frampton marsh in Lincolnshire and Blakeney Point in Norfolk, all areas considered geographically suitable for future field experimentation in the likelihood of isolating a positively N_2-fixing strain of *Microcoleus*.

CHAPTER 4

ISOLATION AND CULTURE OF MICROCOLEUS

4.1 Introduction

This chapter is concerned with the isolation of Microcoleus strains, their growth in a defined, inorganic medium and attempts to grow them in a form morphologically akin to that in the field (i.e. with a communal sheath).

There have been numerous accounts of the occurrence of Microcoleus, the most widely studied having been in intertidal mats and salt marshes. A survey was carried out to locate and thereafter isolate Microcoleus from the upper intertidal areas and salt marshes of Lindisfarne and Budle Bay in Northumbria, the Tetney marshes and Donna Nook in Humberside, Gibraltar Point, Saltfleetby and Thedlethorpe dunes and Frampton marsh in Lincolnshire and Blakeney Point in Norfolk. Samples of sediment and soil were received from a mangrove swamp, Belize (3.2.4) and Varanasi, India (3.2.5) respectively.

In addition three strains of Microcoleus were received: M. chthonoplastes (D778), isolated by **Pearson** et al. (1979) and rendered axenic by R. Rippka at the Pasteur Institute; M. chthonoplastes (D634) collected by B.A. Whitton (1979) isolated and rendered axenic by Al-Mousawi (1984) and M. chthonoplastes D761 collected by B.A. Whitton (1979) and isolated into clonal culture by I. Mahasneh (pers. comm.).

4.2 Morphology of Microcoleus in the natural environment

Microcoleus mats were visible to the naked eye as uneven blue-green patches. Its presence was confirmed microscopically at Gibraltar Point, Blakeney Point, Tetney Marshes, Frampton marshes Lindisfarne and at Church Island.

Cross sections of cores showed Microcoleus mats c. 0.5 mm below the surface overlain with sand and sediment grains and overlying a thin rust coloured layer above either an oxidised brown layer or a grey-black, presumably oxic/micro-oxic layer (Fig. 4.1). The depth and appearence

Fig. 4.1 Sediment core from Microcoleus mats collected at site 1 (14.8.88) and stored in a greenhouse overnight

(a) L.S. (⟵⟶ = 0.2 mm)

(b) Surface view (⟵⟶ = 0.8 mm)

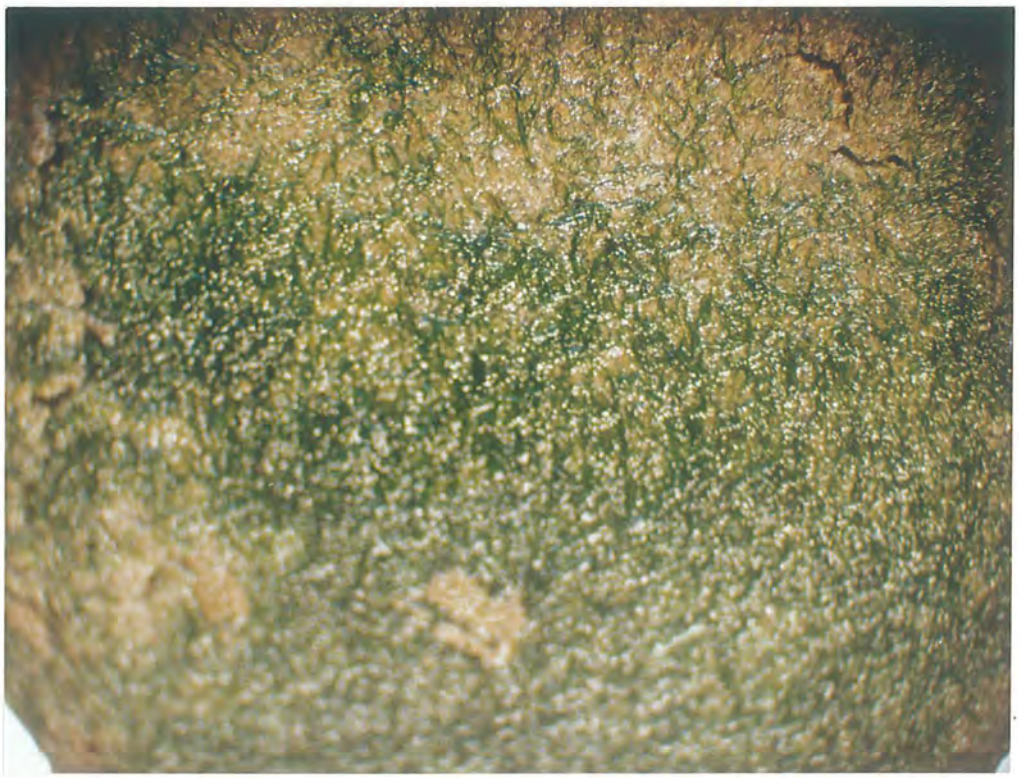
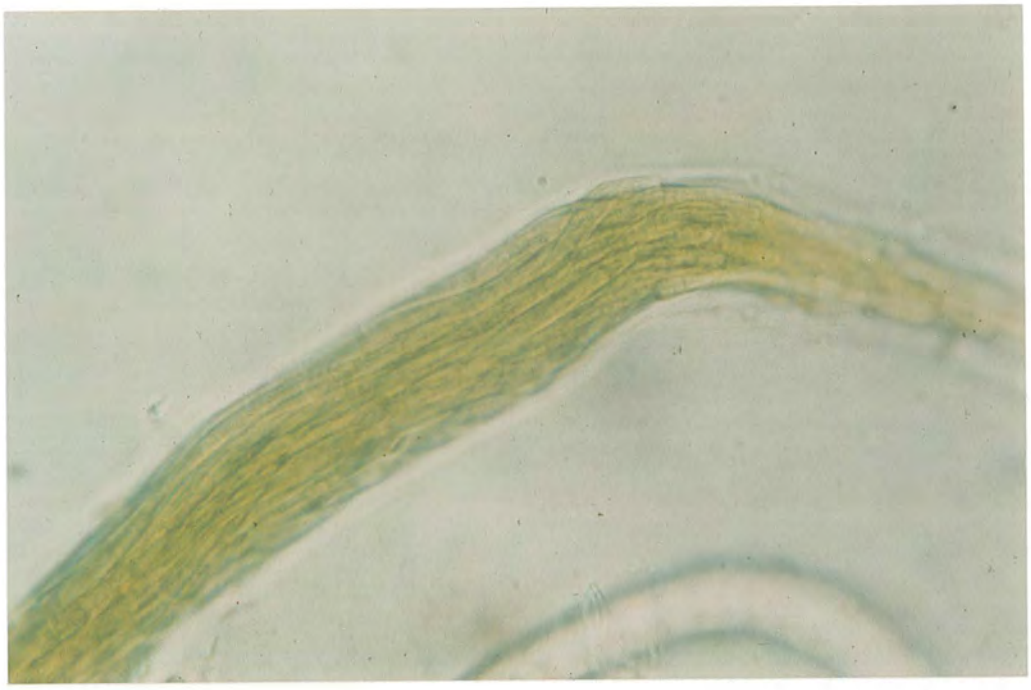


Fig. 4.2 Microcoleus from sediment collected at site 1, Church Island

(a) Numerous trichomes within a colourless sheath (——— = 10 um)



(b) Closed end of sheath stained with ruthenium red (——— = 10 um)

of the these areas varied over a diurnal cycle. Microcoleus sheaths containing blue-green filaments were present in the sediment of Gibraltar Point at depth of up to 16 mm.

In all cases Microcoleus was found to possess a colourless, communal sheath variable in length (600 mm - 3505 mm) and diameter (23 - 161 mm) and generally becoming shorter and wider with depth. Numerous filaments coiled in a rope-like fashion were arranged within the sheath (Fig 4.2a), the diameter of the combined filaments being 50 - 90% that of the sheath and generally increasing with depth. Whilst one end of the sheath was attenuated (Fig 4.2b) the other was open with one or more filaments protruding. When mats were kept 'just' moist with Milli-Q water or submerged in seawater Microcoleus retained their sheaths; however, when mats were submerged in Milli-Q water sheaths were lost and Oscillatoria was co-dominant with Microcoleus within 72 h.

4.3 Isolation

Two Microcoleus strains were isolated using modified GD: Microcoleus D781 from a mangrove swamp in Belize and Microcoleus D782 from usar soil India. In both cases Microcoleus was isolated from sediment (moistened by addition of sterile Milli-Q) placed in a petridish with a microscope coverslip lying on it. After a two week incubation at 30 $\pm$ 3 μmol photon $m^{-2}s^{-1}$ 32.0 $\pm$ 0.5 C, dense growth of Microcoleus was found on the coverslip. Transfer to agar failed to isolate Microcoleus as bacterial growth was prevalent. A sample of enriched cyanobacteria from mangrove swamp soil transferred to liquid medium saw rapid growth of Phormidium with only low levels of Microcoleus and Lyngbya. However, after c. 10 days Phormidium became chlorotic whilst Microcoleus remained a healthy blue-green colour. A sample of this mixed culture was sub-cultured into a range of media. All + N media supported Phormidium whilst - N media favoured either Lyngbya or Microcoleus. Microcoleus grew most favourably in GD_5+N containing 125 mM Ca^{2+} at 32 $\pm$ 0.5°C, in 14(50 μmol photon m^{-2} s^{-1}):10 light:dark. Samples were then gently homogenised before serial dilutions were prepared for streaking and spraying onto agar plates. After c. 48-h single cells or short filaments less than six cells in length (unavoidably heavily bacterised) were transferred to 5 ml volumes of medium in test tubes. A clonal culture of Microcoleus D781 was obtained in this way. A sample of enriched cyanobacteria from usar soil also supported Phormidium in liquid culture but transfer to -N medium as explained above failed to enrich for Microcoleus. However, incubation of the mixed culture in the absence of combined nitogen under micro-oxic conditions (replacing air with N_2) and adding Na_2CO_3 saw a gradual chlorosis in both Phormidium and Lyngbya over a

four week period whilst Microcoleus (D782) remained healthy. On transferring to the above media under the same conditions, Microcoleus could only be isolated in a medium with combined nitrogen.

Since modified GD had been suitable for the isolation of two Microcoleus strains and was able to support five Microcoleus strains from a range of temperate and sub-tropical environments, the medium was employed for the further isolation of Microcoleus strains from all other sites.

Microscopic examination of sediments collected at Gibraltar Point, Frampton Marsh, Tetney marshes, Blakeney Point and Lindisfarne showed several genera of cyanobacteria including Microcoleus. Seven genera of cyanobacteria were enriched from these sites. Nodularia sp. and Pseudanabaena sp. were co-dominant in the absence of combined nitrogen whilst Oscillatoria sp. and Lyngbya sp. were co-dominant in the presence of combined nitrogen. Also present in the latter were traces of Microcoleus, Schizothrix and Spirulina. Despite changes in the concentration of nutrients (Na^+, K^+, Ca^{2+}, Mg^{2+}), as well as in the temperature and PAR, Microcoleus could not be isolated. All attempts to transfer sample to - N medium (as for the isolation of Microcoleus D781) and to a micro-oxic environment (as for the isolation of Microcoleus D782) failed to select Microcoleus.

Mixed cultures containing Microcoleus were subjected to osmotic shocks by changing the salinity of the surrounding medium (2.4.4). Oscillatoria was enriched in + N medium whilst all genera in - N medium became chlorotic after 4 days.

Sediment cores placed at the centre of agar plates saw the growth of heavily contaminated Microcoleus filaments into the surrounding agar. Transfer of agar blocks containing hormogonia and filaments to a fresh agar plate, sloppy agar and liquid culture failed to support growth.

Isolations in modified Erdschreiber medium (Table 2.7) and MN, a marine medium patterned on BG-11 and containing seawater (Waterbury and Stanier, 1978) were also unsuccessful.

Diatoms, mainly as empty frustules were found in all media, both in the presence and absence of combined nitrogen.

4.4 Purification

All attempts to purify Microcoleus D761, D781 and D782 by physical manipulation, homogenization and sonication, the atomizer technique, the pour-plate technique, osmotic shock, phototaxis and using antibiotics were unsuccessful, although the latter eliminated the Gram positive bacteria

leaving strains contaminated by only one or two Gram negative, rod-shaped bacteria.

4.5 Morphology of *Microcoleus* strains in culture

A summary of the strains available and their morphological characteristics is given in Table 4.1.

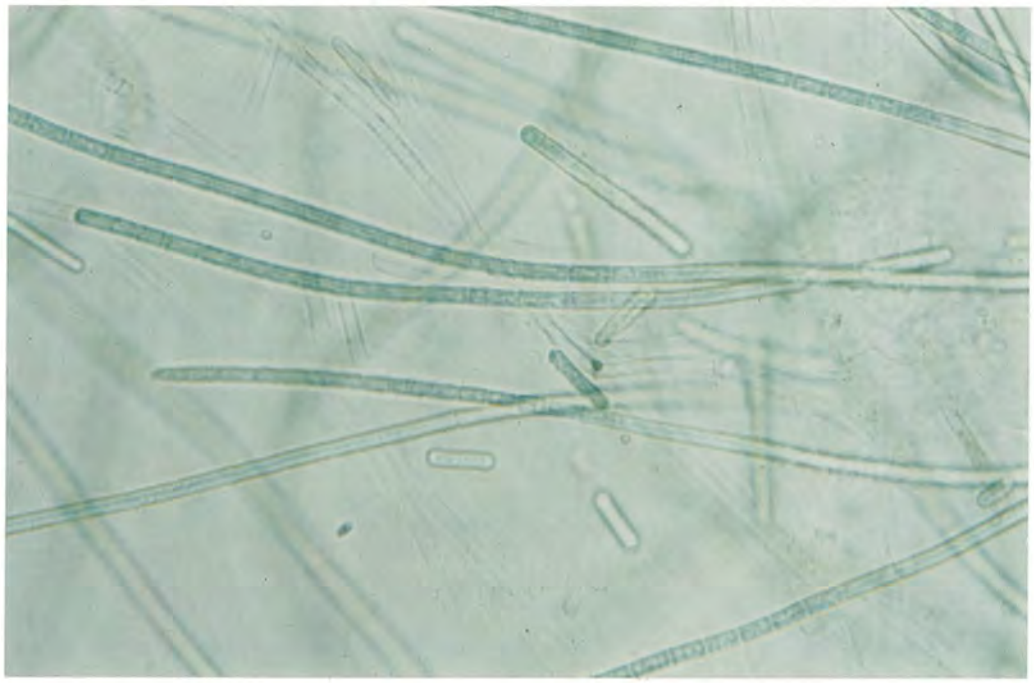
Table 4.1 A summary of the *Microcoleus* strains available and their morphological characteristics.

Microcoleus strain	Medium	Temperature (°C)	Sheath	Cell Diameter (mm)	Length (mm)	Axenic (+/-)
D634	GD_5+N	32.0 ± 0.5	-	4 - 5	4 - 12	+
D761	GD_5+N	32.0 ± 0.5	-	3 - 4	5 - 10	-
D778	GD_5-N	20.0 ± 0.5	-	4 - 5	6 - 7	+
D781	GD_5+N	32.0 ± 0.5	-	3 - 4	4 - 6	-
D782	GD_5+N	32.0 ± 0.5	-	4 - 5	5 - 7	-

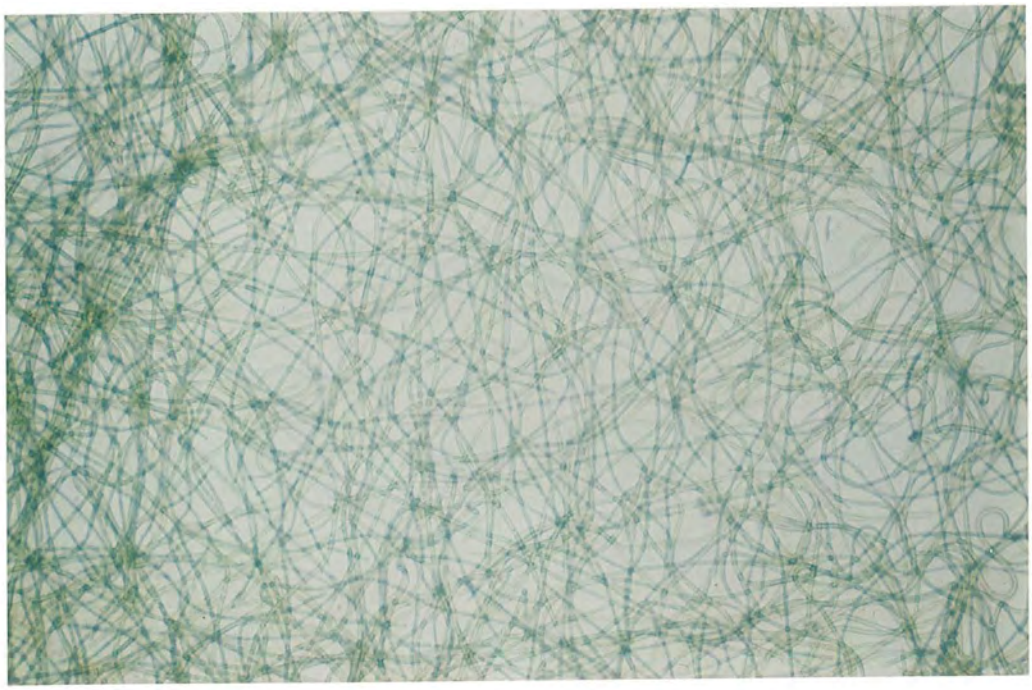
Microcoleus D634, D761 and D781 grew caespitotically on the bed of Erlenmeyer flasks, coalescing to form a complete mat around mid. log and thereafter rising to the surface. Growth continued both in this mat and on all liquid to glass surfaces. Provided the flasks were not disturbed the mat remained flat whilst disturbed mats formed 'gelatinous structures' floating just below the surface of the medium. *Microcoleus* D778 grew as described during early logarithmic growth; however, loose

Fig. 4.3 Morphology of Microcoleus D 634 in liquid medium GD_5-N. 20°C, 14:10 light (50 umol photon m^{-2} s^{-1}):dark

(a) Filaments, hormogonia and sections of empty sheath (——— = 10 um)



(b) 'Lattice like' arrangement of filaments (——— = 50 um)



'wool-like' growth was present thereafter in the body of the liquid when light was incident from below (no incubation area was available in a light:dark cycle with light incident from above). After 1 - 2 days of caespitotic growth Microcoleus D782 was replaced by several small (diameter c. 5 mm), gelatinous structures.

Mats were composed of long, unbranched, slightly coiled blue-green filaments (Fig. 4.3) with empty sections at irregular intervals.

4.6 Sheath

Mucilage was produced by all strains and secreted into the medium (as indicated by the texture of the medium and staining with Indian Ink) during log growth and over the stationary phase.

All strains possessed a sheath around the individual trichomes (Fig. 4.3, 4.4) but lost their communal sheath in culture, whether maintained in liquid or on agar. The structure of the cell envelope of Microcoleus D778 at mid log in medium GD_{30}-N at 20.0 $\pm$ 0.5 C in 16:8 light (50 + 3 mmol photon m^{-2} s^{-1}):dark shows a 230 nm wide peptidoglycan layer surrounding the electron transparent LI. A lamellated sheath 460 nm wide was seen around the outer membrane (Fig. 4.4).

All attempts to grow these strains with a communal sheath, including variations in light periodicity, intensity and quality, altering water availability, varying the nutrient concentrations (Table 2.11) and exposing them to osmotic shock (changing the salinity of the medium by 4‰ every 2 h) were unsuccessful. However, when Microcoleus D634 and D778 were injected to into modified Winogradsky columns (2.4.9) to a depth of 2 mm and incubated in unidirectional light, filaments aligned and intermittently 2 - 3 trichomes were found within one sheath. Similar structures were seen in Microcoleus D634 grown in liquid medium supplemented with 516 mM Na and 125 mM Ca (salinity of 30‰) under electron microscopy, when a thin (c. 350 nm) sheath was found around three trichomes (Fig. 4.5).

Fig. 4.4 T.S. of a filament of Microcoleus D634 at mid-log in liquid medium GD+N at a salinity of 5‰. 32°C, 14:10 umol photon m^{-2} s^{-1}):dark (⟵⟶ = 0.5 µm)

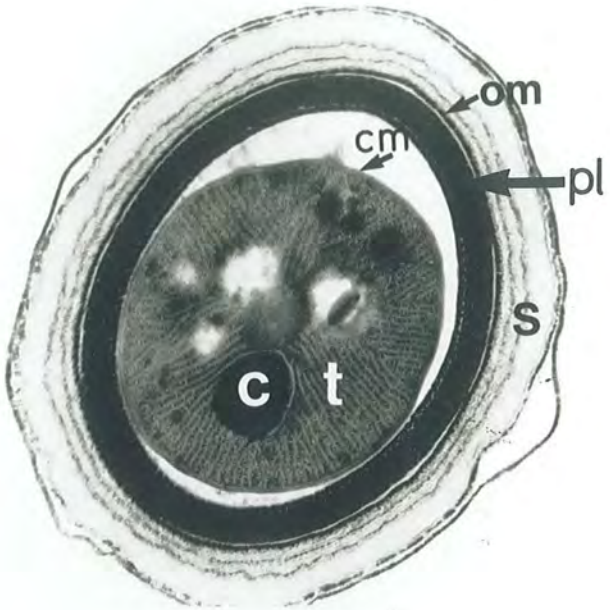
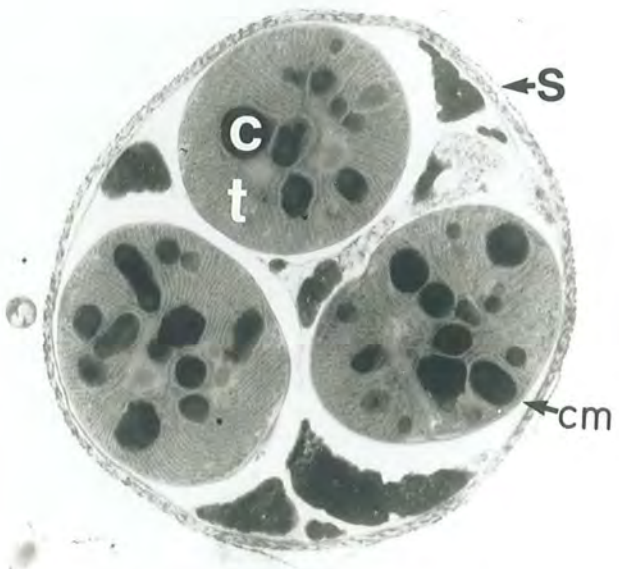


Fig. 4.5 T.S. of Microcoleus D634 at mid-log in liquid medium GD+N containing 125 mM Ca^{2+} at a salinity of 30‰. 32°C, 14:10 light (50 umol photon m^{-2} s^{-1}):dark (⟵ 1.0 µm)



CHAPTER 5

EFFECT OF ENVIRONMENTAL VARIABLES ON MICROCOLEUS STRAINS APPARENTLY UNABLE TO FIX N_2

5.1 Introduction

This chapter is concerned with attempts to grow Microcoleus strains D634, D761, D781 and D782 in a medium free of combined nitrogen and to test for nitrogenase activity indirectly by acetylene reduction activity (ARA). Microcoleus D634, D761, D781 and D782 were compared over a range of salinities, nutrient concentrations, temperature and PAR under oxic and micro-oxic conditions, in order to determine the range and optimum conditions for growth (and possibly nitrogenase activity). Since Microcoleus D634 was axenic emphasis was placed on this strain.

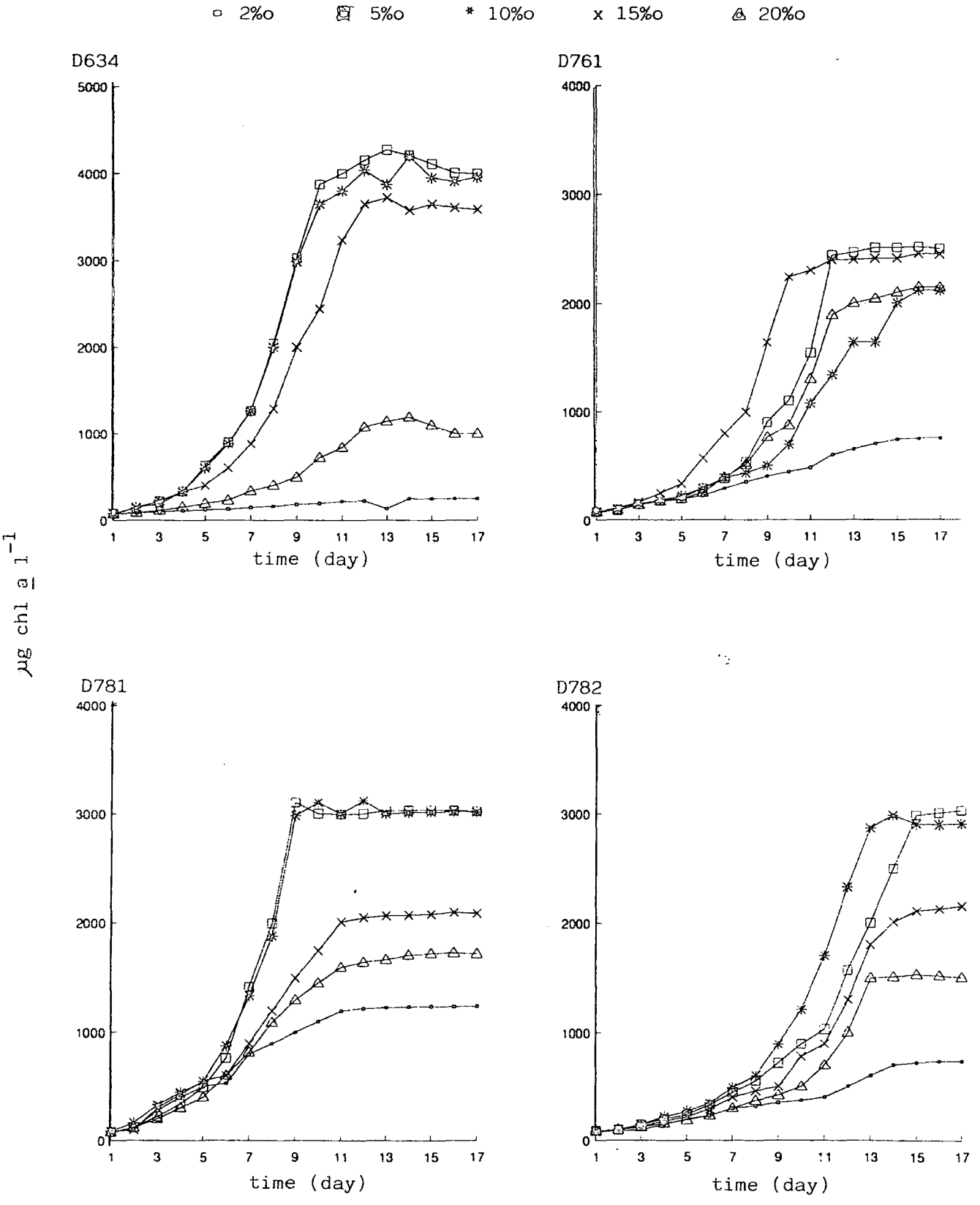
5.2 Growth

Growth (µg chl *a* l^{-1} and mg l^{-1} dry weight) was compared at salinities (addition of NaCl to medium GD+N) of 2, 5, 10, 15 and 20‰ at 32 ± 0.5 C in 14:10 light (50 ± 3 µm photon m^{-2} s^{-1}):dark. Growth, as indicated by the doubling times (t_d) was optimal for Microcoleus D634, D781 and D782 at a salinity of 5‰ and for Microcoleus D761 at 5 and 10‰. (Table 5.1; Figs. 5.1 & 5.2). Chl *a* levels were c. 1% that of dry weight during exponential growth.

Table 5.1 Growth rates of Microcoleus D634, D761, D781 and D782 at salinities of 2, 5, 10, 15 and 20‰. 32 C, 14:10 light (50 μmol photon m^{-2} s^{-1}):dark

Salinity ‰	Mid. log Chl a l^{-1}	day	Growth rates μ	K	G
Microcoleus D634					
2	129.2 ± 13.3	6	0.11 ± 0.01	0.15 ± 0.01	0.49 ± 0.07
5	608.5 ± 40.4	5	0.51 ± 0.02	0.73 ± 0.03	1.37 ± 0.07
10	619.5 ± 54.6	5/6	0.40 ± 0.02	0.57 + 0.03	1.74 ± 0.09
15	490.9 ± 36.3	5/6	0.30 ± 0.01	0.43 + 0.02	2.38 ± 0.08
20	237.2 ± 34.7	6	0.23 ± 0.01	0.33 ± 0.02	3.07 ± 0.10
Microcoleus D761					
2	400.0 ± 20.0	8	0.20 ± 0.01	0.261 ± 0.015	4.80 ± 0.17
5	432.7 ± 28.7	7/8	0.25 ± 0.01	0.357 ± 0.020	2.80 ± 0.14
10	357.8 ± 37.8	6/7	0.26 ± 0.01	0.380 ± 0.019	2.63 ± 0.13
15	330.3 ± 27.7	5	0.37 ± 0.02	0.530 ± 0.021	1.89 ± 0.14
20	368.6 ± 30.1	6/7	0.25 ± 0.01	0.367 ± 0.018	2.70 ± 0.14
Microcoleus D781					
2	500.6 ± 31.0	5/6	0.26 ± 0.01	0.371 ± 0.016	2.60 ± 0.135
5	545.1 ± 30.3	4/5	0.46 ± 0.06	0.650 ± 0.033	1.54 ± 0.11
10	365.0 ± 29.6	4/5	0.38 ± 0.05	0.541 ± 0.034	1.85 ± 0.09
15	501.6 ± 29.1	5	0.28 ± 0.02	0.390 ± 0.030	2.40 ± 0.09
20	521.0 ± 30.0	5	0.20 ± 0.02	0.341 ± 0.029	2.91 ± 0.09
Microcoleus D782					
2	290.6 ± 29.0	6/7	0.10 ± 0.01	0.146 ± 0.007	5.01 ± 0.16
5	492.8 ± 36.8	6/7	0.30 ± 0.02	0.438 ± 0.022	2.28 ± 0.11
10	546.8 ± 39.8	8	0.26 ± 0.01	0.372 ± 0.021	2.69 ± 0.13
15	390.1 ± 29.1	6/7	0.25 ± 0.01	0.360 ± 0.018	2.81 ± 0.13
20	350.6 ± 30.2	6/7	0.20 ± 0.01	0.396 ± 0.019	4.20 ± 0.11

Fig. 5.1 Growth of Microcoleus D634, D761, D781 and D782 in medium GD+N at salinities of 2, 5, 10, 15 and 20‰. 32°C, 50 µmol photon m^{-2} s^{-1}



5.3 Effect of nutrient concentration on growth.

See 2.4.4 for methods

5.3.1 Salinity (addition of NaCl)

Microcoleus D634, D761 and D781 grew at salinities between 1 and 35‰ with optima between 5 - 6‰ (86 - 103 mM Na) 4 - 5‰ (69 - 86 mM Na) and 4 - 5‰ (69 - 86 mM Na) respectively (Fig. 5.2, 5.3), whereas Microcoleus D761 grew at salinities between 1 and 38‰. (17 - 654 mM Na^+) with an optimum between 10 and 16‰ (172 and 275 mM Na) (Fig. 5.3). Optimum salinities were the same irrespective of whether cultures were maintained at salinities of 5, 10, 15 or 20‰ (86, 172, 258 or 344 mM Na) 60 days prior to the experiment.

Neither Microcoleus D634 nor D761 grew in the absence of Na (Fig. 5.4) whereas Microcoleus D781 and D782 grew poorly at mid-log in the absence of Na^+ (Fig. 5.4); phaeopigments were present and no further growth occurred after 6 days in the absence of Na. A minimum of 7 mM Na (salinity of 0.4‰) was required for growth to proceed after 6 days.

Cultures could survive (2.2.4) but not grow at salinities in excess of 35‰: the longer the incubation period the lower the salinity tolerated e.g. 80‰ (1376 mM Na) for 3 h but not 6 h, 60‰ (1032 mM Na) for 6 h but not 12 h, 50‰ (860 mM Na) for 12 h but not 24 h.

Fig. 5.2 Influence of salinity (addition of NaCl) on growth of Microcoleus D634, D761, D781 and D782. 32°C, 14:10 light (50 µmol photon m^{-2} s^{-1}):dark

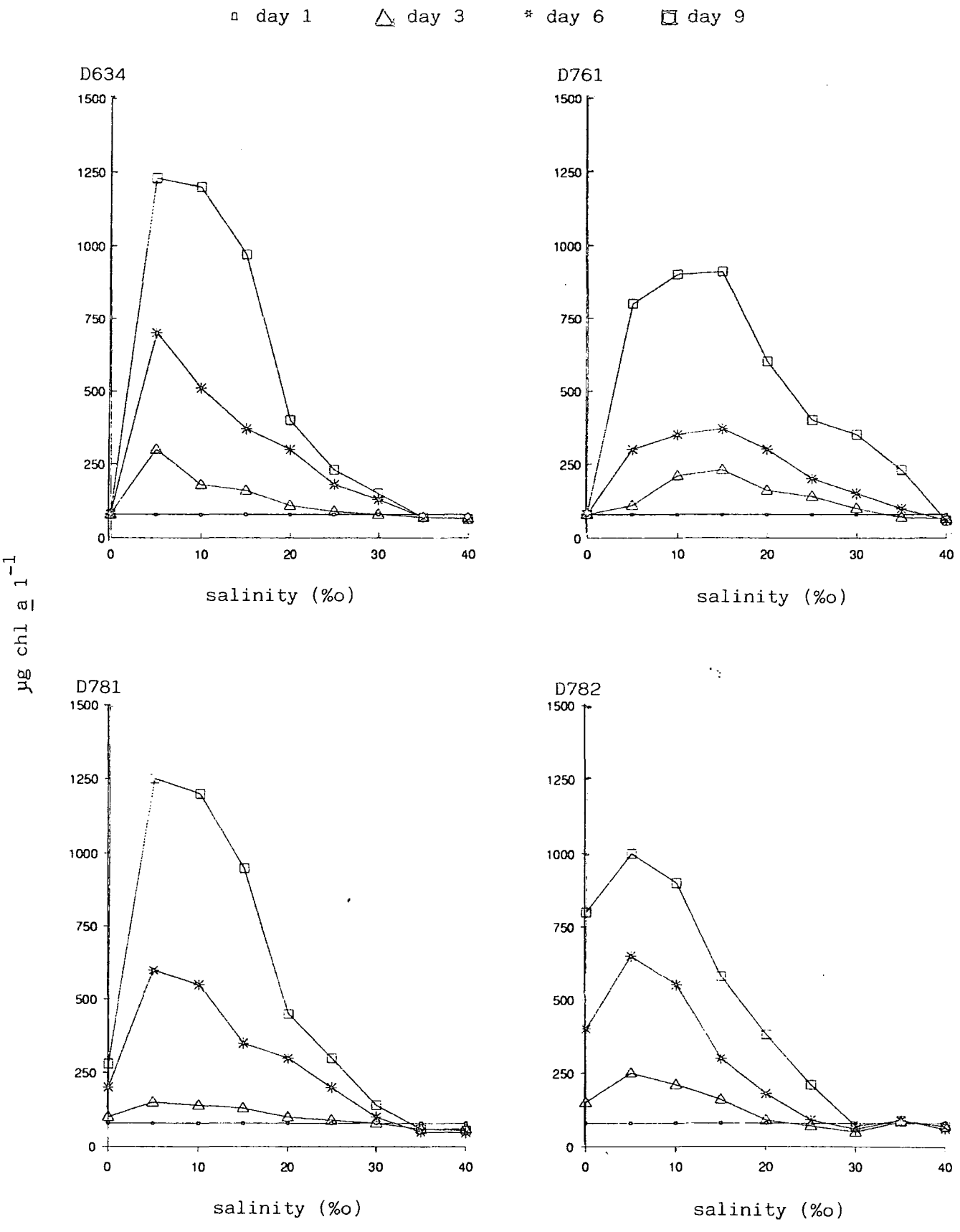


Fig. 5.3 Influence of salinity (addition of NaCl) on growth Microcoleus D634, D761, and D782 at their optimum range. 32°C, 14:10 light (50 µmol photon m^{-2} s^{-1}):dark

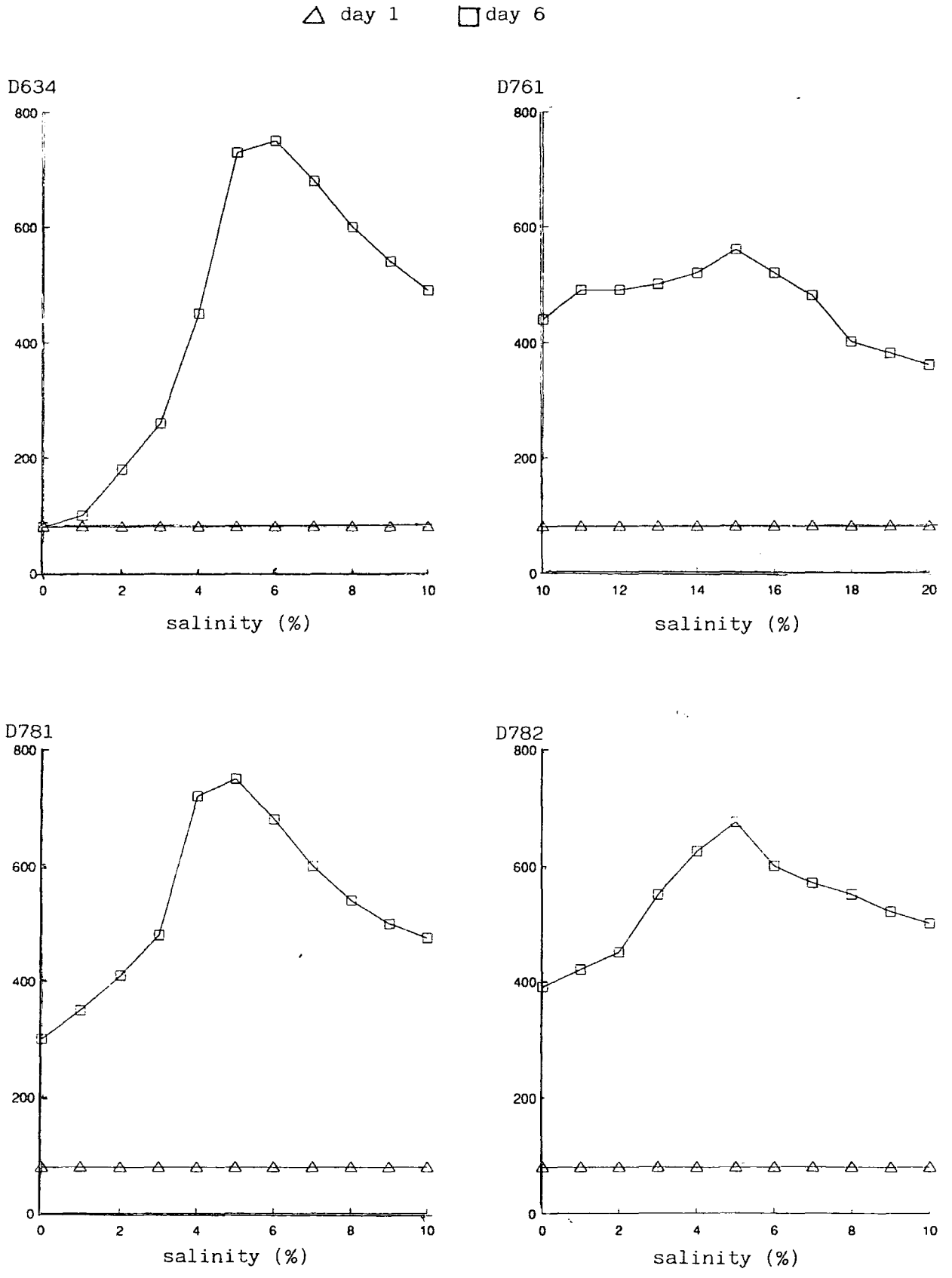
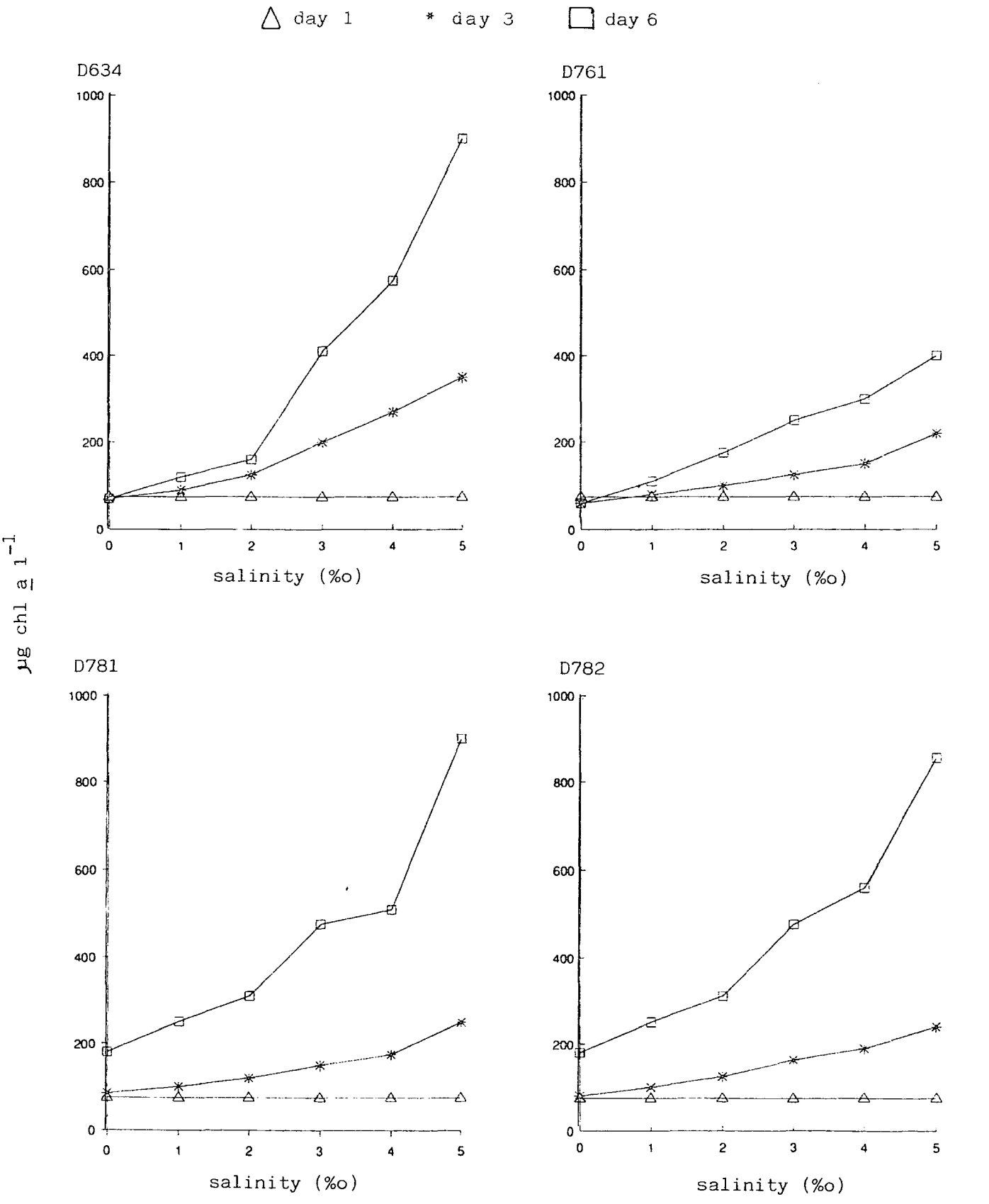


Fig. 5.4 Influence of low salinities (addition of NaCl) on growth in Microcoleus D634, D761, D781 and D782. 32°C, 14:10 light (50 µmol photon m^{-2} s^{-1}):dark



5.3.2 Calcium (as $CaCl_2.2H_2O$)

No growth occurred in the absence of Ca (Fig. 5.5 - 5.8) whilst maximum growth was found between 5 and 100 mM (Fig. 5.5 - 5.8) the precise value depending on the strain and the salinity of the medium (Fig. 5.9). At Ca concentrations in excess of 450 mM a precipitate was found even when phosphorus, nitrogen and sodium (85 mM) were added after autoclaving. AAS showed this to be due to depostition of calcium and sodium.
Although Microcoleus D634 could not grow at salinities (addition of NaCl) $\geq$ 40%; it could survive shorter periods at this and higher salinities, depending on the concentration of calcium present (Fig. 5.9): 2 mM Ca could not support growth at a salinity of 40% (688 mM Na); however as the concentration of calcium increased from 5 - 20 mM so growth increased at salinities of 10 - 30% (172 - 516 mM Na) (Fig. 5.9).

Fig. 5.5 Influence of calcium on growth of Microcoleus D634, D761, D781 and D782 in medium GD_5+N. 32°C, 14:10 light (50 μmol photon m^{-2} s^{-1}):dark

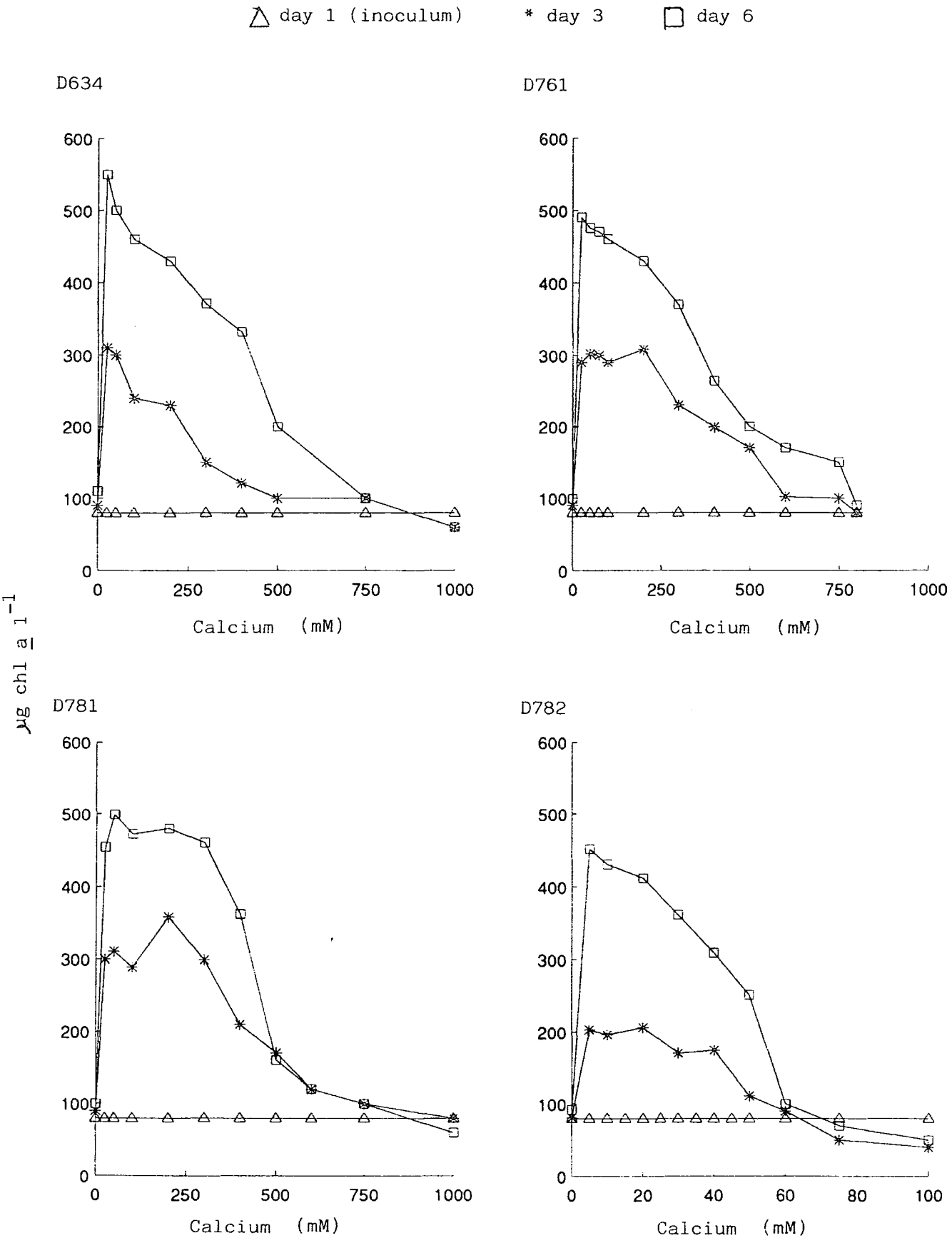


Fig. 5.6 Influence of calcium on growth of Microcoleus D634, D761, D781, D782 in medium GD_{10}+N. 32°C, 14:10 light (50 μmol photon m^{-2} s^{-1}):dark

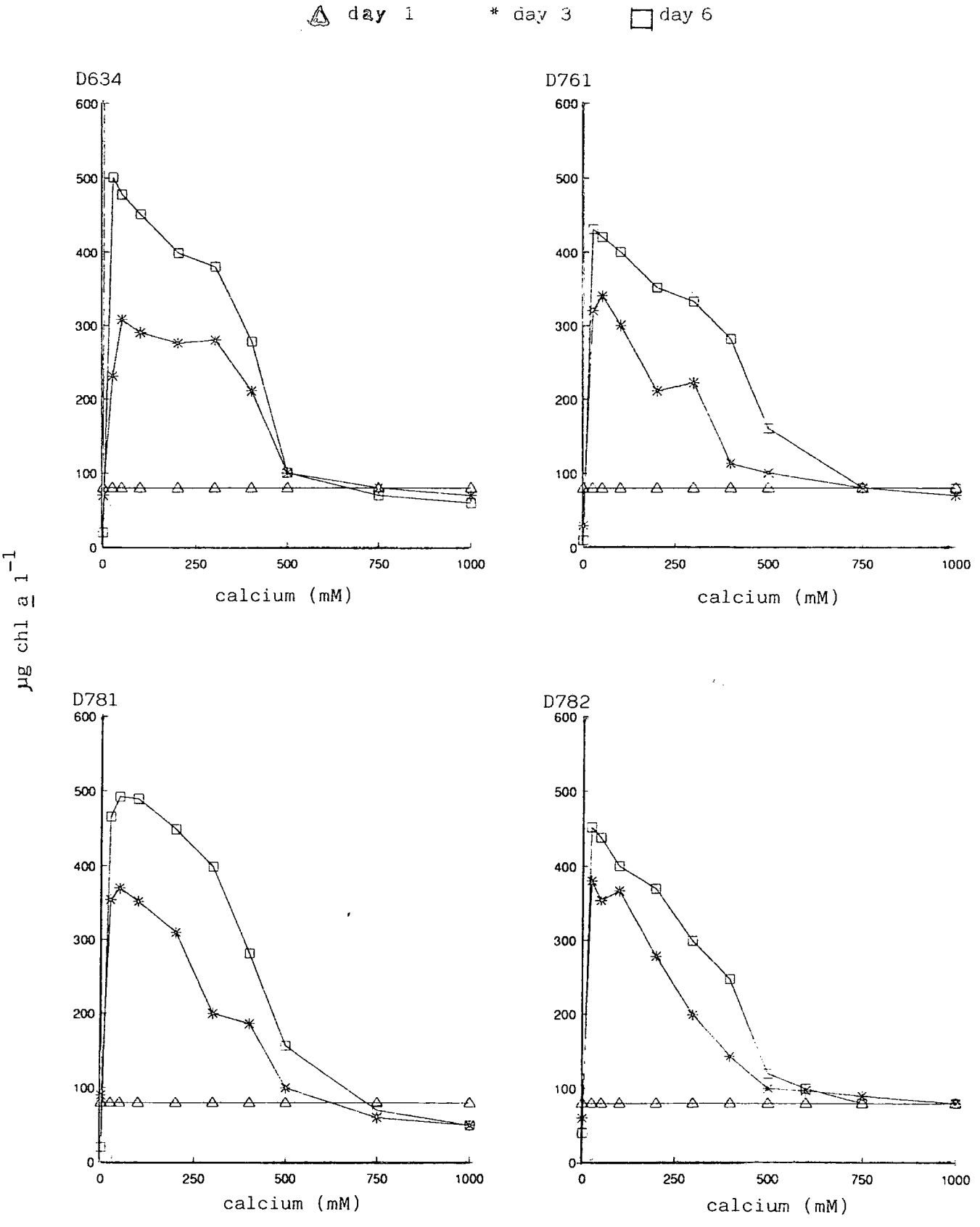


Fig. 5.7 Influence of calcium on % growth of Microcoleus D634, D761, D781 and D782 after 48-h in medium GD_5+N or GD_{10}+N. 32°C, 14:10 light (50 μmol photon m^{-2} s^{-1}):dark

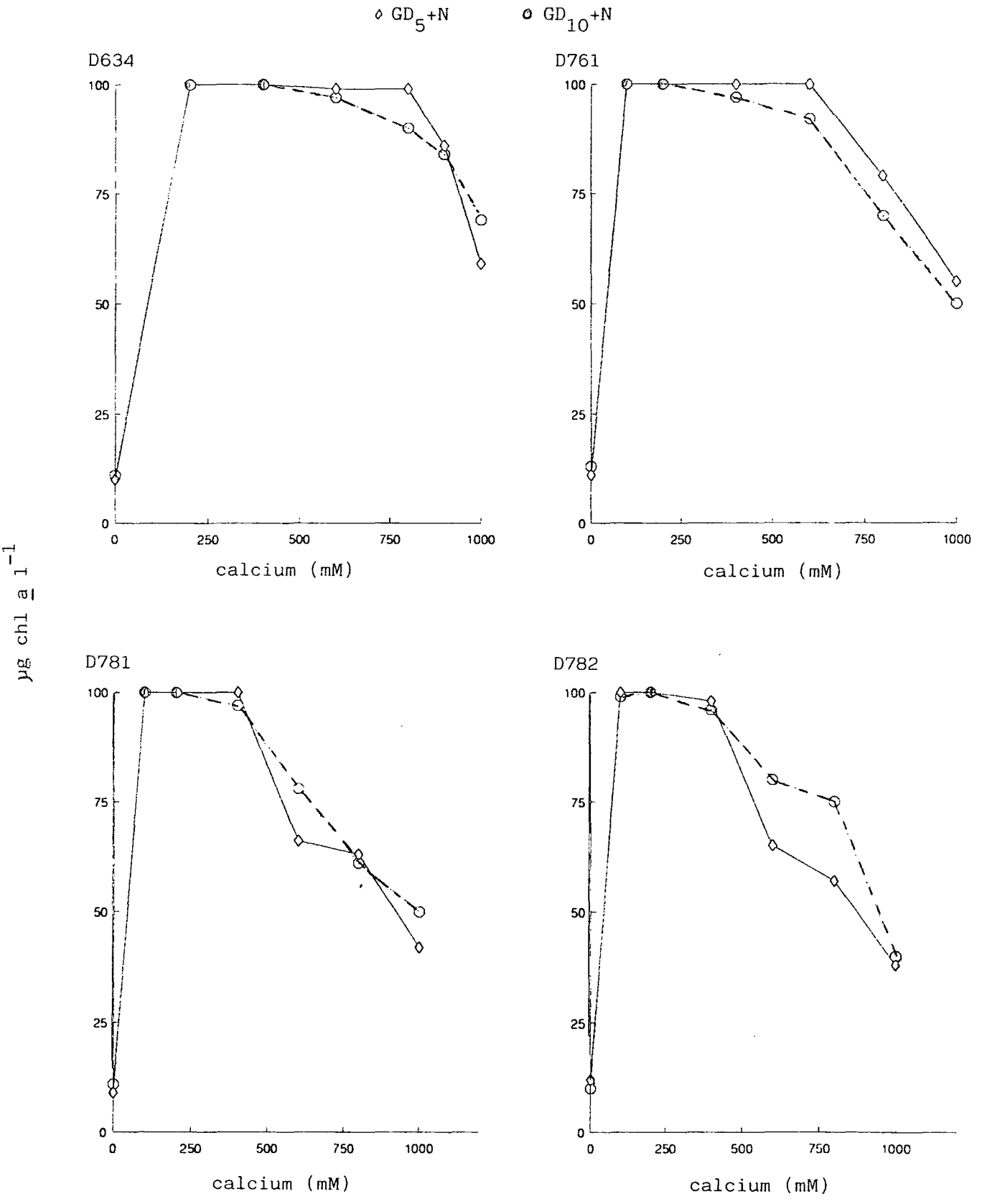


Fig. 5.8 Influence of calcium at a lower range on growth of Microcoleus D634. 32°C, 14:10 light (50 µmol photon m^{-2} s^{-1}):dark

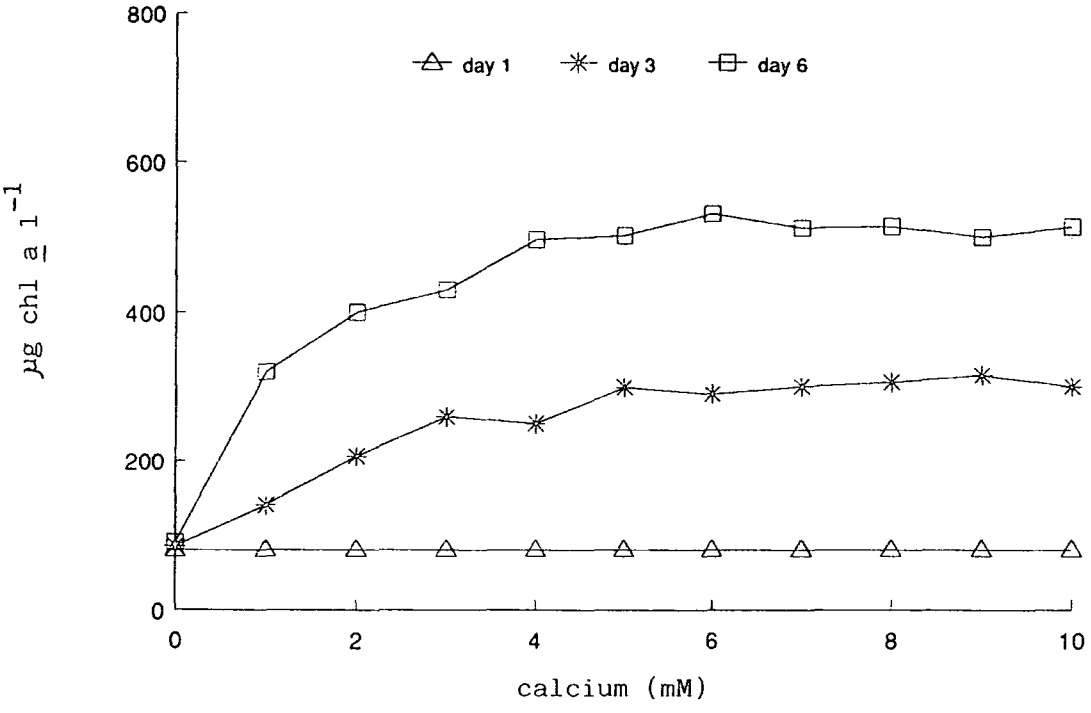
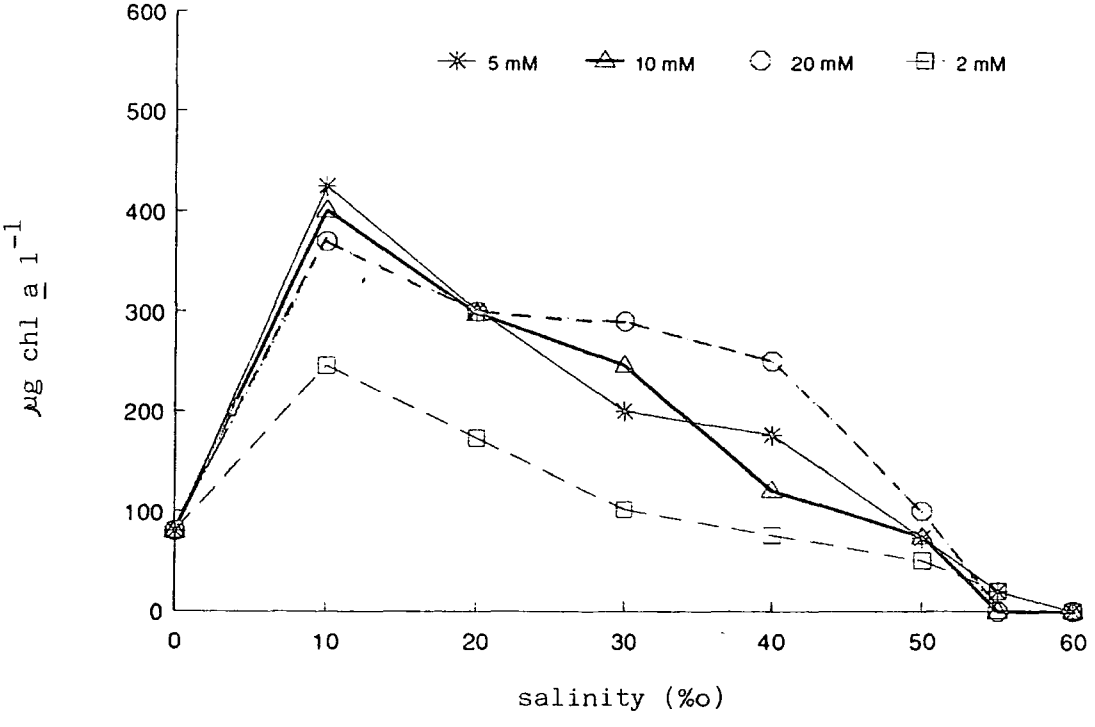


Fig. 5.9 Influence of salinity and calcium concentration on growth of Microcoleus D634. 32 °C, 14:10 light (50 µmol photon m^{-2} s^{-1}):dark



5.3.3 Potassium (as KCl)

A minimum of 50 mM K^+ was required for maximum growth of Microcoleus D634 and D782, 75 mM for Microcoleus D781 and 100 mM K^+ for Microcoleus D761 at salinities of 5 and 10‰ (Fig. 5.10). At K^+ concentrations in excess of 100 mM growth of Microcoleus D634, D761 and D781 was higher at a salinity of 10‰ (172 mM Na) than at 5‰ (86 mM Na), whereas the reverse was true of Microcoleus D782 (Fig. 5.10).

At salinities of 10, 20, 30 and 40‰ (172, 344, 516 & 688 mM Na), growth varied according to the concentration of K^+ (Table 5.11); the higher the concentration of K^+, the higher the concentration of chl a after 6 days. Survival at salinities in excess of 30‰ (516 mM Na) was dependent on the incubation period as well as on the concentration of K^+ (Table 5.2).

Table 5.2 Survival (+/-) growth of Microcoleus D634 at salinities of 30 - 50‰ at 10 or 25 mM K^+. 32 C, 50 mmol photon m^{-2} s^{-1}

Salinity	Incubation period (h)			
‰	6	12	18	24
At 10 mM KCl				
30	+	+	+	+
35	+	+	+	+
40	+	+	+	-
45	+	-	-	-
50	-	-	-	-
At 25 mM KCl				
30	+	+	+	+
35	+	+	+	-
40	+	+	-	-
45	-	-	-	-
50	-	-	-	-

Fig. 5.10 Influence of potassium on growth of Microcoleus D634, D761, D781 and D782. 32°C, 50 µmol photon m^{-2} s^{-1}

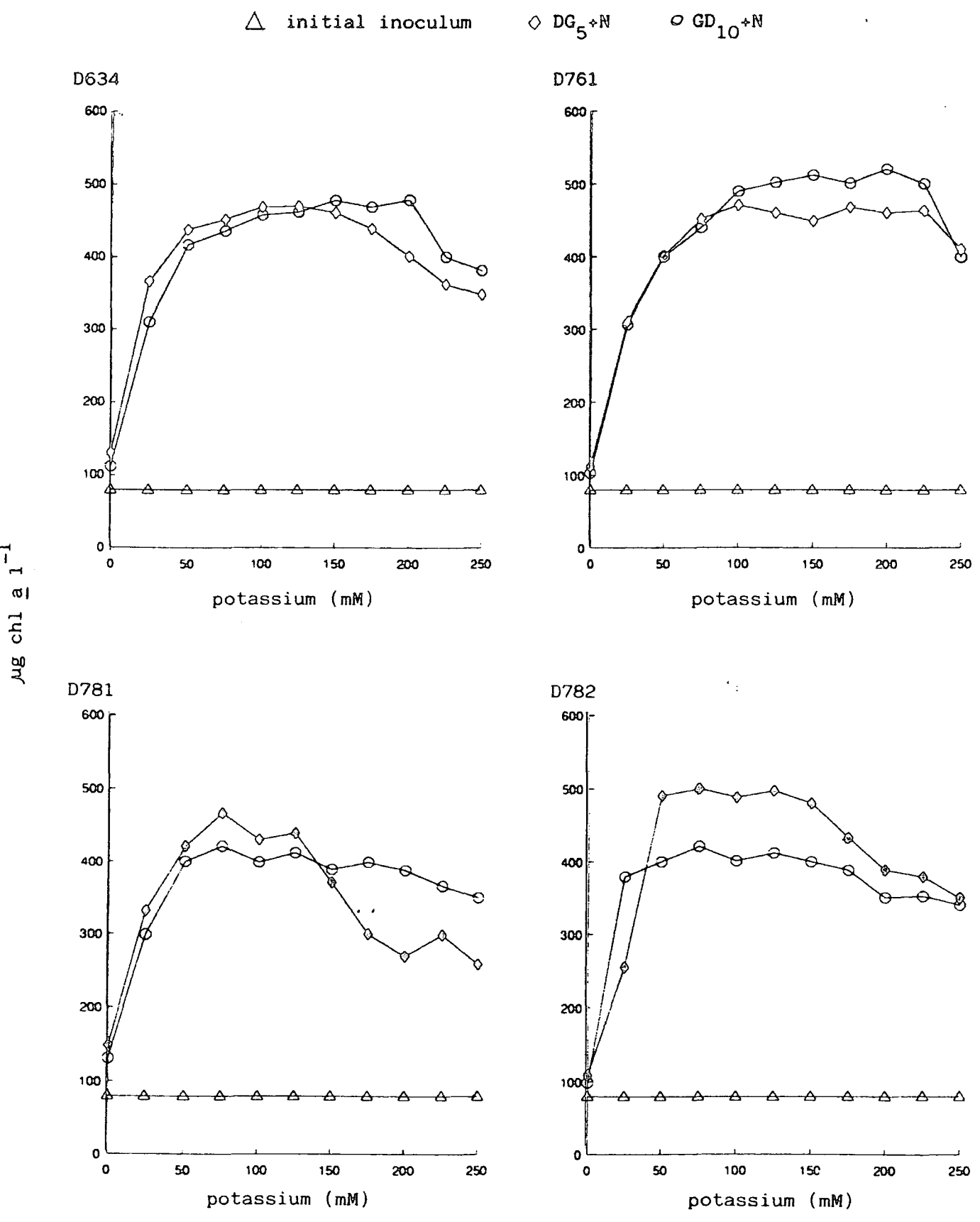
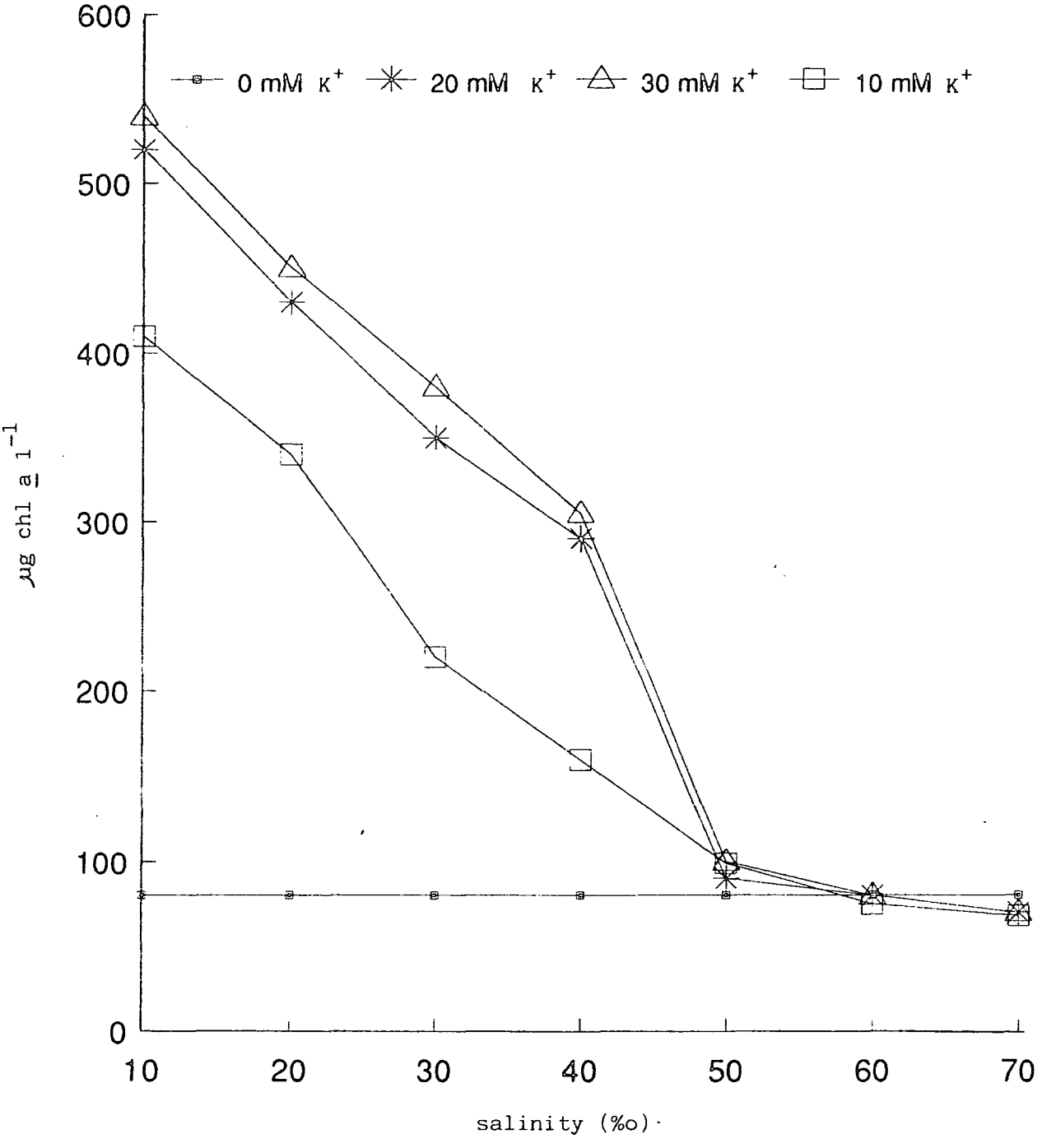


Fig. 5.11 Influence of salinity and concentration of potassium on growth of Microcoleus D634. 32°C, 14:10 light (50 µmol photon m^{-2} s^{-1}):dark



5.3.4 Magnesium (as $MgSO_4.7H_2O$ and $MgCl_2.6H_2O$)

Growth of Microcoleus D634 was influenced by the concentration of Mg^{2+} and the form (Cl^- or SO_4^{2-}) in which it was supplied: when Mg^{2+} was provided as $MgSO_4 7H_2O$ (< 50 mM), growth was higher at a salinity of 10‰ (172 mM Na) than at 5‰ (86 mM Na) (Fig. 5.12); however, the reverse was true when Mg^{2+} was provided as $MgCl_2 2H_2O$ (Fig. 5.12). At concentrations between 75 and 225 mM growth, in response to $MgSO_4 7H_2O$, was higher at 10‰ (172 mM Na); however, there was no significant ($p<0.05$) difference between salinities of 5 and 10‰ (86 - 172 mM Na) when Mg^{2+} was supplied as Cl^{-1} (Fig. 5.12). Further, when Mg^{2+} was supplied in a ratio of 1:1 $Cl^{-1}:SO_4$ a different response again was found (Fig. 5.12).

Growth of Microcoleus D761, D781 and D782 was compared only in response to $MgCl_2 6H_2O$. Growth increased at concentrations of up to 150 mM and declined at concentration in excess of c.450 mM, the precise value depending on the strain and the salinity of the medium (Fig. 5.13).

Growth at salinities between 5 and 30‰ (86 - 516 mM) increased as the concentration of Mg was raised from 10 to 20 mM; however, there was no significant ($p<0.05$) difference in growth between 20 and 30 mM Mg (Fig. 5.14).

Fig. 5.12 Influence of magnesium as $MgCl_2 6H_2O$ and $MgSO_4 7H_2O$ on growth of Microcoleus D634 in media GD_5+N and GD_{10}+N. 32°C, 14:10 light (50 μmol photon m^{-2} s^{-1})

△ initial inoculum ◇ GD_5+N ○ GD_{10}+N

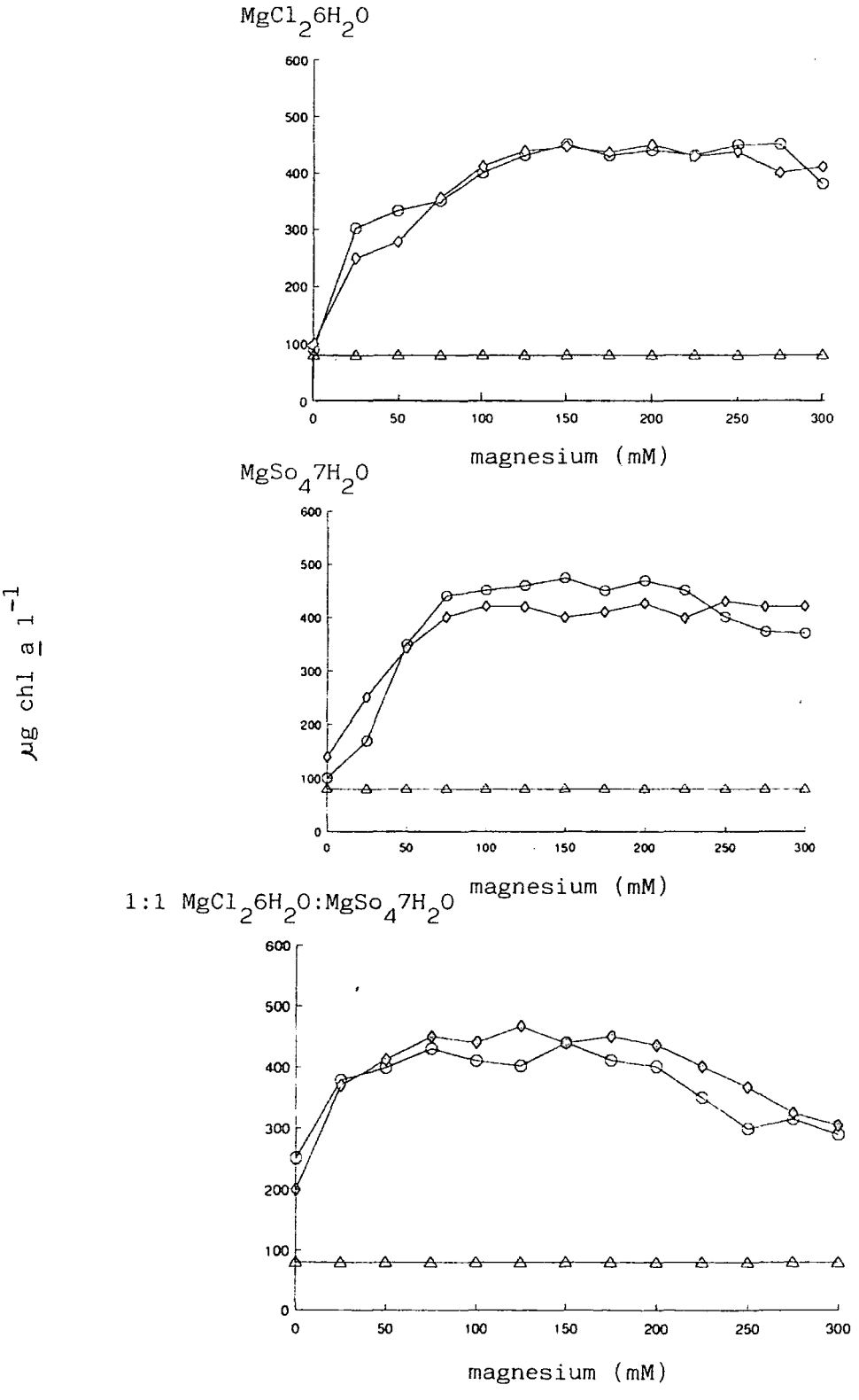


Fig. 5.13 Influence of magnesium (as $MgCl_2 6H_2O$) on growth in Microcoleus D761 D781 and D782 in medium GD at salinities of 5 and 10‰. 32°C, 14:10 light (50 μmol photon m^{-2} s^{-1}):dark

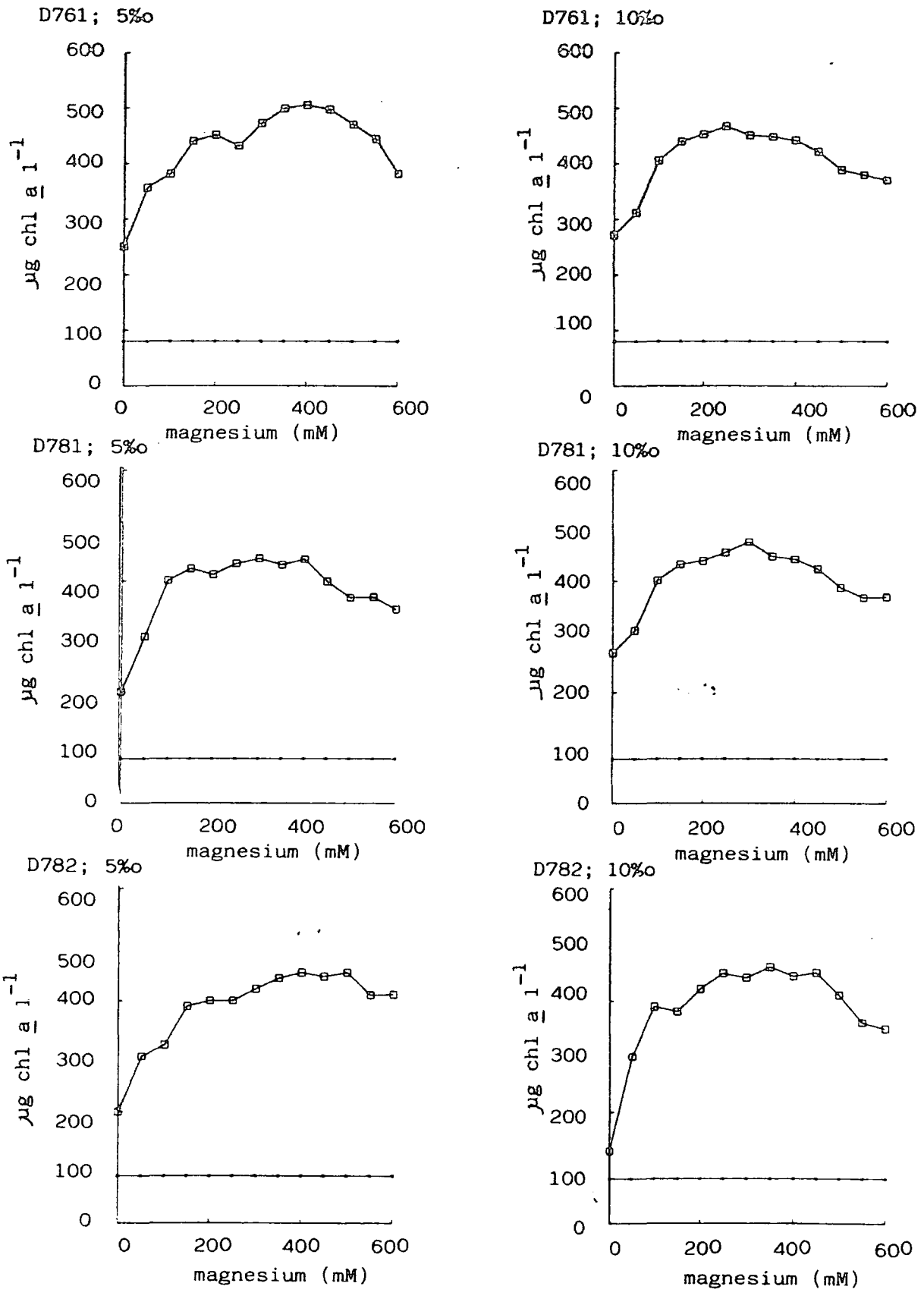
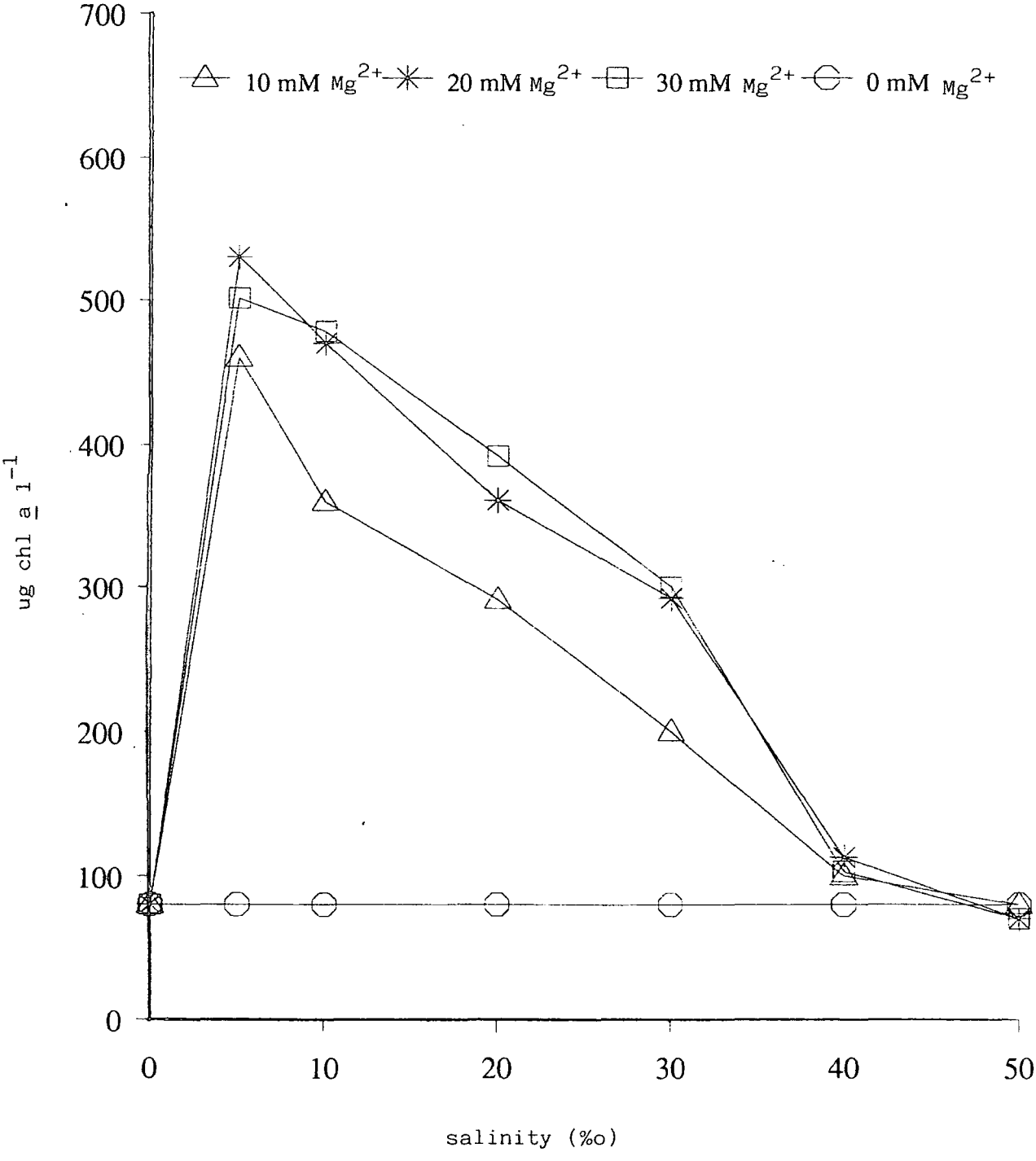


Fig. 5.14 Influence of magnesium (as $MgCl_2 6H_2O$) and salinity (addition of NaCl) on growth of Microcoleus D634. 32°C, 14:10 light (50 µmol photon m^{-2} s^{-1}):dark



5.4 The effect of PAR on growth

The influence of red, blue, green and white light, each at two or more irradiances was compared on % growth (as mg l^{-1} dry weight) of Microcoleus D634, D761, D781 and D782, after 24 h (and a pre-incubation period of 1 h) under the same conditions. All strains failed to grow in either blue or green light; however, both Microcoleus D634 and D761 grew in red light (Fig. 5.15, 5.16). Growth at a PAR of 100 ± 3 µmol photon m^{-2} s^{-1} was higher than at 50 ± 3 µmol photon m^{-2} s^{-1} (Fig. 5.15, 5.16).

Fig. 5.15 Influence of PAR on growth in Microcoleus D634, D761, D781 and D782. 32°C, 103-14 μmol photon m^{-2} s^{-1}

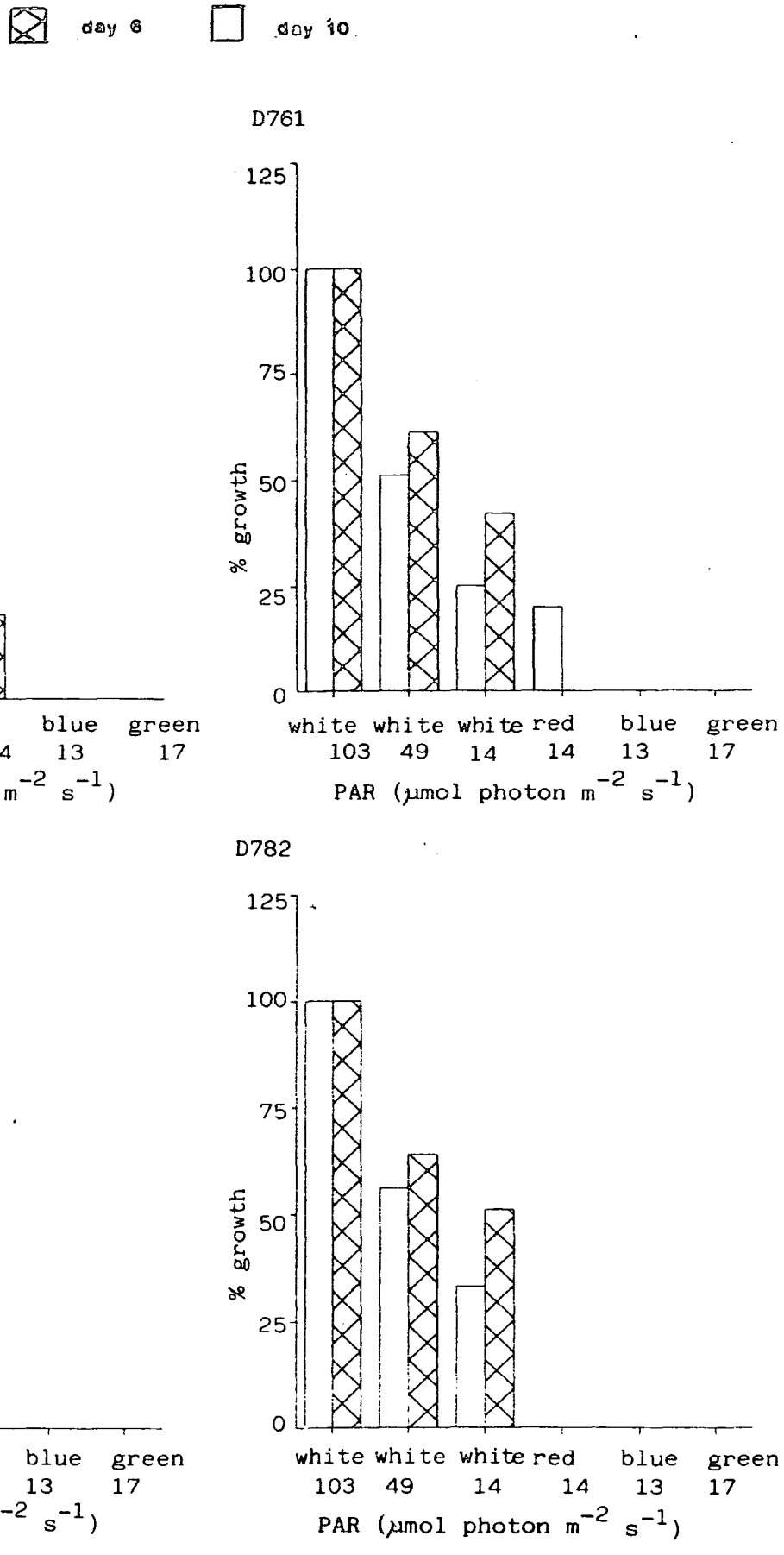
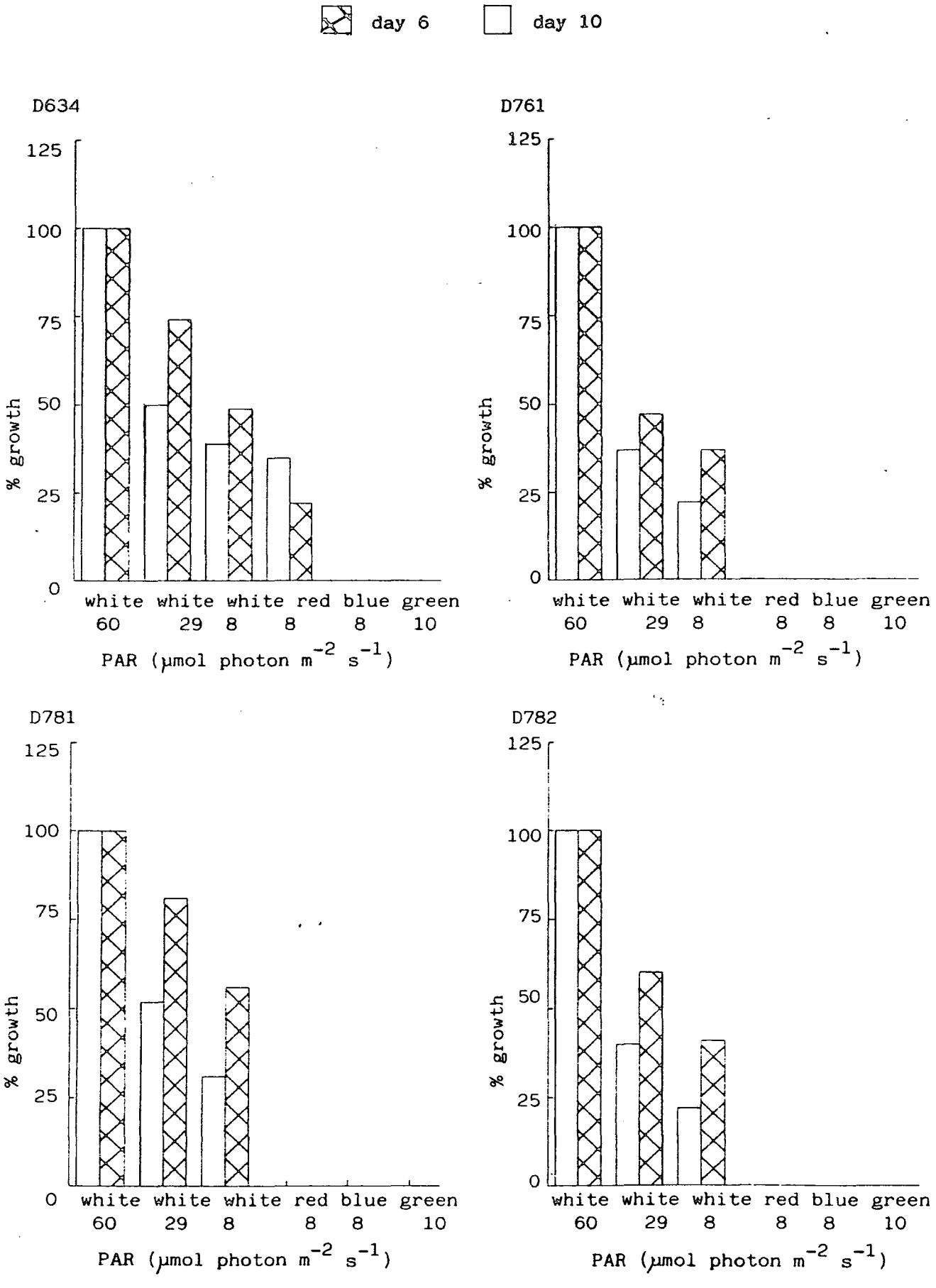


Fig. 5.16 Influence of PAR on growth in Microcoleus D634, D761, D781, D782. 32°C, 60-8 μmol photon m^{-2} s^{-1}



5.5 Effect of temperature on growth

Microcoleus D761, D781 and D782 had optimum temperatures for growth between 28 - 34°C, 30 - 32°C and 32°C respectively, with a 50% decrease in growth 2 C above or below the optimum (Fig. 5.18). Microcoleus D634 had an optimum of 30 - 34°C at salinities of 5, 20 and 30‰ (86, 172 & 516 mM Na) (Fig. 5.17); however, the optimum temperature at a salinity of 10‰ (172 mM Na) was wider (28 - 36°C). Growth decreased by 5 - 25% 2°C above or below the optimum.

All strains grew at 38°C but not at 40°C; however, Microcoleus D634 survived temperatures in excess of 40°C, e.g. 18 h at 45.0 + 0.5 C and 12 h at 55.0 ± 0.5°C. When the PAR was reduced to 30 ± 3 µmol photon m^{-2} s^{-1} higher salinities could be tolerated for longer periods e.g. 24 h at 45°C.

Table 5.3 Survival (+/- growth) of Microcoleus 634 at salinities of 30 - 50‰. 32 C, 100, 50 and 30 µmol photon m^{-2} s^{-1}

Salinity (‰)	Incubation period (h)			
	6	12	18	24
100 ± 4 µmol photon m^{-2} s^{-1}				
30	+	+	+	-
35	+	+	+	-
40	+	-	-	-
45	-	-	-	-
50	-	-	-	-
50 ± 4 µmol photon m^{-2} s^{-1}				
30	+	+	+	+
35	+	+	+	+
40	+	+	+	-
45	+	-	-	-
50	-	-	-	-
30 ± 4 µmol photon m^{-2} s^{-1}				
30	+	+	+	+
35	+	+	+	+
40	+	+	+	+
45	+	+	+	+
50	+	-	-	-

Fig. 5.17 Influence of temperature and salinity on growth of Microcoleus D634.
50 µmol photon m^{-2} s^{-1}

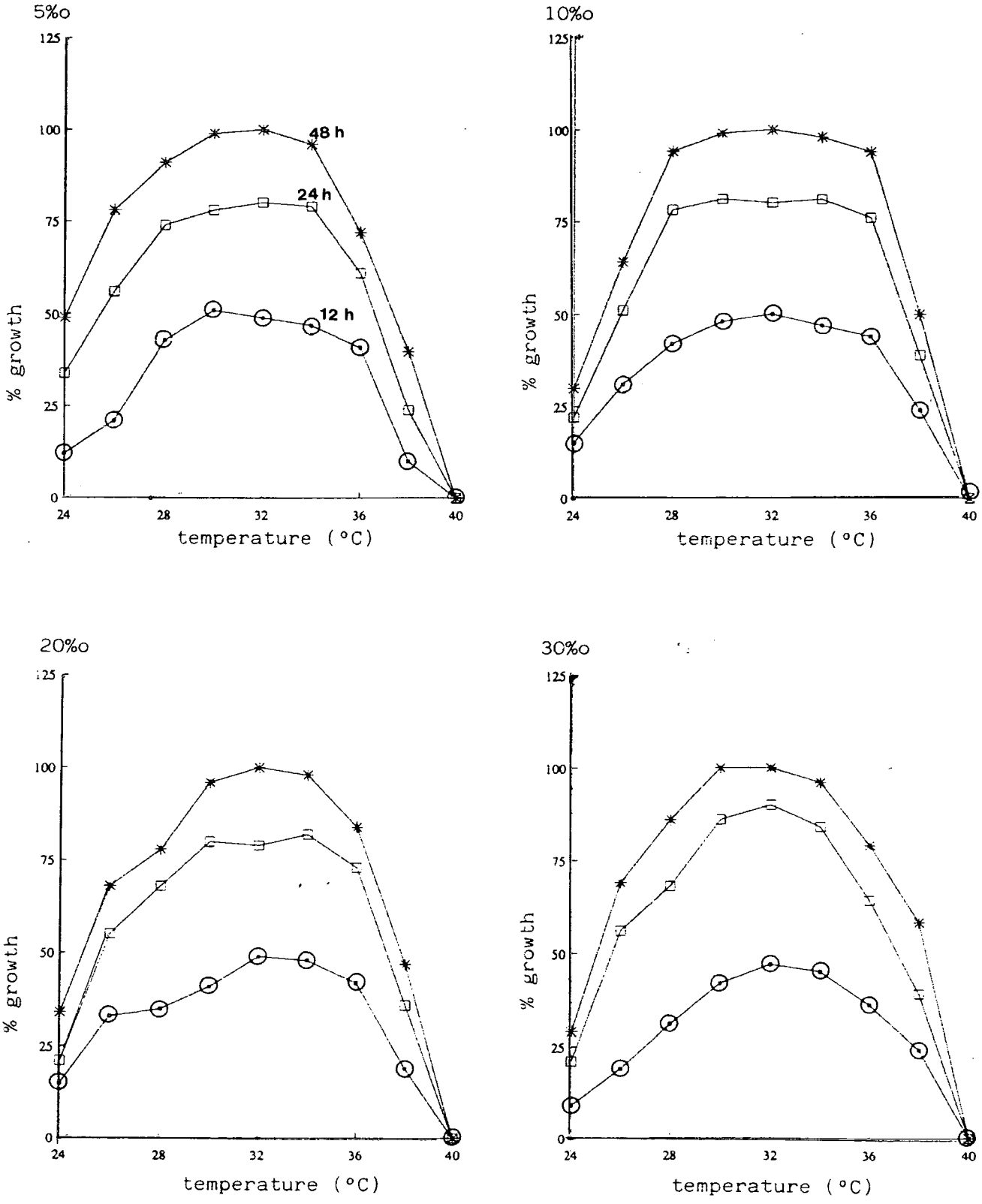
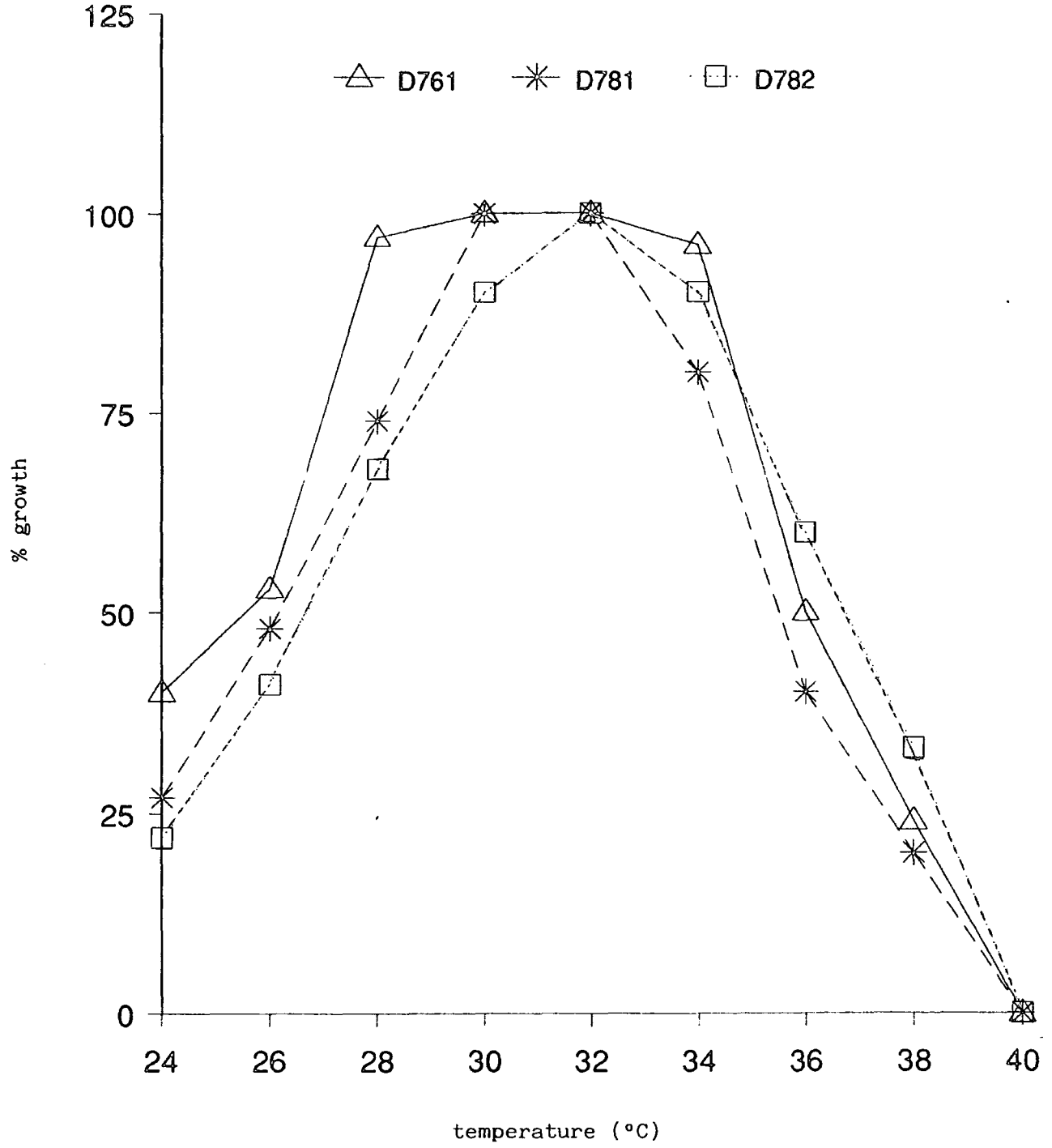


Fig. 5.18 Influence of temperature on % growth of Microcoleus D634, D761 and D781 after a 24-h incubation period. 50 µmol photon m^{-2} s^{-1} .



5.6 Growth in the absence of combined nitrogen

Attempts were made to grow Microcoleus D634, D761, D781 and D782 in the absence of combined nitrogen. Despite the numerous changes made to medium GD (Table 2.11) at pH 7.5 and 8.0 none of these strains grew in the absence of combined nitrogen, either under oxic or micro-oxic conditions when incubated in 14:10 light:dark at 30 ± 4, 50 ± 3 or 100 ± 3 µmol photon m^{-2} s^{-1} at 32.0 ± 0.5°C. Similarly, all attempts to gradually reduce the nitrogen in the medium from 0.5 to 0.4 to 0.3 to 0.2 to0.1 mM NH_4-N over several growth cycles failed to support cells in the absence of combined nitrogen.

5.7 Nitrogenase activity

Microcoleus D634, D761, D781 and D782 were grown to early log and mid. log at salinities of 2, 5, 10, 15, 20 and 30‰ (34, 68, 172, 258, 340 and 516 mM Na) in 0.1 mM NH_4-N and 10 or 20 mM Ca. They were then transferred to the same medium without combined nitrogen (2.4.4) and ARA measured under oxic and micro-oxic conditions (± DCMU) (2.4.7) over a period of 48-h using 2 and 4-h incubation (in case of low levels of activity). The experiment was repeated using inoculum at 476.90 ± 29.67, 805.66 ± 36.77 and 1200.68 ± 40.77 µg chl a l^{-1}. In addition, Microcoleus D634 was grown micro-oxically and ARA examined ±DCMU over 24-h periods during early and mid. log. ARA was also examined on adding sulphide to the medium under micro-oxic conditions (2.4.7) at concentrations of 2.0, 4.0, 6.0 8.0 and 10.0 mM both in the light (30 & 50 µmol photon m^{-2} s^{-1}) and in the dark for 0.5, 1.0, 2.5, 5.0, 10.0 and 20.0-h at pH 7.0 and 7.8. All experiments were carried out at 32.0 ± 0.5°C. ARA was not recorded in any of the strains under any condition.

CHAPTER 6

NITROGENASE ACTIVITY IN *MICROCOLEUS* MATS

6.1 Introduction

Nitrogenase activity (acetylene reduction activity) was examined on *Microcoleus* mats located on Church Island (3.2.3) and known from previous studies (Pearson *et al.*, 1979) to contain a N_2-fixing strain of *Microcoleus*. Fourteen visits were made to these mats between May 1987 and October 1988; the dates and times of experiments both *in situ* and on returning samples to the laboratory are summarised in Tables 6.1 and 6.2. ARA was examined in relation to season, diel cycle and environmental variables including light periodicity, intensity and quality, temperature and tidal submergence.

Table 6.1 ARA experiments carried out on *Microcoleus* mats *in situ*

Experiment	Date	Time
24 h ARA	05/06.06.87	1800 - 1800
	20/21.07.87	1800 - 1800
	03/04.08.87	1800 - 1800
	18/19.08.87	1800 - 1800
	08/09.09.87	1800 - 1800
	14/15.10.87	1800 - 1800
Effects of PAR on ARA	06.06.87	1200 - 1400
	21.07.87	1200 - 1400
	19.08.87	1200 - 1400
Effect of light quality on ARA	19.08.87	0600 - 2200
Effect of temperature on ARA	21.07.87	1100 - 1500
	04.07.87	1100 - 1500
	19.08.87	1100 - 1500
	09.09.87	1100 - 1500
Effect of tidal submergence on ARA	13.08.88	1000 - 1400
		1400 - 1600
		2200 - 2400
	14.08.88	0000 - 0200
		0200 - 0400
Comparison of ARA at sites 1, 2 and 3	20.07.87	1400 - 1600
		2000 - 2200
	21.07.87	0200 - 0400
		0600 - 0800
Effect on ARA of adding DCMU	21.07.87	1800 - 1800

Table 6.2 ARA experiments on *Microcoleus* mats returned to the laboratory from Church Island, Anglesey.

Experiment	Date mat collected	Date of experiment
Effect of light intensity on ARA	22.07.87	23.07.87
	22.07.87	24.07.87
Effect of light quality on ARA	22.07.87	23.07.87
	22.07.87	24.07.87
Effect of temperature on ARA	09.09.87	11.09.87
Effect of Na^+, Ca^{++}, Mg^{++} and K^+ on ARA	19.08.87	22.08.87
	19.08.87	23.08.87
Effect of combined nitrogen on ARA	04.08.87	06.08.88
Effect of phosphorus on ARA	04.08.88	07.08.87
Effect of tidal submergence on ARA	19.08.87	22.08.87
Effect of micro-oxic environments on ARA	14.08.87	15.08.88

6.2 *Microcoleus* mats

6.2.1 Organisms present at sites 1, 2 and 3

ARA on *Microcoleus* mats *in situ* was carried out at three sites (3.2.3) with the majority of experiments at site 1. The succession of organisms at these sites between May and October 1987 are summarised in Table 6.3. A similar trend was seen during the limited visits made in 1986 and 1988.

6.2.2 Concentration of chlorophyll *a*

Core samples were collected for chl *a* analysis (n = 8) at 1000, 1200, 1400 and 1600 during each 24-h ARA period in the field. Samples were pooled and found to have a normal distribution thus parametric statistical methods were employed. Chl *a* levels at sites 1 and 2 were similar between the 6.6.87 and 21.7.87 whilst chl *a* at site 3 was approximately 80% higher; however, chl *a* concentrations in the latter declined thereafter whilst concentrations at sites 1 and 2 increased to a reach a maximum of 168.0 - 190.11 on the 15.10.89 (Table 6.4).

6.2.3 Cross-section of sediment cores

Microcoleus (< 0.5 mm deep) was overlain by sediment and sand < 0.5 deep. Beneath Microcoleus ran a rust coloured layer of variable thickness to which mats adhered strongly especially in September and October. Beneath the rust region lay beige-brown mud intermittently grey and/or black at depths of 1.0 to 10.0 mm. These grey/black areas (presumably micro-oxic and anoxic zones) were most prominent after submersion in sea water, after heavy or pro-longed rain or early in the morning when the sediments were covered with dew.

Table 6.4 Mean chlorophyll a (n = 32) concentration cm^{-2} at sites 1, 2 and 3 of Church island between June and November, 1987.

Date	Mean	CV
Site 1		
06.06.87	43.54 ± 12.09	23.02
21.07.87	68.91 ± 11.05	16.06
04.08.87	83.89 ± 15.78	18.81
19.08.87	123.40 ± 19.78	16.03
09.09.87	177.01 ± 17.00	12.01
15.10.87	168.05 ± 31.89	18.98
01.11.87	101.31 ± 50.16	50.16
Site 2		
06.06.87	34.99 ± 10.89	21.43
21.07.87	64.33 ± 12.67	18.88
04.08.87	90.99 ± 13.01	15.21
19.08.87	136.16 ± 21.31	15.01
09.09.97	210.34 ± 15.67	13.67
15.10.87	190.11 ± 25.14	25.14
01.11.87	112.46 ± 46.63	42.01
Site 3		
06.06.87	70.31 ± 46.31	46.31
21.07.87	126.31 ± 40.01	36.14
04.08.87	120.14 ± 42.30	42.36
19.08.87	100.61 ± 30.16	30.02
09.09.87	66.90 ± 40.16	70.56
15.10.87	40.16 ± 26.31	56.31
01.11.87	10.31 ± 8.61	83.88

Table 6.4 Succession of organisms present in *Microcoleus* dominant mats at sites 1, 2 and 3, Church Island, Anglesey.

Site	Date	Description of site
1	14/15.05.87	Sediment overlain by *Ulothrix flacca*, *Rhizoclonium* sp. and *Enteromorpha* sp. A little *Microcoleus* and *Oscillatoria* detected microscopically. Diatomaceous growth as *Navicula* and *Nitzschia* spp.
2		As site 1
3		Patches of *Microcoleus* visible amongst *Salicornia* plants especially around shoot of plant. Sediment moister than sites 1 and 2. Diatomaceous growth denser with greater number genera (*Navicula* spp., *Nitzschia* spp., *Achnanthes* spp., *Stauroneis* sp. and *Gyrosigma* spp.).
1	05/06.06.87	*Enteromorpha* sp. present in bleached moribund form. *Microcoleus* sparse and patchy in distribution. *Oscillatoria* formed c. 5% of the of total biomass whilst diatomaceous growth less than 1%.
2		As 1
3		As on previous visit. *Salicornia* plants grown in number and height.
1	20/21.07.87	A wide expanse of *Microcoleus* mat present as blue-green powdery patches. *Oscillatoria* c. 5% of the total biomass. Traces of *Spirulina* present.
2		As for 1
3		*Microcoleus* more prevalent to the eye. Diatomaceous growth less dense both microscopically and to the eye. Sediment very moist - difficult to walk on.
1	03/04.8.89	Mats more apparent to the eye, covering a greater surface area and being much less patchy in appearance.
2		A few *Salicornia* plants present.
3		*Salicornia* plants slightly chlorotic. *Microcoleus* less dense than on previous visit (Fig. 6.) with elevated phaeopigment concentrations (41.3 + 4.6%)
1	18/19.8.87	*Microcoleus* more abundant. Patches of *Vaucheria* developing at lower limit of *Microcoleus* mat.
2		As for 1. *Salicornia* plants grown 3 cm.
3		*Salicornia* chlorotic. Few patches of *Microcoleus* visible. Diatoms more abundant.
1	08/09.09.87	Mats much denser forming fairly even, leathery blue-black cover.
2		As 1.
3		*Salicornia* chlorotic. Diatoms more abundant.
1	14/15.10.87	As on previous visit.
2		As 1
3		Traces of *Microcoleus*, mainly as empty sheaths

6.3 ARA of *Microcoleus* mats *in situ* over 24-h periods

To determine when ARA occurred over a diel cycle in natural populations ARA was examined *in situ* over six 24-h periods (Table 6.2) between June and October, 1989. Climatic conditions including light intensity and temperature of the air, sediment and incubation vessel are summarised in Tables 6.5 and 6.6 as well as Figs. 6.2 - 6.7.

ARA occurred at all times during the five 24-h assays; however, no reproducible response in ARA was apparent over the diel cycles (Fig. 6.1). However, ARA was comparatively high on the 5/6.6.87, 18/19.8.87 and 8/9.9.87 with over 70% activity in the dark (Fig. 6. 1, 6.2, 6.5 & 6.7) on days with 7 - 12 h of sunshine (Table 6.5), whereas on cloudy, overcast days with less than 2 h of sunshine (Table 6.5), ARA was fairly constant (Fig. 6.1, 6.3, 6.4 & 6.6) over the diel cycle.

When samples were incubated artificially in the dark (universal bottles covered with foil) during daylight hours ARA increased at sunset and to a lesser extent in the afternoon (1400 - 1800) (Figs. 6.2 - 6.7).

Table 6.5 Climatic data (rainfall* (mm), sun hours (GMT) and cloud cover (fine = $\frac{0}{8}$ to $\frac{2}{8}$ cloud cover; cloudy = $\frac{6}{8}$ to $\frac{8}{8}$; fog = visibility less than 1000 m) during experimental periods (5/6.6.87; 20/21.7.87; 3/4.8.87; 18/19.8.87; 8/9.9.87; 14/15.10.87) and four days prior to each of the given periods.

Date	Rain (mm)		* rainfall	Sun hours	Cloud cover
1. 6.87	3.8	0500-0900, 1400-2100, 2300-2400	+/++	0.0	Cloudy
2. 6.87	14.0	0400-2200	+/++/+++	0.0	Cloudy. Fog 1900-2300
3. 6.87	2.7	0200-0600, 1000-1400, 1700-1900	+/++/+++	1.4	Cloudy. Fog 0500-1100, 1500-1600
4. 6.87	0.0			9.6	Fine 1600-2000, otherwise cloudy
5. 6.87	14.5	1300-2400	+/++/+++/++++	8.1	Cloudy
6. 6.87	5.4	0100-0300, 0600-0800, 1300-2400	++/+++	1.4	Cloudy
16. 7.87	0.0			6.6	Cloudy
17. 7.87	4.2	0900-1800	++/+++	6.1	Cloudy
18. 7.87	31.2	0900-2400	++/+++	0.0	Cloudy
19. 7.87	0.2	0000-0800, 1100-1400, 1600-1700 1900-2200	++	0.1	Cloudy
20. 7.87	0.0			1.1	Cloudy, otherwise fine at 0400, 1300, 1700 and 1800
21. 7.87	0.0			2.6	Cloudy
30. 7.87	2.7	1000-2400	+	0.2	Cloudy
31. 7.87	0.2	0500-0800, 1400-1600	+	1.5	Cloudy
1. 8.87	9.4	0500-0600, 1500-2400	+/++/+++	1.8	Cloudy
2. 8.87	0.0			5.8	Cloudy
3. 8.87	0.0			2.2	Fine, cloudy am.
4. 8.87	0.0			3.3	Fine but cloudy 0800 and 1100
14. 8.87	0.2		+	4.1	Cloudy
15. 8.87	0.1	0100-0500	++	1.9	Cloudy to 1800, then fine
16. 8.87	6.7	0300-0900	+	7.9	Cloudy 0100-1100, then fine
17. 8.87	trace	0000-0200, 1800-2300	++	7.9	Cloudy but fine 1100-1200
18. 8.87	4.0	0100-0300, 0500-0600, 1400-1700	+/++	7.4	Cloudy
19. 8.87	0.7	0900-1100, 2000-2200	+/++	1.6	Cloudy
4. 9.87	5.4	1900-2400	++/+++	9.6	Cloudy 0600-1000, 1800-2400 otherwise fine
5. 9.87	0.6	0000-0700, 1300-1400	++/+++	8.9	Cloudy 1200-1300, otherwise fine
6. 9.87	7.7	0800-1500, 1800-2000	++/+++	1.2	Cloudy
7. 9.87	trace	1900-2100 (showers)	++	8.8	Cloudy otherwise fine 0600-1400
8. 9.87	trace	0000-0200	++	7.1	Cloudy but fine 1600-1700
9. 9.87	1.4	2200-2400	++/+++/++++	0.3	Cloudy
9.10.87	11.2	0000-0500, 0700-2300	++/+++	0.0	Cloudy
10.10.87	17.8	1500-1600, 1700-2200	++	0.4	Cloudy
11.10.87	2.2	0000-0400, 1600-2000	++/+++	5.6	Cloudy
12.10.87	10.4	0000-0300, 1800-2200	++/+++/++++	8.6	Cloudy, but fine 1100-1600
13.10.87	23.5	0500-0900, 1500-1900	++/+++/+++++	7.2	Cloudy
14.10.87	3.0	1900-2400	++/+++	1.5	Cloudy but fine 0800-1200
15.10.87	29.8	0000-1200	++/+++	0.3	Cloudy

* rainfall (+ light ++ medium +++ heavy ++++ very heavy)

Table 6.6 Mean temperature (°C) of the air, sediment and incubation vessels during the experimental periods (5/6.6.87, 20/21.7.87, 3/4.8.87, 18/19.8.87, 8/9.9.87 and 14/15.10.87) (n = 24)

Time	Temperature (°C)			
	sediment	air	incubation vessel controlled	incubation vessel uncontrolled
5/6.6.87				
1800-2200	11.0±0.0	10.0±2.0	11.0±1.0	11.0±1.0
2200-0200	8.0±0.5	8.0±0.0	7.5±0.5	7.5±0.5
0200-0600	8.0±0.0	8.0±0.5	8.0±1.0	8.5±1.5
0600-1000	8.5±0.5	12.0±3.0	12.0±1.0	12.0±3.0
1000-1400	9.0±0.5	14.0±1.0	14.0±0.5	14.0±1.0
1400-1800	9.0±0.5	11.5±1.5	12.0±1.0	12.0±2.0
20/21.7.87				
1800-2200	15.0±0.5	19.5±7.5	20.0±1.5	28.0±8.0
2200-0200	14.0±0.5	11.0±2.0	10.0±1.0	11.0±2.0
0200-0600	13.0±0.5	10.5±1.5	10.0±1.0	9.0±2.0
0600-1000	13.0±0.5	16.5±4.5	15.0±1.0	16.0±4.0
1000-1400	13.5±0.5	18.5±1.5	18.0±1.0	18.0±2.0
1400-1800	13.0±0.5	20.5±5.5	18.0±1.0	18.0±3.0
3/4.8.87				
1800-2200	15.0±0.5	14.5±4.5	15.0±1.0	17.0±7.0
2200-0200	12.0±1.0	9.5±0.5	9.0±0.5	9.0±1.0
0200-0600	11.0±1.0	10.0±1.0	10.0±0.5	14.0±4.0
0600-1000	12.0±1.0	14.0±4.0	12.0±1.0	14.0±4.0
1000-1400	13.0±1.0	18.0±2.0	17.0±1.0	18.0±2.0
1400-1800	13.5±1.0	24.5±6.0	20.0±1.5	27.0±9.0
18/19.8.87				
1800-2200	15.0±0.5	16.0±1.0	16.0±0.5	16.0±1.0
2200-0200	15.0±0.5	13.0±3.0	13.0±1.0	13.0±3.0
0200-0600	14.0±0.5	13.0±3.0	13.0±1.0	13.0±2.0
0600-1000	15.0±0.5	20.0±7.0	20.0±1.0	23.0±8.0
1000-1400	16.0±1.0	22.0±5.0	20.0±1.0	25.0±8.0
1400-1800	17.0±1.0	22.0±5.0	20.0±1.0	24.0±7.0
8/9.9.87				
1800-2200	12.0±0.5	12.0±4.0	12.0±1.5	12.0±4.0
2200-0200	11.0±1.0	8.0±0.0	8.0±0.0	8.0±0.0
0200-0600	10.5±0.5	9.0±1.0	9.0±1.0	9.0±1.0
0600-1000	11.0±1.0	14.0±4.0	12.0±1.5	14.0±4.0
1000-1400	12.5±0.5	19.5±4.5	18.0±1.5	20.0±4.0
1400-1800	12.5±0.5	22.5±2.5	22.0±1.0	22.0±3.0
14/15.10.98				
1800-2200	11.5±0.5	13.0±2.5	14.0±1.0	12.5±2.5
2200-0200	11.0±0.5	10.5±0.5	11.0±1.0	11.0±1.0
0200-0600	11.0±0.5	12.0±2.0	11.0±1.5	12.0±2.0
0600-1000	11.0±0.5	13.5±2.5	14.0±1.0	14.0±4.0
1000-1400	11.5±0.5	17.5±2.5	16.0±1.0	18.0±3.0
1400-1800	12.0±0.5	19.0±3.0	16.0±1.0	18.0±2.0

Fig. 6.1 ARA of Microcoleus mats at Church Island over 6 24-h periods between June and October 1987

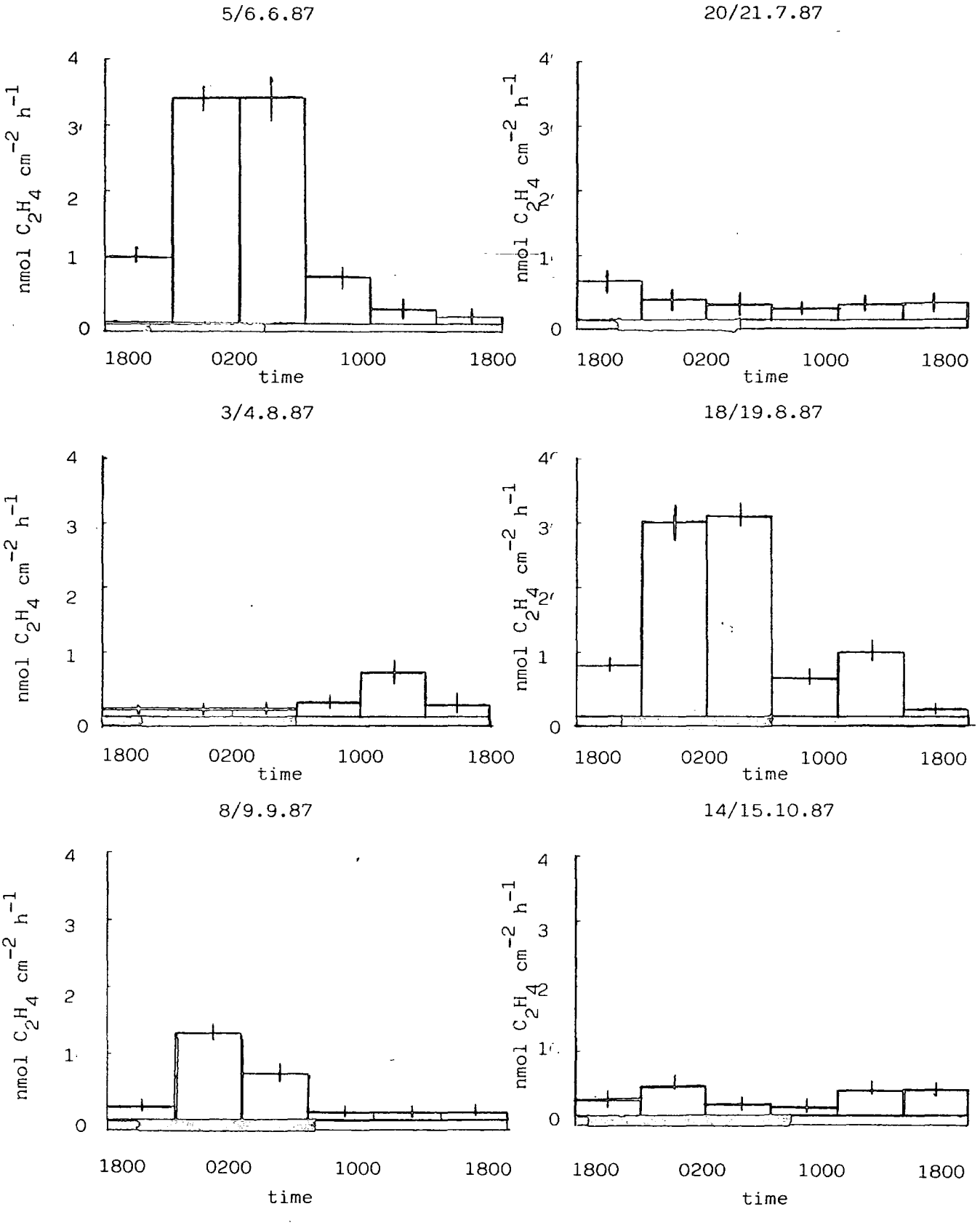


Fig. 6.2 ARA in Microcoleus mats on Church Island between 1800 and 1800 on the 5/6.6.87 when incubated under natural light:dark (a) and in the dark only (b). Temperature (°C) (d), PAR (μmol photon m^{-2} s^{-1} (c)

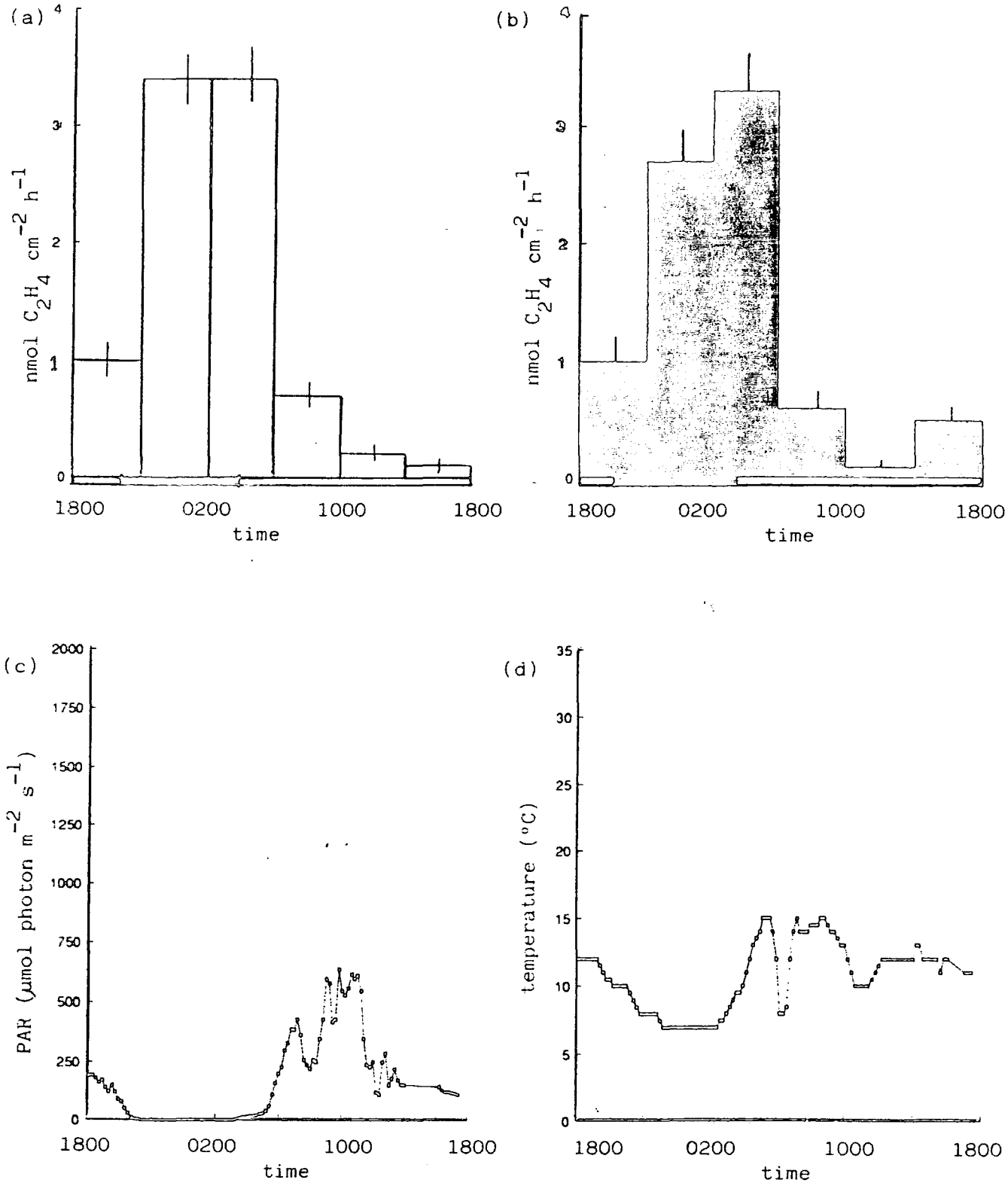


Fig. 6.3 ARA in Microcoleus mats on Church Island between 1800 and 1800 on the 20/21.7.87 when incubated under natural light:dark (a) and in the dark only (b). Temperature (°C) (d), PAR (µmol photon m^{-2} s^{-1}) (c)

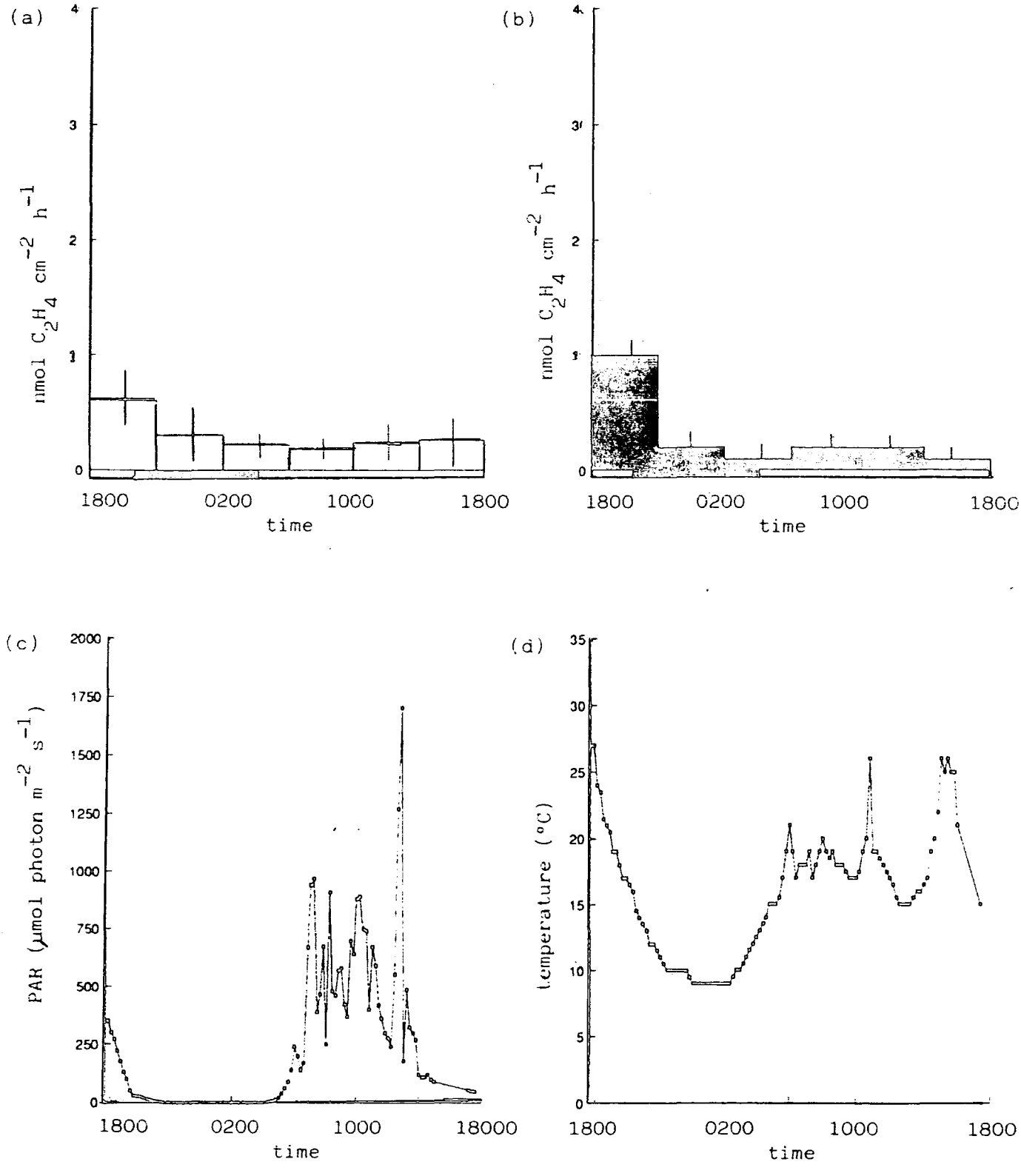



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Fig. 6.4 ARA in Microcoleus mats on Church Island between 1800 and 1800 on the 3/4.8.87 when incubated under natural light:dark (a) and in the dark only (b). Temperature (°C) (d), PAR (μmol photon m^{-2} s^{-1}) (c)

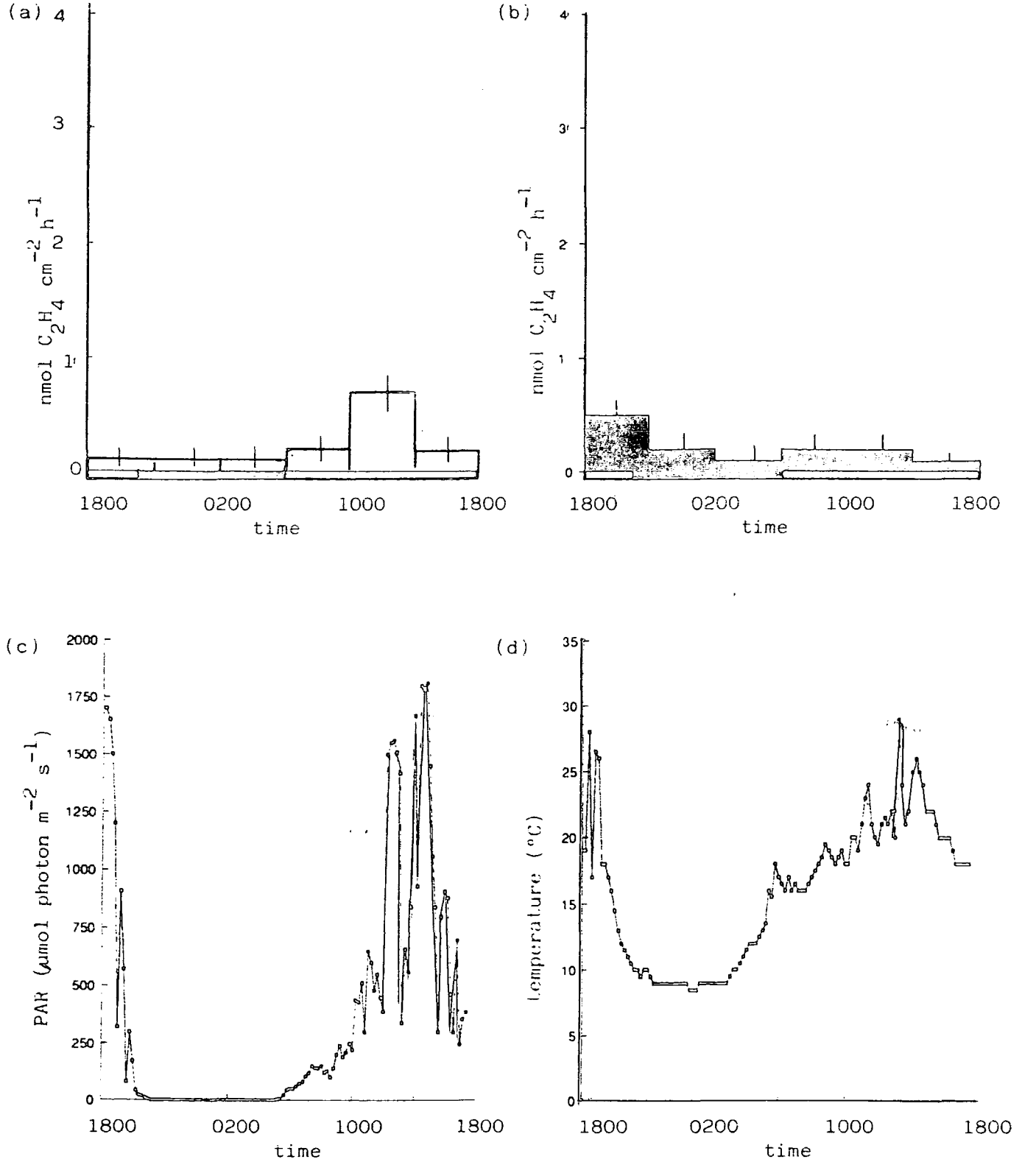


Fig. 6.5 ARA in Microcoleus mats on Church Island between 1800 and 1800 on the 18/19.8.87 when incubated under natural light:dark (a) and in the dark only (b). Temperature (°C) (d), PAR (μmol photon m^{-2} s^{-1}) (c)

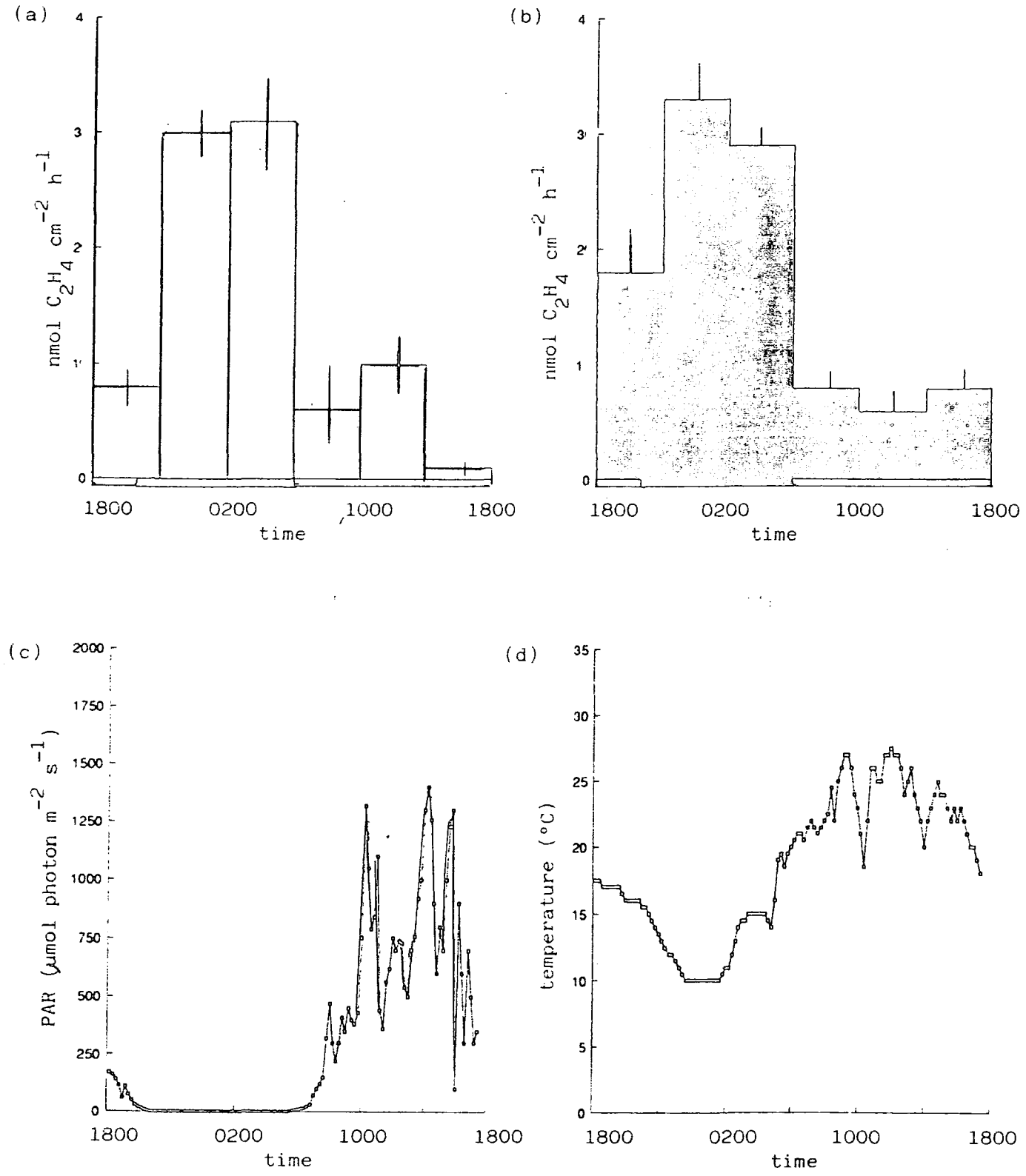


Fig. 6.6 ARA in Microcoleus mats on Church Island between 1800 and 1800 on the 8/9.9.87 when incubated under natural light:dark (a) and in the dark only (b). Temperature (°C) (d), PAR (μmol photon m^{-2} s^{-1}) (c)

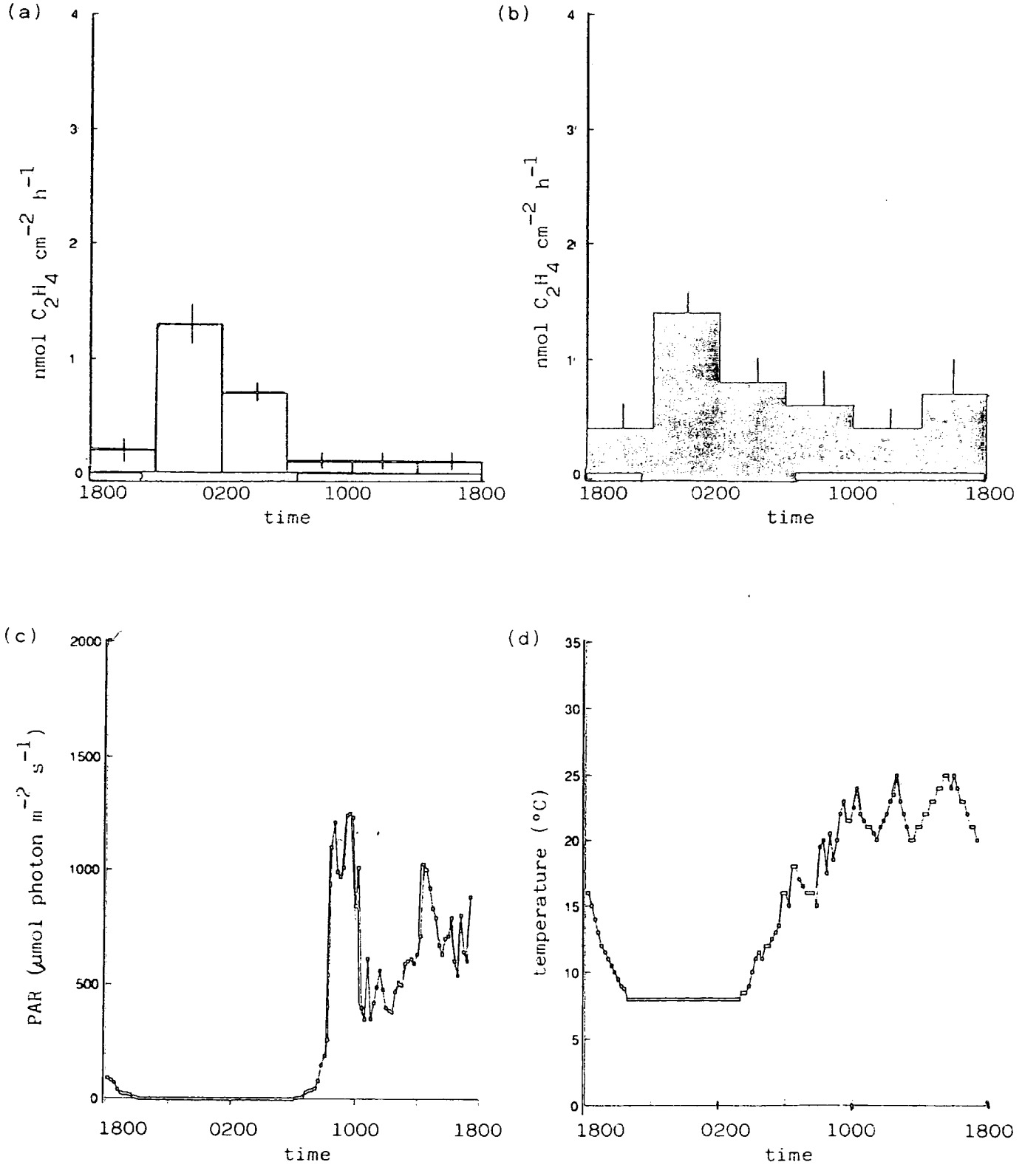
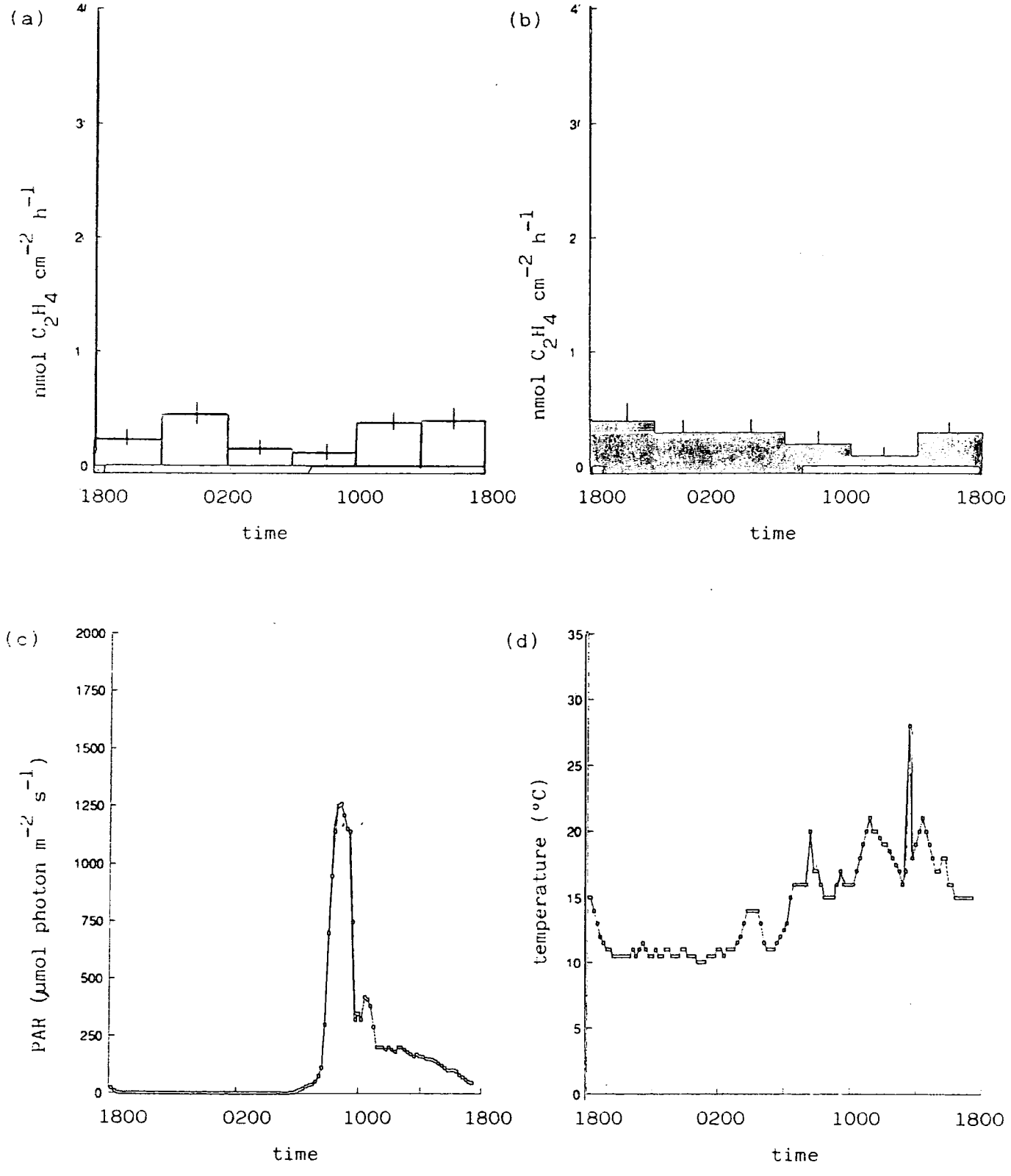


Fig. 6.7 ARA in Microcoleus mats on Church Island between 1800 and 1800 on the 14/15.10.87 when incubated under natural light:dark (a) and in the dark only (b). Temperature (°C), PAR (μmol photon m^{-2} s^{-1}) (c)



6.4 Nitrogenase activity in Microcoleus mats when submerged

Spring tides (>5.0 m) covered sites 1 and 2 whilst tides >4.9 m covered site 3. The time mats are submerged by 5.0 - 6.2 m tides are given in Table 6.7.

Table 6.7 Time (± 5 min.) Microcoleus mats at sites 1 and 2 of Church Island, Anglesey, were covered by spring tides of 5.0 - 6.2 m.

Height of tide m	Time of submergence h	min.	Date
5.0	1	15	20.10.87
5.1	2	15	13.08.88
5.2	2	40	21.10.87
5.3	3	15	20.10.87
5.4	3	50	14.08.88
5.5	4	10	21.10.87
5.7	4	30	30.08.88
5.8	4	50	29.08.88
5.9	4	55	30.07.88
6.2	5	00	29.08.88

To determine the effect of tidal submergence on ARA in situ a 24-h assay was carried out at site 1 on 13/14.08.88 when the mats were covered by 5.1 and 5.4 m tides between 1050/1305 and 2220/23.40 respectively. In addition, at 1400/1600 and 0200/0400 ARA was compared on mats exposed to air with those submerged by seawater and freshwater (Milli-Q). The effect of submergence was also examined on Microcoleus mats returned to the laboratory (2.9.3).

6.4.1 Mats returned to the laboratory

Experiment 1

To determine the influence of salinity on ARA of Microcoleus mats after tidal submersion, mats were pre-submerged in Milli-Q water supplemented with NaCl giving salinities of 5, 10, 20 and 30‰ (86, 172, 344 & 516 mM Na) for 1, 2 and 3 h before ARA was compared immediately afterwards over a 1-h incubation period.

After submersion in at salinities of 10, 20 and 30‰ ARA was significantly higher than at 5‰ (Fig. 6.8a). Pre-submerging for 3 h as opposed to 1 or 2 h led to a significant ($p<0.05$) increase in ARA at 20 and 30‰; however, at 5 and 10‰ there was no significant ($p<0.05$) difference. Grey zones developed 3 - 10 mm below the mats after pre-submerging for 2 h and becoming black after 3 h. Prior to submerging in these media pore waters had a salinity of 6‰ whereas after submersion salinity rose to c. 1 - 3‰ lower than that of the overlying media (within 0.5-h) and remained so over the 3 h preincubation.

Experiment 2

To compare the influence of seawater on ARA after tidal submersion, Microcoleus mats were pre-submerged in seawater diluted with Milli-Q water to salinities of 10 and 30‰. (pH of 7.8 buffered with HEPES) for 1, 2 and 3 h before comparing ARA immediately afterward over a 1-h incubation period. There was no significant ($p<0.05$) difference in ARA at 5 and 10‰ (Fig. 6.8b) after a 1 and 2 h pre-incubation; however, after 3 h ARA was significantly lower at 10‰. ARA at 20 and 30‰ was significantly ($p<0.05$) lower than at 10‰.

Experiment 3, 4 and 5

To determine the influence of Ca, Mg and K on ARA, Microcoleus mats were pre-submerged for 1-h in both Milli-Q water supplemented with NaCl and in seawater diluted with Milli-Q water to salinities of 10, 20 and 30‰ (172, 344 and 516 mM NaCl) each supplemented with 0, 10, 20, 30 and 40 mM Ca, K and Mg (buffered with HEPES to pH of 7.8).

ARA was generally higher under seawater than Milli-Q water (Fig. 6.9). ARA increased significantly ($p<0.05$) on adding up to 20 mM Ca, Mg and K but declined at concentrations in excess of 30 mM (Fig. 6.9).

Fig. 6.8 Influence of presubmerging Microcoleus mats returned to the laboratory in Milli-Q water supplemented with NaCl (a) and seawater diluted with Milli-Q water (b) to salinities of 5, 10, 15 and 20‰ on ARA. 20°C, 50 µmol photon m^{-2} s^{-1}, pH 7.8

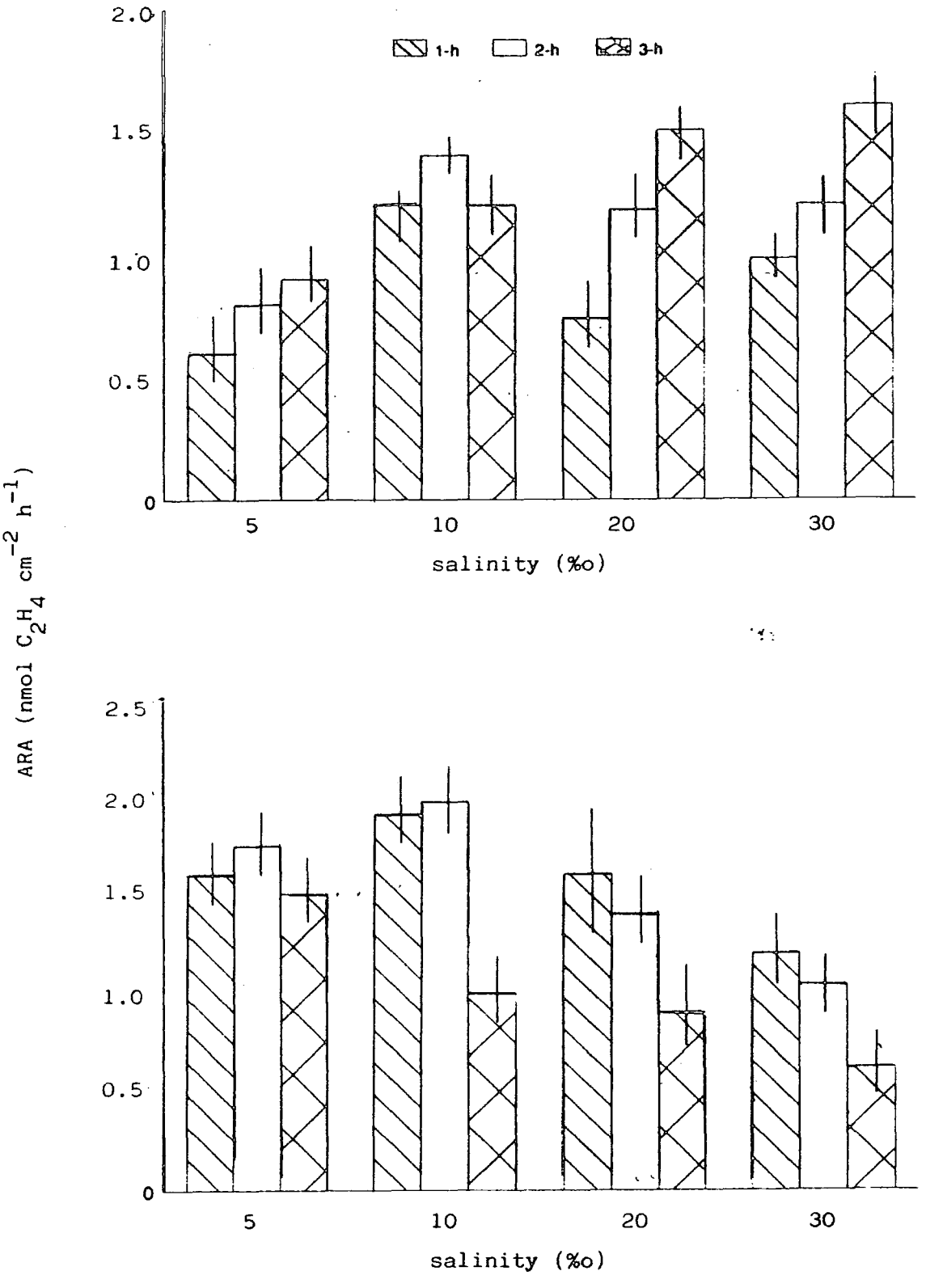


Fig. 6.9 Influence of presubmerging Microcoleus mats (n = 5) in Milli-Q water supplemented with NaCl and seawater diluted with Milli-Q to a salinity of 10‰, each containing calcium, potassium or magnesium, on ARA. 20°C, 50 µmol photon m^{-2} s^{-1}, pH 7.8

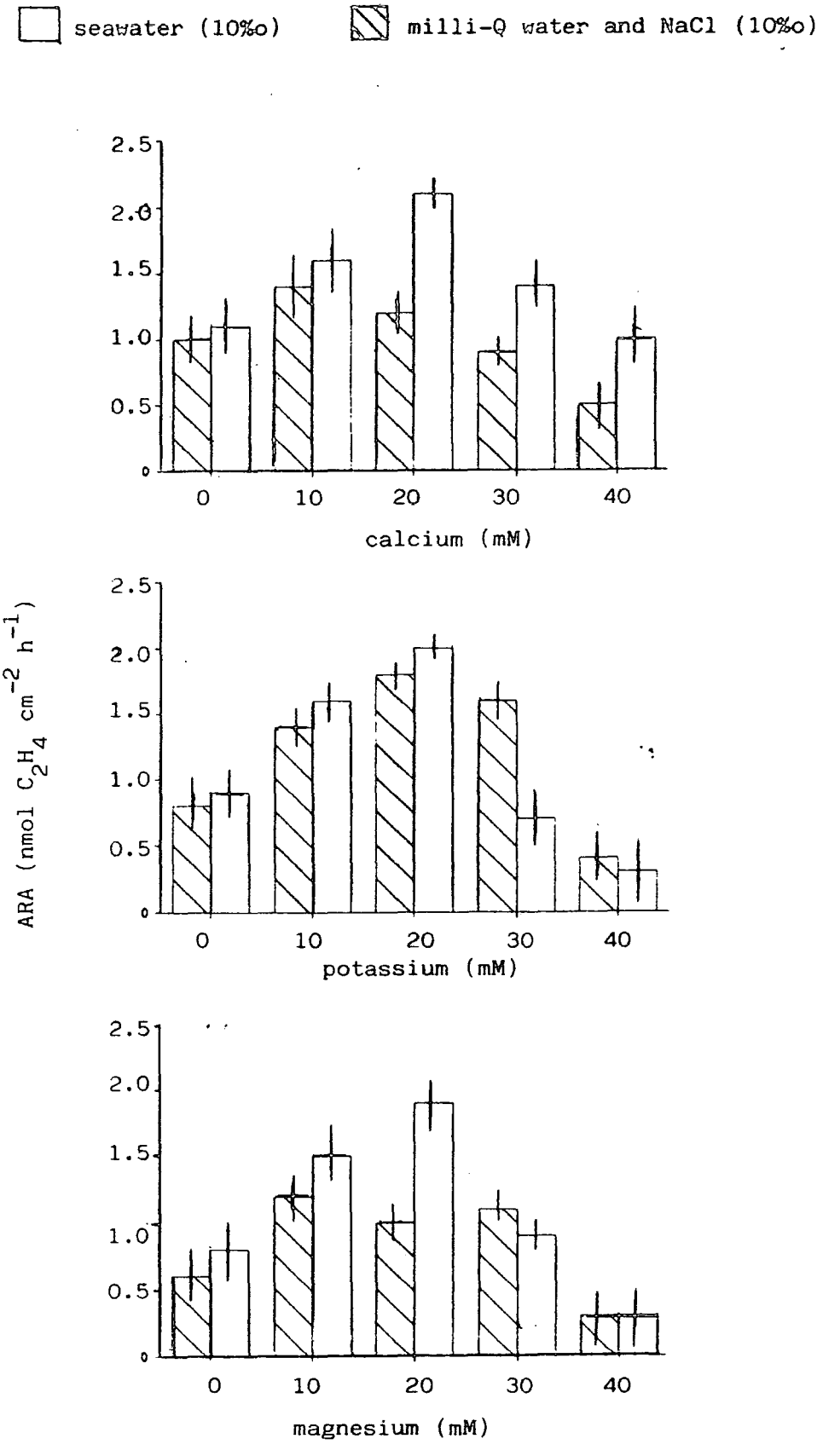
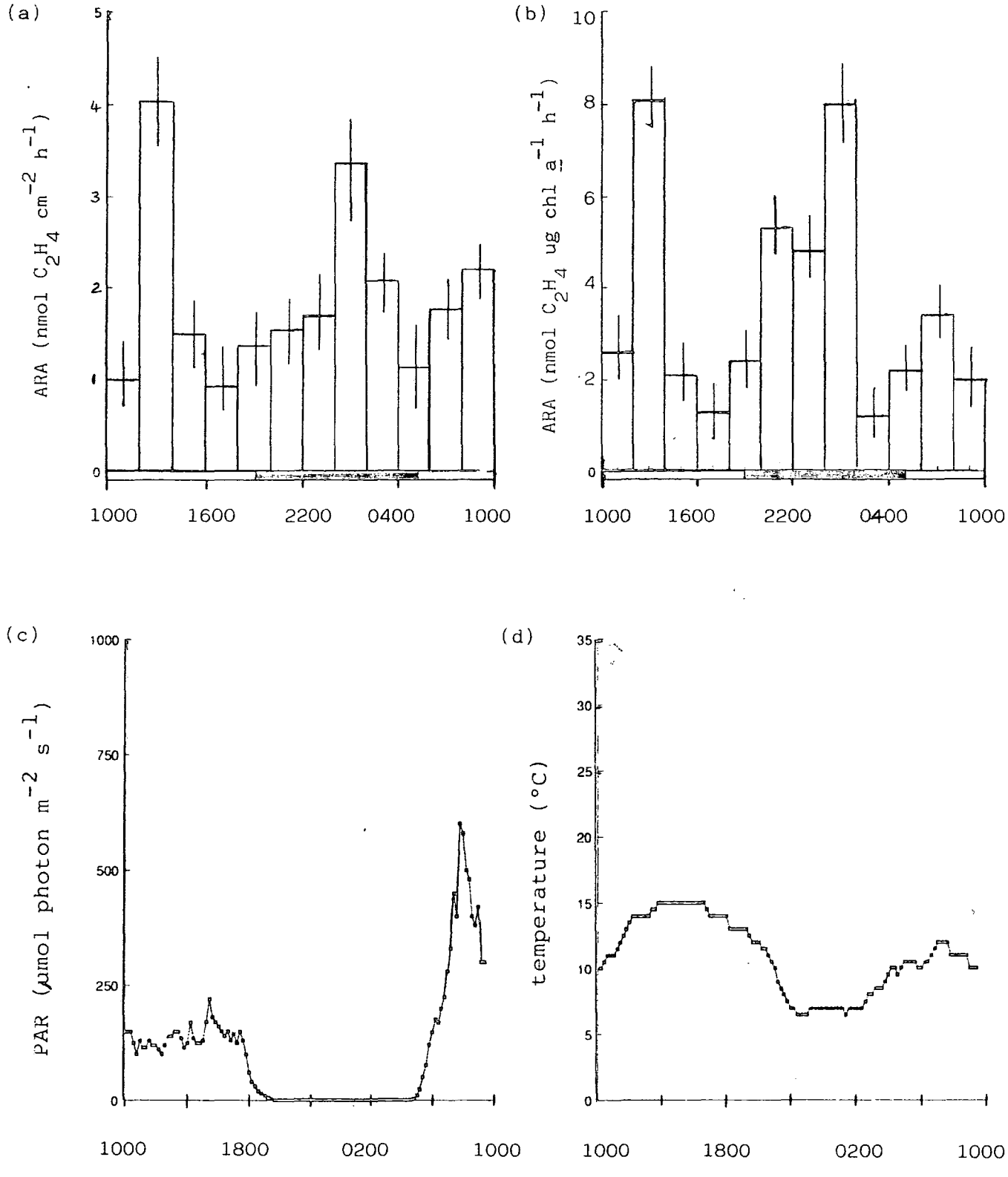


Fig. 6.10 ARA in Microcoleus mats at Church Island between 1000 and 1000 on 13/14.8.8 when mats were submerged by a 5.1m (1050-1305) and 5.4m (2220-2340) tides



6.4.2 ARA on mats submerged by sea water *in situ*

ARA occurred over the whole 24-h period with peaks between 1200 and 1400, the two periods during or after mats were partially or completely submerged by seawater. ARA increase 4 fold when covered by the tide in the morning and doubled after night time submersion (Fig.6.10).

6.5 ARA in the presence of combined nitrogen

Combined nitrogen may or may not be present intermittently in the sediments of sites 1, 2 and 3. To compare the effects of combined nitrogen on ARA, mats were pre-submerged for 2-h in Milli-Q water supplemented with either NH_4-N at 0 - 10 mM (pH 7.8) before removing the liquid and testing for ARA immediately (t = 0) as well as 2 and 4-h later over 1-h incubation periods. In all experiments mats were pre-submerged in Milli-Q -N to serve as a control. ARA was reduced but not inhibited on adding combined nitrogen irrespective of the concentration (Fig. 6.11).

6.6 Effect of PO_4-P on mats returned to the laboratory

To determine the influence of PO_4-P on ARA, *Microcoleus* mats were pre-submerged in Milli-Q water (pH 8.0) supplemented with 0 - 10 mM PO_4-P and ARA measured immediately afterwards over a 1-h incubation period.

ARA increased on adding PO_4-P at concentrations between 0.03 and 7.5 mM, but at concentrations in excess of this activity declined (Fig. 6.12).

Fig. 6.11 Influence of NH_4-N on ARA in Microcoleus mats returned to the laboratory. 20°C, 50 umol photon m^{-2} s^{-1}

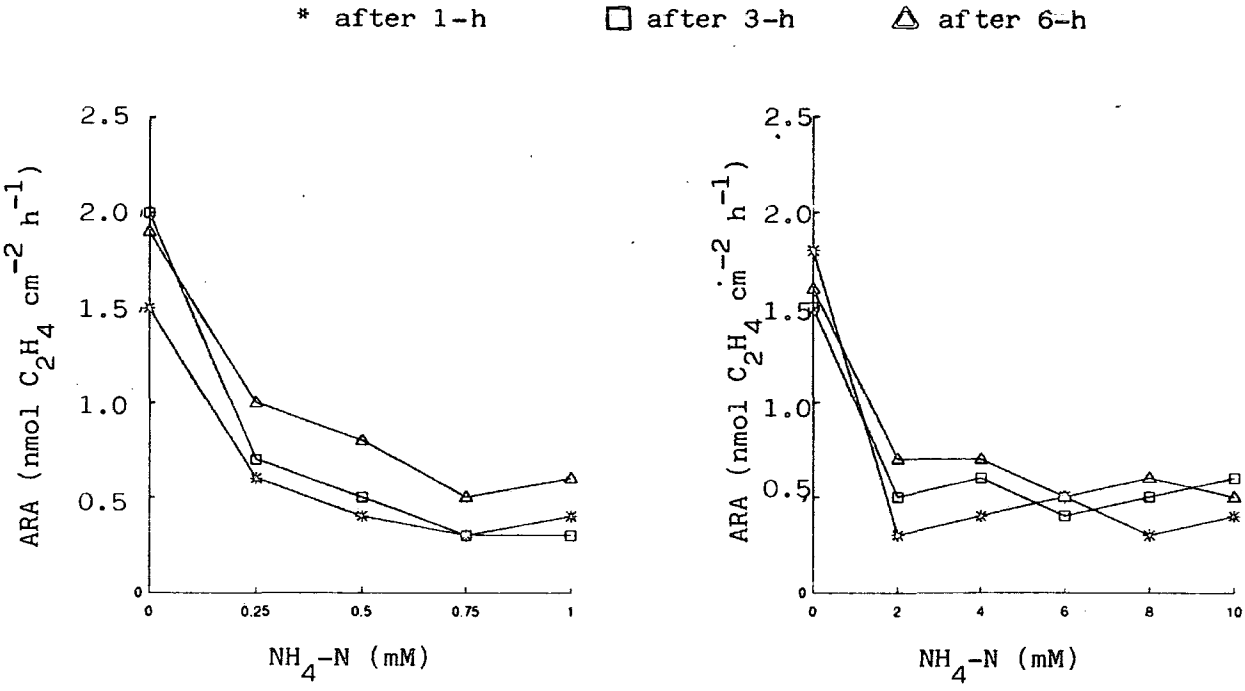
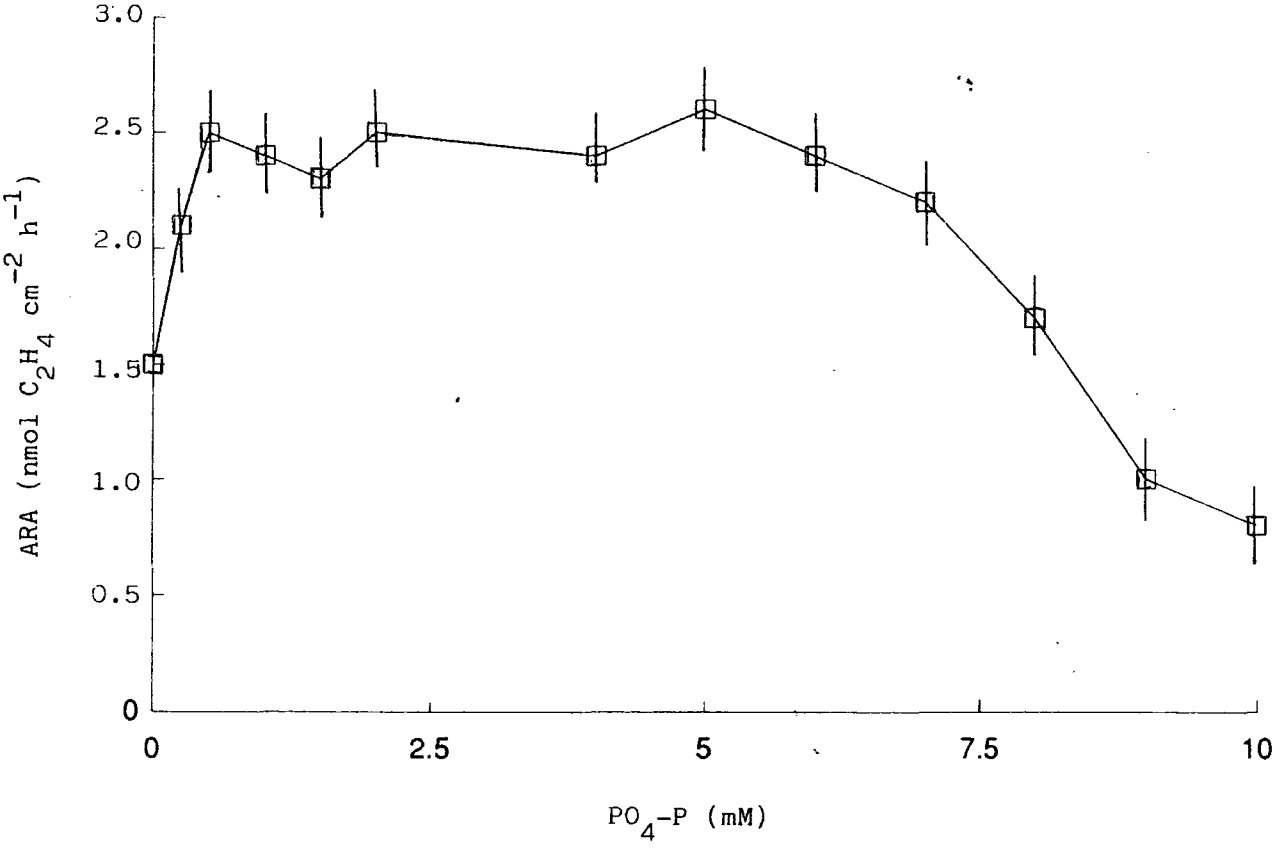


Fig. 6.12 : Influence of PO_4-P on Microcoleus mats returned to the laboratory. 20°C, 50 umol photon m^{-2} s^{-1}



6.7 Effect of PAR on ARA

PAR may change considerably in nature over a few hours (Figs 6.2 - 6.7). To examine its effect on ARA, both in situ and in the laboratory, cores were incubated in universal bottles covered with neutral density or foil and compared at 0, 10, 44 and a 100% intensity.

Experiments were carried out at 1200/1400 and 1400/1600 on the 6.6.87, 21.7.87 and 19.8.87. PAR incident of the incubation vessels during these periods are given in Fig. 6.2 - 6.7. ARA decreased with decreasing PAR (Fig. 6.12); however, on the 19.8.87 there was no significant ($p<0.05$) difference at 10, 44 and 10% PAR (Fig. 6.12).

When the effect of PAR was examined on ARA in Microcoleus mats returned to the laboratory and stored in the greenhouse (2.2.4), ARA varied not only with PAR but was also dependent on the pre-incubation conditions (Fig. 4.13). When samples were collected at 0730 and 1130, ARA increased with increasing PAR; however, at 1530 ARA was highest at 50 m photon m^{-2} s^{-1}, whilst at 1930 ARA activity was highest in the dark (Fig. 4.13).

The influence of light quality (white, blue, red and green) was examined in the field (Table 6.2). On each occasion ARA occurred under white and red light but not in green or blue light. ARA in red light was significantly ($p<0.05$) lower than at a white light of equivalent irradiance.

Fig. 6.13 Influence of PAR on ARA in Microcoleus mats at site 1, Church Island, between 1200 and 1400 on 6.6.87, 21.7.87 and 19.8.87

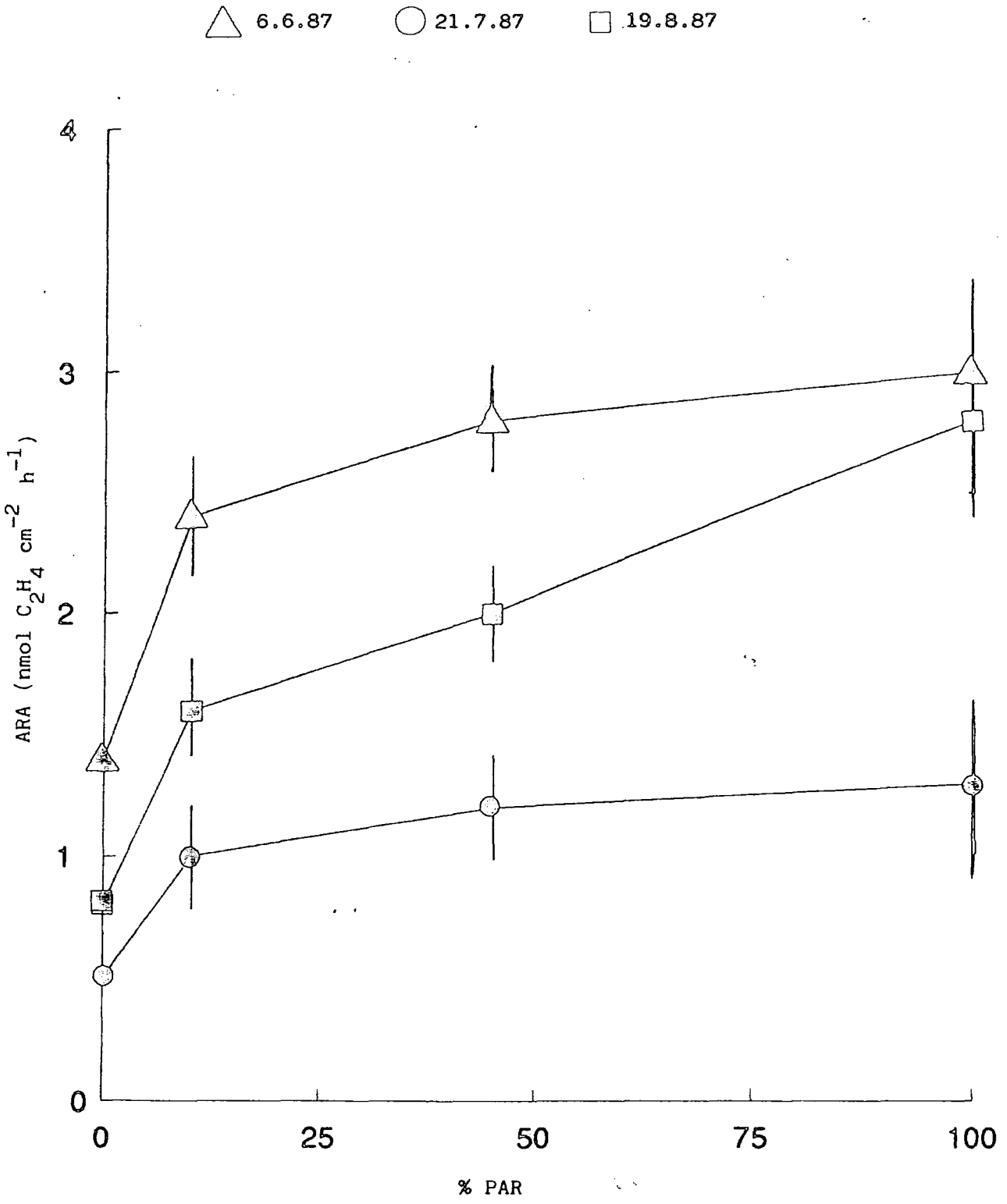


Fig. 6.14 Influence of PAR on ARA in Microcoleus mats returned to the laboratory on the 22.7.87, incubated in the greenhouse and samples collected at 0730, 1130, 1530 and 1930 on 23.7.87. 20°C, 50 umol photon m^{-2} s^{-1}

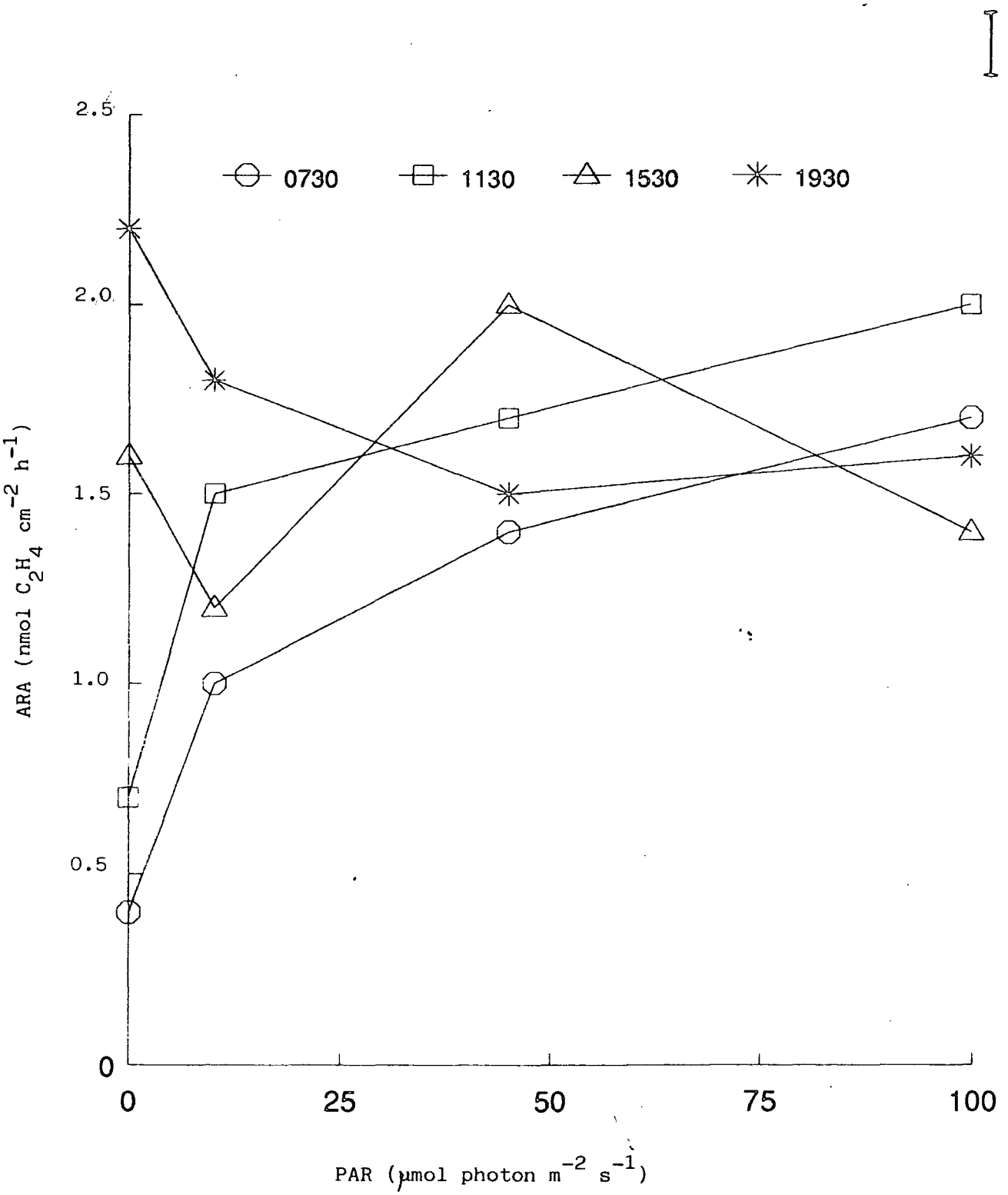
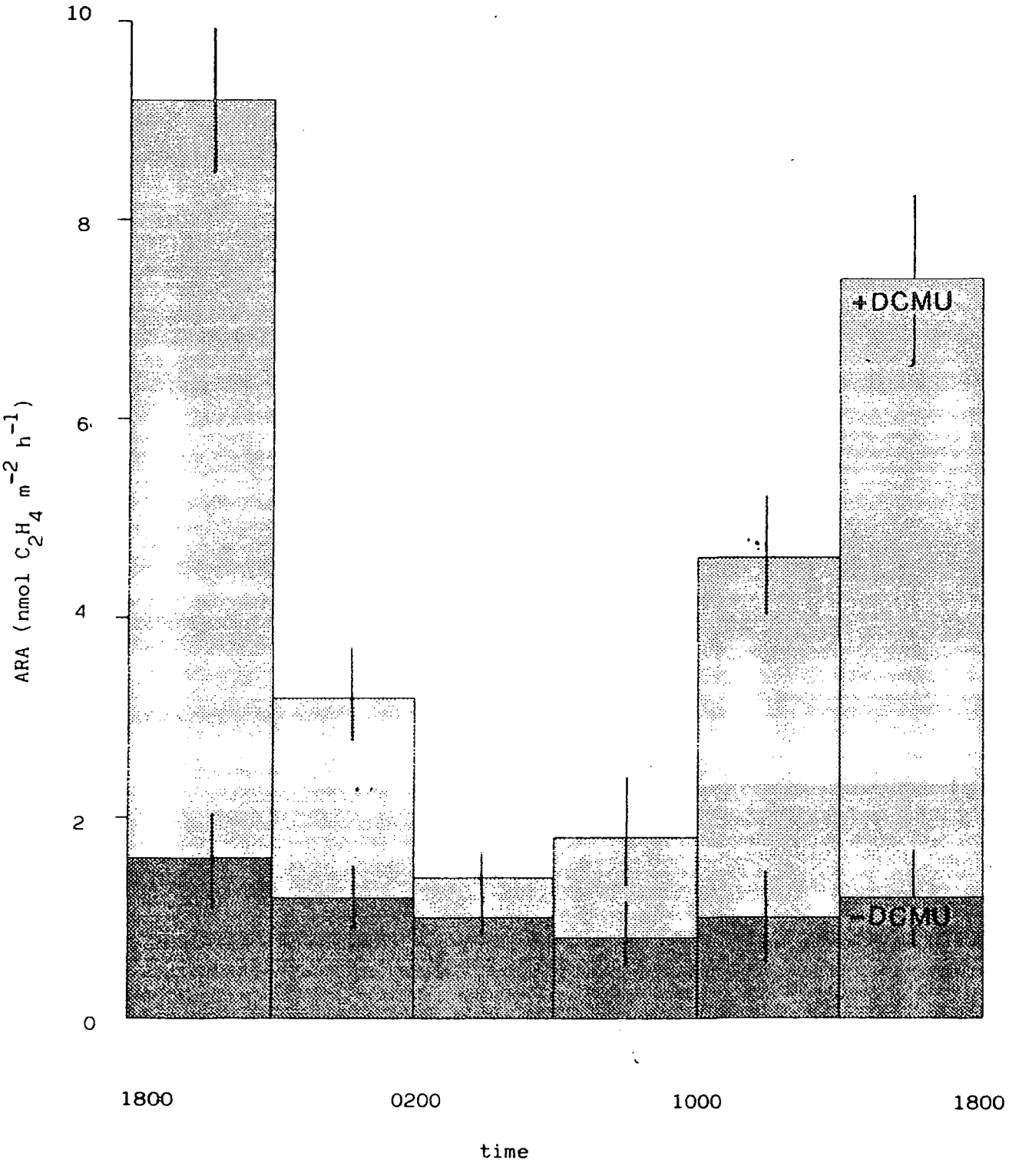


Fig. 6.15 Influence of DCMU on ARA in Microcoleus mats on Church Island between 1800 and 1800 on 20/21.7.87



CHAPTER 7

Nitrogenase activity in Microcoleus D778

7.1 Introduction

This chapter is concerned with the effects of environmental variables such as nutrient concentration, light (periodicity, intensity and quality), temperature and pH on growth and ARA in axenic cultures of Microcoleus D778 under oxic and to a lesser extent micro-oxic conditions.

7.2 Growth

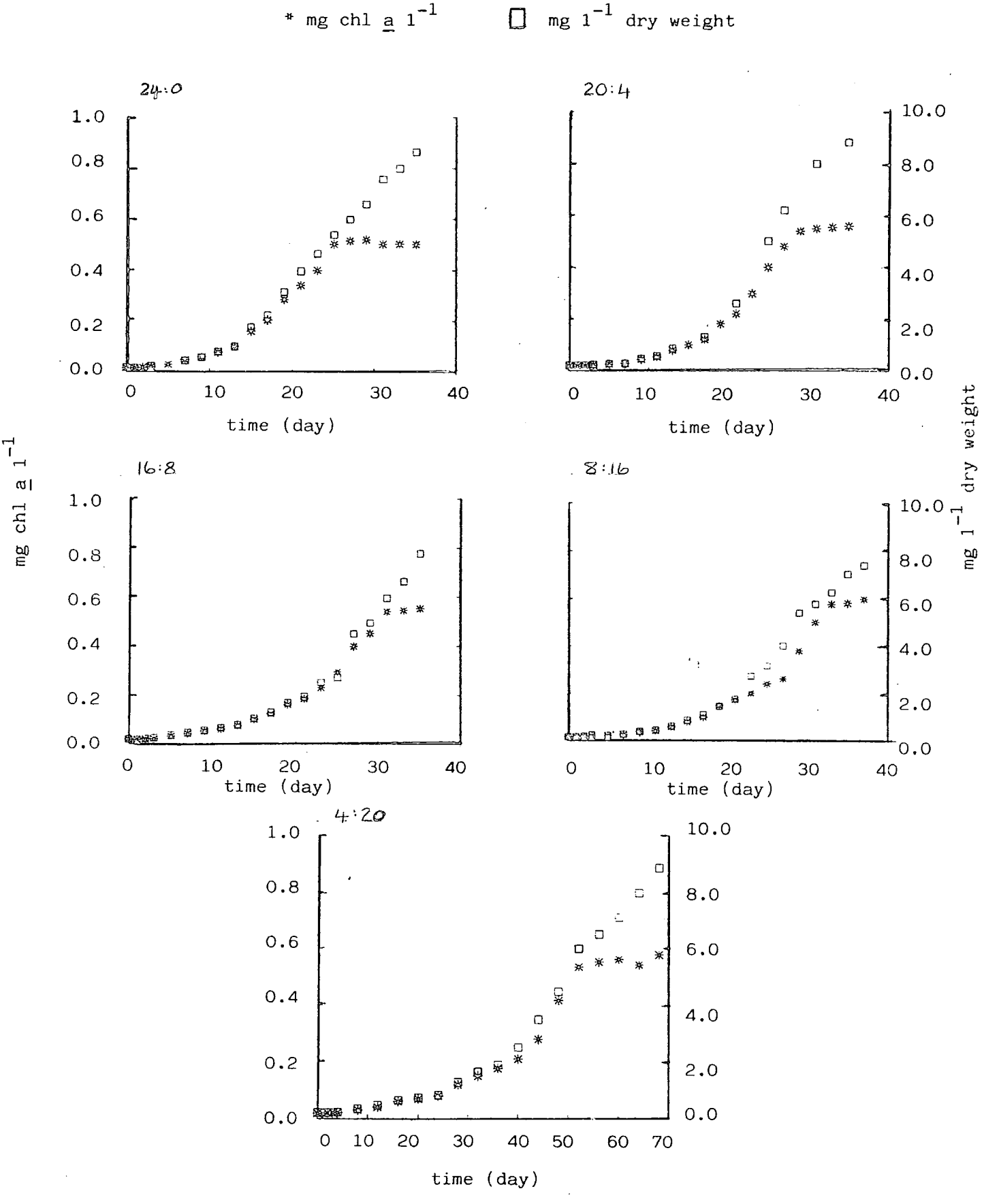
Unless specified otherwise, all experiments were carried out at 20.0 ± 0.5°C at a PAR of 50 ± 3 μmol photon m^{-2} s^{-1}.

Growth (μg chl *a* l^{-1} and mg l^{-1} dry weight) curves were generated at a salinity of 5‰ (86 mM Na) in 24:0, 20:4, 16:8, 8:16 and 4:20 light:dark. Samples were collected at the same time every other day (4-h after commencement of the light phase for cycles involving dark periods) and analysed for chl *a*. A lag phase was apparent under all conditions (Fig. 7.1). Both the lag and log phases increased with decreasing ratio of light to dark (Table 7.1) although there was no significant ($p<0.05$) difference between 20:4 and 16:8 light:dark. The exponential growth rate (K) decreased as the light period decreased (Table 7.1) although rates were similar at 16:8 and 8:16 light:dark (Table 7.1). Chl *a* concentrations were c. 1% that of dry weight during exponential growth (Fig. 7.1).

Table 7.1 Growth rates of Microcoleus D778 at a salinity of 5‰ (86 mM NaCl) under 24:0, 20:4, 16:8, 8:16 and 4:20 light:dark. 20°C, 50 μmol photon m^{-2} s^{-1}

Light:dark	Mid. log		Growth constants		
	μg chl *a* l^{-1}	day	μ	K	td
24:0	1253.6 ± 47.6	18	0.15 ± 0.01	0.22 ± 0.01	4.41 ± 0.39
20:4	1060.4 ± 31.8	22	0.11 ± 0.01	0.16 ± 0.01	6.16 ± 0.27
16:8	1120.6 ± 38.7	25	0.11 ± 0.01	0.16 ± 0.01	5.95 ± 0.25
8:16	1245.0 ± 31.3	22	0.12 ± 0.01	0.17 ± 0.01	5.47 ± 0.29
4:20	970.6 ± 30.3	38	0.06 ± 0.01	0.09 ± 0.06	10.00 ± 0.50

Fig. 7.1 Influence of light: dark cycles on growth of Microcoleus D778 in medium GD_5-N. 20°C, 50 μmol photon m^{-2} s^{-1}



Growth (μg chl *a* l^{-1} and mg l^{-1} dry weight) curves were also carried out at 24:0 and 16:8 light:dark in the presence of either 0.5 mM NH_4-N or NO_3-N. In contrast to cultures grown in the absence of combined nitrogen (Fig. 7.1), only a short lag phase of one day was found in the presence of combined nitrogen (Fig. 7.2). Whilst growth in the presence of combined nitrogen was higher than in its absence (Table 7.2) growth rates in NH_4-N were significantly ($p<0.05$) greater than in the presence of equimolar concentrations of NO_3-N (Table 7.2).

Table 7.2 Growth rates of *Microcoleus* D778 at a salinity of 5‰ in the presence of 0.5 mM NH_4-N ot NO_3-N in continuous light and 16:8 light:dark. 20°C, 50 μmol photon m^{-2} s^{-1}

Light:dark	Mid log		Growth constants		
	μg chl *a* l^{-1}	day	μ	K	td
NO_3-N					
24:0	1256.7 ± 62.8	9	0.27 ± 0.01	0.39 ± 0.03	2.74 ± 0.11
16:8	1421.6 ± 56.9	13	0.21 ± 0.01	0.32 ± 0.02	3.55 ± 0.14
NH_4-N					
24:0	1261.1 ± 37.8	9	0.36 ± 0.01	0.46 ± 0.02	2.41 ± 0.01
16:8	1326.3 ± 53.1	11	0.24 ± 0.01	0.34 ± 0.02	3.03 ± 0.15

7.3 Nitrogenase activity during lag, logarithmic and stationary growth

ARA was compared on alternate days during lag, log and stationary growth on cultures grown in 24:0, 20:4, 16:8, 8:16 and 4:20 light:dark. The first assay was carried out 2-h after inoculation and thereafter at the same time on each occasion.

ARA was highest during the lag phase immediately prior to log growth (Fig. 7.4). However, in 4:20 light:dark ARA was markedly lower and fairly constant with time both in the light and in the dark (Fig. 7.4). ARA decreased steadily from mid. log, terminating at the onset of the stationary phase.

To ensure trends in ARA were similar at different periods of a 24-h cycle, ARA was tested 2, 4, 12 and 14-h after the end of the dark period (in 16:8 light:dark) during lag and logarithmic phases of growth. ARA trends were similar (Fig. 7.4).

Fig. 7.2 Influence of combined N on growth of Microcoleus D778 in medium GD_5+N. 20°C, continuous light and 16:8 light (50 µmol photon m^{-2} s^{-1}):dark

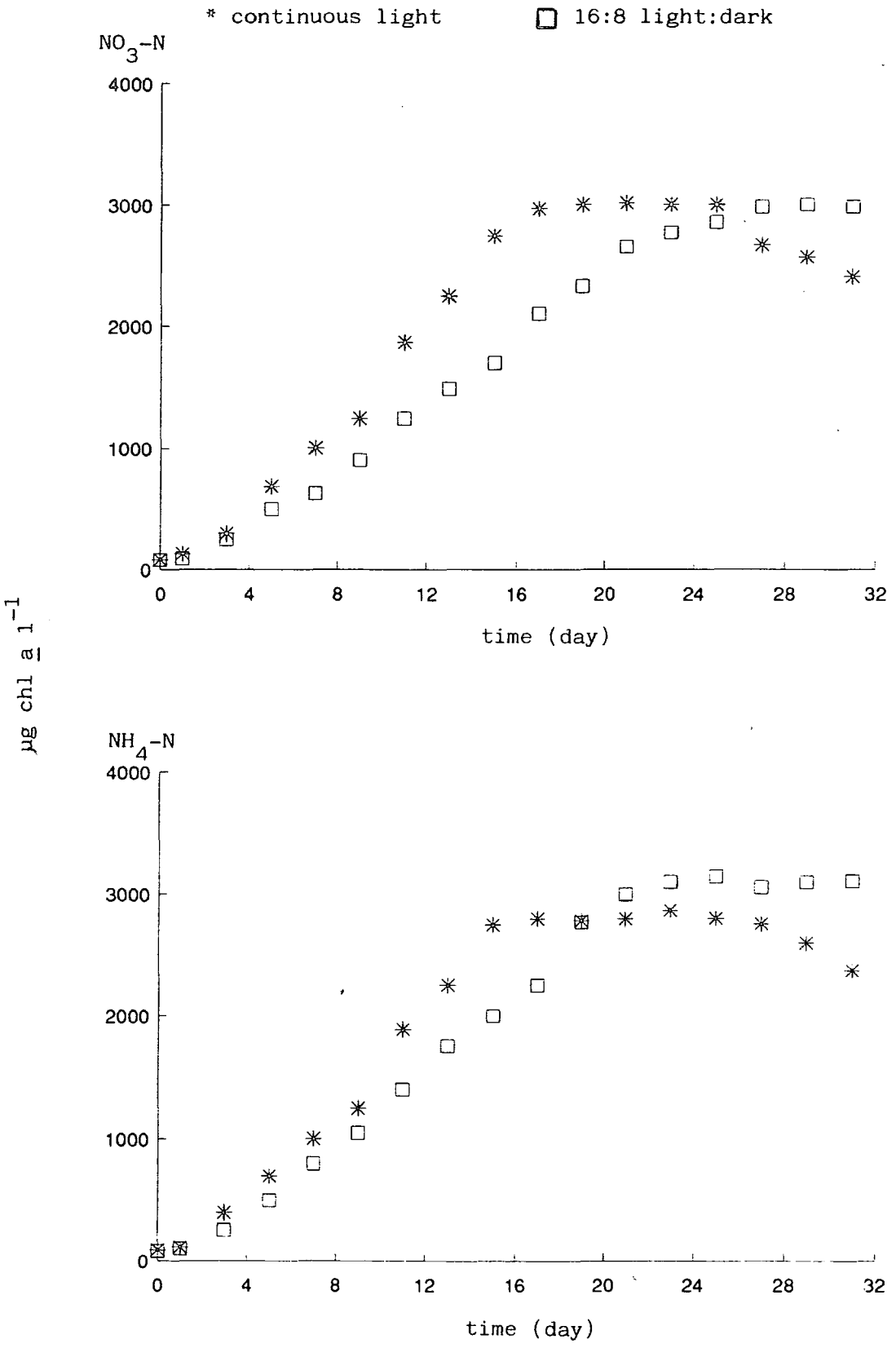


Fig. 7.3 Comparison of ARA in Microcoleus D778 2, 4, 6 and 8-h after dark during lag and logarithmic growth under 16:8 light:dark. 20°C, 50 µmol photon m^{-2} s^{-1}

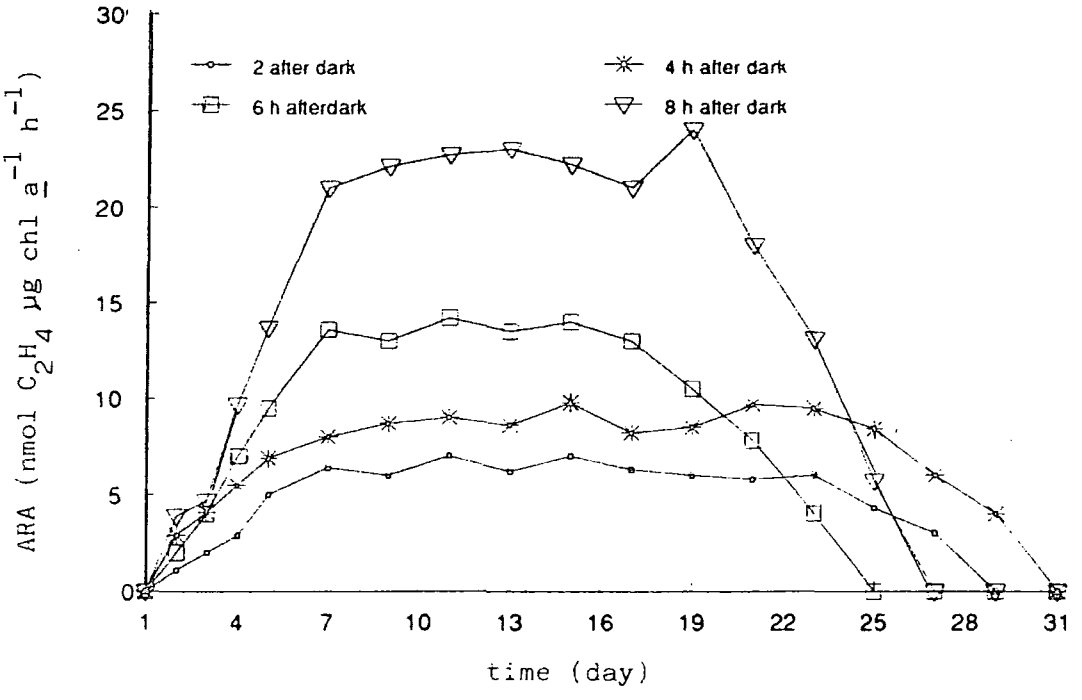
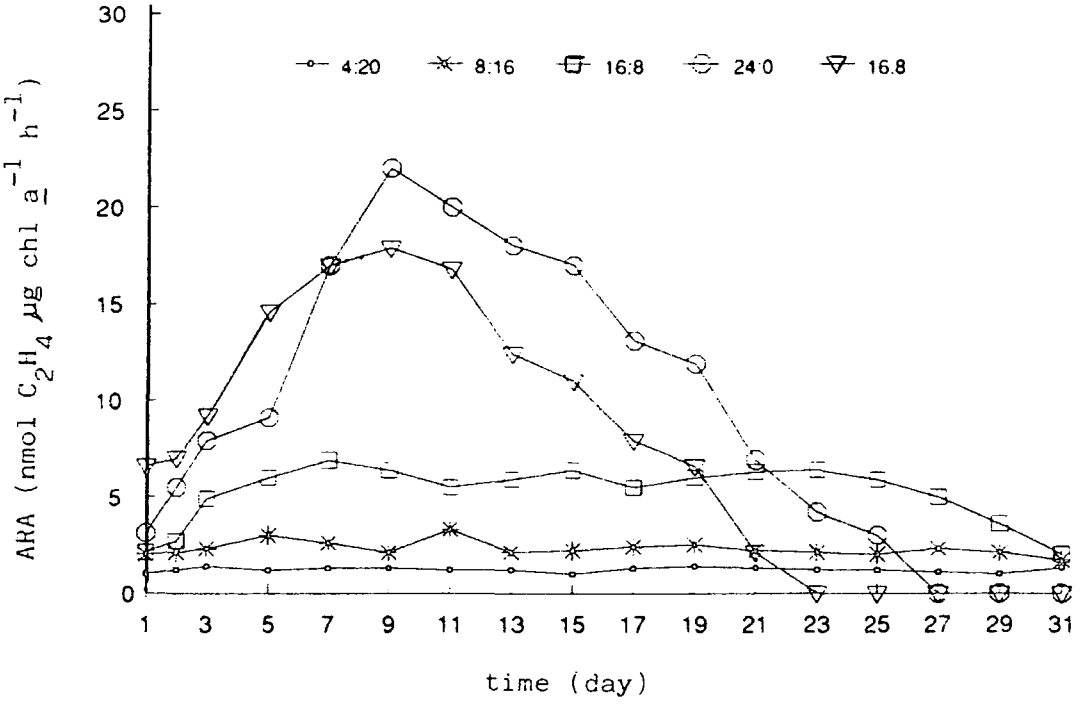


Fig. 7.4 ARA curves for Microcoleus D778 in light:dark cycles. 20°C, 50 umol photon m^{-2} s^{-1}



7.4 Nitrogenase activity over a 24-h period

ARA was compared over 1, 2 and 4-h incubation periods. Trends in ARA were similar using all three incubation periods; however, the shorter the incubation period the more accurately could changes in activity be seen (Fig. 7.5).

ARA was then compared over a 24-h period using 1-h incubation periods on days 10/11, 13/14, 17/18, 19/20 and 20/21 for cultures grown in 24:0, 20:4, 16:8, 8:16 and 4:20 light:dark respectively. ARA was detected at all times (using 1 h incubation periods), during both the light and dark period, with the majority of ARA in the dark during 8:16 and 4:20 light:dark; however, under 20:4 and 16:8 light:dark the majority of ARA occurred in the light (PAR at 50 ± 3 μmol photon m^{-2} s^{-1}) (Fig. 7.6).

Table 7.3 % ARA in Microcoleus D778 both in the light and in the dark during 24-h assays in 24:0, 20:4, 16:8, 8:16 and 4:20 light:dark cycles. 20°C, 50 μmol photon m^{-2} s^{-1}

Light:dark	% ARA	
	light	dark
24:0	100	0
20:4	81	19
16:8	60	40
8:16	28	72
4:20	8	92

Sharp deviations in ARA were observed at irregular intervals (using 1 h incubation periods) under 20:4 light:dark and in continuous light (Fig. 7.6) Under both 20:4 and 16:8 light:dark, ARA more than doubled at the onset of the dark period and in the latter attained levels of ARA unrecorded in the light (Fig. 7.6). In 8:16 and 4:20 light:dark ARA was lower than 0.6 ± 0.1nmol C_2H_4 m^{-2} s^{-1} at all times in contrast to activities of 0.4 - 3.2 ± 0.1 nmol C_2H_4 μg chl $\underline{a}^{-1}$ h^{-1} in 24:0, 20:4 and 16:8 light:dark.

Due to insufficient incubation area examination of ARA over a 48 h period was not possible using 48 1 h periods (n = 4), therefore, 12 2 h periods were used. ARA was similar on each consecutive 24-h period for cultures grown in 16:8 light:dark (Fig. 7.7). However, on transferring cultures to continuous light, ARA patterns changed (Fig. 7.7) such that after 72 h the response was

g. 7.5 Influence of incubation period on ARA in Microcoleus D778 over a 16:8 light:dark cycle. 20°C, 50 μmol photon m^{-2} s^{-1}

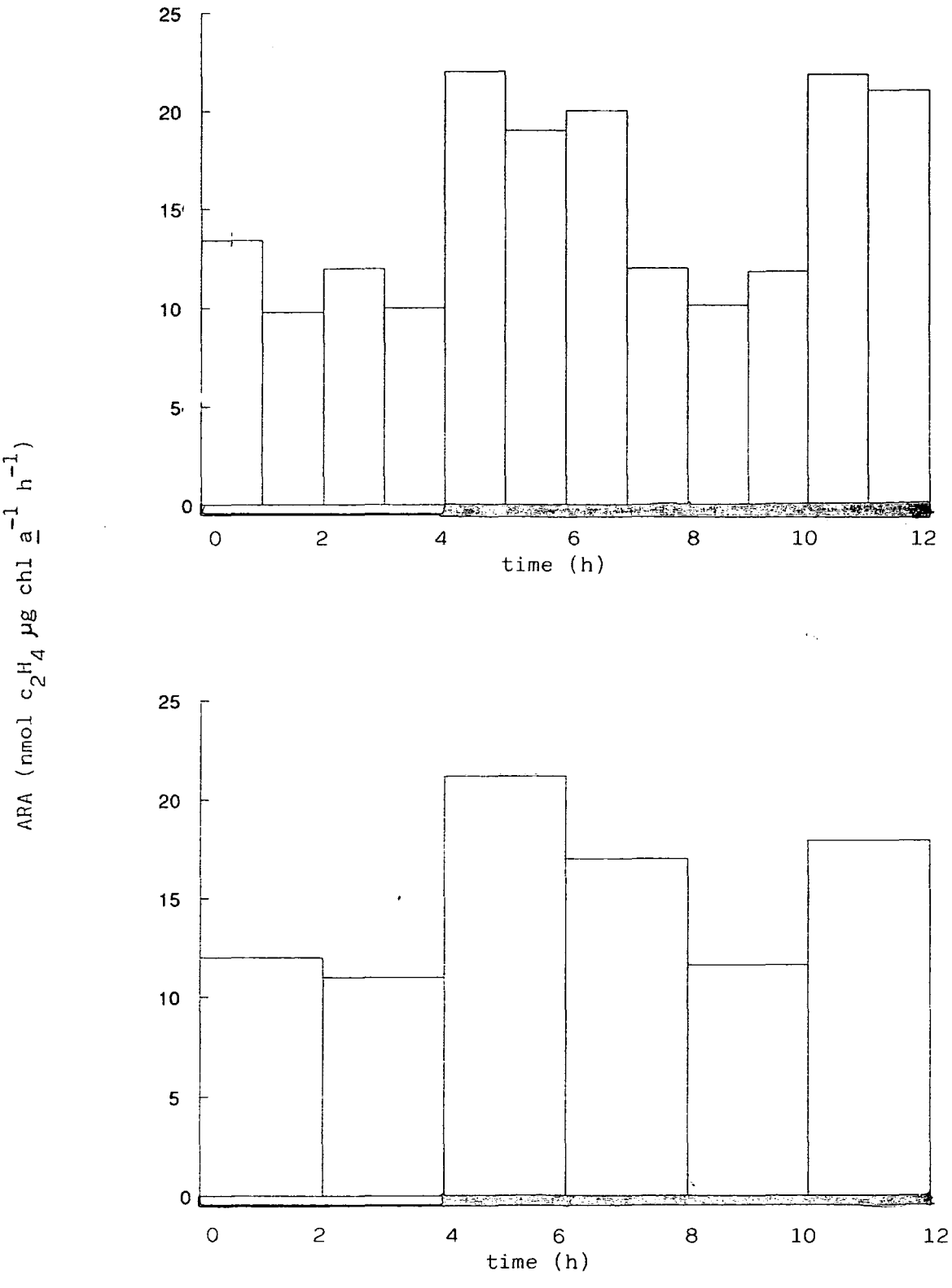


Fig. 7.6 Influence of light:dark cycles on ARA in Microcoleus D778. 20°C, 50 µmol photon m^{-2} s^{-1}, 5%o

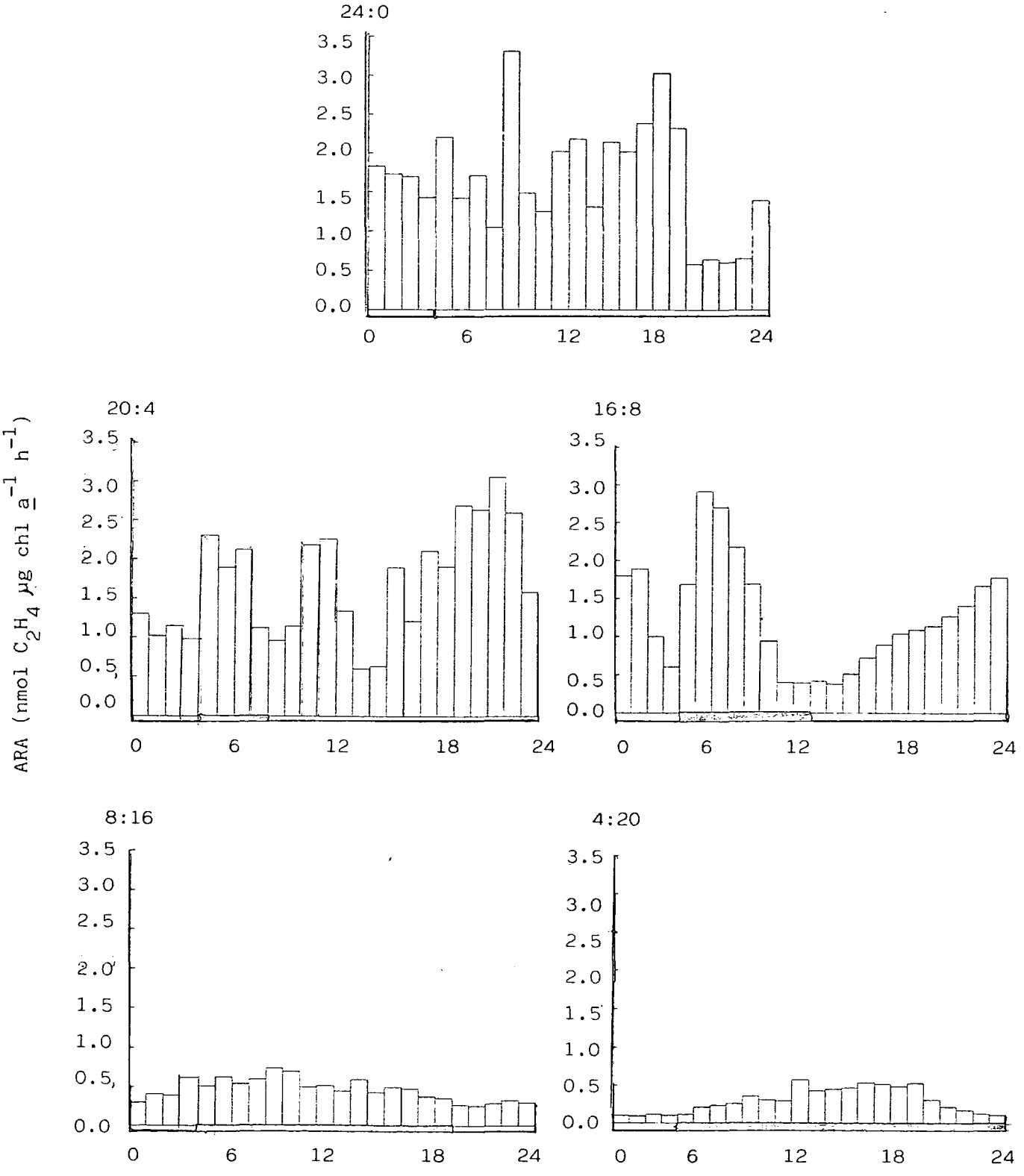
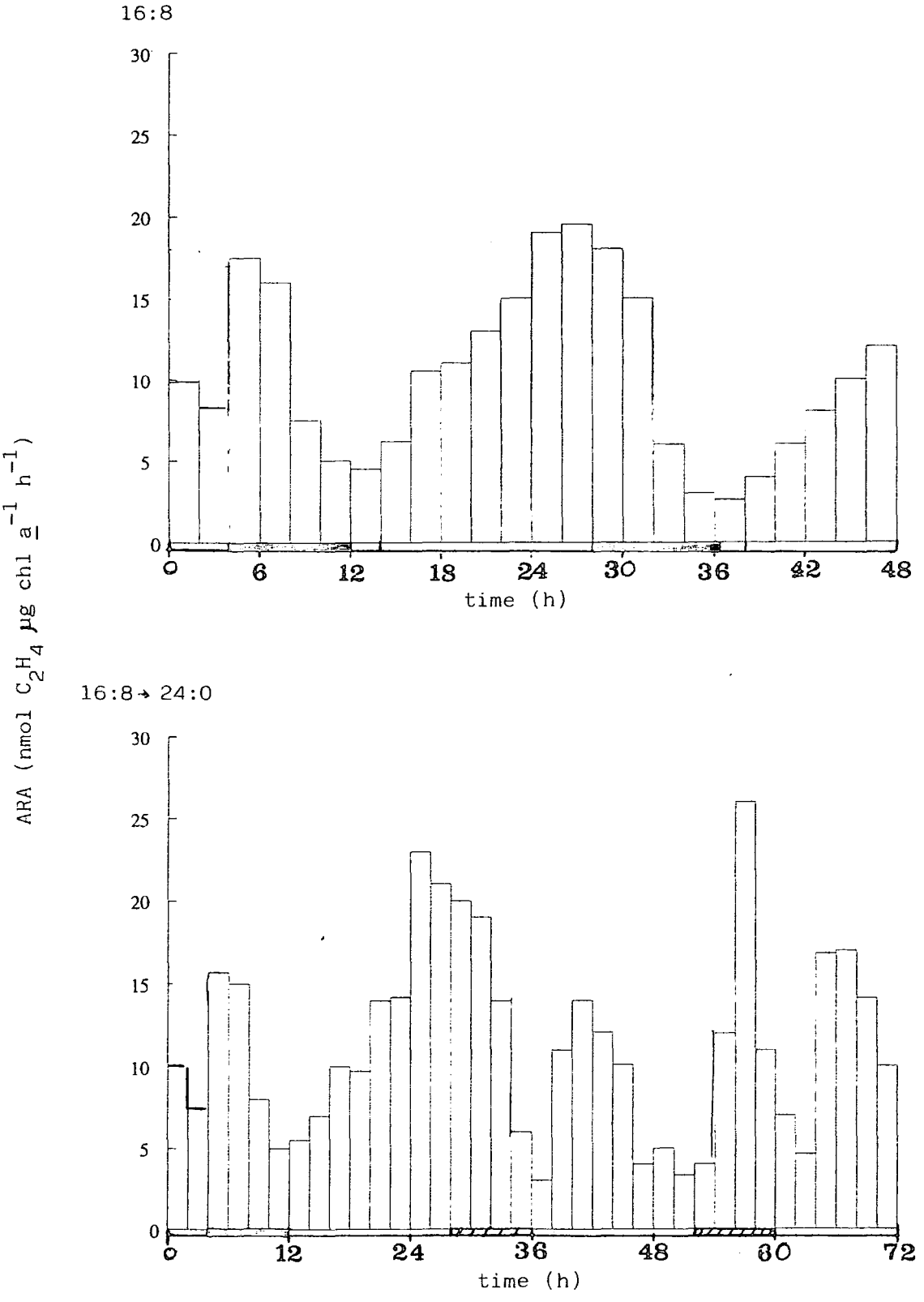


Fig. 7.7 Comparison of ARA in Microcoleus D778 transferred from 16:8 light:dark to continuous light. 20°C, 50 μmol photon m^{-2} s^{-1}



similar to that previously found for cultures grown in continuous light (7.6).

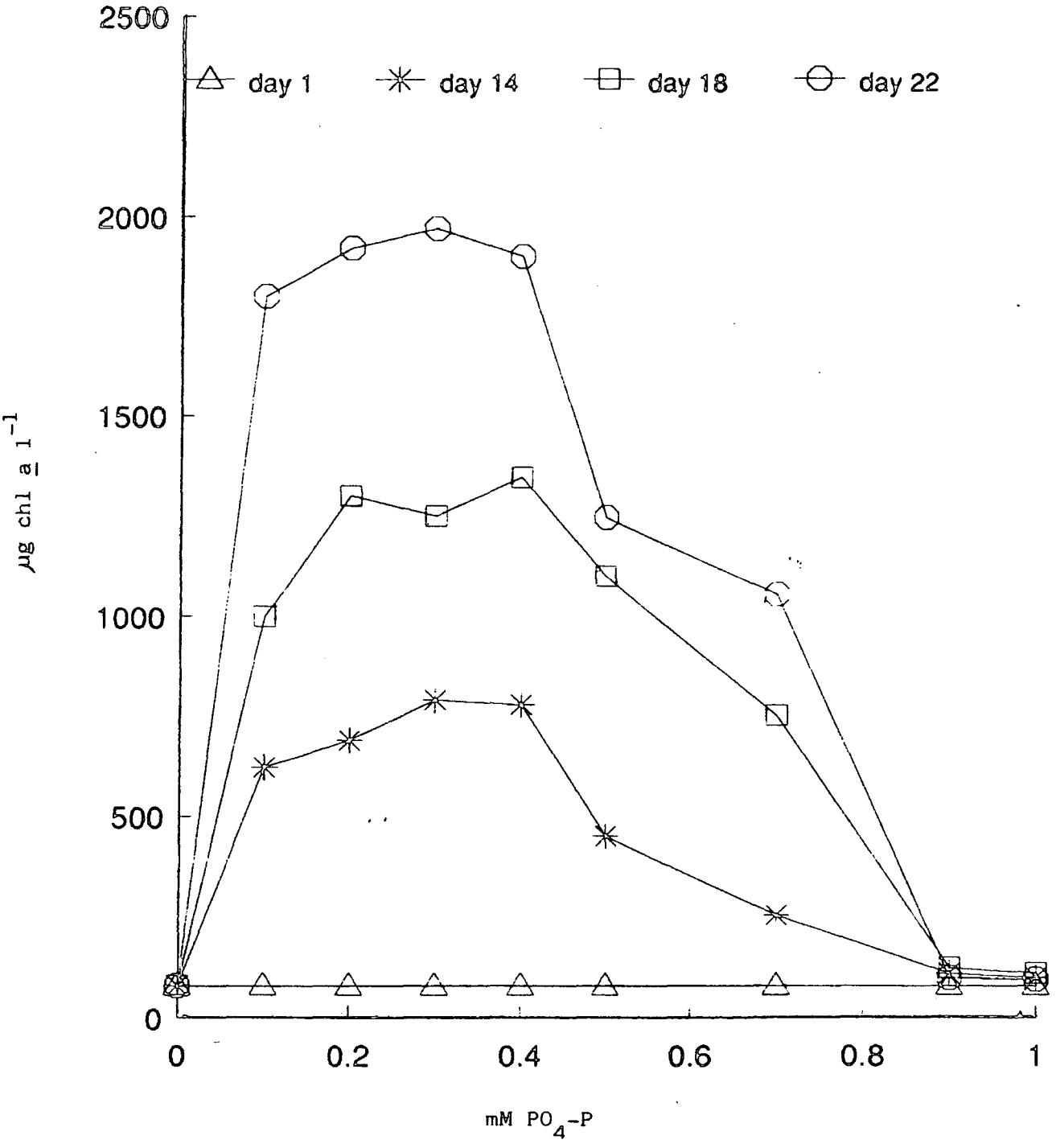
All further experiments comparing growth (unless specified otherwise) were carried out at mid. log in $GD_5 \pm N$ in 24:0 or 16:8 light:dark. ARA was tested at the point of maximum activity corresponding to the highest concentration of chl *a*. (Fig. 7.4).

7.5 Influence of nutrients on growth and ARA

7.5.1 PO_4-P

Cultures were grown in 0.0 - 1.0 mM PO_4-P in the presence and absence of combined nitrogen. Growth and ARA were optimum between 0.1 - 0.4 mM PO_4-P, whereas at higher concentrations a precipitate was formed when the salinity of the medium was in excess of 20‰ (344 mM Na) due to deposition of calcium (as shown by AAS).

Fig. 7.8 Influence of PO_4-P on ARA in Microcoleus D788 in medium GD$_5$-N. 20°C, 16:8 light (50 µmol photon m^{-2} s^{-1}):dark



7.5.2 Salinity (addition of NaCl)

Cultures grew at salinities of 0.5 - 37‰ (9 - 636 mM Na^+) in the absence of combined N and at salinities of up to 40‰ (688 mM) in the presence of combined N, with optima between 5 and 15‰ (86 and 258 mM Na), both in the presence and absence of combined N (Fig. 7.9). Cultures expressed ARA at salinities of 0.5 to 35‰ (9 - 602 mM Na) with optima between 5 and 15‰ (86 - 258 mM Na) (Fig. 7.10 - 7.12).

Table 7.4 Growth rates of Microcoleus D778 at salinities of 5, 10, 15 and 20‰ in medium GD-N. 20°C, 16:8 light (50 µmol photon m^{-2} s^{-}):dark

Salinity	Mid log		Growth constants		
‰	µg chl *a* l^{-1}	day	µ	K	td
5	1060.4 ± 31.3	20	0.11 ± 0.01	0.16 ± 0.01	6.16 ± 0.27
10	880.4 ± 44.0	21	0.11 ± 0.01	0.16 ± 0.01	6.33 ± 0.32
15	876.3 ± 45.1	20	0.11 ± 0.01	0.17 ± 0.01	6.06 ± 0.30
20	381.1 ± 46.3	18	0.13 ± 0.01	0.18 ± 0.01	5.52 ± 0.28

When ARA was compared over a 16:8 light (50 µmol photon m^{-2} s^{-1}):dark cycle at salinites of 5 and 30‰ (86 and 510 mM), total ARA over the 24 h periods was c. 50% lower in the latter. In addition, ARA at 30‰ was never higher than 6.00 ± 0.10 nmol C_2H_4 µg chl *a*$^{-1}$ h^{-1} during 1 h incubation periods, whereas activity rose to 14.3 ± 0.12 nmol C_2H_4 µg chl *a*$^{-1}$ h^{-1} at a salinity of 5‰. At a salinity of 5‰ there was a sudden increase in ARA from 7.16 ± 0.11 - 12.3 ± 0.11 nmol C_2H_4 µg chl *a*$^{-1}$ h^{-1} at the onset of the dark period, whereas at 30‰ there was a significant ($p<0.05$) decrease in ARA (Fig. 7.13).

Fig. 7.9 Influence of salinity on growth of Microcoleus D778. 20°C, 16:8 light (50 μmol photon m^{-2} s^{-1}):dark

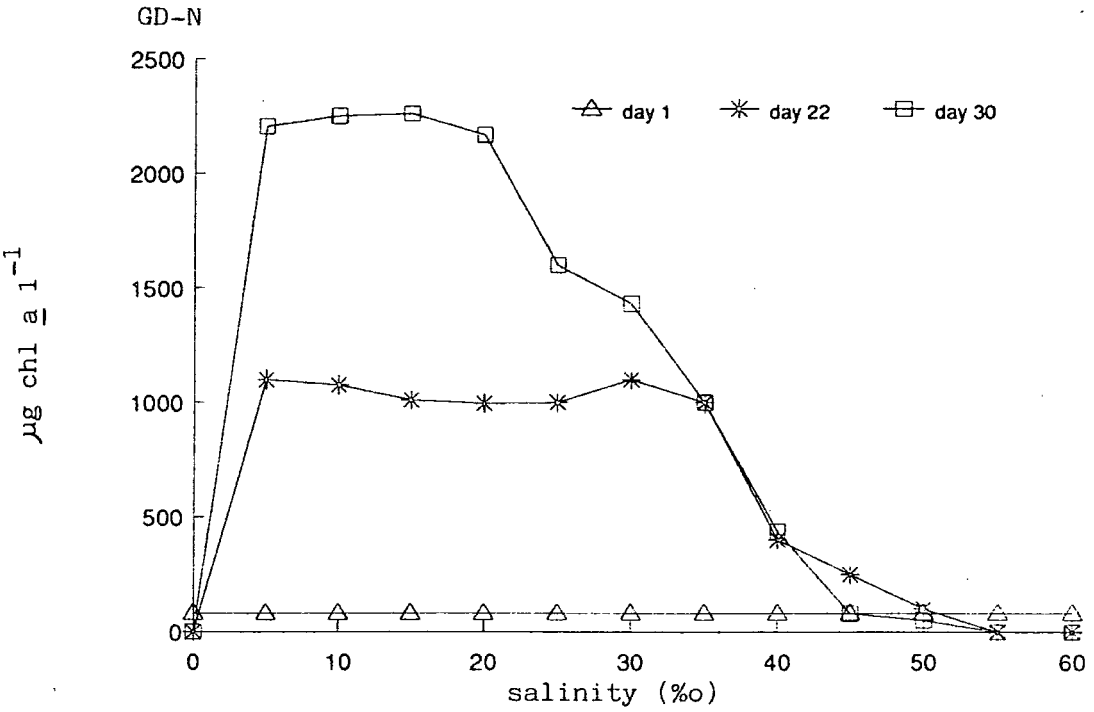


Fig. 7.10 Influence of salinity on ARA. 20°C, 16:8 light (50 umol photon m^{-2} s^{-1}):dark

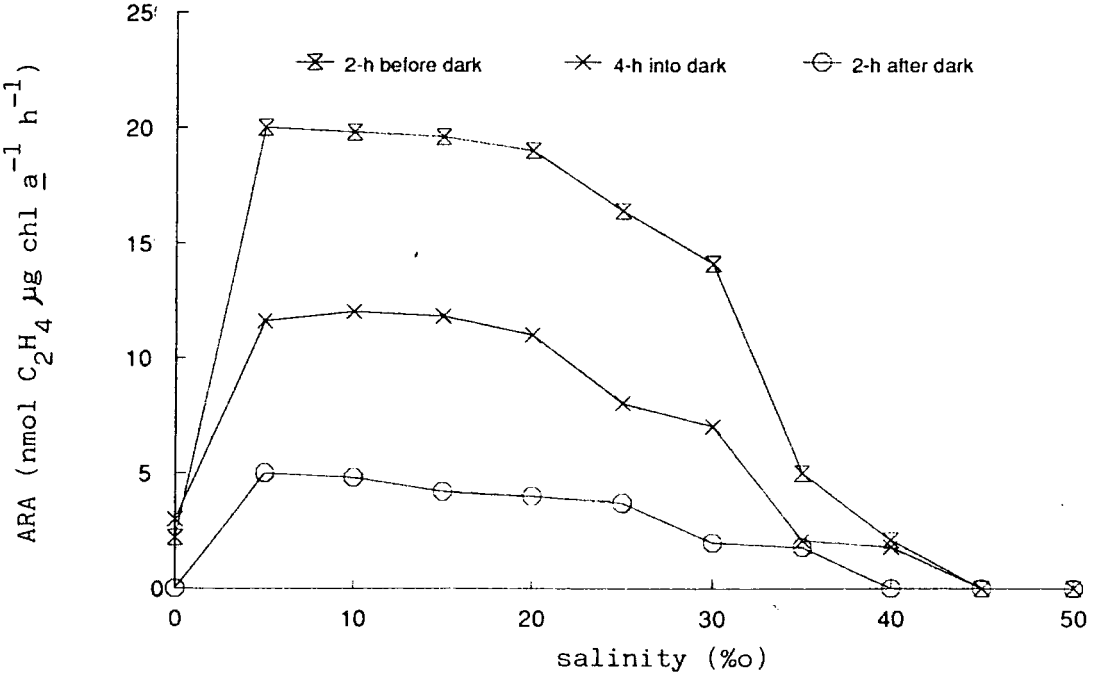


Fig. 7.11 Influence of salinity (addition of NaCl) on ARA in Microcoleus D778 at 12-h intervals on days 8/9. 20°C, 50±3 umol photon m^{-2} s^{-1}

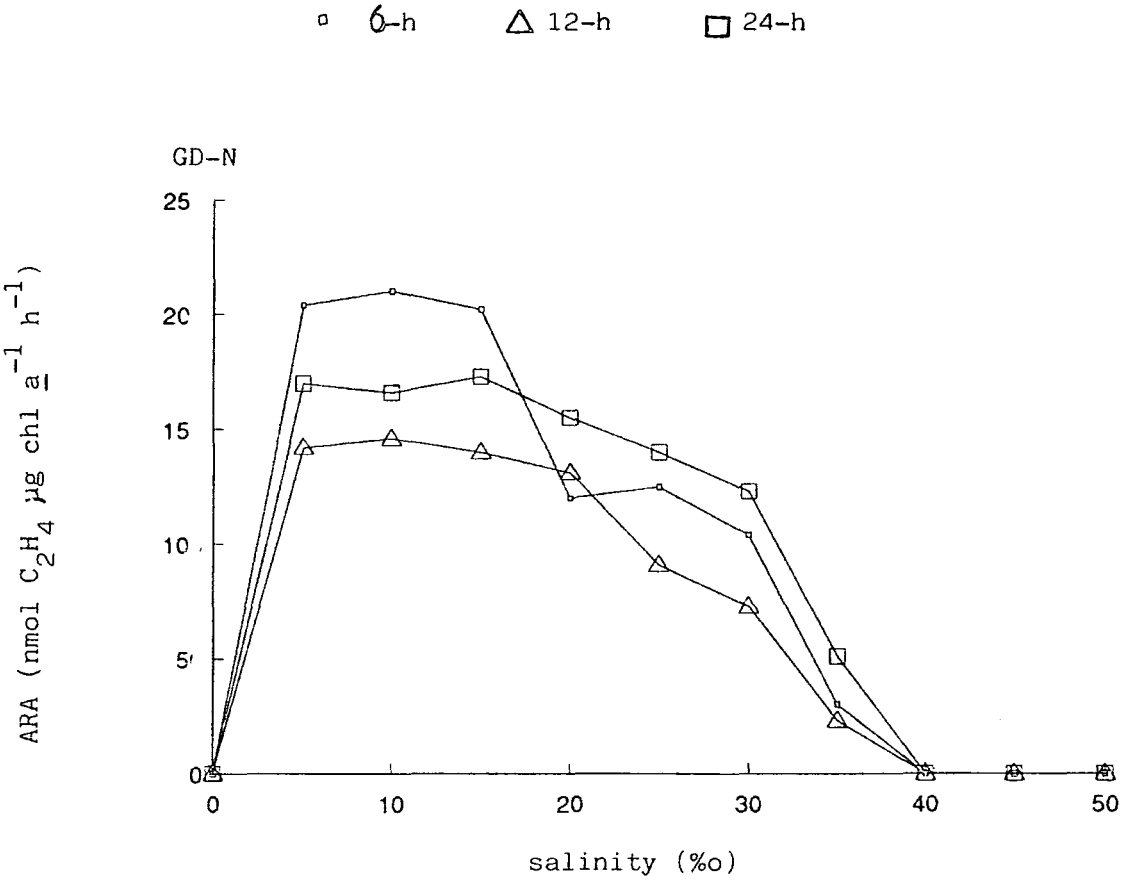
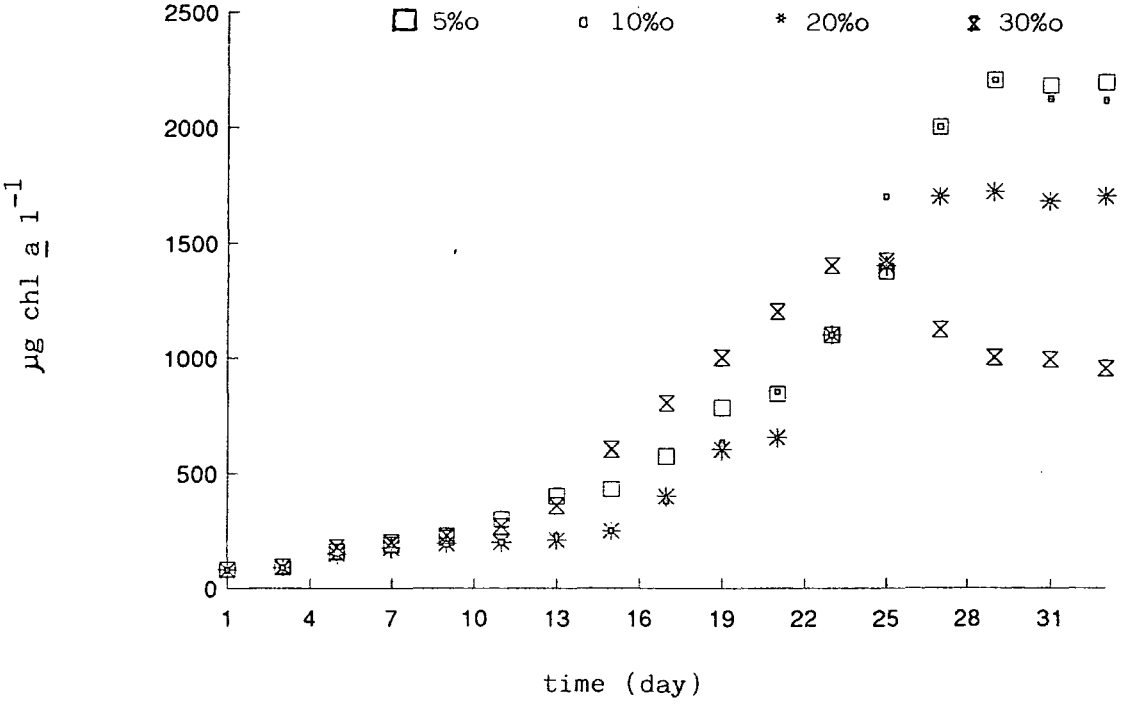
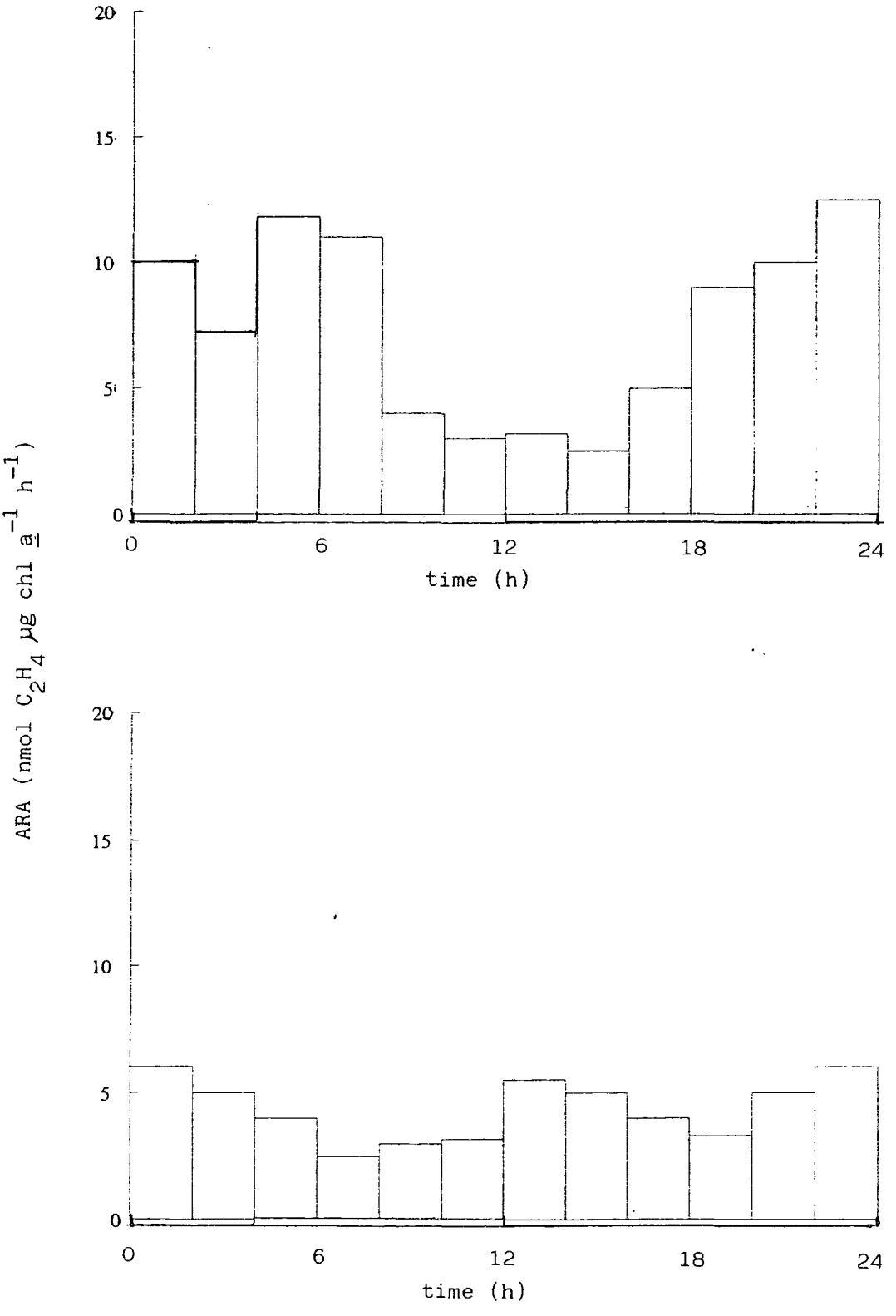


Fig. 7.12 Influence of salinity on growth of Microcoleus D778 in medium GD-N. 20°C, 16:8 light (50 umol photon m^{-2} s^{-1}):dark



ig. 7.13 Influence of salinity on ARA in Microcoleus D778 under 16:8 light (50 µmol photon m^{-2} s^{-1}):dark at 20°C



7.5.3 Calcium ($CaCl_2 2H_2O$)

Growth in the presence and absence of combined N, both in continuous light and 16:8 light:dark, increased at Ca concentrations of up to 45 mM (Fig. 7.26). In the absence of combined N growth was similar up to Ca^{2+} concentrations of 300 mM (Fig. 7.14), whereas in the presence of combined N, growth declined at concentrations in excess of 300 mM Ca (Fig. 7.15). ARA required 50 mM Ca^{2+} for maximum activity in continuous light (Fig. 7.16), whereas in 16:8 light:dark 75 mM Ca was required for maximum activity on day 5 but only 25 mM on day 15 (Fig. 7.16),

Growth at salinities of 5 - 30‰ (85 - 516 mM Na) was compared at different concentrations of calcium (Fig. 7.17). Growth was highest at 25 mM Ca^{2+} with only 80% growth in 200 mM Ca^{2+} and under 50% at 5 mM Ca^{2+}.

Fig. 7.14 Influence of calcium on growth of Microcoleus D778 in medium GD_5-N. 20°C, continuous light and 16:8 light (50 µmol photon m^{-2} s^{-1}):dark

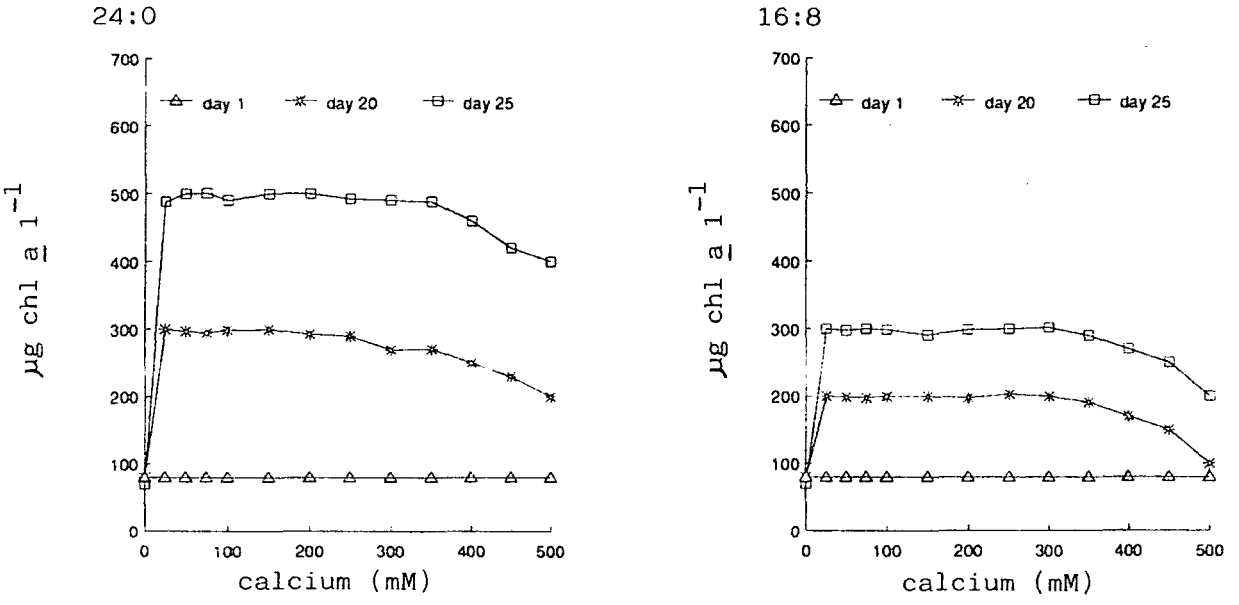


Fig. 7.15 Influence of calcium on growth of Microcoleus D778 in medium GD_5+N. 20°C, 16:8 light (50 umol photon m^{-2} s^{-1}):dark

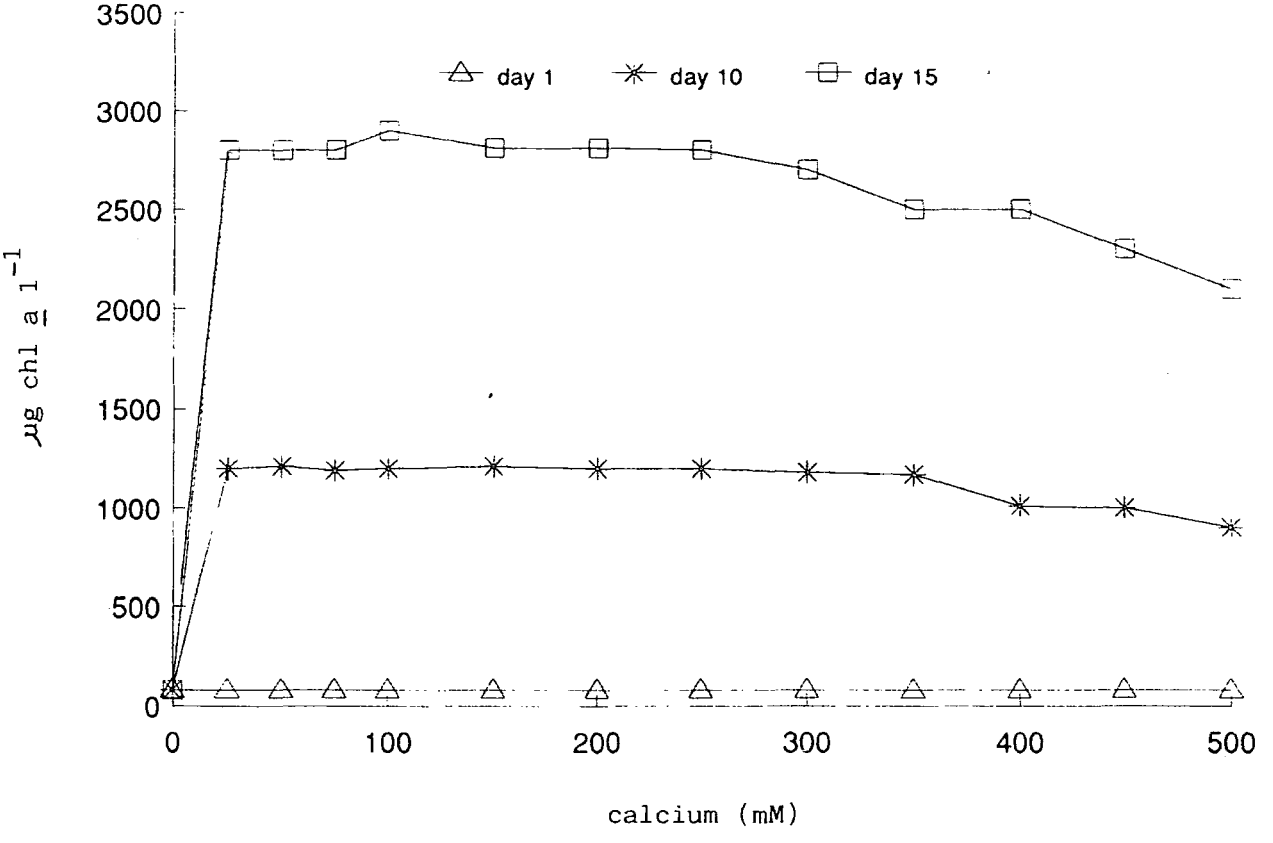


Fig. 7.16 Influence of calcium on ARA in Microcoleus D778. 20°C, continuous light and 16:8 light (50 umol photon m^{-2} s^{-1}):dark

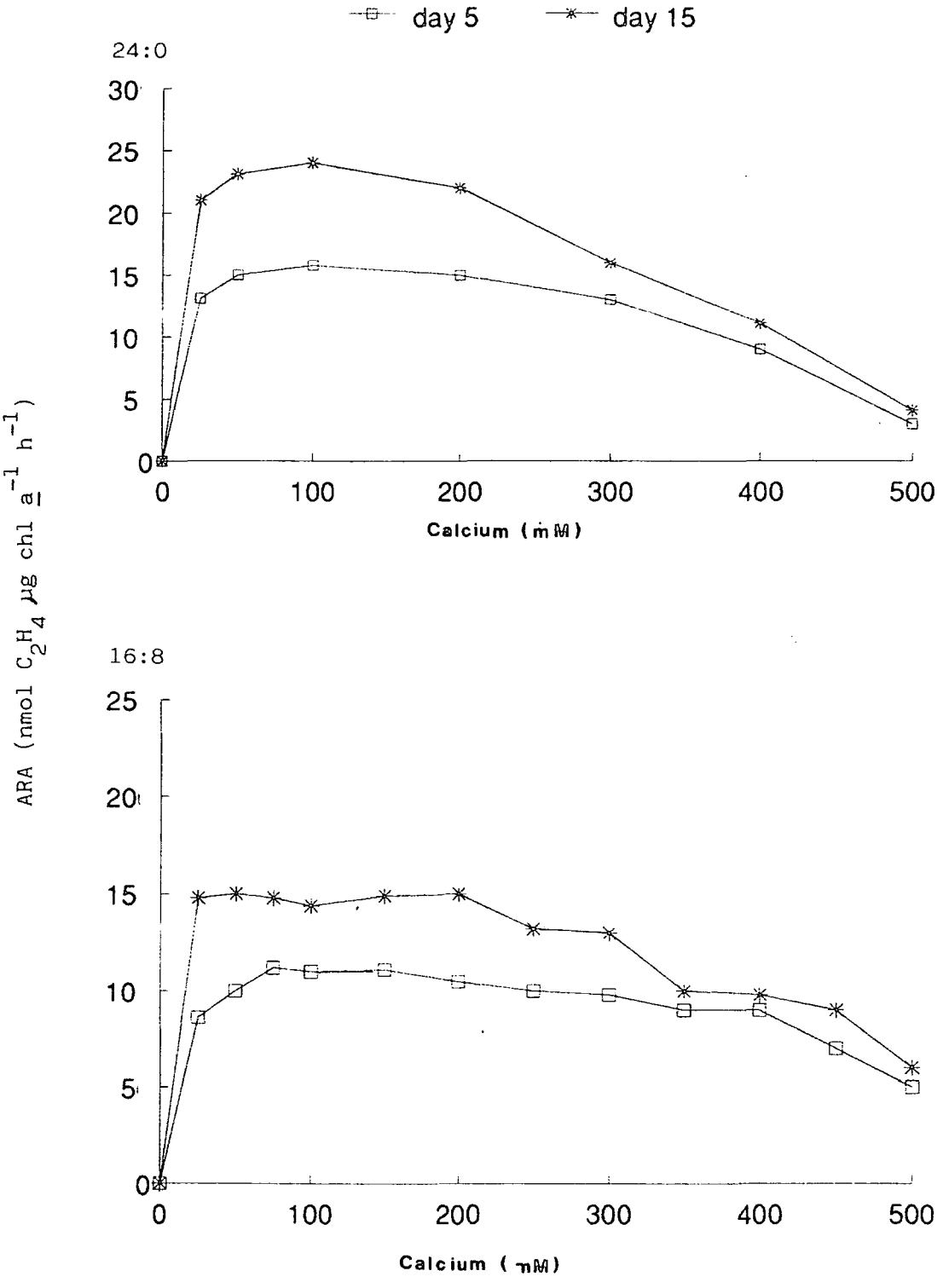
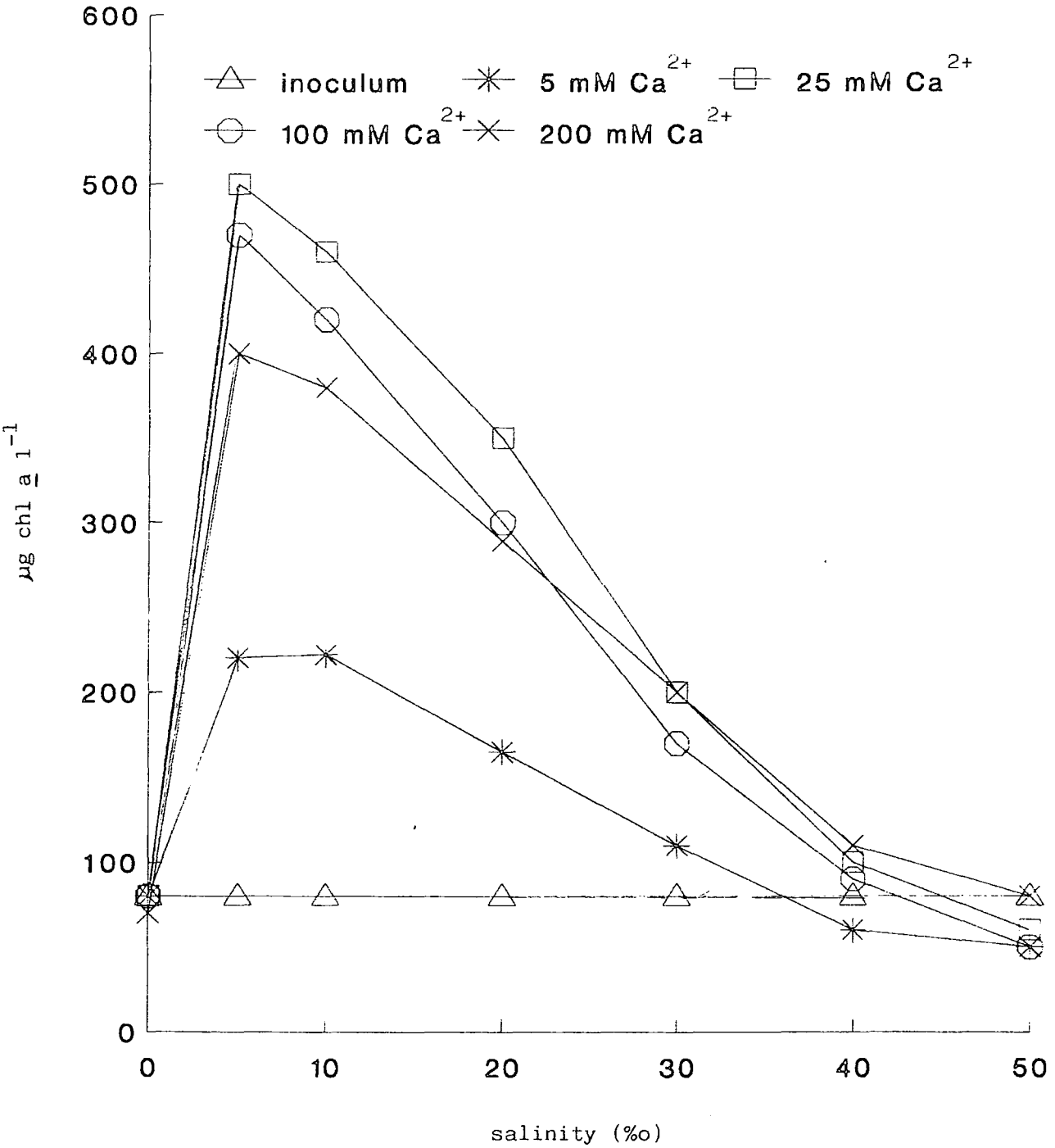


Fig. 7.17 Influence of calcium and salinity on growth of Microcoleus D778. 20°C, 16:8 (50 µmol photon m^{-2} s^{-1}):dark



7.5.4 Potassium (KCl)

In the absence of combined N Microcoleus required 25 mM and 50 mM K^+ for maximum growth under 16:8 and 24:0 light:dark respectively (Fig. 7.18), whereas only 25 mM K^+ was required under continuous light in the presence of combined N (Fig. 7.19).

Growth at salinities of 5 - 30‰ (86 - 516 mM Na) was dependent on the concentration of K^+ (Fig. 7.20). No growth was found a salinity of 40‰ (688 mM Na) in the presence of 10 mM and 200 mM K^+; however, there was a significant ($p<0.05$) increase after 6 days at 25 and 100 mM K^+ (Fig. 7.20).

ARA increased at K^+ concentrations of up to 25 mM, both in continuous light and in a 16:8 light:dark cycle (Fig. 7.21); however, ARA in 15 day old cultures under continuous light declined at concentrations in excess of 25 mM whereas ARA remained constant up to concentrations of 300 mM in 5 day old cultures.

Fig. 7.18 Influence of potassium on growth of Microcoleus D778 in medium GD_5-N. 20°C, continuous light + 16:8 light (50 µmol photon m^{-2} s^{-1}):dark

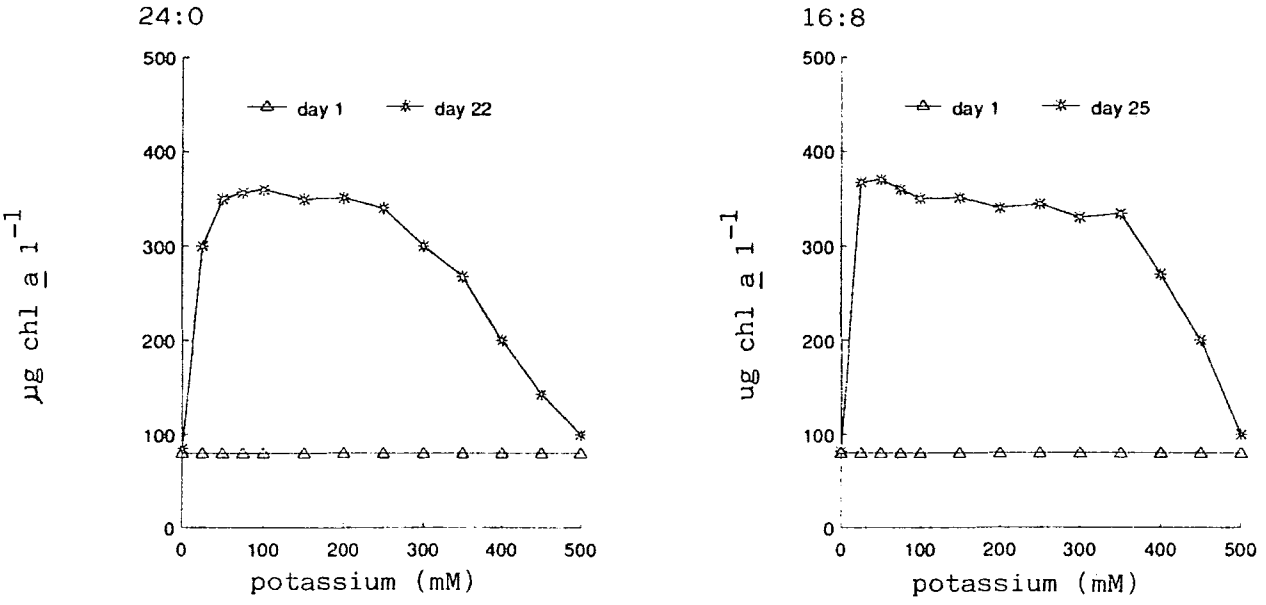


Fig. 7.19 Influence of potassium on growth of Microcoleus D778 in medium GD_5+N. 20°C, 50 µmol photon m^{-2} s^{-1}

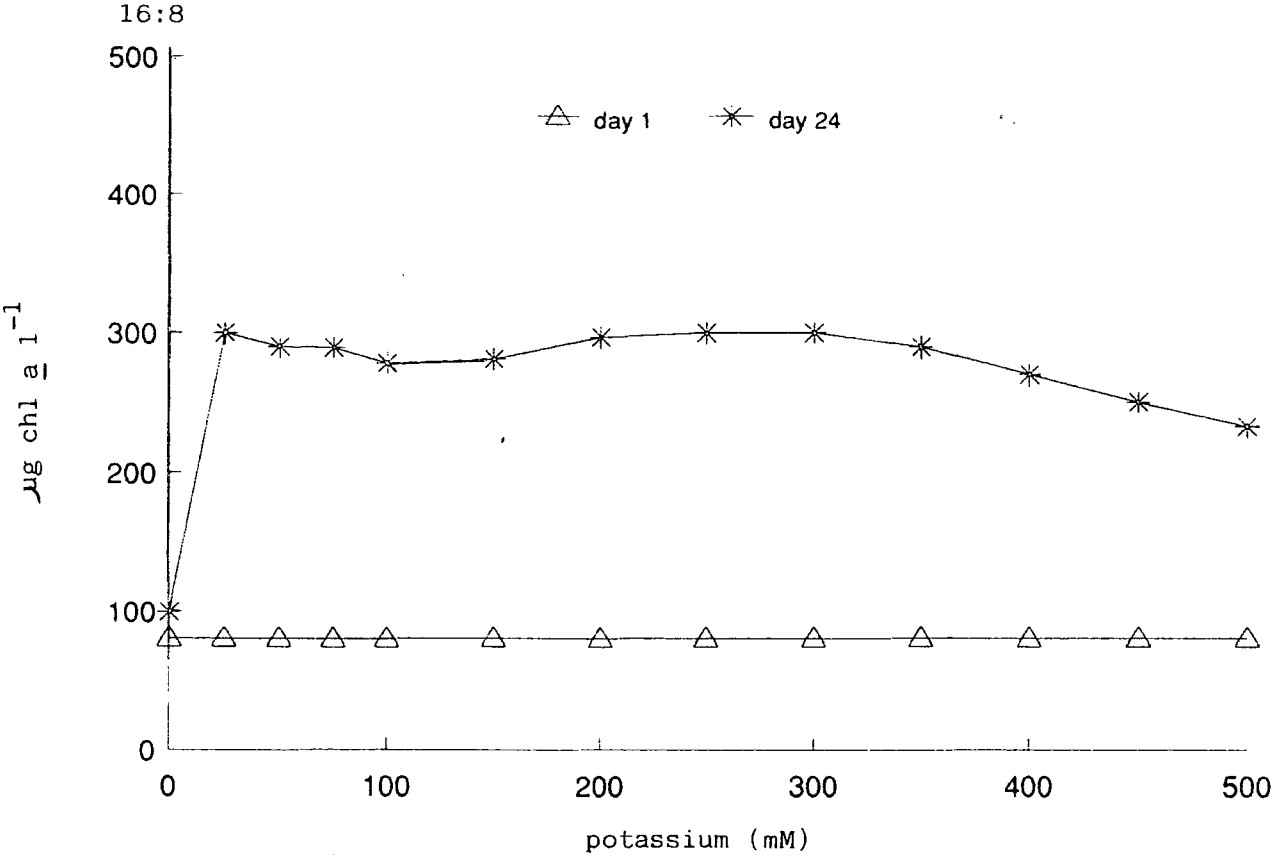


Fig. 7.20 Influence of potassium and salinity (addition of NaCl) on growth of Microcoleus D778. 20°C, 16:8 light (50 µmol photon m^{-2} s^{-1}):dark

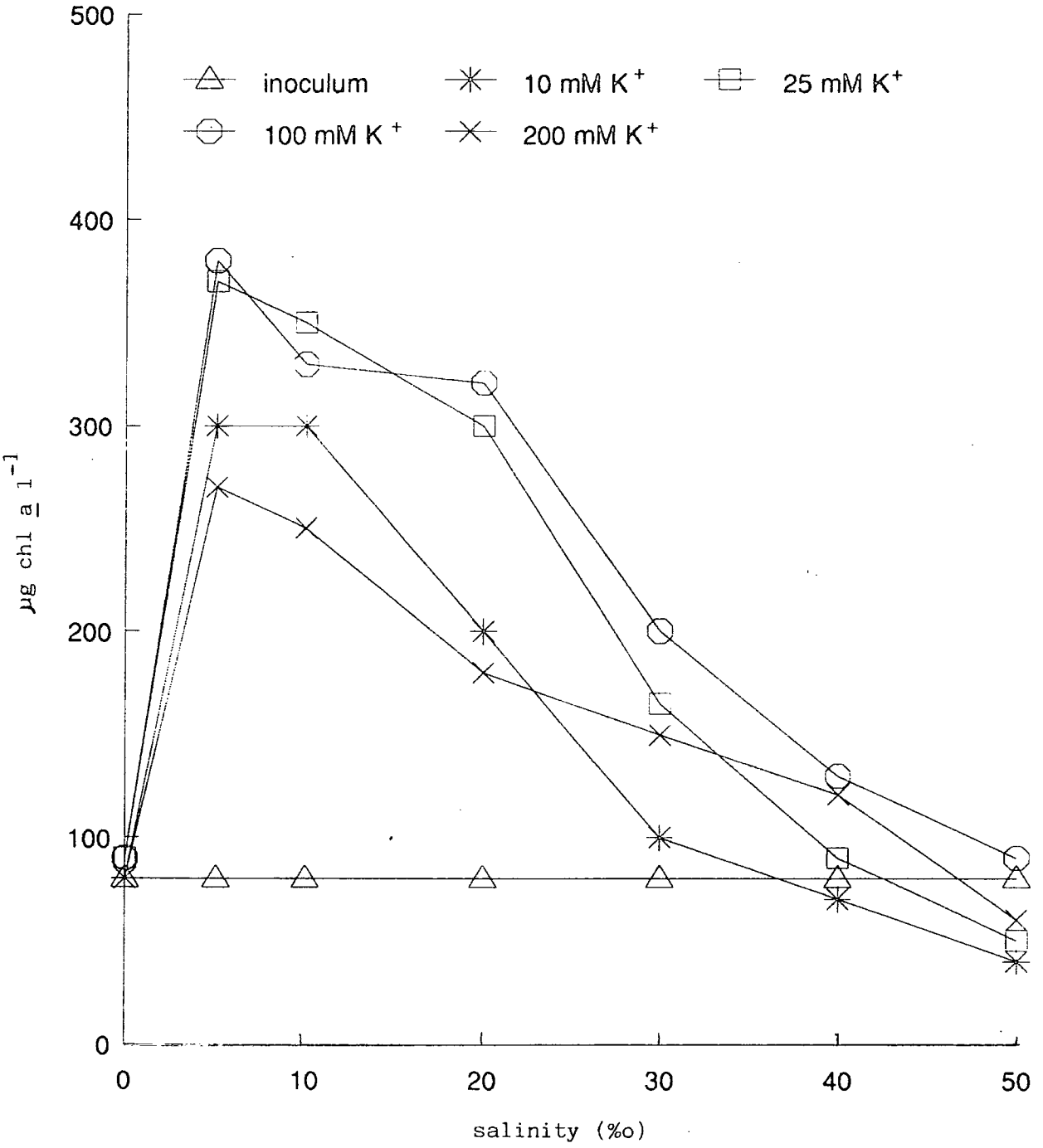
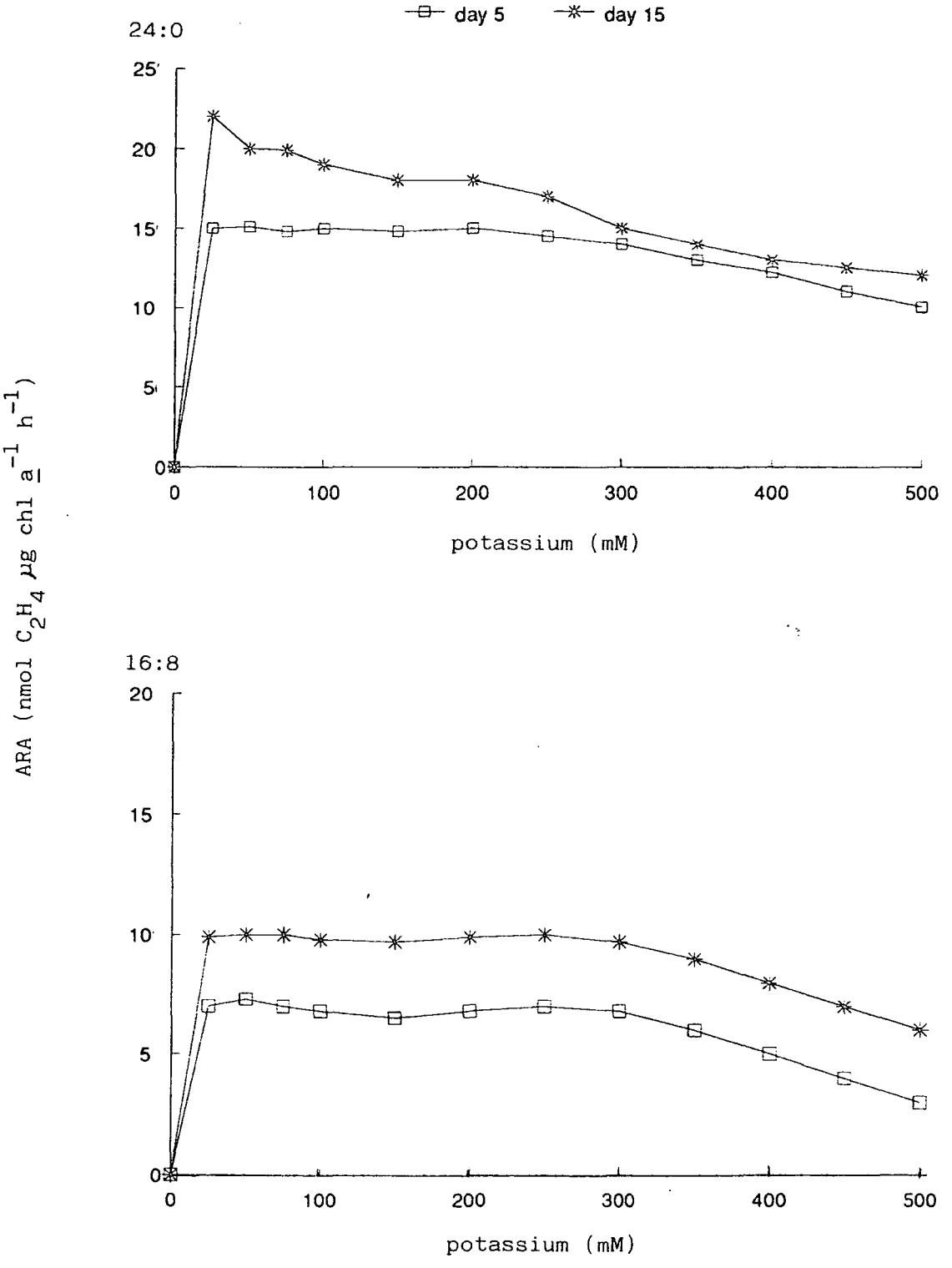


Fig. 7.21 Influence of potassium on ARA in Microcoleus D778. 20°C, continuous light and 16:8 light (50 μmol photon m^{-2} s^{-1}):dark



7.5.5 Magnesium ($MgCl_26H_2O$)

Maximum growth, both in the presence and absence of N, under 16:8 light dark cycle required 25 and 15 mM Mg respectively (Fig. 7.22, 7.26). When growth was compared between 25 and 500 mM Mg, growth in the presence of N under continuous light declined at concentrations in excess of 300 mM, whereas in the absence of combined N growth declined at 200 and 250 mM Mg under continuous light and 16:8 light:dark respectively.

Growth (-N) at salinities of 5 - 30‰ (86 mM - 516 mM Na) increased as the concentration of Mg increased from 5 - 100 mM; however, growth at 200 mM Mg was lower than that at 25 mM Mg (Fig. 7.24). Cultures were able to grow at salinities of 40‰ (688 mM Na) in the presence of 25 and 100 mM Mg, but not at 5 and 200 mM Mg; however, magnesium concentrations of 200 mM raised the salinity by c. 10‰.

ARA in continuous light was highest between Mg concentrations of 25 - 100 mM (Fig. 7.25) whereas, in 16:8 light:dark there was no significant difference in ARA at Mg concentrations between 25 and 250 mM (Fig. 7.25).

Fig. 7.22 Influence of magnesium on growth of Microcoleus D778 in medium GD_5-N. 20°C, continuous light and 16:8 light (50 µmol photon m^{-2} s^{-1}):dark

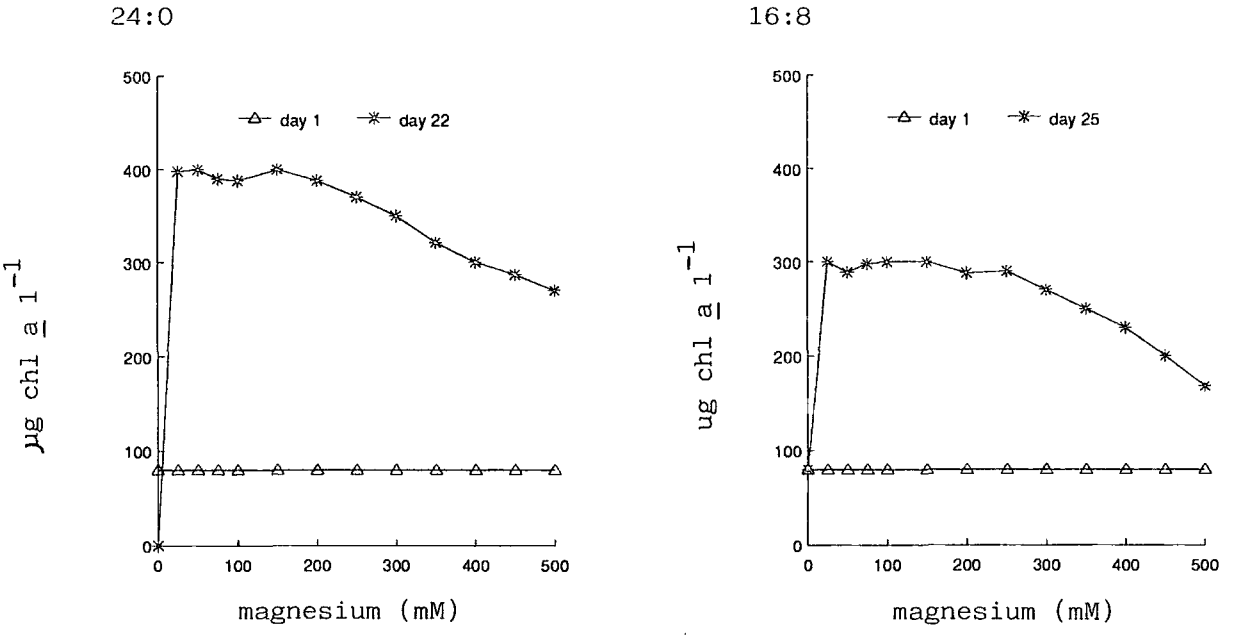


Fig. 7.23 Influence of magnesium on growth of Microcoleus D778 in medium GD_5+N. 20°C, 50 umol photon m^{-2} s^{-1}

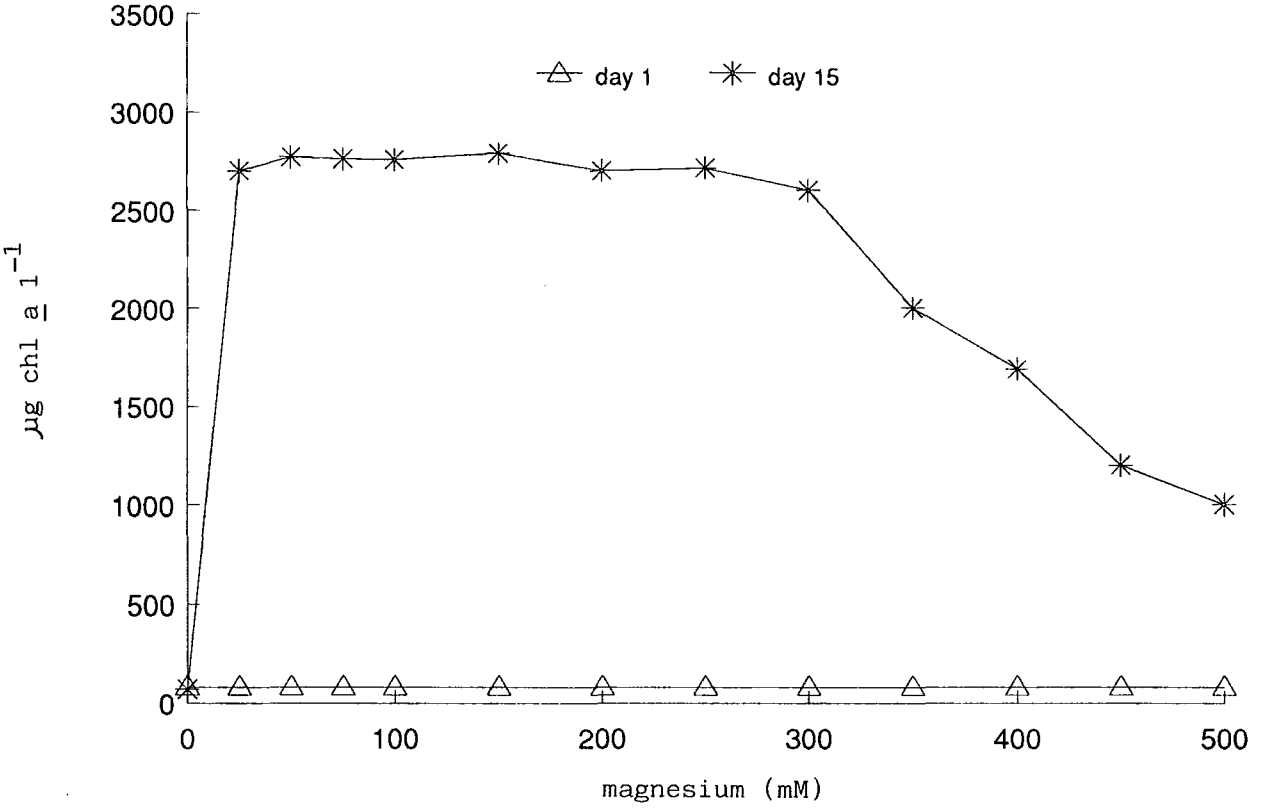


Fig. 7.24 Influence of magnesium and salinity on growth of Microcoleus D778. 20°C, 16:8 light (50 μmol photon m^{-2} s^{-1}):dark

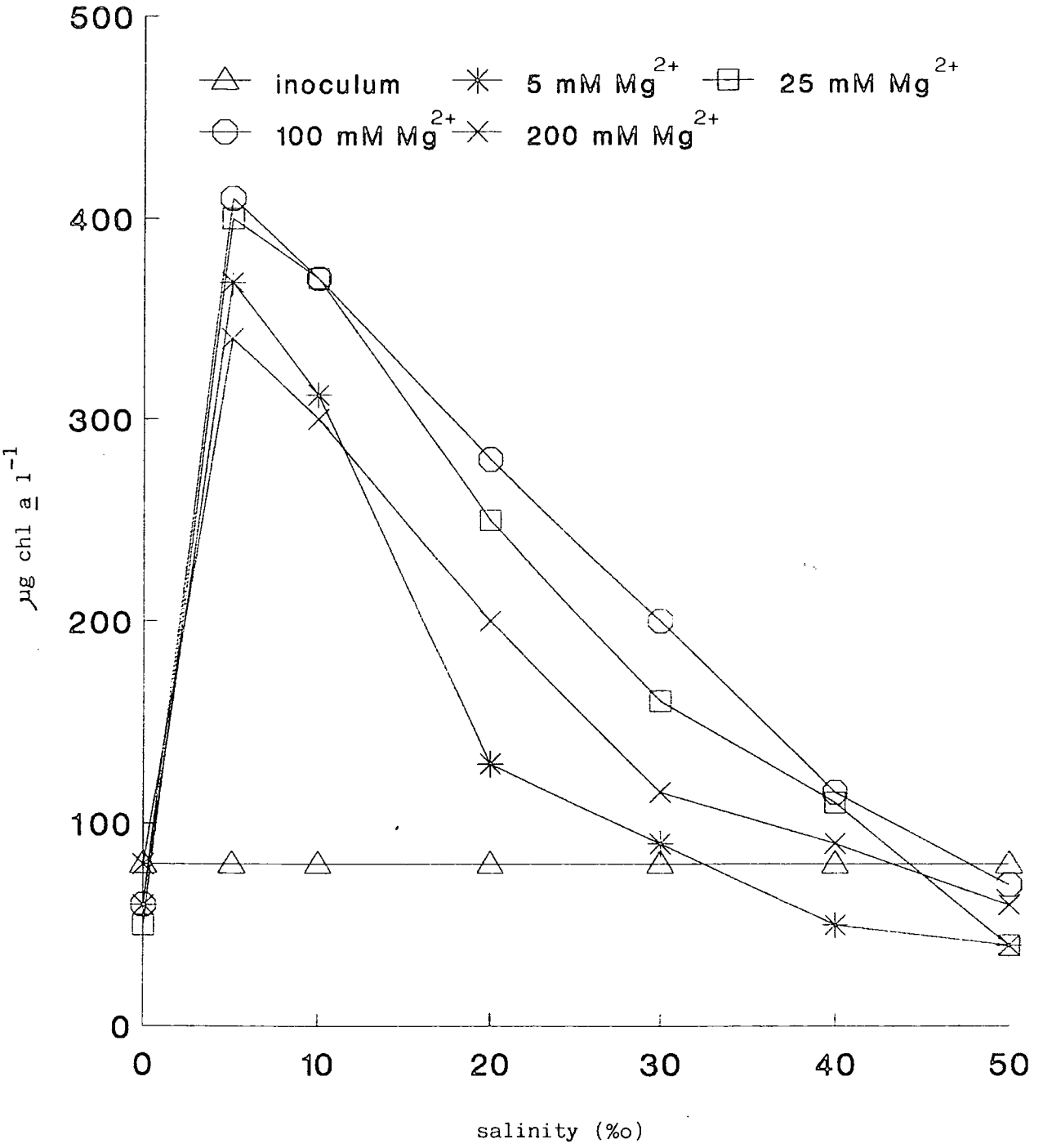


Fig. 7.25 Influence of magnesium on ARA in Microcoleus D778. 20°C, continuous light and 16:8 light (50 µmol photon m^{-2} s^{-1}):dark

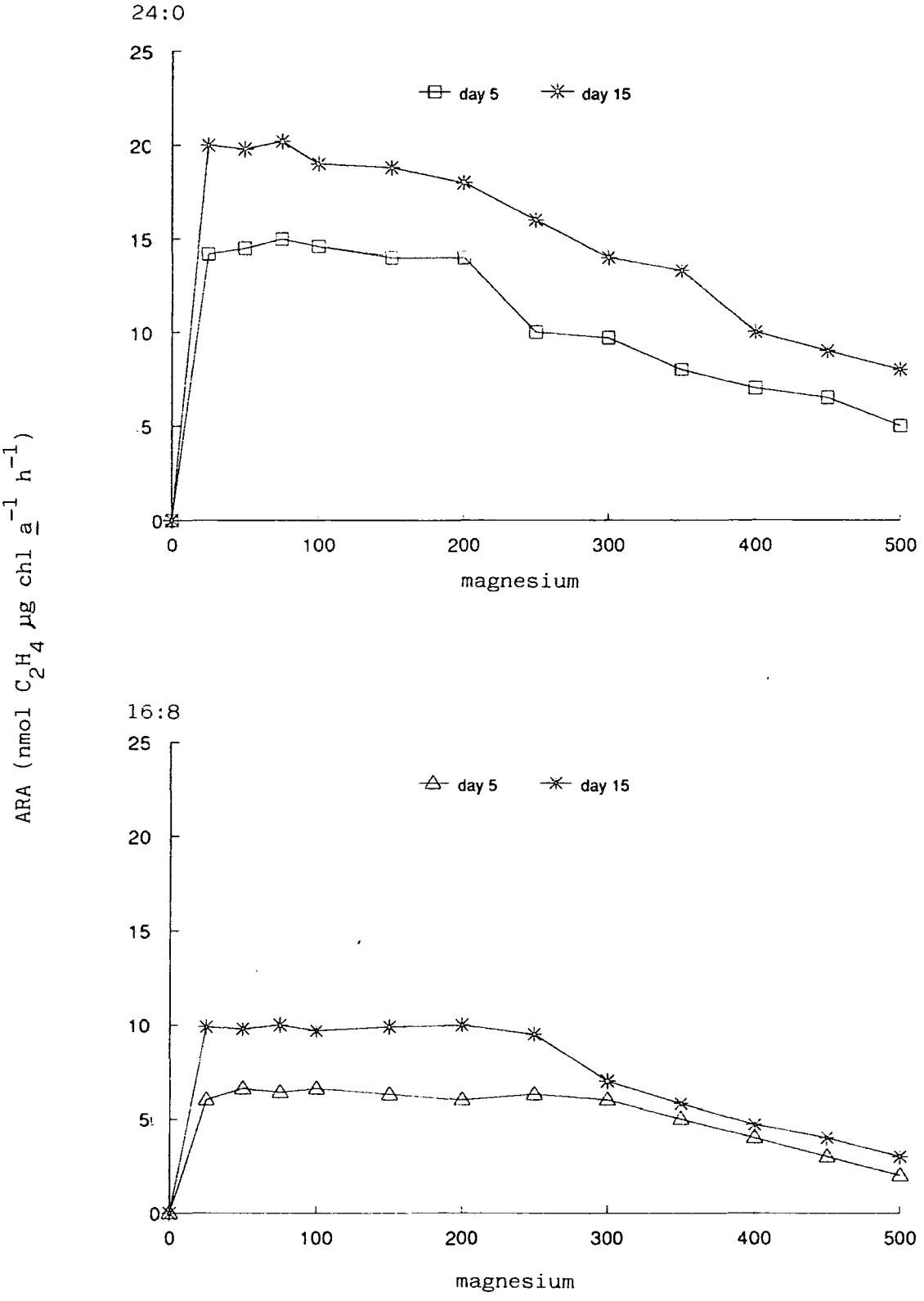
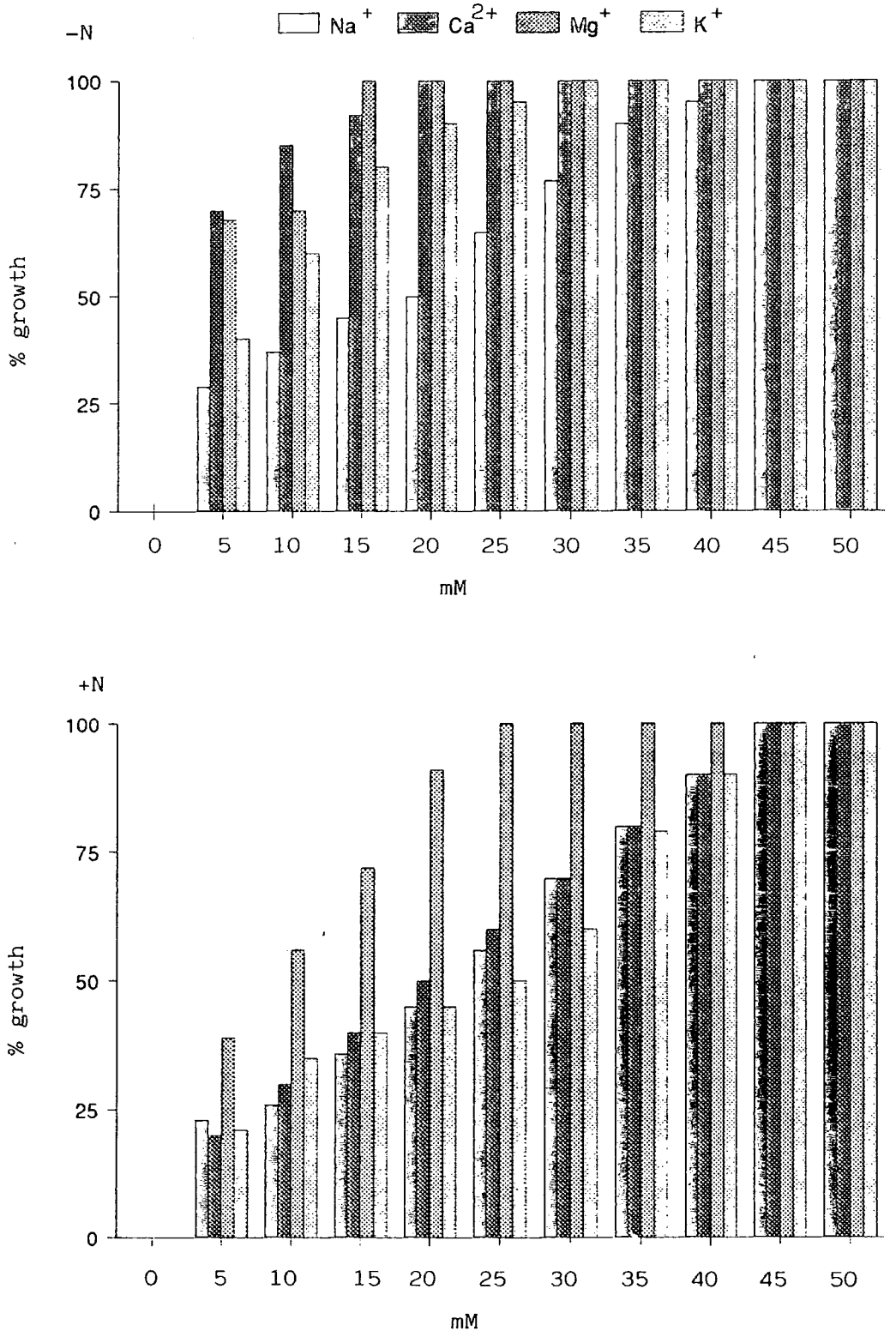


Fig. 7.26 Influence of Na^+, K^+, Ca^{2+} and Mg^{2+} on % growth of Microcoleus D778 after 48-h in 16:8 light:dark. 20°C, 50 µmol photon m^{-2} s^{-1}



7.6 Influence of PAR on growth and ARA

The influence of PAR on ARA was compared 1, 4, 8 and 12 h after dark during a 16:8 light:dark period. 1 h after dark, ARA increased significantly ($p<0.05$) from 0 to 50 to 100 µmol photon m^{-2} s^{-1} whereas 4 h after dark there was a significant ($p<0.05$) increase from 0 - 50 µmol photon m^{-2} s^{-1} but no significant ($p<0.05$) difference between 50 and 100 µmol photon m^{-2} s^{-1}. However, 8 and 12 h after dark ARA decreased considerably from 0 to 50 to 100 µmol photon m^{-2} s^{-1} (Fig. 7.27).

When The effect of red light was compared with that of white light of an equal irradiance 1, 4, 8 and 12 h after an 8 h preincubation in the dark, ARA was similar after 1 and 4 h; however, after 8 and 12 h activity was significantly higher under red light (Fig. 7.28). When cultures were incubated in white light in the presence of DCMU, ARA was always higher under these conditions than in either red light or in thedark, increasingly so the longer the light period (Fig. 7.28).

In contrast to a 16:8 light:dark cycle at 50 µmol photon m^{-2} s^{-1} (Fig. 7.6) when the majority of ARA occurred in the light, the reverse was found under a PAR of 100 µmol photon m^{-2} s^{-1} (Fig. 28b), with over 83% ARA in the dark. In addition, using 1 h incubation periods, sudden increases and decreases in ARA were observed in the latter during the light period.

ig. 7.27 Influence of PAR on ARA in Microcoleus D788. 20°C, 16:8 light:dark

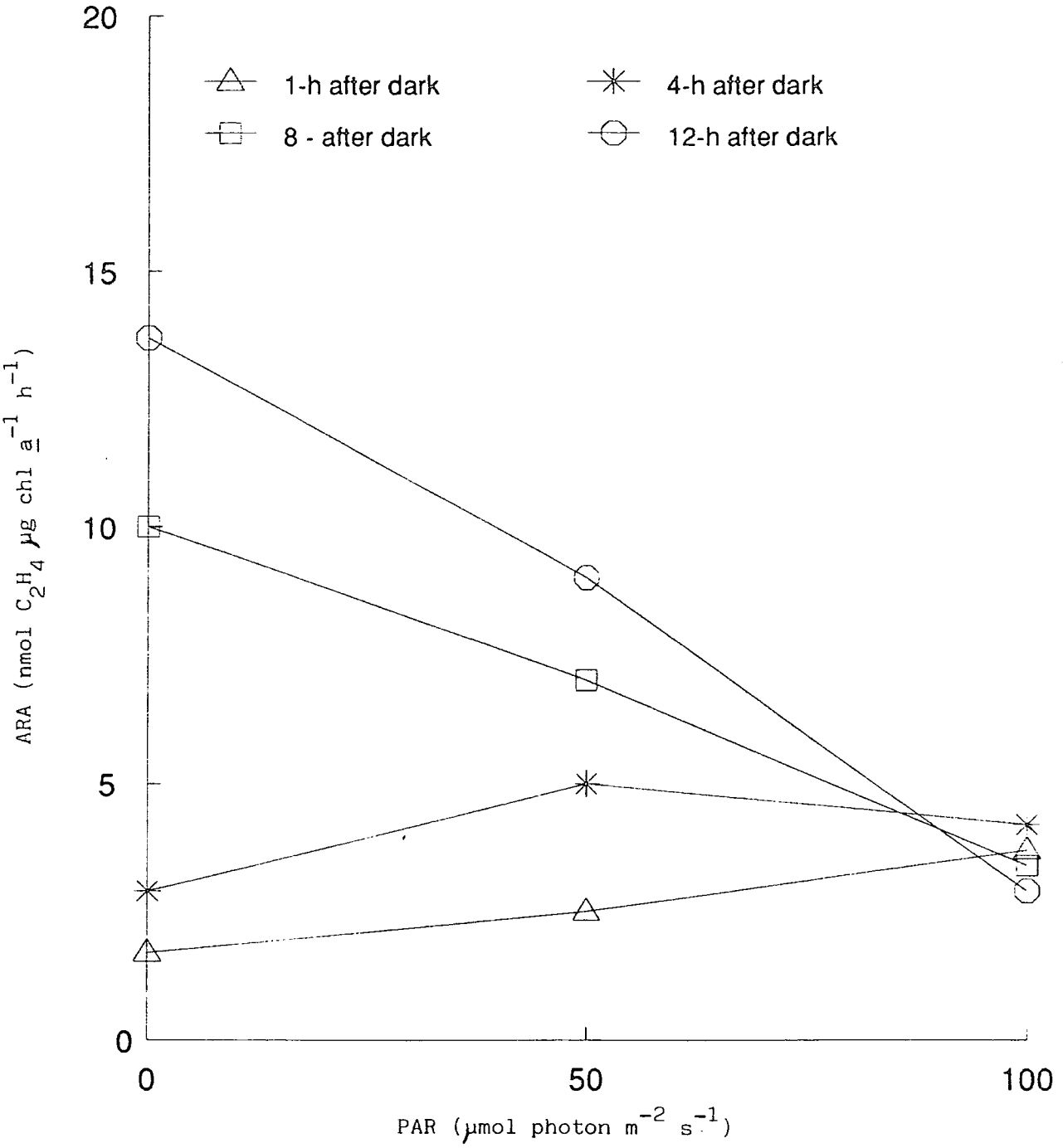


Fig. 7.28 Influence of white light (±DCMU), red light and darkness on ARA 1, 4, 8 and 12-h after an 8-h incubation period in the dark. 20°C, 14 µmol photon m^{-2} s^{-1}

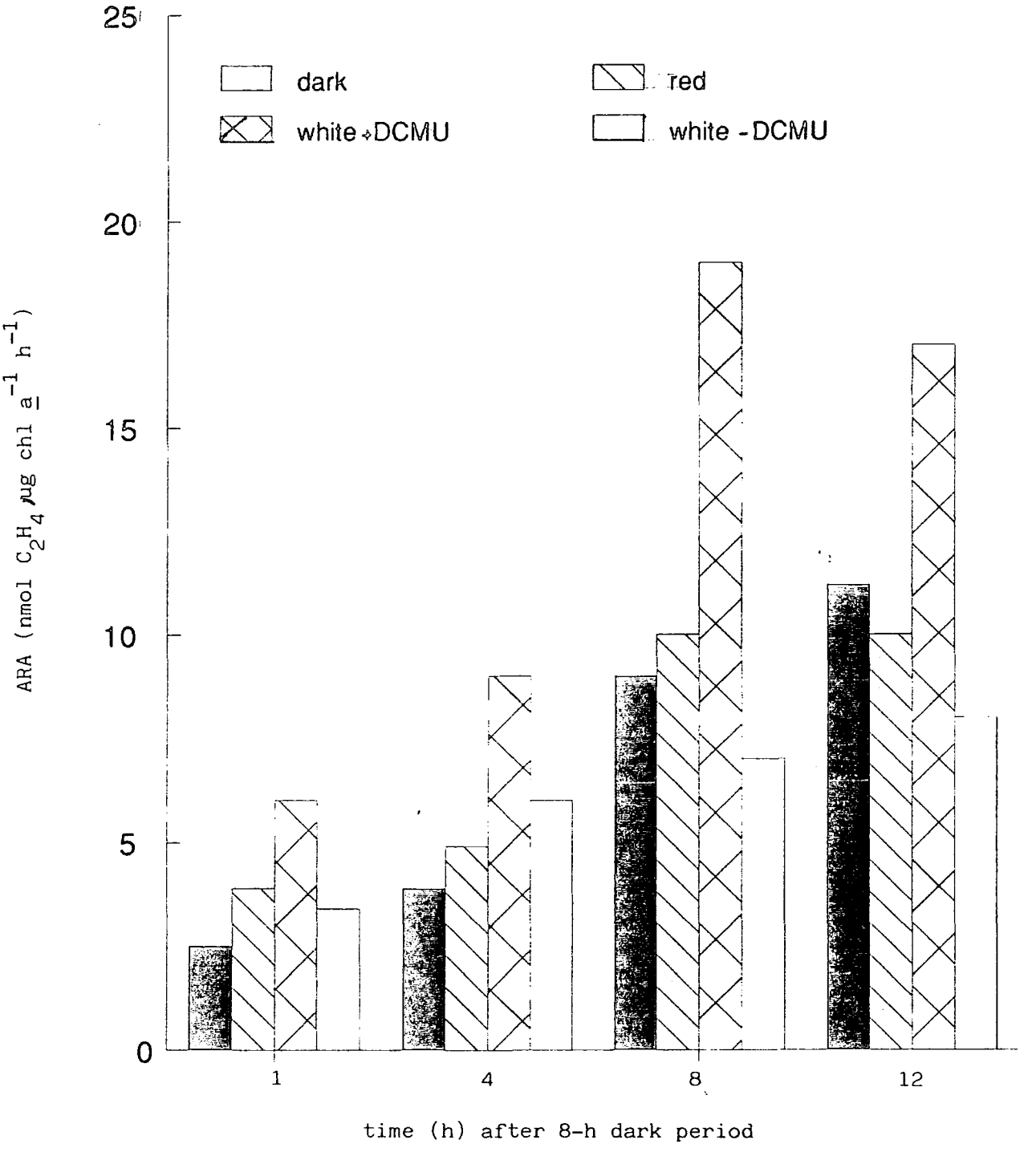
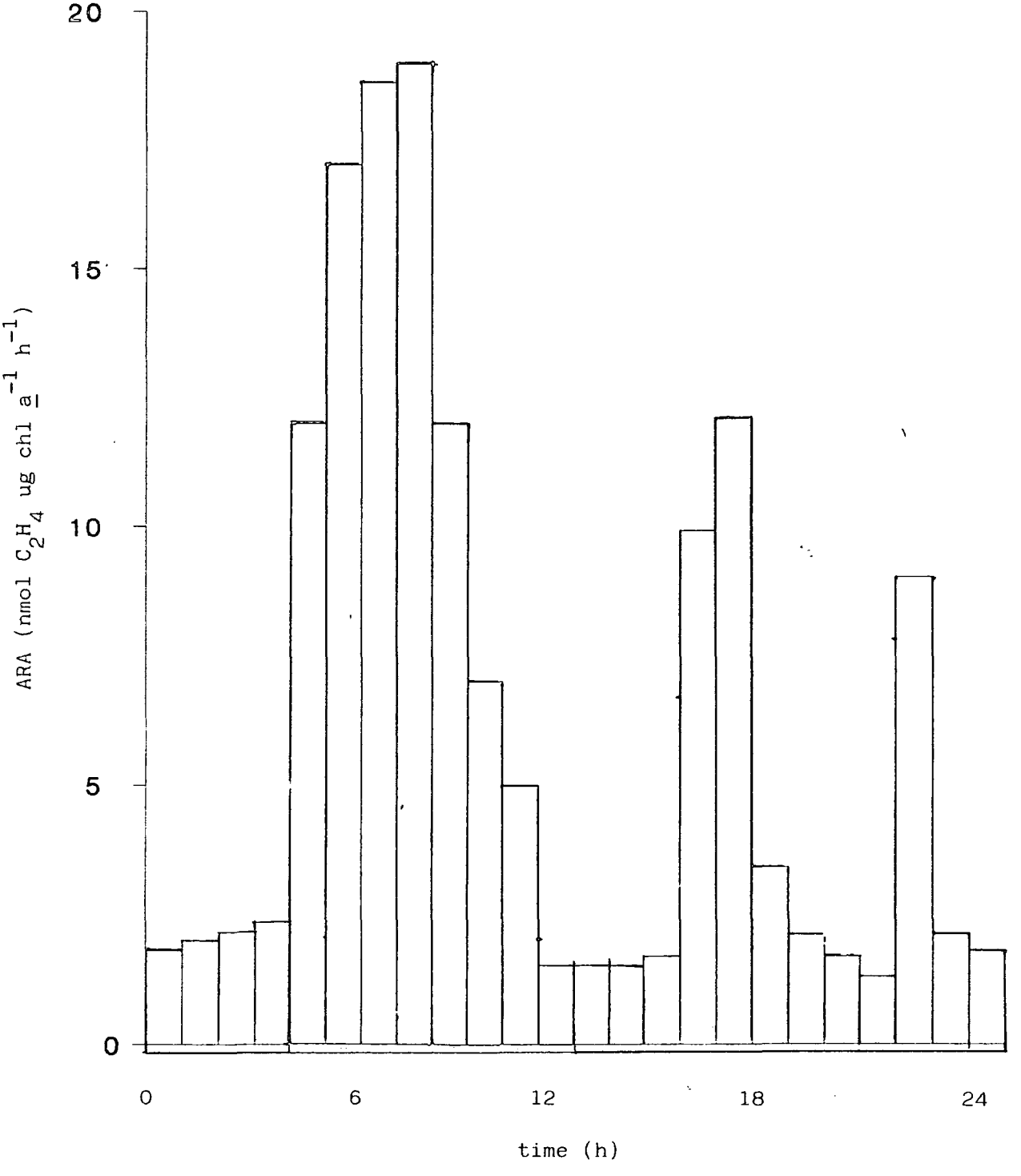


Fig. 7.20b Influence of a 16:8 light:dark cycle on ARA in Microcoleus D778. 20°C, 100 μmol photon m^{-2} s^{-1}



7.7 Influence of temperature on growth and ARA

Cultures grew between 16.0 ± 1.0 and 32 ± 0.5°C with an optimum at 20 ± 0.5 - 26 ± 0.5°C. To determine how high a temperature Microcoleus D778 could survive, cultures were transferred to temperatures between 20.0 and 70.0 ± 0.5°C for up to 6-h. Cultures were then returned to a temperature of 20°C where growth as chl a was compared 48-h later. (Only one temperature could be compared at a time). At 50 ± 3 µmol photon cultures survived 40°C for 6-h, 45°C for 4-h, 50 and 55°C for 2-h but not 60°C whilst at 30 ± 4 µmol photon m^{-2} s^{-1} they survived temperatures of 50°C for 4-h and 60°C for 1-h. Survival at given temperatures depended not only on PAR, but also on the salinity of the medium. At a salinity of 30‰ (516 mM Na), cultures survived temperatures of 35°C for 5-h and 40°C for 4-h; however, at a salinity of 5‰ (86 mM Na) they were able to survive temperatures of 35°C and 40°C for 6-h.

Cultures had a slightly higher (2- 3°C depending on the PAR) temperature tolerance in the presence of combined nitrogen compared with those fixing N_2.

ARA occurred from 16.0 ± 1.0°C to 34.0 ± 0.5°C reaching a peak at 20.0 ± 0.5°C - 26.0 + 0.5°C (Fig. 7.29); however, both the range and optimum temperature for the incubation period (Fig. 7.29). ARA was detected at 4°C but only after a 3-h pre-incubation period and with a 4-h incubation period. To determine how long ARA could be maintained at its highest range, cultures were exposed to temperatures of 32, 34, 36, 38 and 40 ± 0.5°C for 12-h and ARA measured after 1, 3, 6, 9 and 12-h. ARA could not be detected after a 1-h exposure to 36.0 ± 0.5°C or 6-h at 32.0 ± 0.5°C .

Fig. 7.29 Influence of temperature and salinity on ARA in Microcoleus D778 after 12, 24, 36 and 48-h exposure. 50 µmol photon m^{-2} s^{-1}

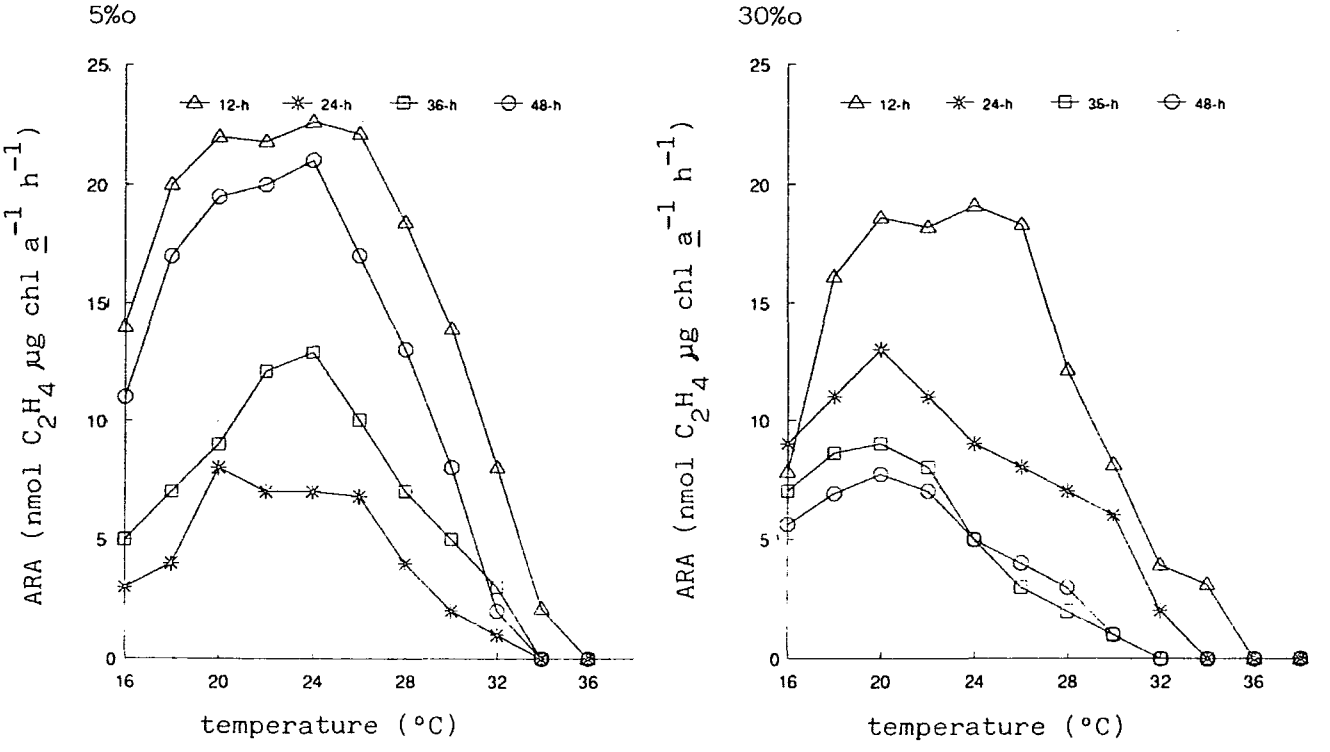
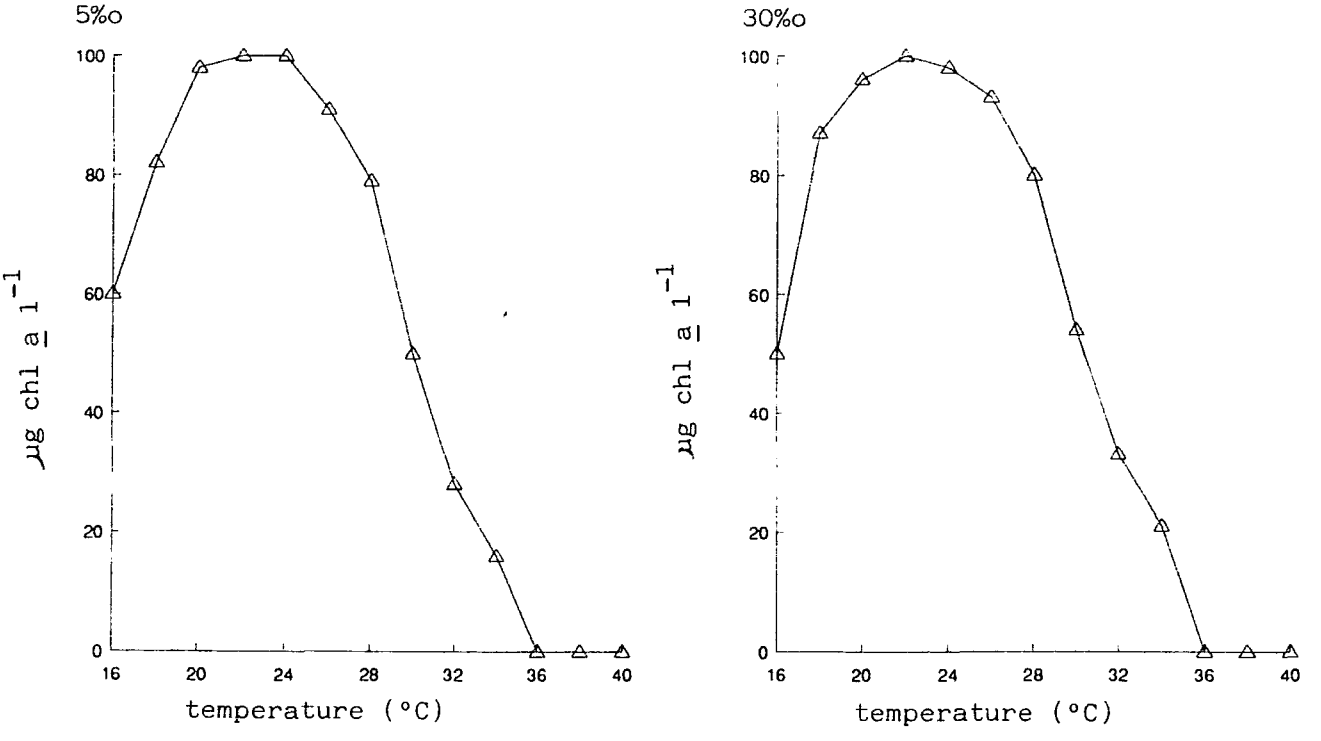


Fig. 7.30 Influence of temperature and salinity on growth of Microcoleus D778 after 48-h



7.8 Influence of pH on ARA

7.8.1 Experimental conditions

Seven buffers (Table 2.6) were used to cover the pH range 7.0 - 10.0. Cultures in continuous light were grown in GD_5-N containing HEPES before the medium was changed to a range of new pH (2.4.4). Pre-incubation periods of of 1 and 2-h were compared; however, as no significant ($p<0.05$) difference was found between any of these, a pre-incubation period of 1-h was selected.

7.8.2 Comparison of buffers

To ensure changes in ARA at different pH was not due to the buffer employed, ARA at a given pH was compared using two or more buffers. No significant ($p<0.05$) difference in ARA was recorded between HEPES, PIPES or TES at pH 7.0; however, at pH 7.5 and 8.0 ARA was significantly lower in TES than in the other two. ARA at pH 8.5 - 10.0 buffered with glycine was always lower than in HEPPS, AMP or CAP. In order to compare ARA over a range of pH the minimum number of buffers was required per pH and as a consequence of the above experiments the following buffers were selected for use at given pH :

pH	buffer
7.0 - 8.0	HEPES
8.1 - 8.7	HEPPS
9.0 - 10.0	AMP
10.1 - 11.0	CAP

7.8.3 Comparison of nitrogenase activity over the pH range 7.0 - 10.0

Cultures were grown to mid. log in 16:8 light:dark at pH 8.0 before transferring to a new pH (2.4.4) ranging from 7.0 to 10.0. ARA was recorded over the whole range of pH tested and showed optimum activity at pH 8.0 (Fig. 7.31). To ensure this pH optimum of 8.0 was not due to pre-experimental conditions (i.e. growth at pH 8.0), cultures were grown to day 22 in 16:8 light:dark at pH 8.0, transferred to pH 7.0, 7.5 and 8.0 (n = 4) for 48-h and ARA compared over 1-h incubations 48 h later at pH 7.0, 7.2, 7.4, 7.6, 7.8 and 8.0. In all cases ARA was highest at pH 8.0.

7.8.4 Influence of pH and salinity on ARA

Environmental variables such as pH and salinity may alter simultaneously under natural conditions. To test the effect of such changes cultures were grown at pH 8.0 at salinities of 5, 10, 15 and 20‰ (86, 127, 344 and 516 mM Na) under a light:dark regime of 16:8. Each was transferred at mid. log to pH

from 7.0 to 10.0. ARA was recorded under all pH values examined, the optimum pH increasing from 8.0 - 8.5 as the salinity increased (Fig. 7.31). However, when the temperature increased from 20°C to 25°C, the optimum pH was found to be between 8.5 - 9.0 (Fig. 7.32).

Fig. 7.31 Influence of pH on ARA in Microcoleus D778. 20°C, 50 umol photon m^{-2} s^{-1}

I p<0.05

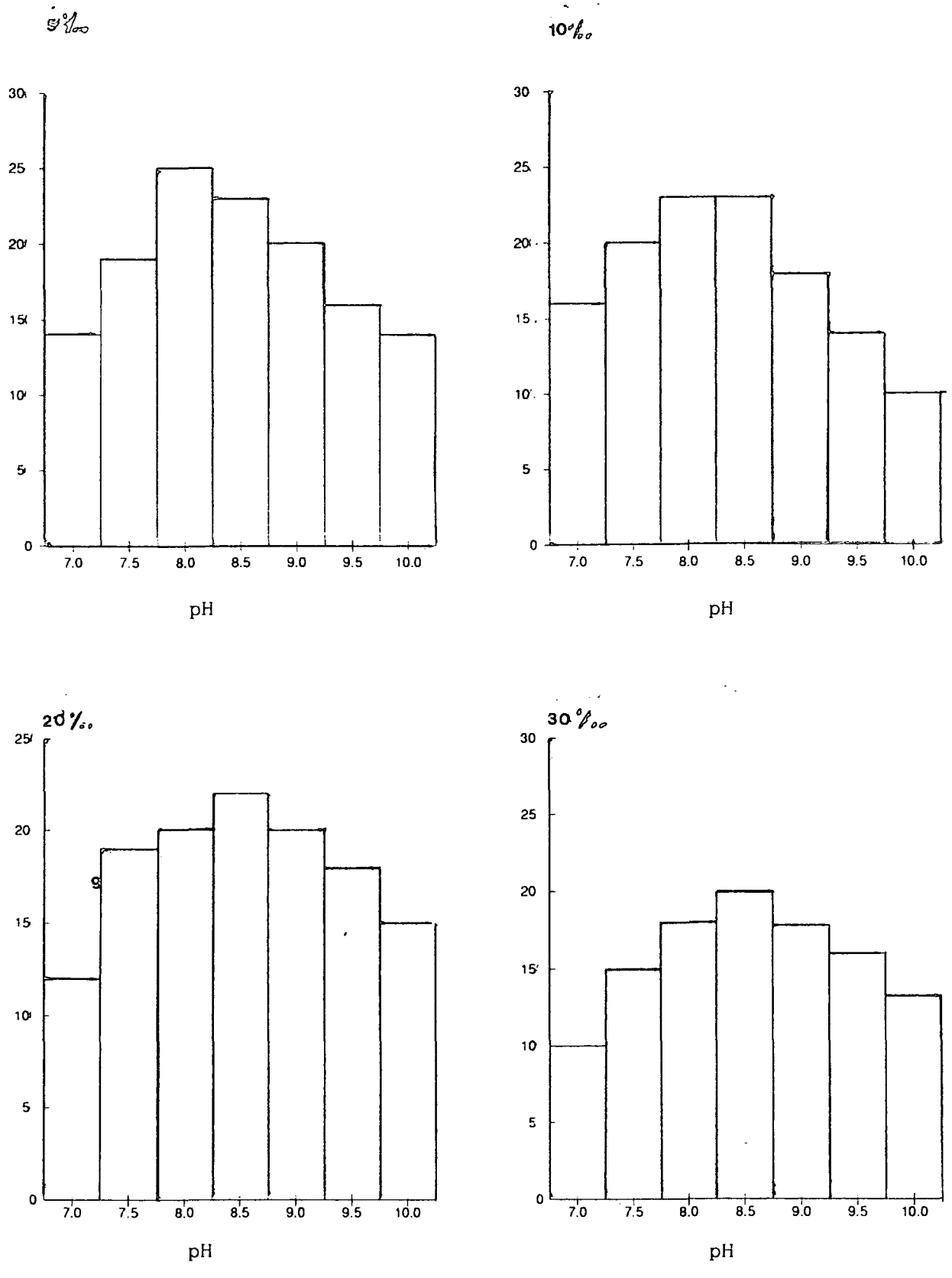
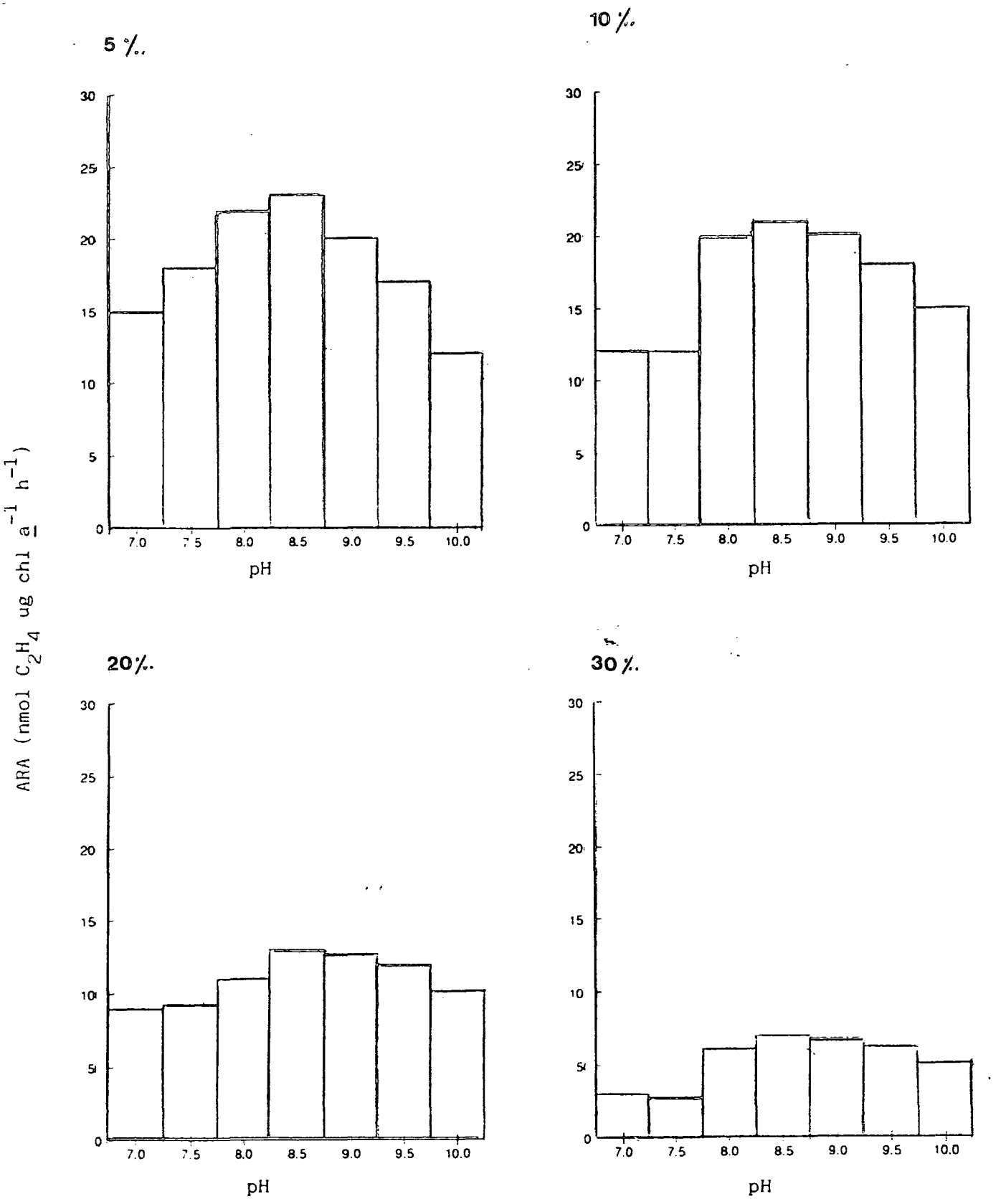


Fig. 7.32 Influence of pH on ARA in Microcoleus D778. 25°C, 50 umol photon m^{-2} s^{-1}



7.8 Influence of reducing conditions on growth and nitrogenase activity

Cultures were grown for 10 days in 16:8 light:dark and ARA compared in the presence and absence of DCMU 1 - 16 h after an 8 h pre-incubation period in the dark. ARA was 2 - 8 fold higher in the presence of DCMU than in its absence, the difference generally increasing with the length of exposure (PAR of 50 umol photon m^{-2} s^{-1}) (Fig. 7.33).

ARA was compared in cultures grown oxically (2.4.7) for 9 and 12 days in 16:8 light:dark before transferring cultures to oxic and micro-oxic conditions ± DCMU (2.4.7). ARA was lowest under oxic conditions in the absence of DCMU, activity doubling on adding DCMU to 9 day old cultures (Fig. 7.34). ARA was higher still under micro-oxic conditions, particularly in the presence of DCMU. A similar pattern was found on 12 day old cultures (Fig. 7.34).

Fig. 7.33 Influence of DCMU on ARA 1-16 h after an 8-h incubation in the dark. 20°C, 50 umol photon m^{-2} s^{-1}

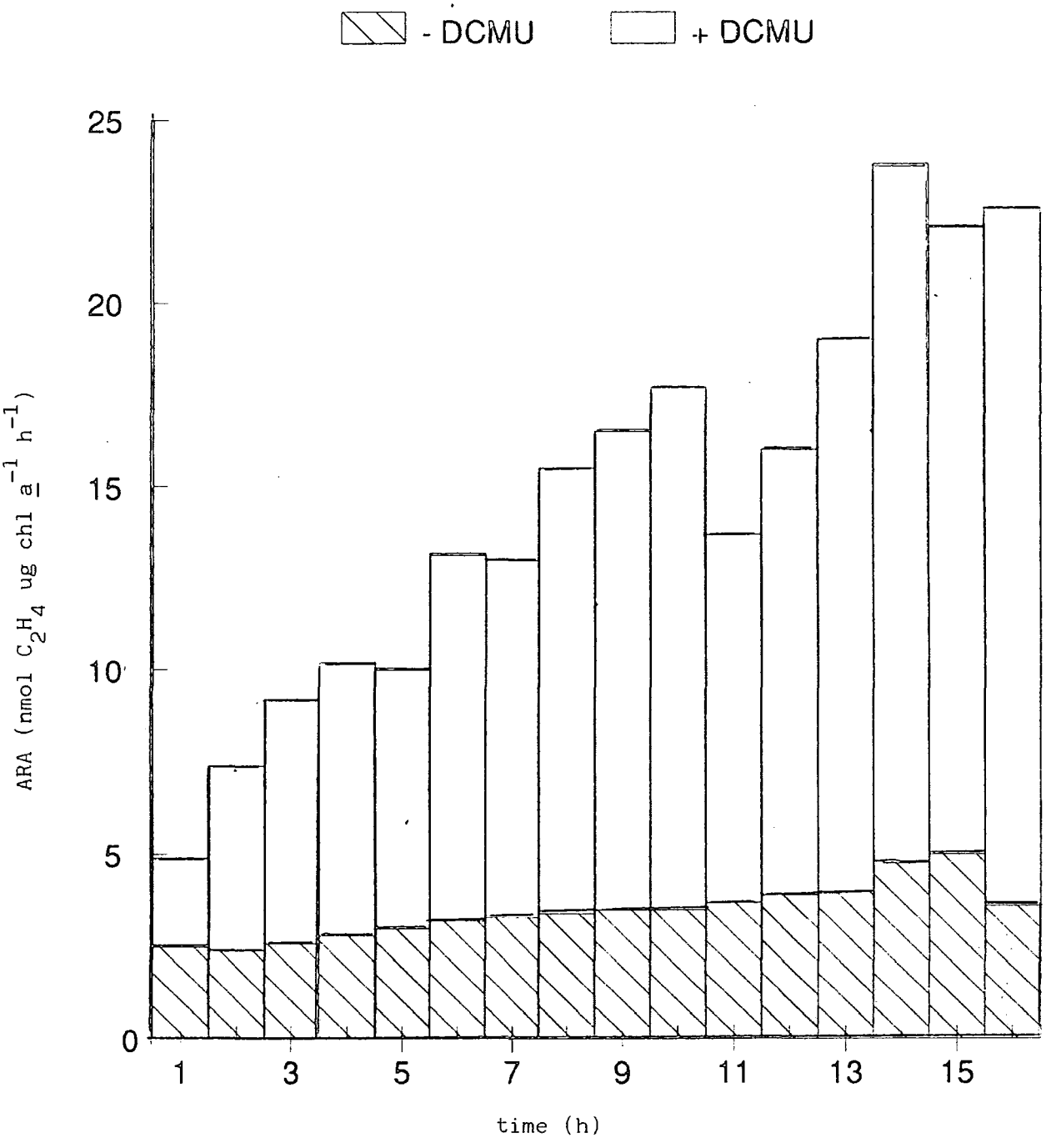
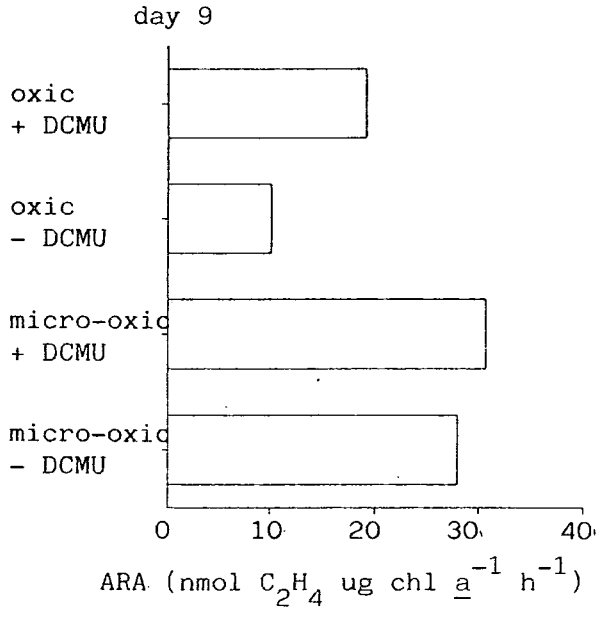


Fig. 7.34 Influence of an oxic and a micro-oxic (±DCMU) environment on ARA in Microcoleus D778. 20°C, 16:8 light (50 umol photon $m^{-2} s^{-1}$):dark



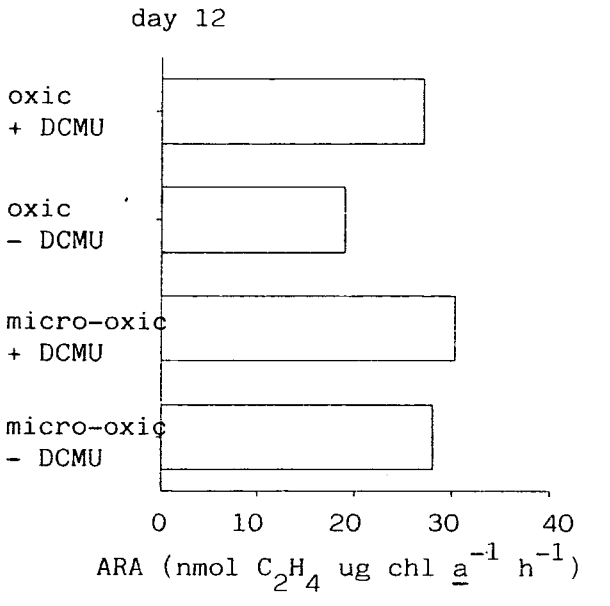
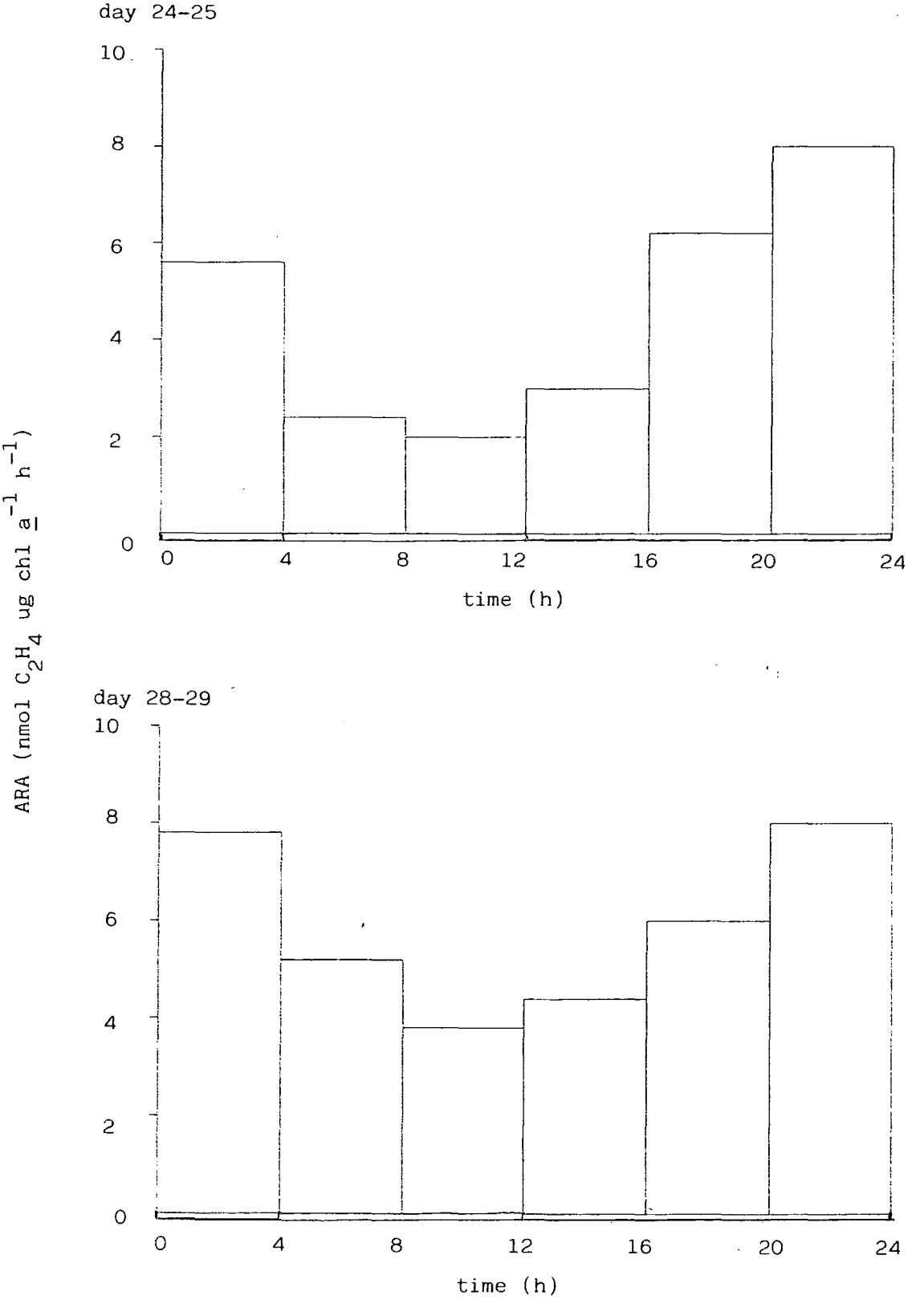


Fig. 7.35 Influence of a 16:8 light:dark cycle on ARA under micro-oxic conditions. 20°C, 50 umol photon m^{-2} s^{-1}



CHAPTER 8

DISCUSSION

8.1 Ability of Microcoleus strains to fix N_2

Microcoleus is one of the most widespread genera of cyanobacteria. It has been studied mainly in intertidal sand and mud-flats where the limited data suggest that it may be one of the most active organisms within the mat. Since intertidal sediments are believed to be environments low in biologically available nitrogen (Bautista & Paerl, 1985; Bohlool & Wiebe, 1978; Carpenter & Capone, 1983; Ryther & Dunston, 1981) the ability of Microcoleus to fix N_2 (as shown by previously by Pearson et al., 1979) is of considerable ecological importance.

Of the five strains used in this study (Microcoleus D634 from an Iraqi salt marsh; Microcoleus D761 and D782 from mangrove areas on Aldabra Atoll and Belize respectively; Microcoleus D778 from the upper intertidal sand and mudflats of Anglesey; Microcoleus D781 from usar soil, India), prelimiary studies showed only Microcoleus D778 to fix N_2 as shown by ARA and growth in the absence of combined nitrogen.

Since communal sheaths are likely to influence the physiology of strains, attempts were made to grow strains in culture in a form morphologically akin to that in the field (i.e. with a communal sheath (Fig. 4.2)).
When Microcoleus mats were submerged in Milli-Q water for a few hours, filaments glided out of the open end of the sheath and after c. 24 h sheaths appeared to bend and 'burst' releasing filaments. However, when the mats were kept dry, 'just' moist or submerged in seawater sheaths remained intact suggesting that water availability and salinity influence its structure. When Microcoleus D634 was grown in standard medium containing 516 mM Na^+ and 125 mM Ca^{2+} (salinity of 30‰), electron microscopy showed a thin (c. 350 nm), communal sheath around three filaments (Fig. 4.5). Castenholz and Halfen (1967), Dias (1968) and Snellen and Raj (1970) working on Oscillatoria sp., Sphaerotilus natans and Leucothrix mucor respectively all found sheaths to be affected by Ca^{2+} deprivation and Walsby (1968) found mucilage trail formation to be observed in the presence of elevated concentrations of calcium. He believed calcium and magnesium ions increased cell and filament aggregation and suggested that these divalent cations may have had an important role in the activation of exoenzymes involved in slime synthesis, the integrity of the

slime layer and motility. Within the thin, communal sheath individual trichomes were not surrounded by sheaths in contrast to that seen for Microcoleus in situ at Church Island and Gibraltar Point under light microscopy, when each trichome had a 0.5 µm sheath. Interestingly, the peptidoglycan layer in single filaments of Microcoleus D634 were 230 nm wide (Fig. 4.4) in contrast to 1 - 10 nm peptidoglycan layer normally found in cyanobacteria; however, Halfen and Castenholz (1971) found a 200 nm peptidoglycan layer in Oscillatoria princeps isolated from a microbial mat. Biochemical analysis of peptidoglycan layers from a range of cyanobacteria (although not Microcoleus) has shown it to be composed of glucosamine, muramic acid:alanine:glutamic acid:diaminopimelic acid in a molar ratio of 1:1:2:1:1 (Drews & Weckesser, 1982) although Jones and Yopp (1979) found the peptidoglycan layer from halophilic strains to contain a higher proportion of glucosamine, suggesting that the structure varies according to the salinity of the environment. This work, although limited shows variation in the cell envelope occur as a consequence of changes in the medium and since the cell envelope, notably the peptidoglycan layer and outer membrane play a role in transporting ions into and out of the cell (Drews & Weckesser, 1982) it may have a bearing on the physiology of the cell.

When Microcoleus D634 and D778 were incubated in dialysis tubes and subjected to osmotic shock (increase of 4‰ every 2 h to a salinity of 32‰ and vice versa over 32 h) cultures survived and although no sheath was found, copious quantities of mucilage was present in the medium. The distinction between sheath and mucilage per se is not one that has been made satisfactorily by any author, although Stewart (1980) and Drews and Weckesser (1982) noted that sheaths were soluble in water, forming a mucilage-like substance. Since liquid environments may result in the solubilisation of the sheath, attempts were made to grow the strains in a solid environment where water availability was low. When Microcoleus D634 and D778 were injected into modified Winogradsky columns at a depth of 2 mm and incubated with light incident from above, filaments lay alongside each other but no more than 2 - 3 filaments were seen within a communal sheath.

When Microcoleus D778 was grown in the presence of combined N for a year and transferred to - N medium it would only grow under certain conditions: at salinities of 5 - 15‰ but not 20 - 25‰; under 30 and 50 µmol photon m^{-2} s^{-1} but not 100 µmol photon m^{-2} s^{-1}; in 16:8 light:dark but not in continuous light; all experiments were carried out at 20°C. Initially, growth rates were much slower than those calculated for cultures maintained permanently in - N. Microcoleus strains D634 and D761 which had been maintained for a minimum of 2

years in 13.8 mM NO_3-N and Microcoleus D781 and D782 for up to 6 months in 0.5 mM NH_4-N, were all grown at 0.1 mM NH_4-N before environmental variables including nutrient concentrations (Table 2.11), PAR and temperature (5.5) were altered in an attempt to grow these strains in a medium free from combined N. Although a large variety of environmental conditions were provided, microbial mats are complex environments and it may be that conditions were unsuitable for it to e.g. adjust its metabolism to protect itself from O_2.

Microcoleus are usually found in calcareous environments therefore growth was attempted under a range of Ca^{2+} concentrations, particularly since Ca^{2+} appears to play a role in cyanobacterial physiology including in vitro PSII activity (Becker & Brand, 1985) and in the protection of nitrogenase activity in unicellular and non-heterocystous filamentous cyanobacteria (Gallon & Hamodi, 1984; Smith et al., 1987). Although growth of all strains increased up to Ca^{2+} concentrations of 25 mM in the presence of N, no growth occurred in its absence.

Since Microcoleus is found in the upper intertidal where salinities vary, growth was compared over a range of salinities. All strains were mixohaline growing at salinities of 0.5 - 30‰ (40‰) (9 - 516 (688) mM NaCl) and surviving periods under euryhaline (salinities of 30 - 40‰; 516 - 688 mM NaCl) and polyhaline (salinities >40‰; > 688 mM NaCl) conditions. The shorter the time of exposure the higher the salinity tolerated. Growth rates also varied according to the concentration and ratio of Na^+ to K^+, Na^+ to Ca^{2+} and Na^+ to Mg^{2+}; however, no growth was found in the absence of combined N.

Stal and Krumbein (1985) and Giani and Krumbein (1986) found Microcoleus chthonoplastes and Phormidium boryanum respectively to fix N_2 only under micro-oxic conditions; however, when Microcoleus D634, D761, D781 and D782 were incubated micro-oxically (2.4.7) and ARA measured under a variety of conditions (5.6), results were negative.

Two media were required for the isolation of Microcoleus D781: the first containing N competitively inhibiting heterocystous forms and the second, a -N medium allowing Microcoleus a competitive advantage over Oscillatoria and Phormidium (chlorotic after 10 days). This suggests an ability on the part of Microcoleus D781 to fix N_2, or alternatively, it may adjust its metabolic rate enabling it to survive better on its intracellular N reserves.

To try and determine further whether or not these strains were able to fix N_2, DNA was isolated in order to look for nif genes. However, the DNA isolated was heavily contaminated with carhohydrate and consequently could not be restricted. No further work was carried out along these lines as it was considered a research area initsef.

8.2 Nitrogen fixation in Microcoleus D778

Microcoleus D778 was isolated by Howsley et al., (1979) from the upper intertidal sand and mud-flats of Church Island, Anglesey (3.2.3). The influence of environmental variables on ARA was compared in situ, on returning mats to the laboratory and on axenic cultures in the laboratory.

8.2.1 Microcoleus mats at Church Island

In order to determine when Microcoleus mats were present on Church Island, monthly observations were made in 1986 and 1988 and twice monthly observations in 1987. Microcoleus mats were formed in late May to early June, replacing mats of Ulothrix flacca, Rhizoclonium sp. and Enteromorpha sp.. Mat development proceeded throughout the summer, reaching a peak in September (Table 6.4) and gradually decomposing thereafter leaving only empty, apparently undegraded sheaths by mid-November.

In order to determine when N_2 fixation occurs under natural conditions, ARA was measured over six diel periods between June and October, 1987. A different response was found on each occasion although generally high activity (with over 70% in the dark) was found on sunny days with 7 - 12 h of sunshine and low, fairly constant ARA over with a slight peak in the afternoon during cloudy, overcast days (1 - 2 h of sunshine).

From diel ARA experiments on Microcoleus mats, Paerl et al., (1989) also found different responses in ARA on different days: on hot, sunny days the majority of ARA occurred in the dark whilst on a cloudy, overcast day, ARA was similar over the diel cycle. On measuring the pO_2 with microelectrodes they found ARA on sunny day to occur when the pO_2 was low, whereas on a cloudy overcast day both the pO_2 and ARA were fairly consistent throughout the 24 h period.

Potts (1977) measured ARA in Microcoleus mats on the Aldabra Atoll (9°S 24′ S, 46° 20′ E) at 22 and 24 nmol C_2H_4 cm^{-2} h^{-1}, whilst Paerl et al., (1989) working on Microcoleus mats on the Shackleford Banks (34° 35′ N, 77° 25′ W) measured ARA over diel cycles between 6 and 23 nmol C_2H_4 cm^{-2} h^{-1}. ARA in the Microcoleus mats at Church Island ((53° 14′ N, 4° 6′ W) were considerably lower (0.1 - 3.4 nmol C_2H_4 cm^{-2} h^{-1}), but comparable with the limited tests carried out by Pearson et al., (1979) on these mats, as well as those recorded by Stal (1985) and M.S.H Griffiths (pers. comm.) for Oscillatoria mats on the Island of Mellum (53° 40′ N, 9° 8′ E) and on marshland near Loughor, (S. Wales)

respectively. Such differences in ARA may be a consequence of different climatic conditions; however, since ARA in the Microcoleus mats at Church Island varied considerably according to the concentration of acetylene administered (Fig. 2.3, 2.4), it is difficult to assess how much variation is due to differences in the acetylene reduction technique e.g. Bautista and Paerl (1985) used 50% C_2H_2, Stal (1985), 15% C_2H_4 and Griffiths et al., (1987) 4% C_2H_4. 10% acetylene was used during the course of this study after comparing activity at concentrations of 1 - 25% C_2H_2 v/v (2.9.2).

From the data at Church Island and those of Paerl et al., (1989), it can be seen that estimates of diel ARA are only valid if ARA is measured over the whole period unlike those of Carpenter et al., (1978) who used daytime measurements to estimate total mat N inputs.

8.2.2 Influence of PAR and light:dark cycles on ARA

When Microcoleus D778 was grown in 8:16 and 4:20 light (50 μmol photon m^{-2} s^{-1}):dark the majority (72 and 92% respectively) of activity occurred in the dark whereas in 20:4 and 16:8 light:dark the majority of ARA (81 and 60% respectively) occurred in the light. In addition, ARA fixed per 24 h decreased as the ratio of light to dark decreased.

Since energy required for photosynthesis in the dark is presumably obtained from catabolism of stored glycogen via the oxidative pentose phosphate pathway, which, coupled to an oxic respiratory phosphorylating electron transport chain serves to provide ATP in the dark (Smith, 1982; Ohmori, 1984), changes in the pattern of ARA over a diel cycle will therefore be a reflection of the ratio of light hours to dark hours and the efficiency of photosynthesis and C assimilation during the light period. When Loogman et al. (1980) grew Oscillatoria agardhii at light saturating levels the overall growth rate increased as the length of light:dark increased but not in direct proportion. As the length of the light period increased the efficiency of light energy incorporated decreased. It may be that the faster growing cells under the longer photoperiod had a higher respiratory rate or alternatively there may be a time dependent inhibition of photosynthesis which increases progressively during the light period due to the build-up of oxidants. The rate of glycogen accumulated in the light and its subsequent mobilization in the dark has been shown to depend on the PAR (Stone & Garif, 1981) and on the duration of the light period (Foy & Smith, 1980; Loogman et al., 1982). Loogman et al., (1982) also found the rate of glycogen synthesis in to decline as the length of the light period in a 24 h cycle increased, with maximal cell

glycogen content requiring a minimum of 8 h per 24 h. Comparatively low rates of rates of ARA in 8:16 and 4:20 light:dark may therefore have been due to insufficient carbon.

When the PAR was increased from 50 to 100 μmol photon m^{-2} s^{-1} during a 16:8 light:dark cycle, >80% ARA occurred in the dark in contrast to only 40% at 50 μmol photon m^{-2} s^{-1}. In addition, there were sharp changes (using 1 h intervals) in ARA during the light period at 100 μmol photon which were not found at 50 μmol photon m^{-2} s^{-1} Fig. 7.6 and 7.28b). Photosynthetic rates may have been depressed at the higher irradiance leading to a temporal separation in photosynthesis and N_2 fixation as found in Synechococcus grown under 12:12 light:dark (Mitsui et al., 1986). This would explain how cultures can fix N_2 in continuous light. However, growth in 20:4 light:dark was significantly ($p< 0.05$) higher than in continuous light. Similarly, Zevenboom and Mur (1984) working on Microcystis aeruginosa found that a 2 h period of darkness per 24 h to be required for maximum growth. Further evidence for a temporal separation between these two systems during the light comes from evidence for the catabolism of glycogen reserves during the light as well as fluctuations in the ratio of ADP to ATP in the light (Gallon et al., 1988).

Since PAR affected the diel pattern of ARA, further tests were carried out both on mats and in vitro.

When the influence of PAR was examined on ARA in Microcoleus mats returned to the laboratory (6.7) a different response was found at different times of the day (Fig. 6.13): in the morning ARA increased with increasing irradiance whereas the reverse was true in the afternoon and evening. Taking into account Revsbech et al (1983), Stal (1985) and Paerl et al. (1989) account of micro-oxic or anoxic conditions in cyanobacterial mats during dark periods and oxic (O_2 up to five times that of supersaturation) during the light one could suppose the decrease in ARA in the afternoon and evening to be due to a build up of photosynthetically produced oxygen, whereas in the morning O_2 concentrations would have been low.

When ARA in Microcoleus D778 was compared at 0, 50 and 100 μmol photon m^{-2} s^{-1}, ARA was generally higher in the dark than at 100 μmol photon $_m^{-2}$ s^{-1}, irrespective of the pre-incubation conditions, whereas activity at 50 μmol photon m^{-2} s^{-1} was higher in the dark after a pre-incubation in the dark but not in the light, presumably a reflection of the level of endogenous C available and on the pO_2. Most temperate cyanobacteria have maximum growth rates at a white light irradiance of 50 - 60 μmol photon m^{-2} s^{-1} (van Lierre et al.,1979; Foy, 1980; Gibson & Smith, 1982; Gibson & Foy, 1983; Wyman & Fay,

1986; L.J. Stal (pers. comm.), although they are able to grow at higher, sub-optimal irradiances (Raps et al.,1983; Wyman & Fay, 1986).

In contrast, ARA in Microcoleus in situ decreased with decreasing PAR. During these incubation periods PAR incident on the mats was < 2000 µmol photon m^{-2} s^{-1}, therefore according to Stals (1985) estimation of the penetration of light through these mats (2.55% PAR via the first mm of dry sand, 0.55% the second mm and 0.55% the third mm whilst 10.64, 3.36 and 0.32% penetrated the first, second and third mm of wet sand respectively), Microcoleus in these mats may not be exposed to a PAR of 100 µmol photon m^{-2} s^{-1} often due to shadowing by the overlying layer of sand/sediment, although % light penetration will vary according to particle size and character.

Isolation was favoured at low PAR (30 µmol photon m^{-2} s^{-1}) in medium GD_5-N supplemented with 125 mM Ca^{2+}. Smith (1988) demonstrated a negative correlation between the intracellular level of Ca^{2+} and incident irradiation. From this, he suggested that Ca^{2+} activates photosynthesis at lower PAR thus indirectly protecting nitrogenase activity against excessive concentration of O_2.

Stal (1985) showed red light to penetrate sediment better than either blue or green light and interestingly, ARA, both in Microcoleus mats and Microcoleus D778 occurred in the presence of both white and red light but not in green or blue light. When ARA in Microcoleus D778 was compared in white and red light of equivalent irradiance with that in the dark 1, 4, 8 and 12 h after an 8 h preincubation in the dark, ARA activity after 1, 4 and 8 h ARA was higher in red light than in the dark, possibly due to an active PSI (it absorbs at λ > 660 nm) thereby producing energy via cyclic photophosphorylation without producing electrons. According to the manufacturers (Lee), the primary red filters used transmitted at of λ of 600 - 700 therefore it must be assumed that the phycocyanin and chl a of PSII which absorb at 610 -625nm and at λ > 660 nm (Fay, 1985) may also have been active. After a 12 h incubation, ARA was higher in the dark than in red light, possibly due to a build-up of O_2 and / or endogenous carbon. During the first 4 h after dark, ARA in white light was significantly ($p<0.05$) higher than in red light However, Myers et al. (1978) working on Anacystis nidulans found a decrease in the maximum rate of photosynthesis in red light but a higher quantum efficiency in far red light. After 8 and 12 h in the light (50 µmol photon m^{-2} s^{-1}), ARA in red light was significantly ($p<0.05$) higher than in white light of equivalent intensity, possibly due to a sufficient store of endogenous carbon or via production of C and via PSI, with only a limited PSII (if any) and therefore a lower concentration of O_2.

8.2.3 Influence of O_2 on ARA

When DCMU was added to the mats *in situ* over a diel cycle (20/21.7.87), understandably there was no significant ($p<0.05$) increase in ARA during the dark; however, activity rose at all times during the light period, particularly in the afternoon and at dusk (Fig. 6.12) presumably due to a lowering of photosynthetically produced oxidants built up during the day. On adding DCMU to *Microcoleus* mats in Shark Bay, Australia, Paerl *et al.* (1989) found ARA to increase both at sunrise and sunset with only a minimal increase in late morning. Increasing ARA at dawn may be due to an increased respiration rate, provided sufficient endogenous carbon was present.

When ARA in *Microcoleus* D778 was compared in the presence and absence of DCMU 1 - 16 h after an 8 h preincubation in the dark, ARA was always higher in the presence of DCMU, increasingly so with time, presumably due to a build up of pO_2.

To further determine whether the pO_2 influences responses in ARA over a diel cycle, cultures were grown both oxically and micro-oxically in 16:8 light (50 μmol photon m^{-2} s^{-1}):dark. The pattern of response differed with ARA increasing markedly at the onset of the dark period under oxic conditions, whereas under micro-oxic conditions ARA decreased in the dark. During dark periods energy is available from endogenous carbon reserves whereas in the light under micro-oxic conditions energy may be obtained via cyclic photophosphorylation using only PSII or alternatively from PSI which is less likely to be inhibitory due to a decrease in the pO_2.

No ARA was found for *Microoccleus* D778 in the dark under anoxic conditions, presumably since catabolism of glycogen proceeded via the oxidative pentose phosphate pathway which relies on oxygenic respiration for electrons. However, Stal and Krumbein (1985) and Stal & Heyer (1986) showed *Oscillatoria* isolated from a microbial mat to generate energy anoxically in the dark by a heterofermentative lactic acid fermentation while *Oscillatoria terebriformis* from an organic rich mat in Hunters Spring, Oregon fermented exogenous glucose or fructose to lactic acid in the dark (Hoare *et al.*, 1971).

8.2.4. ARA over a diel cycle when mats were submerged by the tide

When mats were submerged by the tide (salinity 32‰.) *in situ*, ARA increased by > 100% during or immediately after submersion (Fig. 6.10) possibly due to an increased availability of nutrients, due to a change in speciation and therefore availability of micro-nutrients or due to resulting micro-oxic/anoxic conditions. On re-exposing mats to air during this cloudy, overcast day, ARA returned to a level similar to that before submersion (3.2 ± 0.6 - 6.1 ± 0.9 nmol C_2H_4 cm^{-2} s^{-1}). During previous studies on ARA during cloudy, overcast (cloud cover > 6/8), ARA was similar over the diel cycles (Fig. 6.5, Table 6.5) with no sudden increase in activity in contrast to that found on 13/14.8.88 during tidal submersion. To accurately determine the effect of tidal submergence on ARA several diel cycles need to be examined over a range of climatic conditions and the salinity and nutrient concentrations of pore waters measured as well as the temperature, pH and Eh of sediments.

Several workers including Griffiths *et al* (1987) examining ARA in *Oscillatoria* mat over diel cycles excluded measuring activity during the twice daily tidal submersion; such diel rates are therefore an underestimate and should be viewed with caution.

When mats were returned to the laboratory and artificially submerged in seawater, ARA was higher than under Milli-Q water supplemented with NaCl to an equivalent salinity suggesting that ions other than Na^+ were required to enhance ARA although it is probable that adding one ion without counterbalancing with other ions affected the speciation and availability of ions already present in the sediment. Ca^{2+}, Mg^+ and K^+ when added singly to Milli-Q water (pH 7.8) enhanced ARA in the mats at concentrations of up to 20 mM; however, the concentration in pore waters was unknown.

Growth and ARA occurred between salinities of 0.5 and 38‰ (9 - 516 mM NaCl), with an optimum both for growth and ARA around 5‰ (86 mM NaCl). When ARA was compared at salinities of 5 and 30‰ over a 16:8 light (50 µmol photon m^{-2} s^{-1}):dark cycle a different response was obtained (Fig. 7.13). ARA was lower in the latter with a decrease in activity in the dark in contrast to a doubling in ARA at a salinity of 5‰ at the onset of the dark period. Cyanobacteria are known to synthesize carbon osmolytes in response to changes in salinity (Reed & stewart, 1988). Although no such compounds have been looked for in *Microcoleus*, one could suppose that such compounds, if synthesizes in the dark would be dependent on intracellular C reserves, thus competing with ARA for energy.

Sudden fluctuations in salinity from 10 to 40‰ (172 - 688 mM Na) within a matter of a few minutes as occurred on changing the medium during experiments to examine the effects of salinity are unlikely under natural conditions; however, even when the salinity was altered more slowly (4‰ every 2 h) there was no increase in the salinity tolerated by Microcoleus D778. Jeanjean & Joset (1988) found that a period of 5 - 6 h was required by diatoms from inter-tidal sediments to adapt completely to certain salinities, therefore, increasing the salinity by 4‰ 2 h^{-1} may not have allowed sufficient time for Microcoleus D778 to adapt fully to certain salinities; however, on a sunny day in temperate regions one might expect salinities to increase at a faster rate than this.

Whilst Microcoleus D778 grew at salinities of up to 40‰ (688 mM Na) in the presence of combined nitrogen it could only grow in the absence of combined N at salinities of up to 37‰ (637 mM Na) Whitton & Sinclair (1979) and Reed and Stewart (1983) found N_2 fixation to be more sensitive to salinity than growth, and Tel-Or (1980) showed NaCl to inhibit enzymes directly involved in N_2 fixation. Although Na^+ is not usually accumulated by cyanobacteria (Apte & Thomas, 1983; 1986) a transient uptake may occur in response to hypersaline shock (Reed et al., 1985) which is reduced in the presence of NH_4^+ and / or K^+ (>25 mM) which competitively inhibit the uptake of Na^+ thereby reducing the intracellular level of Na^+ (Apte et al., 1987). Growth of all Microcoleus strains used in this study as well as ARA in Microcoleus D778 was dependent on the ratio of Na^+ to Ca^{2+}, Na^+ to K^+ and Na^+ to Mg^{2+}.

In considering the effect of NaCl both the effect of Na^+ and Cl^- need to be considered since inhibition of Ribulose bisphosphate carboxylase activity in Aphanothece halophytica was due to the concentration of Cl^- and not Na^+ (Incharoensaki & Takabe, 1988). They found the intracellular level of Cl^- increased from 35 - 150 mM when the extracellular increased from 500 - 2000 mM. Therefore, inhibition of growth and ARA at salinities of 40‰ (688 MM NaCl) may be due to the extracellular concentration of Cl^-. Growth of Microcoleus D634 at different salinities varied according to the form of Mg^{2+} supplied, i.e. whether as Cl^- or as SO_4^{2-} (Fig. 5.12). Since Cl^- is the most abundant ion in seawater (Long & Mason, 1983) its influence needs to be considered.

Combined nitrogen may or may not be present intermittently in sediments either as a consequence of the precipitation of nitrous oxides in rain or dew or from tidal inundation. NO_3-N and NH_4-N concentrations in the open ocean are 1 mM (Guerrero & Lara, 1987) and 0.05 - 2.0 µM (Valklump & Martens, 1983) respectively with somewhat higher concentrations for coastal waters (Culberson

et al., 1982). However, Howarth and Marino (1988) found ammonium levels in sediments to be generally much higher than in water columns. Sorenson & Rasmussen (1987) found NH_4-N to be the most abundant form of N in intertidal sediments with NO_3-N generally absent; however, their measurements did not take into account diel variation in the pO_2 of microbial mats which may oxidise NH_4-N to NO_3-N. ARA in Microcoleus D778 was reduced at NH_4-N concentration of 0.25 mM but not inhibited. Similarly, when Microcoleus mats were submerged in 0.25 mM NH_4-N ARA was reduced by c. 50% but activity was not inhibited even at NH_4-N concentrations of up to 10 mM; however, the precise concentrations of N available to the organism was not known. When Henrickson (1980) cited in Kaplan (1983) added 5 - 20 ppm N to sediment cores of cyanobacteria, N was found to absorb onto the organic matter. In mud with 12% organic matter only 50% of the added N was recovered in pore water. Paerl et al (1989) failed to suppress ARA in Microcoleus mats on adding 1 mg N^{-1}) although concentrations in the pores were only 0.1 - 0.2 mg N^{-1}. Van Raalte et al., (1974) and Casselman et al., (1981) found concentrations of soluble ammonium > 20 - 100 μM and extractable levels of ammonium > 10 - 20 μg^{-1} respectively to inhibit nitrogen fixation in salt marsh sediments. Paerl et al. (1989) pointed out that the concentration of NH_4^+ in oxygen depleted microzones bordering microbial aggregates exceed those measured in porewater. Hence the potential for periodic variations in ARA by NH_4^+ can not be discounted.

8.2.5 Influence of pH on ARA

pH (as well as Eh) affects the supply rate, chemical speciation and recycling of trace elements and according to Rueter and Peterson (1987) may alter pattern of primary productivity and nitrogen metabolism. In general, the capacity of soil and sediment for most micro-nutrients is highest under neutral or just alkaline conditions. Hence variations in the pH of Microcoleus mats (pH increase as a consequence of photosynthesis) may alter the availability of micro-nutrients and consequently affect nitrogenase directly or indirectly via photosynthesis.

The optimum pH for ARA in Microoceleus D778 was dependent on the salinity of the medium as well as on the temperature. At a salinity of 5‰ (86 mM Na) pH was optimal at 8.0, whereas as a salinity of 10‰ (172 mM Na) pH was optimal at 8.0 and 8.5 and at a salinity of 20 and 30‰ (344 and 516 mM Na respectively) ARA was highest at pH 8.5. At a temperature of 25°C, the optimum pH varied between 8.0 and 9.5 depending on the salinity (addition of

NaCl) (Fig. 7.31, 7.32) Blumivald et al. (1984) and Erber et al. (1986) showed Na^+ to be involved in the regulation of pH in coccoid cyanobacteria by means of a Na^+/H^+ antiporter.

8.2.6 Influence of temperature on ARA

Although growth of Microcoleus D778 was optimal between 20 and 26°C, optimum temperatures for ARA varied with salinity and length of exposure (Fig. 7.29, 7.30).

When ARA was compared over a 16:8 light (50 µmol photon m^{-2} s^{-1}):dark cycle at 20 and 28°C, 60% activity occurred in the light with rates varying from 3 - 17 nmol C_2H_4 µmol photon m^{-2} s^{-1} in the light and 14 and 26 nmol C_2H_4 µmol photon m^{-2} s^{-1} in the dark, whilst in the latter 90% of activity occurred in the dark with rates in the dark varying between 10 and 24 nmol C_2H_4 µmol photon m^{-2} s^{-1} and those in the light between 2 and 6 nmol C_2H_4 µmol photon m^{-2} s^{-1}. Temperature could therefore influence diel ARA possibly accounting for the fall in rates during sunny afternoons; however, temperature does not seem to be the main factor responsible for ARA since the highest rates recorded in the field were between 2200 and 0200 on 5/6.6.87 when the temperature was 8 $\pm$ 1°C.

8.3 Concluding remarks

It would appear from this study that not all Microcoleus strains are able to fix N_2 (no ARA or growth in the absence of N). However, the possibility can not be eliminated without determining whether or not nif genes are present.

Microcoleus strains could not be cultured in a form morphologically akin to that in the field i.e. with a communal sheath, although limited success was found in standard medium containing 125 mM Ca^{2+} and 516 mM Na^+ (salinity of 30‰) when a thin, communal sheath was found around three trichomes. The absence of a communal sheath is likely to have influenced the physiological responses of strains and possibly inhibited N_2 fixation although Microcoleus D778, the N_2 fixing strain did not posses a communal sheath.

From six diel ARA assays on Microcoleus mats at Church Island, Anglesey, a a different response was found on each occasion. However, when the effect of environmental variables such as light periodicity, PAR, pO_2, temperature, salinity, nutrient concentration and pH were examined on mats, both in situ and on returning samples to the field as well as on axenic cultures of

Microcoleus D778 (isolated from these mats), all these variables were found to interact and influence ARA although the concentration of O_2 was probably the single, critical factor determining the ability to fix N_2 at any given time.

SUMMARY

1. A study was carried out to determine how widespread N_2 is in the cyanobacterium Microcoleus, both in the laboratory and in the field. The research was extended to compare the influence of environmental variables on on both N_2 fixing and non-fixing strains of Microcoleus isolated from a range of habitats.

2. Isolation of strains was favoured in standard medium containing 86 mM Na^+ and 125 mM Ca^{2+} (salinity of c. 5‰) under a micro-oxic atmosphere at 20°C, 16:8 light (30 µmol photon m^{-2} s^{-1}):dark.

3. Five strains were used during this study: Microcoleus D634 from an Iraqi salt marsh; Microcoleus D761 and D782 from mangrove areas on Aldabra Atoll and Belize respectively; Microcoleus D778 from the upper intertidal sand and mudflats of Anglesey, Wales; Microcoleus D781 from usar soil, Varanasi, India.

4. Under natural conditions Microcoleus possesses a communal sheath surrounding numerous filaments; however, these were lost in culture, leaving only a 0.5 µm sheath around individual trichomes. Since the sheath is likely to influence the physiology of the strains, attempts were made to grow the culture in vitro in a form morphologically akin to that in the field. Limited success was found in Microcoleus D634 on incubating in standard medium supplemented with 516 mM Na^+ and 125 mM Ca^{2+} (salinity of 30‰) and on injecting into modified Winogradsky columns when 2 - 3 filaments were found within a single, thin sheath.

5. All strains were mixohaline growing at salinities of 0.5 - 30‰ and surviving periods under euryhaline (30 - 40‰) and polyhaline (< 40‰) conditions. The shorter the time of exposure the higher the salinity tolerated. Growth rates varied according to the concentration and ratio of Na^+ to K^+, Na^+ to Ca^{2+} and Na^+ to Mg^{2+}.

6. Tests were carried out to determine which strains grew in the absence of combined N. Despite numerous changes in the nutrient (Na, K, Ca, Mg, Fe, P) concentration under 30 and 50 µmol photon m^{-2} s^{-1} at 28 and 32°C Microcoleus D778 was the only strain to grow in the absence of combined nitrogen. Further, acetylene reduction activity (ARA) was not detected in Microcoleus D634, D761, D781 or D782 6, 12, 24, 48 and 72-h after removal of 0.1 mM NH_4-N

from the medium either in the light (50 µmol photon m^{-2} s^{-1}) or in the dark over a 14:10 light:dark cycle at 28.0 and 32.0°C under both an oxic and micro-oxic (± DCMU).

7. Since Microcoleus D778 was the only strain found to fix N_2 the majority of work thereafter was concentrated on this strain. The strain had been isolated from the upper intertidal sand and mud-flats of Church Island by Pearson et al., (1979). Here, Microcoleus mats formed in late May to early June (1986, 1987, 1988) replacing mats of Ulothrix flacca, Rhizoclonium sp. and Enteromorpha sp.. Twice monthly observations showed mats to develop throughout the summer reaching a peak in September (1987, 1988) and gradually decomposing thereafter leaving only empty, apparently undegraded sheaths by mid-November. Species of the cyanobacteria Oscillatoria and Spirulina and of the diatoms Navicula and Nitzschia were also present in the mats.
The influence of environmental variables (light intensity, quality and periodicity; temperature; pO_2; pH; salinity; nutrients (Ca, Mg, K) were compared on N_2 fixation (indirectly by the ARA) in axenic cultures of Microcoleus D778 and on Microcoleus mats in situ as well as on mats returned to the laboratory.

7. In order to determine when N_2 fixation occurs under natural conditions, ARA was measured over six diel periods between June and October, 1987. A different response was found on each occasion although generally high activity (over 70% in the dark) was found on sunny days with 7 - 12 h of sunshine and low, fairly constant ARA over with a slight peak in the afternoon during cloudy, overcast days (1 - 2 h of sunshine).

8. N_2 fixation in non-heterocystous cyanobacteria is known to be O_2 sensitive and the above response suggest that this may be the case in the field. Micro-oxic conditions (± DCMU) enhanced ARA of Microcoleus in situ and in vitro, the magnitude of the response depending on the PAR and temperature as well as on pre-incubation conditions.

9. To determine whether the ratio of light:dark affected the ARA response in the field, Microcoleus D778 was grown in 24:0, 20:4, 16:8, 8:16 and 4:20 light (50 µmol photon m^{-2} s^{-1}):dark at 20°C. Under 8:16 and 4:20 light:dark 72 and 92% respectively of the total ARA fixed per 24-h period occurred in the dark whereas in 20:4 and 16:8 light:dark the majority (81 and 60%

respectively) of ARA occurred in the light. However, when the PAR was increased to 100 μmol photon m^{-2} s^{-1} during a 16:8 light:dark cycle, 80% ARA occurred in the dark.

10. Sediments are believed to transmit red light more readily than blue or green light. Experiments on mats in situ and on cultures in the laboratory comparing the effects of red (600 - 700 nm) and white (± DCMU) light of equivalent intensity on ARA gave a different response depending on the pre-incubation conditions.

11. Since the pH of the mat is likely to increase during the photosynthetic period, the influence of pH on ARA was examined on Microcoleus D778. Optimum pH varied with salinity and temperature: the optimum pH for ARA activity was 8.0 - 8.5 at salinities of 5 and 10%. and 8.5 at 20 and 30%.; however, at 25°C although the optimum pH at 5%. remained unaltered, those at 10, 20 and 30%. changed to pH of 8.0 - 9.0, 8.5 - 9.5 and 8.0 - 10.0 respectively.

12. Microcoleus mats at Church Island were submerged by spring tides. ARA was examined in situ on the 13/14.8.88 when mats were submerged by the tide (salinity of 32%.). On both occasions ARA increased by 70% during and immediately after submersion. The increase in ARA could have been due to either a micro-oxic environment and/or to a change in the concentration, speciation and availability of nutrients. Artificial submersion of Microcoleus mats returned to the laboratory saw an increase in ARA on adding Ca^{2+}, Mg^{2+} and K^{+} whilst salinities (addition of NaCl) enhanced ARA at 5 and 10%., but rates declined at 20 and 30%..

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