Iron accumulation by blue-green algae from saline environments

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IRON ACCUMULATION BY BLUE-GREEN ALGAE FROM SALINE ENVIRONMENTS

by

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

Ihsan Ali Mahasneh

TO MY PARENTS, WIFE, DAUGHTER AND FAMILY

I OWE EVERY THING FOR THEIR PATIENCE AND HARD WORK

ABSTRACT

A study was made on morphological and cytological features of four Rivulariaceae strains from marine, partially saline and freshwater environments using three versions of saline and freshwater media (standard, - Fe, low P). A brackish Anabaena sp. was used for comparison. Subculture to - Fe medium led to marked changes in morphology and cytology. These were (base to apex): a series of new heterocysts apical to the original one, intra-thylakoidal vacuolation, lack of cyanophycin granules, presence of polyphosphate granules, pale colour, brown sheath, short hair and low hairiness. With low P medium, the changes were the opposite.

Hairs did not form in Calothrix D253 in saline medium, whether the cultures were in - Fe or low P medium. Downshift from saline to freshwater medium led to the synchronized development of hairs in most trichomes, whereas the reciprocal upshift led to loss of hairs.

The Fe content was studied in three Rivulariaceae and Anabaena in batch culture in high and low P media, which eventually led to Fe- and P-limitation, respectively. The content reached a maximum by 24 h in all strains, decreasing subsequently from 1.80 - 0.06 % dry weight.

Siderophore production was tested in four Rivulariaceae and Anabaena in saline and freshwater media. Two Calothrix strains and Anabaena produced a siderophore in freshwater medium. Influence of Fe status on nitrogenase activity was tested in two Rivulariaceae and Anabaena in saline and freshwater media during batch culture. Peaks of nitrogenase activity in the former came three to five days after the Fe peak, by which stage Fe content had dropped to within the range 0.6 - 0.3 % dry weight. In contrast, peak activity in Anabaena came within a day of the Fe peak. Addition of Fe to Fe-limited cultures led to rapid increase in activity.

The significance of these data on morphology, cytology and physiology and possible interactions between Fe- and P-limitation are discussed.

t	time
s	seconds
min	minute
h	hour
d	day
µm	micrometre
µg	microgram
mg	milligram
g	gram
ml	millilitre
L	litre
µM	micromolar
M	molar
N	normal
°C	degrees celsius
HEPES	N-2-hydroxypiperazine-N'-2-ethanesulphonic acid
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
NTA	nitrilotri-acetic acid
EDDHA	ethylenediaminedi(o-hydroxphenylacetate)
DTPA	di-ethylenetriaminepenta-acetic acid
CDTA	(1,2-diaminocyclohexane-N,N-tetra-acetic acid
CCCP	carbonyl cyanide-m-chlorophenylhydrazone
DNP	2,4-dinitrophenol
n	number of measurements
$\bar{x}$	mean
r	correlation coefficient
AAS	atomic absorption spectrophotometry

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1 INTRODUCTION

1.1 Blue-green algae in saline environments

Blue-green algae are widespread in saline environments, being especially abundant in intertidal areas (Whitton and Potts, 1982) and they may play an important role in productivity (Wiebe et al., 1975). However availability of Fe may be a limiting factor for their growth (1.32).

Since this thesis is concerned with Fe in saline environments, a few remarks on the influence of salinity on distribution of algae may be useful. It is questionable whether most of the intertidal blue-green algae can be clearly separated from freshwater forms, on the basis of their salt tolerance. For instance, Fan (1956 in Fogg et al., 1973) concluded that Calothrix scopulorum was a form of the freshwater species C. parietina. Indeed, physiological studies (Stewart, 1964) have shown that marine isolates of C. scopulorum and Nostoc entophytum can grow well at salinities approaching those in which freshwater isolates grow. In a comparison of marine and freshwater algae Stam & Holleman (1979) provided evidence to show that marine isolates were generally more halotolerant than their freshwater counterparts and they suggested that these differences could be genetically determined. Most physiological information about intertidal blue-green algae is in relation to their nitrogen fixation activities (Whitton & Potts, 1982). Although there are many reports referring to Fe-limitation in natural phytoplankton populations (Menzel & Ryther 1961; Van Baalen 1962; Lewin & Chen 1971; Entsch et al., 1983) there are none in intertidal algal communities. As a representative family of blue-green algae, Rivulariaceae was chosen for the present study. Further details of their growth may be found in Sinclair (1977), Wood (1984) and Whitton (1987).

1.2 Iron in the environment

1.21 Speciation, transformation and solubility

Although it is the fourth most abundant element in the earth's crust, Fe occurs only at a very low dissolved concentration in saline and fresh waters. Iron may exist in solution in either the divalent, Fe^{2+}, or the trivalent, Fe^{3+}, form (Stumm & Lee, 1960). In anaerobic environments Fe exists predominantly as Fe^{2+}, and at pH values close to neutral it forms insoluble polymers of hydroxides, carbonates, silicates and phosphates (Page

& Huyer, 1984). However the amount of Fe taken into, or retained in solution, is generally greater for Fe^{2+} than for Fe^{3+} (Stumm & Lee, 1960). In most natural waters the solubility of Fe^{2+} is controlled by the solubility of $FeCO_3$, $Fe(OH)_2$ and FeS, whereas other insoluble Fe(II) salts are of less significance in natural waters (Hutchinson, 1957).

The oxidation-reduction cycles are important components of the biogeochemical cycling of the element (Stumm and Lee 1960). Trivalent Fe may undergo reduction to Fe^{2+} if a suitable reductant is available. For example, H_2S components, which are thermodynamically unstable in the presence of dissolved oxygen, are capable of reducing Fe^{3+}. However the tendency for this to take place increases with decreasing pH (Stumm & Lee, 1960). A dynamic cycle in which Fe^{3+} is reduced to Fe^{2+}, and then oxidized back to Fe^{3+} through interactions with oxygenated organics has been suggested (Morgan & Stumm, 1965). Conversely, it has been suggested that Fe^{2+} produced through biological reactions and chemical or photo-reduction of Fe^{3+} (McMahon, 1969) is not always oxidized immediately (Huntsman & Sunda, 1980). Several organic compounds, as well as increased chlorinity, have been shown to slow the oxidation rate remarkably (Kester et al., 1975) and humic and tannic acids have been shown to reduce Fe^{3+} to Fe^{2+}, particularly at low pH values (Theis & Singer, 1974).

Although Fe was thought to be extremely insoluble in aerated natural waters, it may be far more soluble than was previously thought. For instance, Byrne & Kester (1976) found that freshly-precipitated hydrous Fe oxide has a solubility in seawater of $1 - 2 \times 10^{-8}$ M L^{-1}, six orders of magnitude more than calculated by Cooper (1937). They proposed that $Fe(OH)^+$ and $Fe(OH)^{2+}$ were the dominant soluble forms at pH 8.0 although these data seem to contradict those of Kester et al. (1975; see 1.44).

The distinction between the dissolved and particulate fractions is an arbitrary one, usually made on the basis of filtration. It is common practice to define "dissolved" material as that which will pass through a filter membrane with a pore diameter of approximately 0.5 - 1 µm. The Fe fraction passing through a HA Millipore filter (pore size 0.45 µm) is usually considered as the "soluble" fraction (Brooks et al., 1967). This consists of truly soluble complexes of ferric Fe plus colloidal ferric hydroxide and phosphate particles. Colloidal Fe is still a controversial subject in the literature and it is not clear whether to consider it as dissolved or soluble.

1.22 Concentrations of Fe in natural waters

Many reports give concentrations of Fe in natural waters, but only a few examples are quoted here in order to give a broad idea. The Fe concentration in near-shore surface waters ranges between 0.2 - 4.8 µg L^{-1} and those of open-ocean surface waters vary between 0.4 - 4.1 µg L^{-1} (Chester & Stoner, 1974). Haraguchi & Fuwa (1982) measured 0.1 - 61.8 µg L^{-1} Fe in open-ocean surface waters, using graphite furnace atomic absorption spectroscopy. Aston & Chester (1973) reported 3 µg L^{-1} and 670 µg L^{-1} Fe as the means of Fe concentrations in most sea and River water respectively.

1.3 Iron in algal physiology

1.31 Role of Fe

As with other organisms Fe plays many roles in algae (Kelly, 1974). The biological importance of Fe is due in part to its ability to exist at the two redox states (Neilands, 1974); it can accept or donate electrons according to the oxidation potential of the reactions (Raymond et al., 1984).

Iron is an indispensible component of photosynthesis (Price, 1968; Spiller & Terry, 1980; Hardie et al., 1983). It affects the synthesis of the major photosynthetic pigments chlorophyll a and c-phycocyanin (Oquist, 1971), and it is incorporated into ferredoxin and the Fe-sulphur proteins which play a role in photosystem I (Bengis & Nelson, 1977). Iron functions both as a structural component and as a cofactor for enzymic reactions. It affects synthesis and function of all cytochromes, the cytochrome oxidase complex, catalase, peroxidase and ferredoxins; examples for blue-green algae include Hiyama et al. (1985) and Cohn et al. (1985). Iron is also involved in nitrogen assimilation, since ferredoxin is required as the electron donor for both nitrate and nitrite reductase activities (Huntsman & Sunda, 1980, Verstreate et al., 1980). Moreover Fe is a major component of nitrogenase, with 34 atoms of Fe in each molecule (Eady & Smith, 1979), and more is required in associated electron-carrier proteins as a cofactor for nitrogenase (Mortenson & Thornley, 1979).

A number of studies have shown the ecological importance of Fe in blue-green algae, in particular because it can affect the ability of these organisms to compete with the other microflora present in natural ecosystems

(Haibach et al., 1985). Iron appears to play an important role in blue-green mats in intertidal sediments (Skyring & Johns, 1980), presumably by concentrating Fe on the cell wall; when algal cells migrate to form a new layer, the Fe remains behind with the mat's debris. Hartman (1984) has speculated that Fe^{2+} might have acted as an electron donor, instead of sulphide, in microorganisms which formed microbial mats in the Precambrian era. The ability of some blue-green algae to secrete siderophores (Fe chelator) appears to play an important role in their distribution as well as that of other micro-algae (1.41).

1.32 Iron as a limiting nutrient

Iron is often a key factor limiting algal growth, both in freshwaters (Shelske, 1962; Sakamoto, 1971; Goldman, 1972) and perhaps even more so in marine environments (Menzel & Ryther, 1960, Davies, 1970; Glover, 1977; Huntsman & Sunda, 1980; Brand et al., 1983; Entsch et al., 1983). The addition of Fe to natural assemblages can lead to stimulation of growth and photosynthesis (Sakamoto, 1971; Miller et al, 1974). However, a reduction of growth and photosynthesis following Fe addition can also sometimes occur as a result of a decrease in soluble P (Harrison & Morel, 1983), especially where the assemblages are close to P-limitation.

Fe-limitation may also be a key factor influencing the broader distribution of micro-algae, as between coastal and open waters (Ryther & Kramer 1961) because Fe is associated with land drainage and is therefore present at higher concentrations in coastal compared with open waters.

Many techniques have been used to determine whether Fe is a limiting nutrient by analysis of physiological state. These include the measurement of macromolecules in the cell (Healey & Henzded, 1980), element composition (Rhee & Gotham, 1980; Harrison & Morel, 1986), kinetic parameters associated with nutrient uptake (Zevenboom et al., 1982) and measurement of photosynthetic products both in marine and freshwater (Konopka & Kshnur, 1980) environments.

1.33 Algal Fe content

Algae can concentrate within their cells elements which are present in minute quantities in their environment. Knauss and Porter (1954) showed that the amount of trace elements, such as Fe, Mn and Zn taken up by

Chlorella pyrenoidosa is proportional to the concentration present in the culture medium. Similarly, Hayward (1969) showed that the amounts of Fe, Mn and Zn present in the cells of Phaedactylum tricornutum depend upon the external concentration of these elements in the culture medium. The internal concentration was higher in cells of P. tricornutum, grown in full strength ASP_2 compared with those grown in half-strength medium.

Although much is known about the Fe composition of other algae, only two reports are available about Fe concentrations in blue-green algae. In the first report, the minimum concentration of Fe in healthy cells of Synechococcus sp. was 0.06 % of dry weight compared with 0.01 % for extremely Fe-deficient cells, whilst healthy cells grown in batch culture were able to accumulate up to 0.18 % Fe, suggesting a possible mechanism for storage of Fe (Entsch et al., 1983). In the second report, the average of Fe in dried cells was 3626 ppm Jones and Carr (1978). The Fe content of eukaryotic microalgae also varies considerably. For instance, the Fe content ranged between 0.17 - 4.59 % dry weight in Phaedactylum tricornutum (Hayward, 1969) and 0.024 - 3.45 % dry weight in Chlorella (Knauss & Porter, 1954). Hayward (1968) showed that the amount of Fe per cell in Phaeodactylum tricornutum was not a constant quantity, ranging from 0.02 - 3.14×10^{-11} g Fe. As the external concentration of Fe increases the concentration per cell increases although at higher concentrations this may include Fe in particulate matter attached to the cell wall rather than incorporated into the cytoplasm. Goldberg (1952) has reported the minimum Fe content for phytoplankton division of Asterionella japonica to be 5.6×10^{-6} mg Fe/cell, which is in excess of that reported by Hayward (1968).

1.34 Iron-deficiency in blue-green algae, especially Rivulariaceae

In addition to the general chlorotic effect of Fe deficiency (Price, 1968), a spectral shift of the in vivo chlorophyll a absorption peak from 679 to 673 nm has been reported for Fe-deficient Anacystis nidulans (Oquist, 1971, 1973). Hardie et al. (1983) have shown that Fe starvation in Agmenellum quadruplicatum led to a decrease in photosynthetic activity and an accumulation of intracellular glucose followed by shortages of endogenous nitrogen and carbon; in spite of that, A. quadruplicatum, remained fully viable for extended periods of Fe deprivation. Iron starvation led to accumulation of polysaccharide, followed by degradation of ribosomes, and

finally the thylakoid membranes underwent structural modifications and rearrangement before they too began to break down. Carboxysomes did not appear to be affected during periods of Fe deprivation, but polyphosphate bodies may have been partially degraded. Guikema & Sherman (1984) and Pakrasi et al. (1985) also showed that Fe deficiency in Anacystis nidulans led to alterations in the composition and function of thylakoid membranes in Anacystis nidulans R2. A striking response to Fe-limitation has been shown for Nostoc MAC, where a soluble Fe-containing ferredoxin was displaced by a non-Fe-containing flavodoxin (Hutber et al., 1977).

Calothrix viguieri D253 produced hairs under Fe-deficient conditions in freshwater media and the sheaths had a much deeper brown colouration than other cultures (Whitton et al., 1973; Sinclair & Whitton, 1977). The latter authors also showed that six out of 16 strains of Rivulariaceae produced hairs in response to Fe deficiency. Heterocyst frequency increased and hairs and intra-thylakoidal vacuoles developed in C. parietina D184 under Fe-deficiency (Douglas et al., 1986). A functional significance has been suggested for hairs formed in response to P deficiency, but not so for those in response to Fe deficiency (Whitton, 1987). However the possibility has been raised that hair formation under Fe deficiency might perhaps result from an interaction in some cell process leading to P deficiency in spite of the presence of a high environmental concentration of P (Douglas et al., 1986; Whitton, 1987).

The addition of Fe to Fe-deficient cultures can lead to a quite rapid response; hairs were shed and hormogonia released in various Rivulariaceae strains from both laboratory and field (Sinclair and Whitton, 1977). Carboxysomes and phycobiliosomes increased rapidly within 6 h and new membranes were synthesized within 12 - 18 h (Sherman & Sherman, 1983). When Fe was restored to Fe-deficient Agmenellum quadruplicatum, the intracellular pigment levels increased rapidly and growth resumed.

1.4 Factors influencing availability and transport of Fe

1.41 Influence of chelating materials

(i) Natural chelators

Organic ligands are especially important in that they form dissociable complexes with Fe ions, helping to keep them in solution and to maintain concentrations of the free iron at optimum levels; in other words they act

as metal buffers (Sauchelli, 1969). The many types of chelate include humic, citric, tartaric, ascorbic and amino acids, but these are not always abundant, stable or sufficiently effective (Sauchelli, 1969; Sedlack et al., 1983).

Interactions between humic acids and Fe affect significantly the chemical equilibria and availability of Fe in natural waters (Shapiro, 1957). Under aerobic conditions and alkaline pH, there is almost complete precipitation of Fe in the absence of organic chelates, making it unavailable to phytoplankton (Davies, 1970). The addition of organic chelates may stimulate algal growth by making relatively insoluble Fe more available for uptake. Johnson (1964) and Shapiro (1957) found that addition of organic acid-Fe complexes increased Fe availability to phytoplankton, and Lange (1970) suggested that the stimulation of blue-green algal growth by fulvic acids was due to organic acids holding Fe in solution. Conversely Prakash & Rashid (1968) demonstrated that Fe-humic acid complexes stimulated the growth of marine phytoflagellates and suggested stimulation was not due to increased Fe availability but may be due to use of humic acid as a heterotrophic substrate. Giesy (1976) showed that humic acids stimulated algal growth in the dark, with and without aeration, suggesting their use as a heterotrophic substrate. However, it might be argued that if Fe is to be a limiting factor for primary production in oceanic waters (Menzel & Ryther, 1961) then formation of Fe^{3+}-containing organic complexes (Johnston, 1964 & Lewin & Chen, 1971) or the direct association of Fe-colloids with the cell surface (Goldberg, 1952; Lewin & Guillard, 1963) may be necessary to permit sufficient uptake.

(ii) Artificial chelators

One of the major advances made in the development of media for the culture of microalgae was the introduction of chelating agents as a means of controlling the concentrations of trace metals necessary for growth. Their principal role is to maintain Fe in solution in forms adequate for algae nutrition (Johnston, 1964). In the absence of chelators, the alkaline pH of the culture medium results in almost complete precipitation of Fe (Davies, 1970). EDTA, the most widely used artificial chelator, forms stable chelates with Fe^{3+} and less stable complexes with Fe^{2+} (Stumm, 1985). However it was not effective in chelating Fe in either stored seawater or autoclaved culture medium; in both cases part of the soluble Fe was

converted into a particulate form (Lewin & Chen, 1974). Alternatively, adding more chelator to marine media is not an advantage as, at pH values above 8.0, EDTA actively binds Mg and Ca. In addition, under some conditions sterilization may result in the formation of double Ca or Mg salts, which are less soluble than Na-Ca-EDTA salts and may cause large precipitates in the medium and lead to metal deficiencies (Provasoli et al., 1957). Anderson and Morel (1980) showed that the Ca concentration in the medium would affect the affinity of EDTA and NTA for Fe^{3+}, in the same way that Ca affects the equilibrium concentrations of Fe^{3+}.

Synthetic chelators, especially EDTA, can stimulate the growth of phytoplankton populations in nature (Johnston, 1964; Barber et al., 1971) and in cultures (Johnston, 1964; Barber & Ryther, 1969). Barber et al., (1971) suggested that phytoplankton may be Fe-limited due to the dominance of Fe oxide in seawater, a condition which may be improved by addition of EDTA. This solubilizes hydrous ferric oxide and makes the Fe more available to the phytoplankton (Lewin & Chen, 1971). Conversely, Jackson & Morgan (1978) claim that EDTA is unlikely to improve Fe transport to the cell because the FeEDTA dissociation or exchange kinetics are presumably slow relative to the diffusive flux of Fe oxide. Alternatively, chelators may stimulate growth through depression of toxic metal activities, such as Cu^{2+} (Anderson & Morel, 1978 Jackson & Morgan, 1978).

(iii) Siderophores

The solubility of Fe^{3+} at natural pH is too low to support anything approaching maximum growth rates (Neilands, 1982). Microorganisms under these conditions are, therefore, presented with the problem of obtaining the Fe they need for growth. A common strategy for microorganisms to obtain Fe from their environment is to excrete chelating agents, called siderophores, which possess a very high affinity for Fe^{3+} and relatively low affinity for Fe^{2+} (Neilands, 1981). The term "siderophore" refers to two general classes of microbial ferric-chelating compounds, secondary hydroxamates and catechols (Neilands, 1973). The production of siderophores has been shown in Nostoc muscorum and Lyngbya sp. (Lange, 1974), Anabaena sp. and A. cylindrica (Simpson & Neilands, 1976), the marine blue-green algae Agmenellum quadruplicatum and Coccochloris elabens (Armstrong & Van Baalen, 1979), Anabaena cylindrica (McKnight & Morel, 1980), in the bloom-forming A. flos-aquae (Murphy et al., 1976; McKnight & Morel, 1980) and in Anabaena sp.

(Goldman et al., 1983; Clark et al., 1987). Although most siderophores from blue-green algae appear to contain hydroxamate groups, the only one whose structure has been chemically determined is schizokinen from Anabaena sp. (Simpson & Neilands, 1976, Lammers & Sander-Loehr, 1982). Schizokinen has been detected in rice field soil, where it presumably arises from blue-green algal inhabitants (Akers, 1983).

The siderophores are taken up by specific, largely unknown, transport systems (Neilands, 1977; 1981). Two possible mechanisms have been suggested for the removal of Fe from siderophores; hydrolysis of the siderophores followed by Fe removal, or in situ reduction and release of the Fe^{2+} (Neilands, 1981; Moody & Dailey, 1984).

(iv) Interactions between all types of Fe-chelators

In presence of humic acids Fe is associated with both humic substances and hydroxyl ions at alkaline conditions, forming an apparently homogeneous solution and only dissociates from humic substances at low pH values (Tryland & Gjessing, 1975). Nevertheless, Tryland & Gjessing found that filtration of humic water with EDTA added resulted in a significant increase in the concentration of Fe which was filtrable fraction . In the pH range 5 - 7, about two thirds of the Fe originally "connected" to the humic substances had passed through the filter, indicating that Fe can be removed from organic complexes by EDTA. Lange (1970) showed that fulvic acid, when used in favourable ratios with essential nutrients, was often much more effective than with artificial combination, whilst Giesy (1976) found that humic acids and EDTA, in combination, had a greater effect on growth than when each was present singly.

The ability of algae to obtain chelated Fe depends largely on the metal-hydroxamate complex and the metal : chelator molar ratio. Chelators with high stability constants (for example schizokinen and desferrioxamine) bind an equimolar concentration of Fe (Molar & Morgan, 1972), whilst chelators with smaller stability constants (for exmple EDTA) bind less and do not compete with siderophores (Baily & Taub, 1980). This is supported by the observation that algal chelators approach the effect of an artificial EDTA plus citrate combination (Lange, 1974). Siderophores produced by bacteria and blue-green algae can remove Fe from FeEDTA, making it available to these organisms (Anderson & Morel, 1982).

1.42 Influence of temperature

It has been well established that temperature can influence the rate of diffusion, carrier-mediated uptake, and cell metabolism in general (Huntsman & Sunda, 1980). Miller (1985) reported that most prokaryotes alter their membrane lipid composition in response to changes in temperature. Such alterations are believed to result in membranes with physical characteristics which permit proper membrane function within a particular temperature range.

1.43 Influence of light

Iron is usually added to media in a soluble chelated form such as Fe(III)EDTA (Provasoli et al., 1957). Such chelates are thought to allow Fe to be photo-reduced and released as soluble Fe^{2+} (Hill-Cottingham, 1955) which can then be taken efficiently and utilized by algae, either directly or after oxidation to ionic Fe^{3+} (Owens & Chaney, 1971). The maximum rate of uptake by Thalassiosira weissflogii was achieved in the presence of chelating agents (especially CDTA and EDTA) and particularly in the light (Anderson & Morel, 1982). Finden et al. (1984) showed that Fe(III) oxide, with light-induced reduction, provided a continuous supply of soluble Fe^{2+}. Conversely, light did not affect Fe uptake by T. weissflogii in the absence of chelators (Anderson & Morel, 1980). However, no detectable Fe uptake by T. weissflogii was found in either light or dark in the presence of the very strong chelators, EDDHA and desferrixamine B (Anderson & Morel, 1982). Similarly, poor growth of phytoplankton in the presence of excess EDDHA has been reported by Johnston (1964). It would thus appear that the stable Fe(III)EDDHA complexes are not appreciably photodegraded or reduced.

Generally, light influences nutrient uptake in many ways: it provides the energy necessary for active transport, supplies the carbon skeletons through photosynthesis which act as a sink for incorporated ions, stimulates the growth which results in increased nutrient uptake and provides the energy for production of charged polymers which establish Donnan potentials (De Boer, 1981).

1.44 Influence of pH

pH is known to be a key factor in regulating the speciation of Fe and, consequently, Fe availability in natural waters. For instance, in the marine environment at pH values above 7.0 more than 95 % of trivalent is

present as $Fe(OH)_3$ and more than 90 % of divalent is present as $FeOH^+$, whilst below pH 7.0 less than 20 % of divalent is present as Fe^{2+} whereas only about 2.7 % of trivalent is present as Fe^{3+} (Kester et al., 1975).

The solubility of Fe is influenced directly by pH and by the standard reduction potential (E^o) of the metal ion and both can vary widely from outside to the inside the cell (Morel and Hudson, 1985). The absorption of Fe by Chlamydomonas mundana was much great at neutral or slightly alkaline pH than at acidic one (MaCiasr, 1965).

1.45 Influence of salinity

Salinity may affect metal uptake, partly due to shifting chemical equilibria (Sunda et al., 1978) and partly to the effect of salinity has on both organic and inorganic complexation. Increasing salinity, for instance, reduces organic complexation of many metals due to increased competition for available sites (Mantoura et al., 1978). Kester et al., (1975) reported that two factors are involved in the effect of changes in salinity on Fe^{2+}; changes in concentration of chloride and sulphate-complexing ligands; and changes in stability constants with ionic strength. They showed that in river water at pH 8.0, divalent Fe exists primarily as Fe^{2+}, whereas in marine water the $FeOH^+$ species is predominant. In contrast, however, trivalent Fe exists predominantly as $Fe(OH)_3$ in both marine and river water. Obviously, changes in speciation lead to changes in availability of Fe and, ultimately, uptake by algal cells.

1.46 Influence of ionic interaction and antagonism

The effect of other metal ions an Fe absorption by plants appears to be quite important. High concentrations of cations (Ca, Cu, Mn, Zn) have been found to reduce Fe uptake and are thought to be a major factor causing Fe chlorosis (Brown, 1956; Lingle et al., 1963). Since all cationic trace metals exhibit the same general coordination properties and no ligand can be completely specific for one metal, it has been suggested that toxic metals act by binding competitively tosites of transport, assimilation or utilization of essential metals (Morel & Morel-Lanrens, 1983). The best example of that is Cd which acts as an antagonist of Fe transport in the Thalassiosira weissflogii, both by binding competitively the site of Fe transport and interfering with some intracellular sites of Fe assimilation or utilization (Harrison & Morel, 1983).

1.47 Influence of external concentration of Fe

There are marked differences in the abilities of different algae to grow at low concentrations of ionic Fe (Brand et al., 1983; Murphy et al., 1976). Phaeodactylum tricornutum was able to produce a similar cell density at both low (0.045 mg L^{-1}) and high (4.2 mg L^{-1}) concentrations of Fe (Hayward 1968 & 1969). However, Knauss & Porter (1954) showed that the amount of Fe taken up by Chlorella pyrenoidosa is proportional to the concentration present in the culture medium.

1.48 Influence of phase of growth

The physiological changes during the different phases of growth in batch culture may influence the metal's uptake. Exponentially growing cells generally take up metals at a higher rate than cells in the stationary phase (Morel et al., 1978; Bates et al., 1983) although Cain et al. (1980) observed the opposite effect in green alga Scenesdesmus obliquus. Phytoplankton species in the ocean adjust their nutrient uptake rates according to variations in availability (Sunda & Huntsman, 1985; Harrison & Morel, 1986). Sunda & Huntsman (1986) postulated a number of mechanisms by which algal species in oceanic waters may achieve optimal growth:

(a) required metals are taken up more effectively at low concentrations;

(b) intra-cellular metal pools are used more efficiently;

(c) a lower maximum growth rate is maintained which reduces the required rates of uptake;

(d) the functional enzymatic requirements are switched from metaloenzymes to non-metal enzymes, i.e. replacement of the Fe containing enzyme ferredoxin with the a non-metallic enzyme flavodoaxin (Entsch et al., 1983).

1.5 Mechanisms of Fe uptake

The algal cell surface is composed of a mosaic of adsorption sites, consisting of carboxylic, sulphydryl, phosphatidic, amino and other groups. Some are believed to be "physiologically inert", whilst others are "physiologically active" and lead to transport across the cell membrane.

As many types of algae (diatoms, green algae, blue-green algae, dinoflagellates, coccolithophorineae, prasinophytes) exhibit similar properties of metal accumulation it has been suggested that the uptake process is similar in all these groups (Fisher et al., 1983, 1984). A

general model of metal transport by microorganisms has been proposed (Morel & Hudson, 1985): the cell either synthesizes ligands which bind the metals strongly at the cell surface or uses the activity of surface bound enzymes to precipitate metals extracellularly. The reduction of sulphate to sulphide (by membrane-associated sulphate reductase) and the diffusion of O_2 and H_2O through the cell membrane provides a highly reactive region where the metal can be complexed and precipitated (Stumm, 1985).

Although Fe transport in microbial cells has been studied extensively (Neilands, 1982, 1983), and a model of transport in eukaryotic algae suggested (Anderson & Morel, 1982) there are few particular reports of Fe transport in blue-green algae. It has usually been assumed that algae take up soluble ionic species of Fe. However, the stable form of Fe in oxic waters of neutral or near-neutral pH is $Fe(OH)_3$ and the ionic form is relatively scarce (1.21). Algal growth may therefore cease or be severely curtailed once initial growth had used up the small amount of available Fe. The role of chelating materials in Fe transport has been discussed (1.41). In the explanation of how algal cells can free out the chelated Fe; there are three possible hypotheses for removal of Fe from chelates (Anderson and Morel, 1984):

(a) The complex is reduced and dissociated at the membrane surface:
(b) Chelates are taken up intact and the Fe released within the cell;
(c) The Fe-ligand chelate dissociates outside the cell and the ferric complex is recognized by receptors on the cell surface and transported across the membrane.

Jackson & Morgan (1978) concluded that the dissociation of FeEDTA is too slow to supply Fe to the algal cell, which could make the dissociation step unnecessary for uptake. They cited experiments which showed that EDTA did not cross the phytoplankton cellular membrane. However, Anderson & Morel (1982) showed that a membrane-bound metal binding complex, phytotransferrin, transferred Fe across the membrane by a process not directly coupled to either photosynthetic or respiratory activity.

Although it has been suggested that, like higher plants, some macroalgae may use a mechanism which involves reduction of Fe^{3+} to Fe^{2+} before uptake (Marley, 1981), no similar mechanism has yet been proposed for blue-green algae. A Fe^{3+} reductase has been characterized in a photosynthetic bacterium (Moody & Daily 1985) which is believed to remove Fe from ferric siderophores. Plasmids, as well as chromosomes, have been reported to be

involved in specifying elements of the ferric transport system in microorganisms (Neilands, 1982).

1.6 Aims

Iron availability is a key factor for algal growth particularly in saline environments (1.32). Because availability is very low (1.4), algae may develop particular strategies to deal with this problem. Rivulariaceae was chosen for study because they are one of the larger groups of blue-green algae, being widespread, often nitrogen-fixers and show morphological flexibility in response to nutrient limitation. A brackish strain, Calothrix D253, was selected for intensive study. For certain purposes, however, additional species (up to 4 strains) from the same family were used. A brackish alga Anabaena D697 was used for comparison.

The aims of study were to investigate:

(a) Morphological and cytological responses to Fe-limitation;

(b) Accumulation of Fe under various physiological conditions and in particular whether an "overplus phenomenon" occurs;

(c) Whether the strategy of producing chelating materials (1.41) under Fe-limitation is present in Rivulariaceae;

(d) Influence of Fe status on nitrogenase activity, to see the responses under limitation and subsequent addition of Fe.

2 MATERIALS AND METHODS

2.1 General laboratory procedures

Erlenmeyer flasks were used for liquid media to maintain stock cultures, prepare inocula and in all experiments. They were plugged with silicon bungs (Type S28, Sanko Plastic Ltd, Osaka, Japan). Pre-sterilized plastic petri dishes (Sterilin, England) were used for solid media.

All glassware was washed with detergent, rinsed throughly with hot tap water and then soaked in 10 % Analar HNO_3 for at least eight hours before being rinsed eight times with glass distilled water and dried at 100° C. Apparatus for phosphate analysis was given preliminary washes in 10 % H_2SO_4 and HCl separated by six rinses in distilled water before being HNO_3 washed. Pipettes and tips were soaked in 10 % HNO_3 for at least one hour before being rinsed eight times in glass distilled water and dried at room temperature.

Silicon bungs were cleaned after each experiment by soaking in a 2 % solution of phosphate free detergent (Decon 90, Decon Laboratories Ltd, England) for 20 - 30 min before being rinsed eight times in glass distilled water and dried at 40° C.

2.2 Chemicals

Apart from HEPES and PIPES (Sigma), all chemicals used in the preparation of media were of Analar grade, and obtained from British Drug Houses Ltd (BDH), Poole, England.

2.3 Media

Apart from a chelating agent (EDTA), and a buffer (HEPES) the liquid media used in this study are inorganic. All salts were prepared in high

concentrations (stock solutions) in Milli-Q water obtained from a Milli-Q water system (Millipore Corp.)

2.31 Seawater

Seawater was collected from the Northumberland 1° 39'E 55 35'N (NU 223324) coast near Seahouses and stored in plastic carboys at 4° C in the dark. The water was passed through a GF/C filter before use.

2.32 Synthetic saline media

At the start of the project saline media were made by supplementing either ADP(1)Fe(0.4) or Chu-10D(-N) medium with the required percentage of seawater. Subsequently a modified version of the ASP_6 medium (James, 1974) (referred to here as ASP_6M) was used: the modifications were:

(a) $NaNO_3$ omitted when used to grow forms capable of fixing nitrogen: this is referred to as $ASP_6M(-N)$:

(b) Tris buffer, shown to be toxic to several algae (Mclachlan, 1963), replaced by HEPES:

(c) K_2 glycerophosphate replaced by K_2HPO_4:

(d) Vitamins (vitamin B_{12}, biotin and thiamine) omitted, as a preliminary experiment showed that their absence, separately and in combination, did not affect the growth (visual obs ervaion) of strains used;

(e) Concentration of Co increased to 0.01 mg L^{-1} due to its requirement by N_2-fixers:

(f) Addition of Br, I, and Ni on the basis of their requirements by blue-green algae (Bony, 1966; Almon & Böger, 1983).

The 30 % $ASP_6M(-N)$ was prepared as follows:

(a) NaCL, $CaCl_2$, KCl and $MgSO_4$ were treated as variables and salinity was changed by adjusting their proportions in the original recipe. Thus, 30 %

refers to salt percentage^{S} only, while other components were treated as constants.

The composition of $ASP_6M(-N)$, and 30 % $ASP_6M(-N)$ along with concentrations of each element in the original medium is shown in Table 2.1 and 2.2. In some experiments, however, concentrations of some components were varied: details are discussed with each experiment. Throughout the thesis the saline medium is refered to as 30 % $ASP_6M(-N)$, while freshwater medium to Chu-10D(-N), unless stated otherwise.

Table 2.1 Composition of saline media (mg L^{-1} of salts).

	(i)	(ii)	(iii)
Salt	ASP_6	$ASP_6M(-N)$	30 % $ASP_6M(-N)$
NaCl	24000.0	24000.0	7200.0
$MgSO_4.7H_2O$	8000.0	8000.0	240.0
KCl	700.0	700.0	210.0
$CaCl_2$	150.0	150.0	45.0
$NaNO_3$	300.0	-	-
Na_2glycerophosphate	100.0	-	-
$K_2HPO_4.3H_2O$	-	7.36	7.36
Tris Buffer	1000.0	-	-
HEPES	-	600.0	600.0
Vitamine B_{12}	$5.3x10^{-5}$	-	-
Biotin	0.0005	-	-
Thiamin	0.005	-	-
$Na_2EDTA.2H_2O$	5.0	2.54	2.54
H_3BO_3	5.70	5.70	5.70
$FeCl_3.6H_2O$	0.245	1.94	1.94
$MnSO_4.4H_2O$	0.82	0.82	0.82
$ZnSO_4.7H_2O$	0.110	0.110	0.110
$CoSO_4.7H_2O$	0.024	0.048	0.048
$CuSO_4.6H_2O$	-	0.0185	0.0185
$NiSO_4.7H_2O$	-	0.012	0.012
$Na_2MoO_4.2H_2O$	0.5	0.5	0.5
KBr	-	0.085	0.085
KI	-	0.10	0.10
S $^{o}/oo$	28.49	28.49	8.55

(i): as reported by James (1974).
(ii), (iii): as modified by B. A. Whitton and I. A. Mahasneh (1984). Algology Lab., Durham University

Table 2.2 Composition of saline media (mg L^{-1} elements).

Element	(i) ASP_6	(ii) $ASP_6M(-N)$	(iii) 30 % $ASP_6M(-N)$
Na	9485.7	9485.7	2845.71
Cl	15768.23	15768.23	4730.47
Mg	789.13	789.13	236.7
K	367.11	374.83	112.45
Ca	40.89	40.89	12.27
S	1040.88	1040.88	312.26
N	49.4	-	-
P	9.84	1.0	1.0
B	0.997	0.997	0.997
Fe	0.051	0.40	0.40
EDTA	3.93	2.0	2.0
Mn	0.201	0.201	0.201
Zn	0.025	0.025	0.025
Co	0.005	0.01	0.01
Cu	-	0.0042	0.0042
Ni	-	0.0025	0.0025
Mo	0.198	0.198	0.198
Br	-	0.0567	0.0567
I	-	0.0764	0.0764

Table 2.3 Amount of Fe in the components (as impurities) of saline medium using AAS-graphite furnce. Concentrations are in final medium.

Components	Fe (mg L^{-1})
NaCl	0.0011
$MgSO_4$	0.0044
KCl	0.0033
$CaCl_2$	0.0042
Trace elements	0.0014
K_2HPO_4	0.0034
Na_2EDTA	0.0090
HEPES	0.0017
Milli-Q water	0.0005
Sum of the above	0.0290
Whole medium	0.0300

The amounts of Fe present in salts of saline medium as impurities are given in Table 2.3. The sum of these was 0.03 mg L^{-1} and Fe additions to the medium were based on this.

2.33 Freshwater media

Chu-10D(-N) (Sinclair & Whitton, 1977) was used as the basis for constructing a freshwater medium, but the concentrations of Fe (0.5 mg L^{-1}) and P (1.78 mg L^{-1}) in the original recipe were changed to 0.40 and 1.0 mg L^{-1} for Fe and P, respectively, in order to be the same as those in saline medium. The EDTA concentration was changed to maintain the normal molar ratio (1 : 0.96). The detailed composition is shown in Tables 2.4 and 2.5.

Table 2.4 Composition of freshwater media (mg L^{-1} of salts)

Salt	ADP(1)Fe(0.4)	Chu-10D(-N) (modified)
K_2HPO_4	7.36	-
KH_2PO_4	-	7.36
$MgSO_4.7H_2O$	200.0	25.0
$Na_2SiO_3.5H_2O$	-	10.88
NaCl	230.0	-
$NaHCO_3$	-	15.85
$CaCl_2.2H_2O$	66.2	-
KCl	19.1	35.83
HEPES	600.0	600.0
$FeCl_3.6H_2O$	1.94	1.94
$Na_2EDTA.2H_2O$	2.54	2.54
$MnCl_2.4H_2O$	-	0.045
$MnSO_4.H_2O$	0.51	-
$NaMoO_4.2H_2O$	0.063	0.0068
$ZnSO_4.7H_2O$	0.055	0.055
$CuSO_4.5H_2O$	0.0198	0.0198
$CoCl_2.6H_2O$	0.01	-
$CoSO_4.7H_2O$	-	0.0105
$NiSO_4.7H_2O$	0.012	-
H_3BO_3	0.715	0.715

Table 2.5 Composition of freshwater media (mg L^{-1} of element)

Element	ADP(1)Fe(0.4)	Chu-10D(-N)
P	1.0	1.0
S	26.0	3.25
Cl	171.0	-
Na	90.5	8.44
K	10.02	2.24
Ca	18.1	9.77
Mg	19.7	2.47
Si	-	2.5
Fe	0.4	0.4
Mn	0.125	0.012
Mo	0.025	0.0025
Zn	0.013	0.12
Cu	0.005	0.002
Co	0.0025	0.002
B	0.125	0.125
Ni	0.0025	-

2.34 Solid media

1.0 % agar (w/v) was added to the mineral medium, autoclaved, allowed to cool to about 40° C and poured into pre-sterilized petri dishes in a laminar flow cabinet. Solid media were kept at 4° C in dark. These were used mainly for isolation and purification of cultures and for routine testing for bacterial or fungal contamination.

2.35 Buffers

Media were buffered by HEPES, chosen on the basis of its lack of interference in biological systems (Smith and Foy, 1974). HEPES has a pKa of 7.5 at 25° C, allowing it to be useful in the range 6.8 - 8.2, spanning the pH of saline and fresh waters. The media used, therefore, were bufferred (2.5 mM HEPES) at pH 7.8, similar to the pH of the natural environment. Neither addition of Fe nor Na_2EDTA to the medium caused any change in the pH.

Use of pH probes

In a preliminary experiment the ability of four different electrodes to measure pH in the presence and absence of HEPES was tested. The pH and the mV of solutions of saline and freshwater media were measured over 60 min with pH buffer solutions of pH 4.0, 7.0 and 9.0. There were marked differences between electrodes used to measure the pH of Chu-10D(-N) (with 0.75 mM and 2.5 mM HEPES) (data not shown). For instance, a decrease of 0.25 pH units was recorded when an Orion Ross combination probe was used, whilst a Russell combination probe gave a very stable reading. Measurements of the pH of saline medium showed much smaller differences between electrodes.

2.36 Cehlating agent

Different concentration of EDTA (as Na_2EDTA) was used for chelation in culture media throughout the work, details are given with each case.

2.37 Sterilization

Media were sterilized by autoclaving at 121° C and 10$\frac{1}{2}$ $\kappa N\ m^{-2}$ (15 lb in^{-2}) for 15 min and allowed to cool and re-equilibrate with atmospheric gases at room temperature for at least six hours. When the liquid media contained a high concentration of P, this was autoclaved separately to avoid precipitation and added aseptically to each flask. For the influence of sterilization on Fe solubility see 3.1.

2.4 Laboratory experimental conditions

2.41 Illumination

In the shaking tanks continuous illumination was supplied from beneath by warm white fluorescent tubes. In growth rooms continuous illumination was supplied from above by a bank of cool white fluorescent tubes. Light

quantity was measured using a Macam Quantum meter model Q101 (Macam Photometric Ltd. Livingston, Scotland). The light quantity in the tank in ranged from 90 - 110 µmol photon m^{-2} s^{-1} PAR. To avoid the influence of light variations during long-term experiments, flasks were randomized twice a day and no flask was incubated near the edges of the tank.

2.42 Temperature

Growth rooms were available at temperatures of 15, 20 and 32° C. Tanks were available at range of 15 - 32° C.

2.43 Standard experimental conditions

Experiments were carried out, unless stated otherwise, in Erlenmeyer flasks containing 50 ml of medium at 32° C, 90 - 110 µmol photon m^{-2} s^{-1} and continuous shaking in tanks. A shaking mechanism moved the flasks approximatly 30 mm horizontally 75 times a minute.

2.5 Algal cultures

2.51 Origin and environmental background of the strains

Strains were isolated from saline environments in Aldabra Atoll and Jordan. *Dichothrix* sp. D696 and *Anabaena ambigua* D697 were isolated from a sample collected from a saline pool (Cinq Cases pool-3, Aldabra Atoll). *Calothrix* strains D704 and D705 were isolated from a sample of sediment collected from the intertidal zone of the Gulf of Aqaba, Jordan. All isolates are refered to by their Durham culture number.

Three strains (*Calothrix viguieri* D253, *C. parietina* D550, *Calothrix* D603) were also used from Durham Culture Collection. Details of all strains are given in appendix 3.

2.52 Test for purity

The pure cultures of the strains isolated were examined before each subculturing, and each experimental inoculum was tested as follows:

(a) Algal material was examined microscopically, if no contaminants could be seen, then;

(b) Small pieces of algal material were spread on agar plates of bacterial and fungal test media. This test was carried out in a laminar flow cabinet, itself tested periodically. These media were used as described by Hoshaw and Rosowski (1973). The media were: nutrient broth, peptone glucose, yeast extract and SST. Plates were incubated in the dark at 32° C for two weeks and if no contaminants were visible:

(c) Algal material was inoculated in a routine medium enriched with 0.05 % sucrose + 10 mg L^{-1} N (as NH_4). Flasks were incubated in the dark and light at 32° C for 2 - 10 days. If cultures did not appear cloudy or have obvious bacterial growth they were re-examined as described in (a) above. If no contaminants could be seen in any of these tests it was concluded that the tested strain was axenic.

2.53 Subculturing and maintainence

Standard aseptic techniques were used for subculturing, using a horizontal laminar flow cabinet (Microflow Pathfinder). For experimental subculturing a Gilson Pipettman with sterile tips was used. For routine subculturing of stock cultures, however, a sterile wire loop was used to transfer algal material.

Pure cultures were preserved cryogenically in liquid nitrogen soon after their isolation into an axenic state. Stock cultures, however, were

maintained in liquid media contained in 100-ml straight neck conical flasks closed with silicon bungs and incubated in the growth room.

2.54 Mass determination

The washed algal material was dried in silicon crucibles at 105° C for 24 h. On removal from the oven they were allowed to cool in a desiccator and were weighed on an Oertling R51 balance to five decimal places.

2.55 Preparation of element-limited inocula

Throughout the thesis (unless stated otherwise) Fe-limited inocula were prepared by omission of Fe and reduction (X 0.75 and X 0.25 for saline and freshwater media) of chelating agent (EDTA), combined with use of high P(10 mg L^{-1}). A moderate Fe-limited inocula (for studies in Chapters 5, 7 and 8.1) was prepared by twice subculturing (4 days each) in P(10 mg L^{-1}) Fe(0.2 mg L^{-1}) before use in the experiment. Phosphorus-limited inocula were prepared by twice (2 days each) subculturing in P (0.05 mg L^{-1}) Fe(0.4 mg L^{-1}) before use in the experiment.

Iron- and/or P-limited cultures were pooled and resuspended in the desired medium with the element to be tested omitted. The algal material was converted to a uniform suspension by passage through progressivly finer needles (1.1, 0.63 and 0.5 mm diameter, Sabre Gilette Surgigcal Ltd. England). Algal material were shaken vigorously before aliquots of homogenate were distributed into flasks.

Standard criteria for limitation

The culture was considered to be limited by an element if there were marked change in morphological and cytological features which could only be reversed by the addition of that element.

2.6 Analytical procedures

2.61 Iron

2.611 Routine use of Atomic Absorption Spectrophotometry

All Fe determinations were performed on a Perkin-Elmer (PE) model 5000 Atomic Absorption Spectrophotometer with HGA 500 graphite furnace for the low level concentrations, using matrix-matched standards and blanks. All determinations were performed using an air-C_2H_2 flame at flow rate of 35 each. The limit of detection of Fe was found to be 0.03 and 0.005 mg L^{-1} for the flame and furnace, respectively.

2.612 Algal digestion

Dried algae were digested in 4-ml of 4.0 M HNO_3 (AAS grade) on a Tecam dri-block for 45 min at 110° C and then allowed to cool to room temperature. The clear solution was separated from the pellet by centrifugation, transferred to a 10-ml volumetric flask and made up to 10-ml with Milli-Q water before being analyzed on AAS (2.611).

2.62 Phosphorus

Filtrable reactive phosphorus (FRP) in the medium was analyzed according to Eisenreich et al. (1975). Total algal phosphorus was determined by digestion according to Batterton & Van Baalen (1968).

2.7 Assays for chelating activity and siderophore

(i) Biological assay

This was carried out by subculture the algae in absence of both Fe and EDTA (Boyer et al., 1987).

(ii) Chemical assay for presence of siderophore

This made use of the perchlorate method of Clark et al. (1987). Inocula were first washed three times and subcultured twice in a version of Chu-10D(-N) medium lacking Fe or EDTA: the cultures were then allowed to grow to the stationary phase. A known volume of culture filtrate was concentrated to 20 % initial volume. The assay was carried out by dissolving the filtrate (containing any siderophore) in 5 mM $FeCl_3$ in 0.14 M $HClO_4$. Any particulate material was removed by filtration and the spectrum measured (each 10 nm) using a Shimadzu (model UV-150-02) double beam spectophotometer.

2.8 Nitrogenase activity

Nitrogenase activity was estimated using the acetylene reduction assay technique (Hardy et al., 1973). The cultures were maintained in the normal medium in Erlenmeyer flasks. The total volume of flasks was 128 ± 1-ml. All experiments were performed under standard growth conditions (2.44). The assay started when the silicon bungs were replaced by gas-tight suba-seal bungs (W. Freeman and Co., Barnsley, U.K.). 15-ml of acetylene (BOC) was injected into each flask, which was shaken slightly before equilibration of pressure by another needle. Samples were then incubated under the same experimental conditions. Two controls were always used:

(a) media + C_2H_2

(b) alga minus C_2H_2

After incubation with C_2H_2 for 60 minutes, the gas mixture was collected in 1-ml disposable syringes and the samples were analyzed by injection into a Varian Aerograph 1400 gas chromatograph equipped with a hydrogen flame ionization detector. N_2 was used as the carrier gas at a rate of 45-ml min^{-1} and the 3.0 mm x 2.0 m column was packed with "Porapak" R (Water Associates Inc., U.S.A). The operating conditions were: detector

temperature, 150° C: column temperature, 100° C; hydrogen flow rate, 30-ml min^{-1}: air, 300-ml min^{-1}.

Ethylene and acetylene peaks were identified on recorder traces by the retention time and quantified with standard curves constructed from high purity ethylene standards (BDH Poole, Dorset, England) on the day of use. The results of nitrogenase activity were expressed as nmol C_2H_2 mg dry wt^{-1} min^{-1}.

In order to test whether ethylene was present a contaminant in the acetylene and for possible leakage of hydrocarbons from the suba-seal bungs (Postgate, 1972); blanks with and without acetylene were also injected.

2.9 Microscopy

2.91 Light microscopy

Algal material were examined using a Nikon Fluophot type 109 microscope. A Nikon M-350 camera was used for photography with Kodak Technical pan 2415 film for black and white and Kodak Ektachrome Tungsten "professional" film for colour pictures.

2.92 Electron microscopy

The fixative (pH 7.0) for routine fixation was: 2.5 % (v/v) glutaraldehyde, 1.0 % (v/v) formaldehyde and 0.05 M PIPES. Material was incubated overnight at room temperature, followed by a rinse of Milli-Q water and an overnight soak in PIPES buffer (pH 7.0). A three to five-minute wash in 0.05 M PIPES buffer completed the fixation.

Fixed material was washed twice in Milli-Q water (15 minutes each) before being stained in 1 % osmium tetroxide for one hour at room temperature. Stained material was washed in Milli-Q water before being passed through a dehydration series consisting of two 10-min changes in 10 %, 20 %, 50 %, 75

%. 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 a LKB Ultratome III Ultra-microtone and collected on acid etched (4 % HCl for 1 minute) copper palladium grids. Sections were then stained following Reynolds (1963) for 20 minutes in uranyl acetate, rinsed with distilled water and counterstained in lead citrate for the same length of time. The grids were then washed in distilled water before being dried and examined under a Phillips EM 400 transmission electron microscope. Kodak electron microscopy film was used for all electron-micrographs.

2.93 Morphological characters

Morphological characters were selected for observation and quantification. Dimensions were normally measured on 10 examples of typical filaments, the measurments were rounded to the nearest 0.5. For studies in Chapters 5, 7 morphological changes were scored when they were first appeared.

(i) Trichome

(a) Basal width (width of the widest vegetative cell in the basal zone);

(b) Apical width (width of the narrowest apical vegetative cell);

(c) length of vegetative part of trichome (length between basal and apical living cell);

(d) A hair was defined as "a region of trichome where the cells are narrow, elongated, highly vacuolated and usually apparently colourless" (Sinclair & Whitton, 1977). The extent to which a particular trichome was hairy was expressed as a percentage of the total trichome length (including the hair). The % hairiness of a sample was expressed as the percentage of the total trichomes which possessed hairs, based on a count of 40 trichomes.

(ii) Heterocyst

The formation of secondary heterocysts in Rivulariaceae (adjacent to original basal heterocyst - see 4.3) was expressed as percentage of total trichomes. Heterocyst frequency in Anabaena D697 was measured on non-homogenized material.

(iii) Cellular inclusions

The extent of the presence of cellular inclusions was expressed in terms of the percentage of the cell profile (i.e. area) of a typical trichome, which appeared to be covered by a particular structure. Cyanophycin granules were usually identified by their characteristic refractive appearance (Fuhs, 1973). To confirm the presence of granules in young filaments they were stained with Schneider's acetocarmine (saturated solution of carmine in 45 % acetic acid). A drop of acetocarmine was placed on a drop of dense algal suspension and left for at least five minutes for post-vital staining. After placing coverslips on the slide cyanophycin granules were examined at a magnification 1000 X. Polyphosphate granules were identified using the method of Ebel et al. (1958) as described by Fuhs (1973). Fresh material was soaked for 15 min in 10 % (w/v) $Pb(NO_3)_2$ in 0.1 N HNO_3. The material was then washed thoroughly with Milli-Q water and treated with 10 % $(NH_4)_2S$ for 30 s and then thoroughly washed with distlled water. Polphosphate granules appeared dark-brown to black under the optical microscope.

The term intra-thylakoidal vacuole refers to structures best confirmed with the electron-microscope, but for which a provisional identification can also be made with the light microscope, on account of their distinct colourless appearance.

3 FACTORS INFLUENCING THE APPARENT SOLUBILITY OF Fe IN MEDIUM

3.1 General remarks

Provasoli et al. (1957) pointed out that although Fe, when added with EDTA, is present in solution in culture medium, it forms a precipitate during autoclaving, especially in marine medium. A preliminary experiment in the present study showed that a brown precipitate (presumably containing Fe) was found, especially in saline medium. Studies were planned to investigate the factors influencing the apparent solubility of Fe in saline and freshwater medium. The apparent solubility of Fe was assessed by measuring % of Fe which passed a GF/C filter, together with centrifugation for comparison. For experimental purposes, it was assumed that full solubility occurs when less than 2 % of Fe was retained on the filter.

3.2 Factors influencing apparent solubility

(i) Influence of pH

The first experiment was carried out to test the influence of pH on solubility in saline medium. Buffering was carried out as described in 2.35 and samples were prepared as if for a normal experiment, with flasks being autoclaved (2.37). Samples were left on the bench for 6 h and then either filtered through GF/C filters or centrifuged (4000 r.p.m. for 10 min). The filtrates were then acidified (0.025-ml HNO_3) before Fe was measured as in 2.61.

After both filtration and centrifugation the solubility of Fe decreased (Table 3.1) with increase in pH (pH before autoclaving). Full solubility was obtained when the pH was adjusted to 2.0 before sampling the medium.

Table 3.1 Influence of pH on apparent solubility of 0.4 mg L^{-1} Fe Fe in saline medium. Iron and 2.0 mg L^{-1} EDTA were added to medium prior to autoclaving.

pH	% of apparent Fe in solution	
	filtration	centrifugation
6.0	84.6	89.2
7.0	44.1	55.0
7.8	10.0	16.1

(ii) Influence of EDTA and time of exposure in saline medium

A preliminary experiment showed that the solubility of Fe decreased with time. In a more detailed experiment, therefore, the influence of EDTA and time exposure on solubility of Fe was investigated in saline medium.

Medium was autoclaved and left to stand for 6 h before Fe and EDTA additions. Samples were then incubated under standard experimental conditions (i.e. in light) over 3, 6 and 25 h.

Table 3.2 Influence of EDTA and time of exposure on the apparent solubility of 0.4 mg L^{-1} Fe in saline medium. Iron and the different EDTA concentrations were added to autoclaved medium at the start of the experiment.

EDTA (mg L^{-1})	Fe : EDTA molar ratio	% of apparent Fe in solution over time			
		0	3	6	25 h
2.0	1 : 0.96	97.3	54.7	43.8	26.5
4.0	1 : 1.92	98.1	92.0	81.1	61.9
6.0	1 : 2.88	99.3	94.2	87.6	97.3
8.0	1 : 3.84	-	96.4	92.0	97.3
10.0	1 : 4.8	98.7	92.0	92.0	97.3
20.0	1 : 9.6	99.1	94.2	92.0	97.2

The results (Table 3.2) show that the minimum EDTA required to keep Fe in solution in saline medium is 6.0 mg L^{-1}.

(iii) Influence of EDTA and time of exposure in freshwater medium

The influence of EDTA and time of exposure on solubility of Fe was also tested in freshwater medium. The experiment was carried out as in (ii) with the exception that no sampling was made at t = 0. The minimum EDTA required to keep Fe in solution in freshwater medium (Tables 3.3) is 4.0 mg L^{-1}.

Table 3.3 Influence of EDTA and time exposure on the apparent solubility of 0.4 mg L^{-1} Fe in freshwater medium. Iron and the different EDTA concentrations were added to autoclaved medium at the start of the experiment.

EDTA (mg L^{-1})	Fe : EDTA molar ratio	% of apparent Fe in solution over time (h)		
		3	6	25 h
2.0	1 : 0.96	98.9	98.9	65.2
4.0	1 : 1.92	98.9	98.9	98.9
6.0	1 : 2.88	98.9	98.9	97.2
8.0	1 : 3.84	98.9	98.9	97.2
10.0	1 : 4.8	98.9	98.9	97.2
20.0	1 : 9.6	99.0	99.0	97.2

(iv) Influence of light and dark incubation in saline and freshwater media

An experiment was carried out to test the influence of light on solubility of Fe in saline and freshwater media. Saline medium was prepared as in (ii) and freshwater medium as in (iii), but with only one

EDTA concentration (2.0 mg L^{-1}). Samples were incubated in light or dark for 25 h before filtration.

Table 3.4 Influence of light and dark incubation (25 h) on apparent solubility of 0.4 mg L^{-1} Fe in two media. Iron and 2.0 mg L^{-1} EDTA were added to autoclaved medium at the start of the experiment.

Medium	% of apparent Fe in solution	
	light	dark
saline	26.5	70.7
freshwater	65.2	97.2

Saline medium and the presence both enhanced the reduction in the apparent solubility of Fe.

(v) Influence of salts (in dark) in saline medium

The influence of each salt on solubility of the Fe was tested. The salts and/or solutions were prepared separately at their normal concentrations, buffered at pH 7.8, autoclaved and left to stand for 6 h before Fe (0.4 mg L^{-1}) and EDTA (2.0 mg L^{-1}) were added. Solubility of Fe was followed over 25 h incubation in the dark (Table 3.5).

Table 3.5 Influence of chemical components of saline medium on the apparent solubility of 0.4 mg L^{-1} Fe. Iron and 2.0 mg L^{-1} EDTA were added to autoclaved medium at the start of the experiment.

tested substance	% of apparent Fe in solution over time (h)			
	0	3	6	25 h
Milli-Q water	98.9	99.4	100.1	98.9
NaCl	95.1	91.0	91.0	93.0
$MgSO_4$	87.3	83.6	87.3	87.3
KCl	107.1	107.5	99.0	103.5
$CaCl_2$	95.7	103.5	99.0	107.1
trace elements	101.3	101.3	101.3	99.0
K_2HPO_4	103.5	99.0	99.0	103.5

Although $MgSO_4$ had the greatest effect, it was still less than the influence of all salts in combination (Table 3.5). These results indicate that a high EDTA concentration should be used to maintain Fe in solution during accumulation studies.

(v) Influence of type of filter

The next step was to select a filter for use in accumulation studies. The filters tested were: GF/C (1.2 µm), Millipore (0.45 µm) and Nuclepore (0.2 µm). The experiment was carried out as in (ii) for saline medium and as in (iii) freshwater medium, with the exception that high EDTA was used 6.0 mg L^{-1} for former and 4.0 mg L^{-1} for latter. The solubility of Fe was followed over 25 h. There was no difference in results, so GF/C used for the rest of study.

4 INFLUENCE OF Fe-LIMITATION ON MORPHOLOGY AND CYTOLOGY

4.1 General remarks

The morphology of Rivulariaceae is often influenced markedly by conditions of nutrient limitation (1.3). It was essential, therefore, to identify these changes in order to obtain criteria for use in further experiments.

A series of experiments was planned using various modifications of a saline and a freshwater medium. Saline medium refers to 30 % $ASP_6M(-N)$, and freshwater medium refers to Chu-10D(-N), unless stated otherwise. Three versions of each were used for studies in this chapter: standard as in 2.3 and - Fe and low P medium as in 2.561. Strains of Rivulariaceae were used, together with Anabaena D697. Inocula were taken from exponential cultures, which include many free hormogonia. A few general comments on the appearance of the cultures may be helpful.

Calothrix viguieri D253: dark green; more clumped in saline than freshwater medium.

Calothrix parietina D550: green-brown; floating and attached in saline and freshwater medium.

Calothrix sp. D603: black-brown; floating in freshwater medium.

Dichothrix sp. D696: green-yellow; attached and floating in saline medium.

Calothrix sp. D704: bright green; formed firmly attached bushy colonies in 100 % $ASP_6M(-N)$ medium.

Anabaena ambigua D697: bright green; fully floating during the lag and exponential phases, but tended to form ball- and net-like growths during the stationary phase.

The microscopic appearance was recorded, and descriptions made using the stages of development proposed by Livingstone and Whitton (1983). The main strains (D253, D550, D696, D704) will be described first, with details of Anabaena D697 at the end of 4.4.

4.2 Morphological and cytological changes in standard medium

4.21 Optical microscopy

The first experiment was carried out in standard medium; saline medium was used to grow cultures of Calothrix D253, Dichothrix D696, Calothrix 704 (100 % $ASP_6M(-N)$), while freshwater medium was used to grow Calothrix D550 and also Calothrix D253. The results for the various stages are given in Tables 4.1-4.8, A1.1-1.2, Fig. 4.1-4.2, A1.1.

Stage I

There was no obvious change in dimensions of the trichome of strains. The hormogonia resembled each other in dimensions (3 µm wide), with the exception of Calothrix D704 (1 µm wide). Calothrix D550 had hormogonia with gas vacuoles; these were lost within 24 h of their release.

A change in granulation was obvious in all strains. Polyphosphate granules increased and cyanophycin decreased slightly. Hormogonium production was at a maximum, and the release of hormogonia was similar in all cases. It was facilitated by development of specialized cells (necridia); these degenerative structures were biconcave, pale and colourless. All cultures resembled each other in that the sheath was firm and there were no intra-thylakoidal vacuoles in vegetative cells (see 4.3), no secondary heterocysts and no hairs on the trichomes; after 48 - 72 h growth, the hormogonia had tapered slightly and developed a terminal heterocyst, a change which is considered as the beginning of stage II.

Stage II

As the length of trichome increased, increase in width was restricted to the basal area. All strains resembled each other in that there were no secondary or intercalary heterocysts.

Polyphosphate granules appeared to decrease more rapidly in Calothrix D550 than the other strains; the loss was in each case first apparent in the apical region of trichome. There was an increase in cyanophycin which was most pronounced in Calothrix D253 and Calothrix D550.

Production of hormogonia decreased gradually until they ceased to form. Vacuolation in apical region of the trichomes was recognized as the end of stage II; this was the first indication of hair formation.

Stage III

The development of the hair was similar in each strain, including elongation and vacuolation of a few apical vegetative cells. The extent of hair development varied among strains, as illustrated by the hair dimensions, percentage of hairiness and the percentage of trichome as hair. The increase in length of trichomes varied between strains, with Calothrix D704 the least.

All strains resembled each other in that the polyphosphate granules were absent and cyanophycin increased markedly in both basal and apical areas of the trichomes. Secondary heterocysts were developed immediately above the (original) basal heterocyst.

A deep brown sheath developed in Calothrix D253.

Table 4.1 Dimensions (µm) of morphological charcters of Calothrix D253 in standard saline medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	86.5	7.5	5.5	-	-	<1
1	85.5	7.5	5.5	-	-	<1
2	96.5	7.5	5.5	-	-	<1
3	110.5	7.5	5.5	-	-	<1
4	161.5	7.5	5.5	-	-	<1
6	180.5	7.5	5.5	-	-	<1
8	219.5	8.5	5.5	-	-	<1
10	250.5	8.5	5.5	-	-	<2
12	375.5	10.5	5.5	-	-	<2
14	475.5	10.5	5.5	-	-	<2

Table 4.2 Dimensions (µm) of morphological characters of Calothrix D253 in standard freshwater medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	83.5	7.5	5.5	-	-	<1
2	97.5	7.5	5.5	-	-	<1
4	175.5	7.5	5.5	-	-	<1
6	196.5	7.5	5.5	-	-	<1
8	245.5	7.5	5.5	-	-	<1
10	262.5	8.5	5.5	-	-	<1
14	345.5	8.5	5.5	25.5	1.5	<2

Table 4.3 Dimensions (µm) of morphological charcters of Calothrix D550 in standard freshwater medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	62.5	7.5	4.5	-	-	<1
1	100.5	7.5	4.5	-	-	<1
2	125.5	7.5	4.5	-	-	<1
3	126.5	7.5	5.5	-	-	<1
4	212.5	7.5	5.5	-	-	<1
6	237.5	7.5	3.5	100.5	1.5	<1
8	125.5	7.5	3.5	321.5	1.5	<1
10	100.5	10.5	3.5	470.5	1.5	<1
14	75.5	10.5	3.5	675.5	2.5	<1

Table 4.4 Dimensions (µm) of morphological characters of Dichothrix D696 in standard saline medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	75.5	7.5	5.5	-	-	<1
2	87.5	7.5	5.5	-	-	<1
4	145.5	8.5	5.5	-	-	<1
6	195.5	10.5	6.5	-	-	<1
8	280.5	12.5	6.5	25.5	2.5	<1
10	310.5	12.5	6.5	120.5	2.5	<1

Table 4.5 Granulation (% of cell profile) and frequency (%) of morphological changes in Calothrix D253 in standard saline medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome as hair
	basal	apical	basal	apical	production			
0	30	10	-	-	80	-	-	-
2	70	30	-	-	80	-	-	-
4	70	30	-	-	60	-	-	-
6	60	20	-	-	30	-	-	-
8	30	20	-	-	20	-	-	-
10	20	10	20	10	-	-	-	-
12	-	-	30	20	-	+	-	-
14	-	-	30	20	-	+	-	-

Table 4.6 Granulation (% of cell profile) and frequency (%) of morphological changes in Calothrix D253 in standard freshwater medium.

day	polyphosphate		cyanophycin		hormogonia production	vacuolation	hairiness	trichome as hair
	basal	apical	basal	apical				
0	30	10	-	-	80	-	-	-
2	70	30	-	-	80	-	-	-
4	70	30	-	-	60	-	-	-
6	60	20	-	-	20	-	-	-
8	20	20	10	10	20	-	-	-
10	10	10	20	10	-	-	-	-
12	10	10	30	20	-	+	-	-
14	-	-	30	20	-	+	10	29

Table 4.7 Granulation (% of cell profile) and frequency (%) of morphological changes in Calothrix D550 in standard freshwater medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	10	-	30	-	-	-	-	-
2	50	20	30	10	80	-	-	-
4	10	-	20	-	60	-	+	-
6	-	-	20	10	20	-	20	29
8	-	-	20	10	20	-	30	72
10	-	-	20	10	20	-	50	82
12	-	-	80	20	20	-	80	90

Table 4.8 Granulation (% of cell profile) and frequency (%) of morphological changes in Dichothrix D696 in standard saline medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	20	-	10	10	80	-	-	-
2	70	20	20	10	80	-	-	-
4	60	10	20	10	80	+	-	-
6	40	5	30	10	50	+	-	-
8	20	-	40	10	20	+	10	-
10	-	-	60	10	-	+	30	9
20	-	-	80	20	-	+	80	30

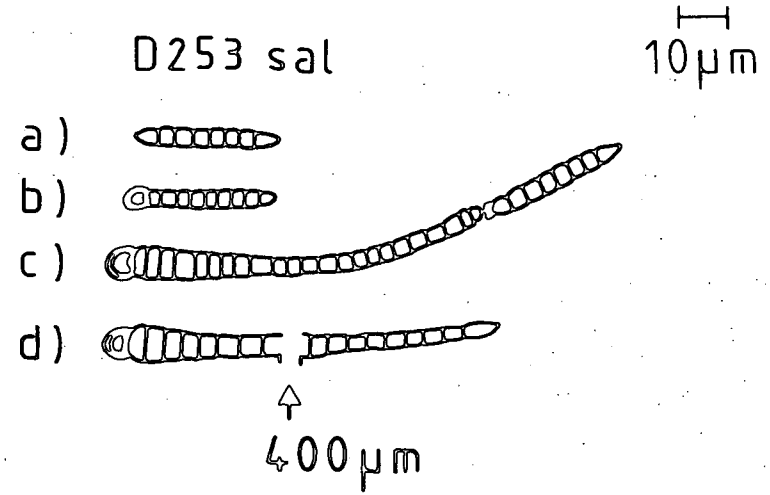
Fig. 4.1 Morphological changes of strains during growth in standard medium in batch culture: Calothrix D253 and Dichothrix D696 in saline medium. Calothrix D253 and Calothrix D550 in freshwater medium

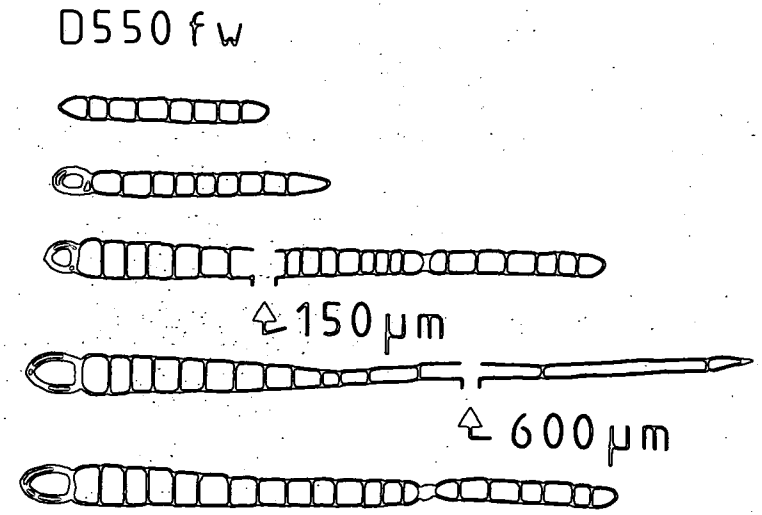
a) Hormogonium

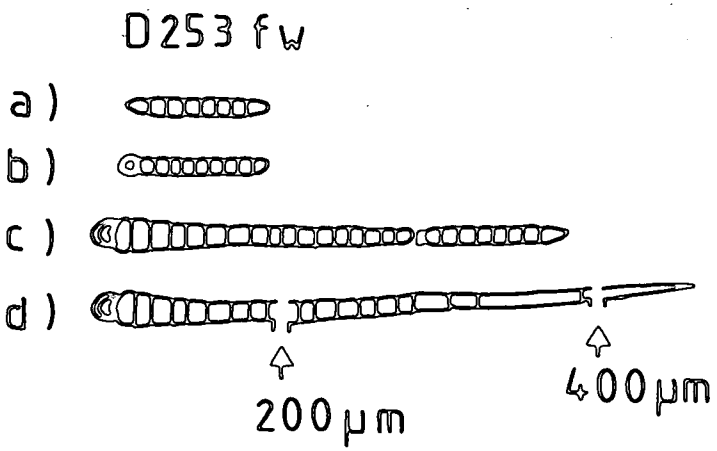
b) Young trichome with heterocyst

c) Hormogonial release from mature trichome with necridium

d) Mature trichome after ceasing hormogonial release


D253 sal
10 µm
a)
b)
c)
d)
400 µm


D550 fw
150 µm
600 µm


D253 fw
a)
b)
c)
d)
200 µm
400 µm

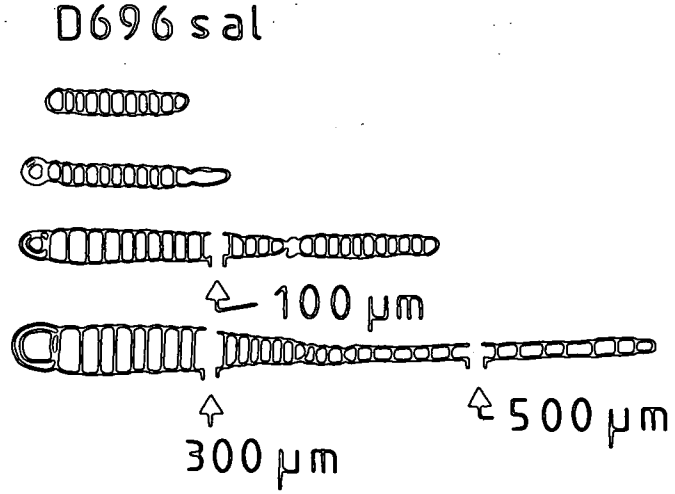

D696 sal
100 µm
300 µm
500 µm

Fig. 4.2 Optical micrograph of strains grown in standard medium

Vegetative cell (VC), hormogonia (Ho), necridium (N), heterocyst (Het), Cyanophycin (C), gas vacuoles (GV) and akinete (AK)

Scale bar = 10 µm

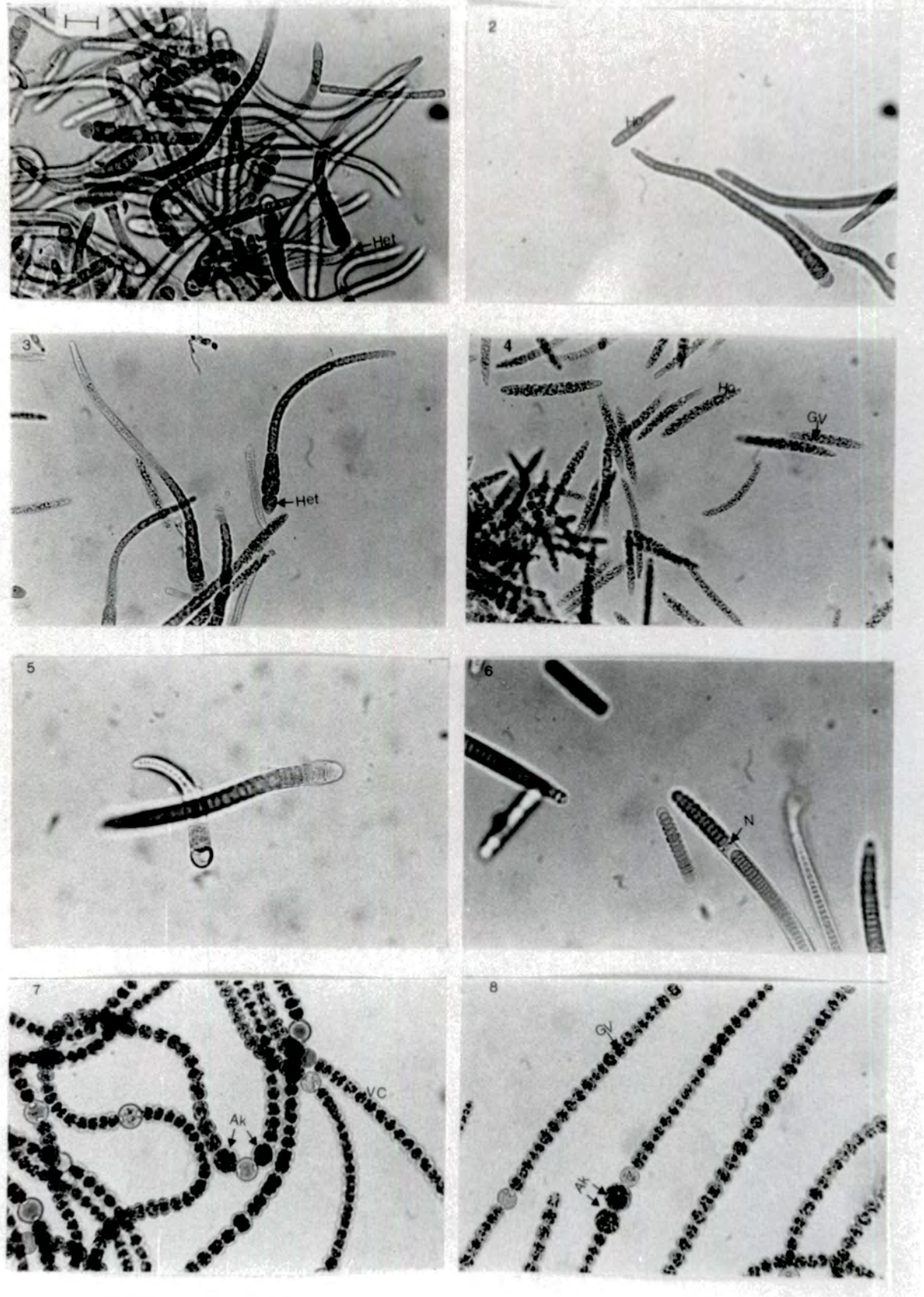
1) *Calothrix* D253 in saline medium

2) *Calothrix* D253 in freshwater medium

3-4) *Calothrix* D550 in freshwater medium

5-6) *Dichothrix* D696 in saline medium

7-8) *Anabaena* D697 in saline medium


1
Het
2
Ho
3
Het
4
Ho
GV
5
6
N
7
Ak
VC
8
GV
Ak

4.3 Morphological and cytological changes under Fe-limitation

4.31 Optical microscopy

It is not clear which factor limits growth in batch culture using standard media (4.2). Fe-limitation may have been implicated during the experiments reported in 4.2, as the formation of secondary heterocysts and a deep brown sheath has been reported previously in Calothrix D253 in - Fe medium (1.32). Therefore a further experiment was carried out to identify morphological and cytological responses to Fe-limitation in order to relate these to physiological responses. The medium used here was different from that in 4.2: omission of Fe, reduction of EDTA and containing high (10 mg L^{-1}) P concentration in order to avoid interaction beteen Fe- and P-limitation. The results for various stages are given in Tables 4.9-4.15. A1.3-1.4, Fig. 4.3-4.5, A1.1.

Stage I

The cultures were in general similar to those in standard medium, but a few differences were noted. Production of hormogonia was less and cyanophycin granules decreased more rapidly. Intra-thylakoidal vacuolation appeared in some hormogonia of Calothrix D253 and in many vegetative cells of the tapered trichome of Calothrix D550. The colour of vegetative cells was pale in all strains.

Stage II

The differences from standard medium became more pronounced. A deep brown sheath developed in Calothrix D253. Production of hormogonia was much less in all strains, especially Calothrix D550. Cyanophycin granules

decreased until they became absent, whereas polyphosphate granules decreased more slowly than in standard medium.

All strains resembled each other in developing secondary heterocysts. Formation of secondary heterocyst was accompanied by degeneration of the basal (collapsed) heterocyst, which appeared disconnected with the secondary heterocyst, although it was sometimes covered by the same sheath.

A marked difference in the colour of vegetative cells and the amount of intra-thylakoidal vacuolation (in vegetative cells) from that in standard medium was also observed. For instance the colour was pale-green in three strains and even paler in Calothrix D550. The latter had the most intra-thylakoidal vacuolation (in vegetative cells), which appeared evenly distributed along the trichomes and increased in size toward the apical zone. Vacuolation in apical region of the trichomes was recognized as the end of stage II; this was the first indication of hair formation.

Stage III

Marked differences were obvious in the appearance of cultures as with standard medium. The hair was shorter and its frequency was less. Although hair length varied between strains, the width was quite similar apart from Dichothrix D696, which was about twice that of the others. The length of the transition region between vegetative cells and mature hairs also varied between strains, as Calothrix D550 had a distinctive zone; other strains had either a short zone or an abrupt transition.

In comparison with standard medium polyphosphate granules continued to be present, particularly in the basal cells of the trichome. In contrast with standard medium, frequency of secondary heterocysts increased sharply, though varied between strains. Many trichomes had up to four adjacent basal heterocysts.

Colour of vegetative cells differed markedly from that in standard medium (4.2).

Table 4.9 Dimensions (µm) of morphological characters of Calothrix D253 in - Fe saline medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	85.5	8.5	4.5	-	-	<1
1	85.5	8.5	4.5	-	-	<1
2	94.5	8.5	4.5	-	-	<1
3	120.5	10.5	5.5	-	-	<1
4	165.5	10.5	5.5	-	-	<1
6	160.5	10.5	5.5	-	-	<1
8	180.5	10.5	5.5	-	-	<1
10	180.5	12.5	5.5	-	-	3.5
12	250.5	15.5	5.5	-	-	3.5
14	260.5	15.5	5.5	-	-	3.5
16	280.5	16.5	5.5	-	-	3.5

Table 4.10 Dimensions (µm) of morphological characters of Calothrix D550 in - Fe freshwater medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	62.5	7.5	5.5	-	-	<1
2	125.5	7.5	5.5	-	-	<1
4	140.5	7.5	5.5	15.5	2.5	<1
6	225.5	7.5	2.5	32.5	2.5	<1
8	175.5	7.5	2.5	35.5	2.5	<1
10	170.5	7.5	2.5	35.5	2.5	<1
12	175.5	7.5	2.5	35.5	2.5	<1

Table 4.11 Dimensions (µm) of morphological characters of Dichothrix D696 in - Fe saline medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	82.5	12.5	6.5	-	-	<1
2	87.5	12.5	6.5	-	-	<1
4	110.5	12.5	6.5	-	-	<1
6	120.5	12.5	6.5	-	-	<1
8	165.5	12.5	6.5	25.5	-	<1
10	212.5	10.5	5.5	25.5	2.5	<1
12	250.5	10.5	5.5	35.5	3.5	2.5
14	274.5	10.5	5.5	50.5	3.5	2.5
22	285.5	10.5	5.5	60.5	3.5	2.5

Table 4.12 Granulation (% of cell profile) and frequency (%) of morphological changes in Calothrix D253 in - Fe saline medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	10	-	10	10	80	-	-	-
2	80	50	-	-	50	-	-	-
4	80	50	-	-	15	+	-	-
6	60	50	-	-	10	+	-	-
8	60	50	-	-	-	+	-	-
10	50	50	-	-	-	+	-	-
12	40	30	-	-	-	+	-	-
14	40	20	-	-	-	+	-	-

Table 4.13 Granulation (% of cell profile) and frequency (%) of morphological changes in Calothrix D253 in -Fe freshwater medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	10	-	10	10	80	-	-	-
2	80	40	-	-	30	-	-	-
4	80	40	-	-	10	+	+	9
6	60	40	-	-	-	+	5	14
8	50	30	-	-	-	+	10	18
10	30	20	-	-	-	+	10	25
12	30	10	-	-	-	+	20	29
14	30	10	-	-	-	+	20	23

Table 4.14 Granulation (% of cell profile) and frequency (%) of morphological changes in Calothrix D550 in - Fe freshwater medium.

day	polyphosphate		cyanophycin		hormogonia	intrathylakoidal vacuolation	hairiness	trichome as hair
	basal	apical	basal	apical	production			
0	30	10	10	-	90	-	-	-
2	10	-	80	30	80	+	-	-
4	-	-	80	20	30	+	10	10
6	-	-	80	10	5	80	20	12
8	-	-	60	10	-	80	20	17
10	-	-	50	10	-	80	20	17
12	-	-	50	10	-	80	20	17

Table 4.15 Granulation (% of cell profile) and frequency (%) of morphological changes in Dichothrix D696 in - Fe saline medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	20	-	30	30	70	-	-	-
2	50	10	10	10	30	-	-	-
4	50	10	-	-	10	+	-	-
6	50	10	-	-	10	+	-	-
8	40	10	-	-	-	+	5	13
10	30	10	-	-	-	+	5	11
12	30	10	-	-	-	+	10	12
14	30	-	-	-	-	+	20	13
22	20	-	-	-	-	+	20	17

Fig. 4.3 Morphological changes of strains during growth in - Fe medium in batch culture: Calothrix D253 and Dichothrix D696 in saline medium and Calothrix D550 in freshwater medium

a) Hormogonium

b) Young trichome with heterocyst

c) Hormogonial release from mature trichome with necridium

d) Mature trichome after ceasing hormogonial release

e) Hormogonial release after addition of Fe to Fe-limited culture

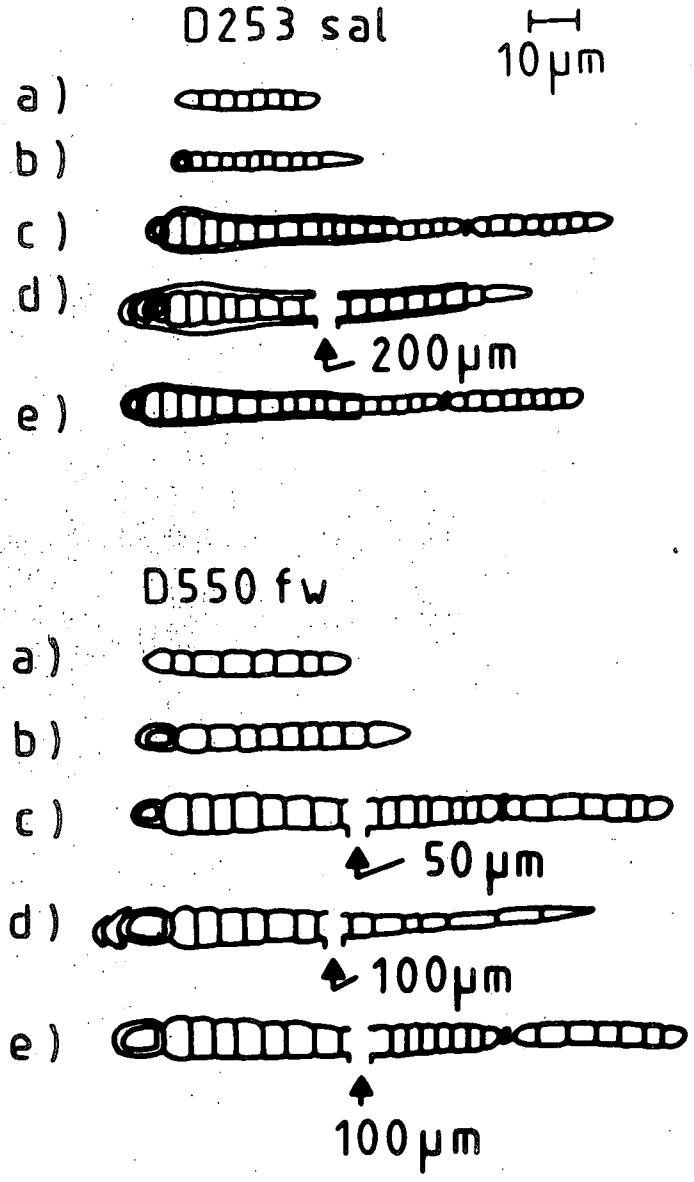

D253 sal
10 µm
a)
b)
c)
d)
200 µm
e)
D550 fw
a)
b)
c)
50 µm
d)
100 µm
e)
100 µm
D696 sal
a)
b)
50 µm
c)
225 µm
d)
200 µm

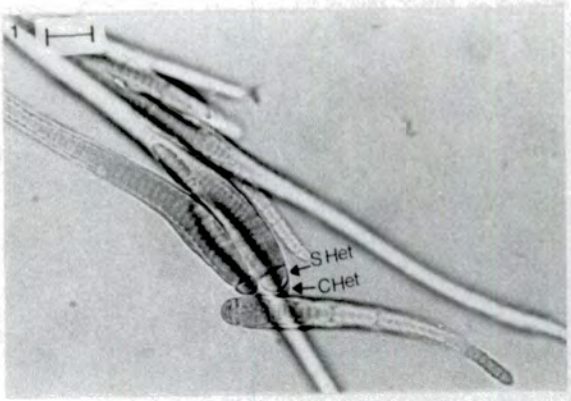
Fig. 4.4 Optical micrograph of strains grown in - Fe medium. Vegetative cell (VC), hormogonium (Ho), necridium (N), heterocyst (Het), hair (Hr), degenerated trichome (DT), akinete (Ak) in Anabaena D697, collapsed heterocyst (C Het), secondary heterocyst (S Het), sheath (S), gas vacuoles (GV), intra-thylakoidal vacuolation (IV), cyanophycin (C), polyphosphate granules (Pp). Scale bar = 10 µm

1-5) Calothrix D253 in saline medium

6-7) Dichothrix D696 in saline medium

8-12) Anabaena D697 in saline medium

13-16) Anabaena D697 after polyphosphate staining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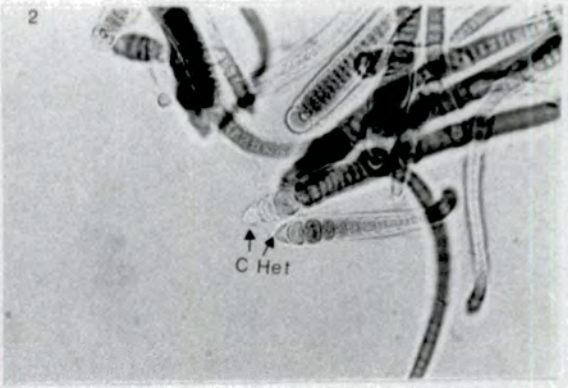
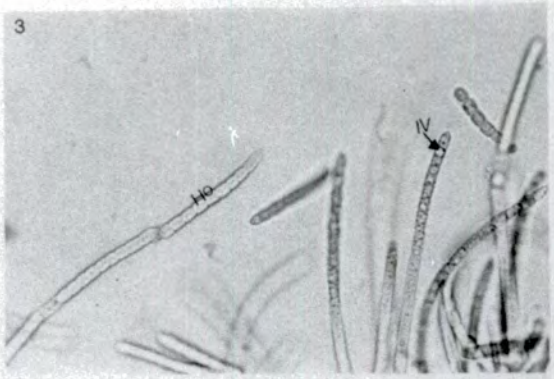
SHet
CHet


2
C Het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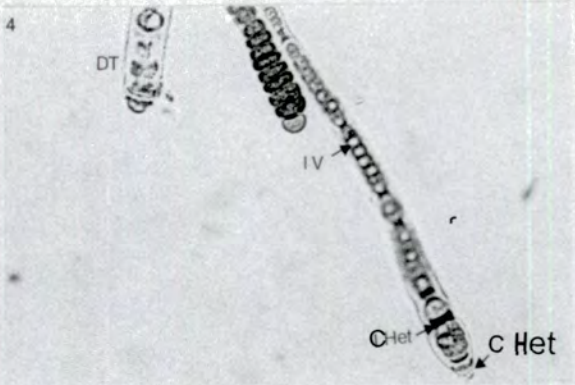
Ho
IV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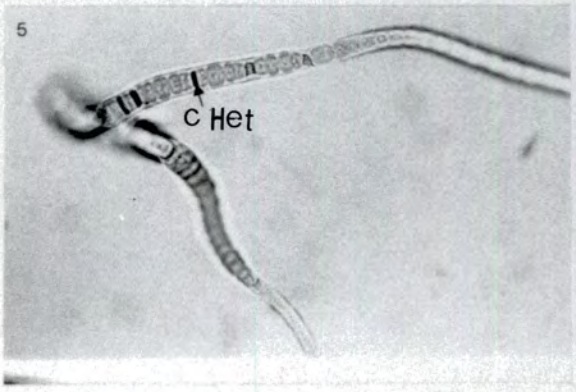
IV
CHet
C Het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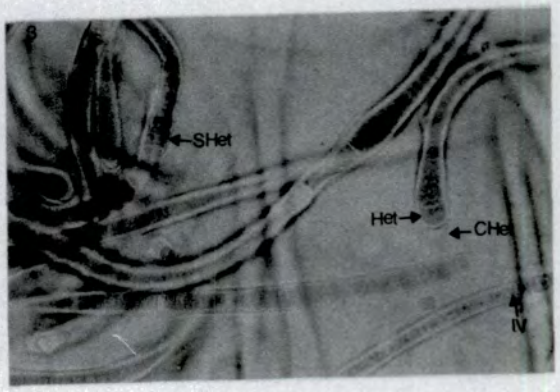
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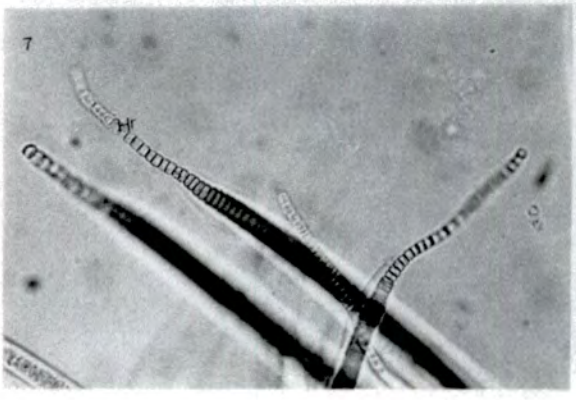
SHet
Het
CHet
IV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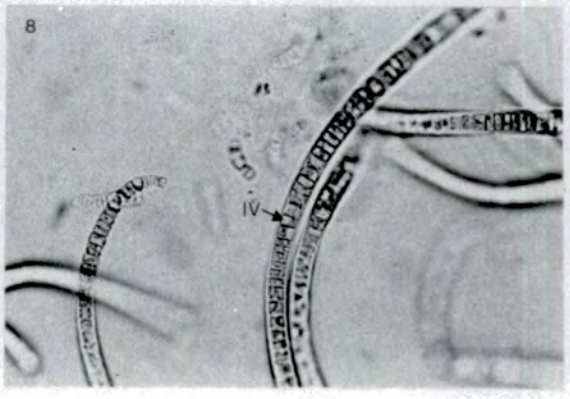
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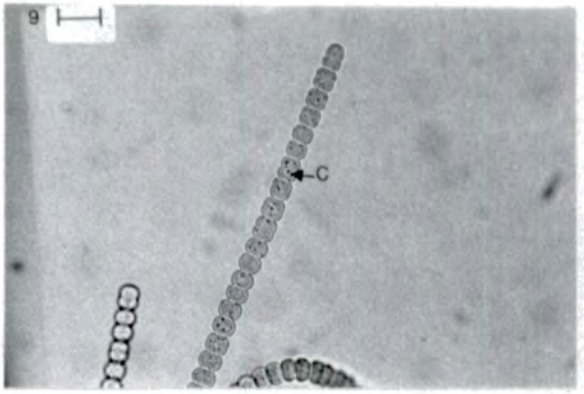
IV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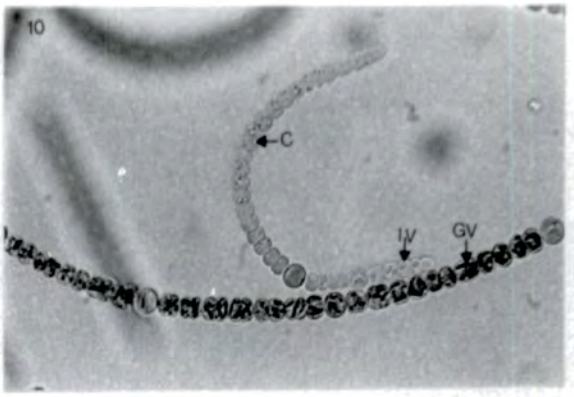
C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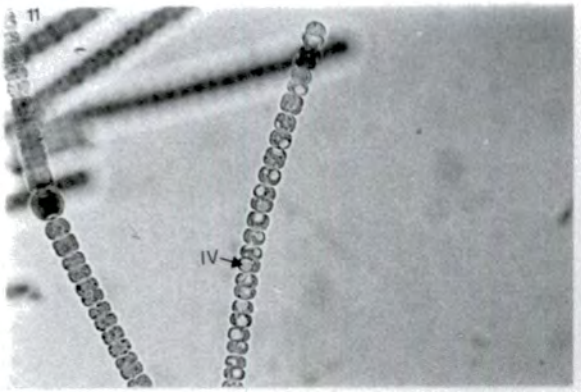
C
IV
GV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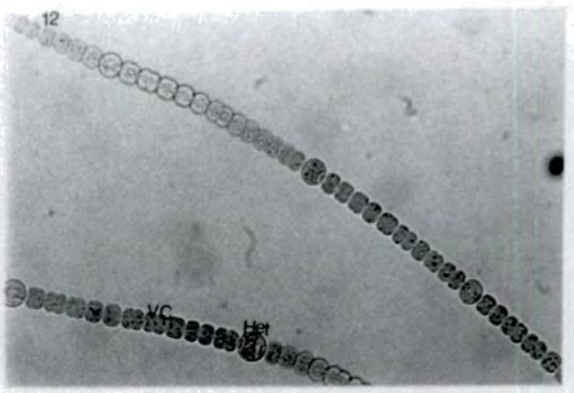
IV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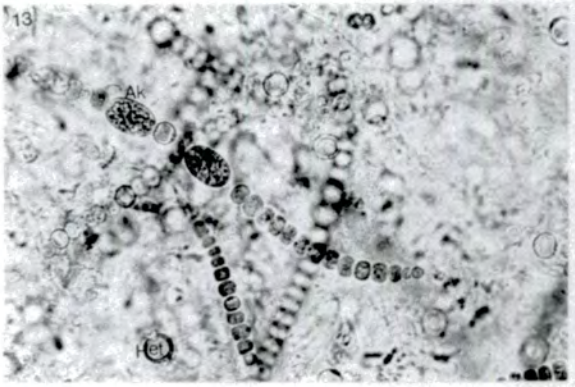
VC
Het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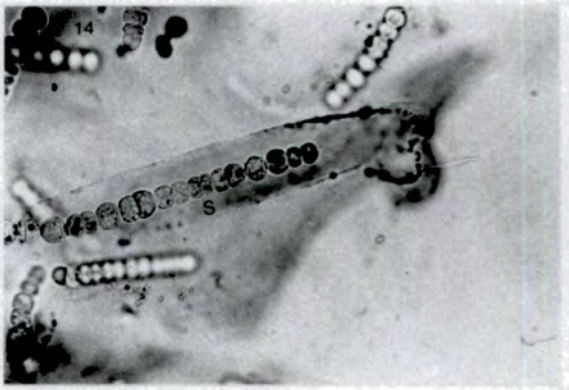
Ak
Het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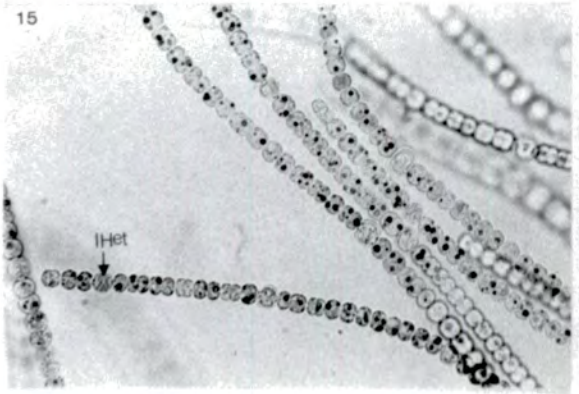
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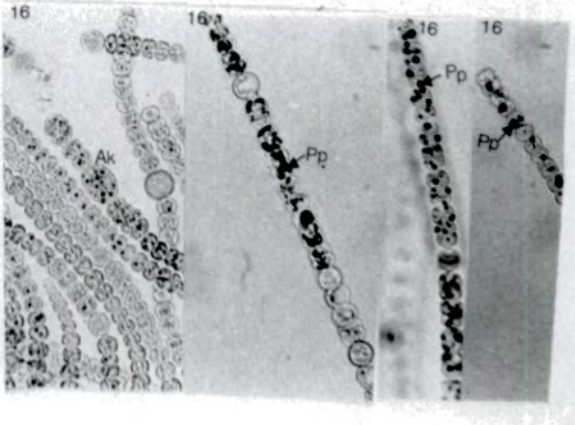
IHet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Ak
16
Pp
16
Pp
16

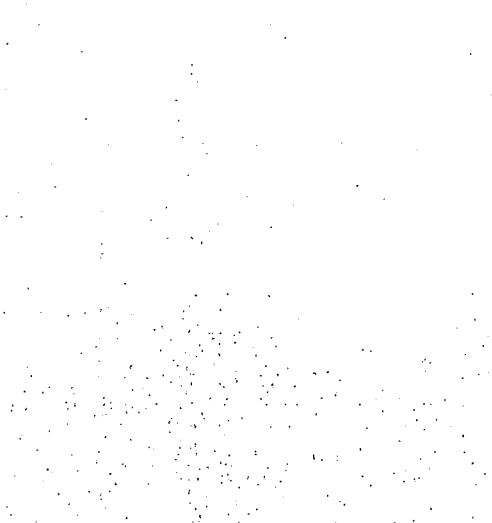
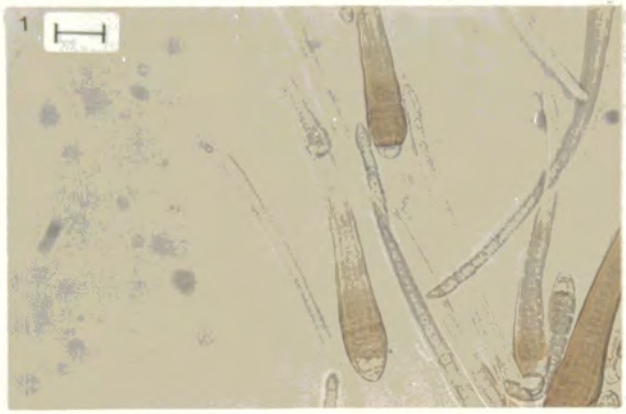


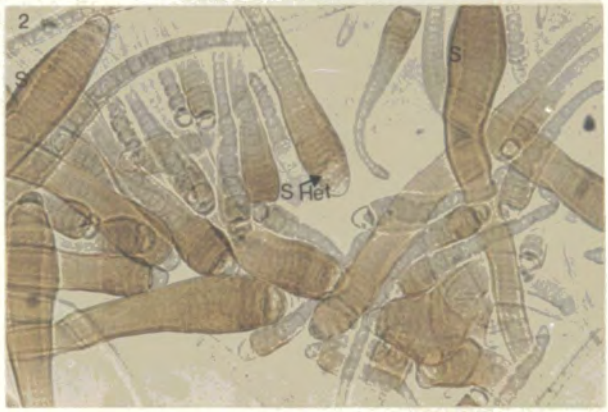
Fig. 4.5 Optical micrograph of Calothrix D253 grown in - Fe medium Hormogonium (Ho). necridium (N). brown sheath (S) intra-thylakoidal vacuolation (IV). Collapsed heterocyst (Het). secondary heterocyst (S Het). Scale bar = 10 µm

1-2) Calothrix D253 in saline medium

3-8) Calothrix D253 in freshwater medium



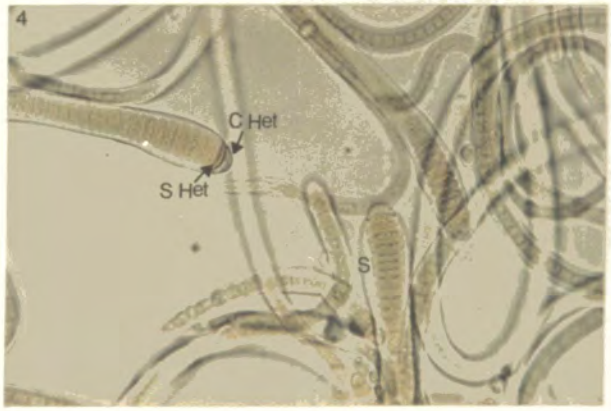
1


S Het

2


Ho
IV

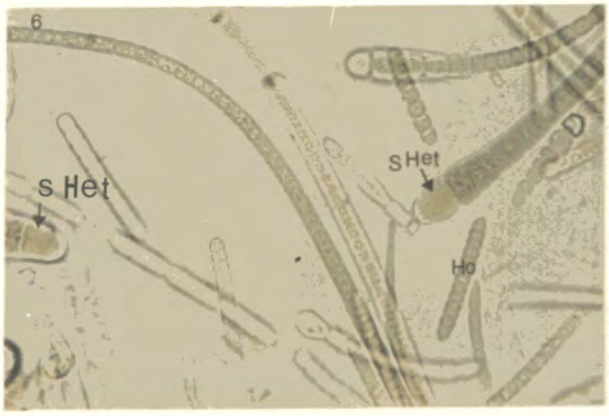
3


C Het
S Het

4


S Het
S Het
C Het

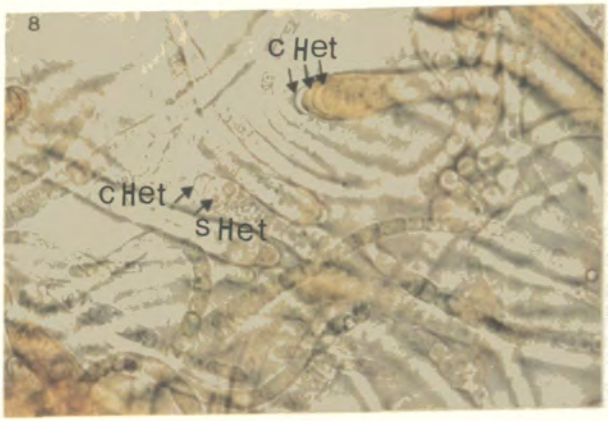
5


S Het
S Het
Ho

6


S Het
C Het
S Het
C Het
N

7


C Het
C Het
S Het

8

4.32 Recovery from Fe-limitation

Addition of Fe (0.4 mg L^{-1}) to Fe-limited cultures (from 4.31) resulted in marked changes in morphology and cytology (Tables 4.16-4.19, A1.5-1.6). Where hairs were formed as a result of Fe-limitation, they were shed within the first 24 h, apparently by lysis of the lowest hair cells. All strains resembled each other in that vegetative cells returned to their normal colour, although a few trichomes in the centre of masses of filaments were slower than the rest. The intra-thylakoidal vacuoles were lost from vegetative cells. After 24 h most basal (collapsed) heterocysts were lost. All strains resembled each other in that cyanophycin granules reappeared in the apical area (i.e. immediately below where hair fell off), whilst polyphosphate granules remained unchanged. The next stage of hormogonium development was distinguished in Calothrix D550 by formation of gas-vacuoles after 24 h.

All strains resembled each other in that their normal colour had returned between 48 and 72 h. Hormogonia production was obvious in all strains. By this time basal (collapsed) heterocysts had been lost, leaving the other heterocyst which was present before addition of Fe (i.e. no new heterocyst).

There was no obvious response of the Fe-limited cultures to addition of either P or trace elements. This was clear evidence that cultures were Fe-limited.

Table 4.16 Dimensions (µm) of morphological characters of Calothrix D253 after addition (day 12) of Fe to Fe-limited culture in saline medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0 (day 12)	219.5	12.5	5.5	-	-	3.5
1	212.5	12.5	5.5	-	-	3.5
2	219.5	10.5	5.5	-	-	<1
3	250.5	10.5	5.5	-	-	<1

Table 4.17 Dimensions (µm) of morphological characters of Calothrix D550 after addition (day 11) of Fe to Fe-limited culture in freshwater medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0 (day 11)	175.5	7.5	3.5	35.5	2.5	<1
1	170.5	7.5	5.5	-	-	<1
2	175.5	7.5	5.5	-	-	<1
3	205.5	7.5	5.5	-	-	<1

Table 4.18 Granulation (% of cell profile) and frequency (%) of morphological characters of Calothrix D253 in saline after addition (day 12) of Fe to Fe-limited cultures

day	polyphosphate		cyanophycin		hormogonia	hairiness
	basal	apical	basal	apical	production	
0 (day 12)	40	30	-	-	-	-
1	40	30	-	5	-	-
2	40	30	5	10	-	-
3	40	30	5	10	5	-
4	40	30	5	20	20	-

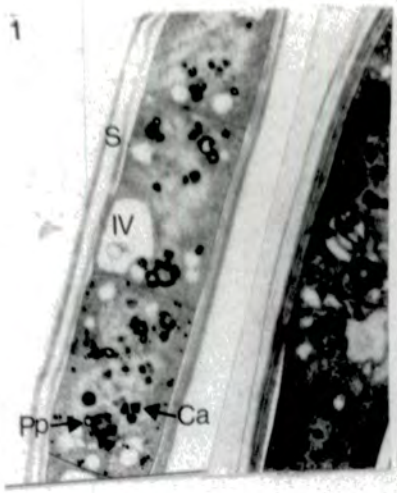
Table 4.19 Granulation (% of cell profile) and frequency (%) of morphological characters of Calothrix D550 in saline after addition (day 11) of Fe to Fe-limited cultures

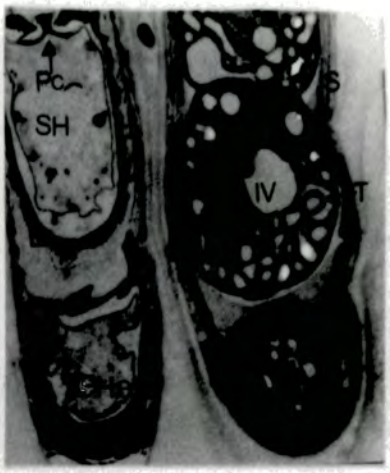
day	polyphosphate		cyanophycin		hormogonia	hairiness
	basal	apical	basal	apical	production	
0 (day 11)	50	10	-	-	-	20
1	50	10	-	10	-	<1
2	40	10	-	10	<10	-
3	40	10	<5	10	80	-

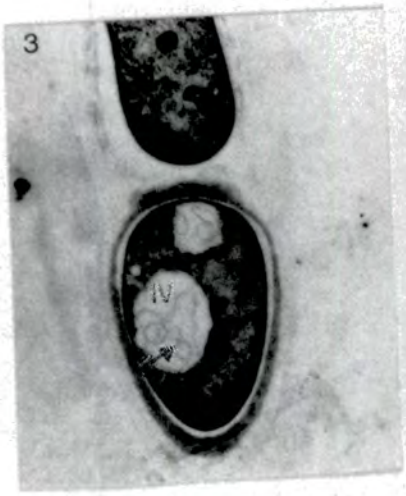
Fig. 4.6 Electron micrograph of Calothrix D550 grown in - Fe freshwater medium

1) apical region of trichome shows intra-thylakoidal vacuoles (IV), carboxysomes (Ca), polyphosphate granules (Pp), sheath (S), x 7000

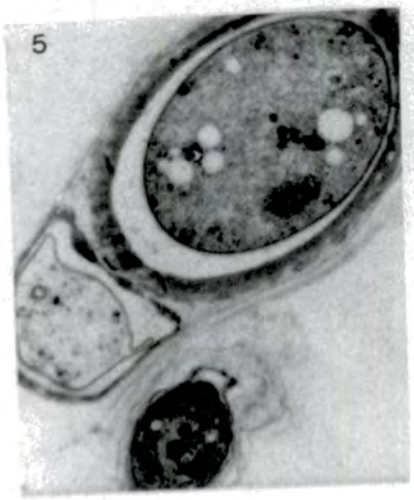
2-7) basal region of trichome shows collapsed heterocyst (C Het), secondary heterocyst (S Het), pore channel (PC), intra-thylakoid vacuoles (IV), polyphosphate granules (Pp) envelope (E), sheath (S), lipid bodies (Lb)
no. 2-7 were taken at x 7000; 7000; 9000; 9000; 9000 and 4300 respectively


1
S
IV
Pp
Ca


Pc
SH
S
IV
T


3
IV


4
C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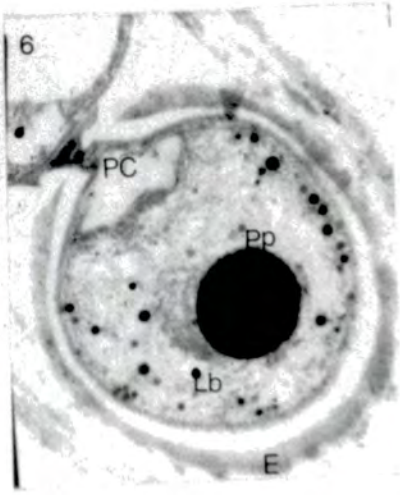

6
PC
Pp
Lb
E

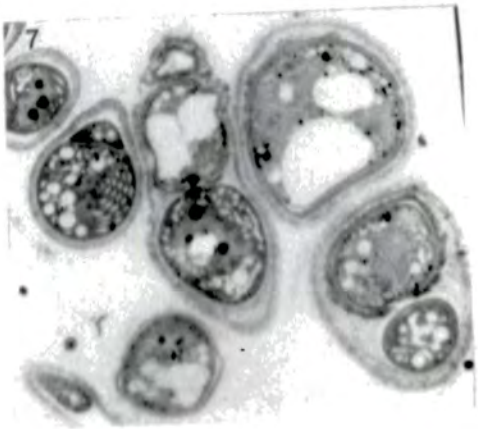
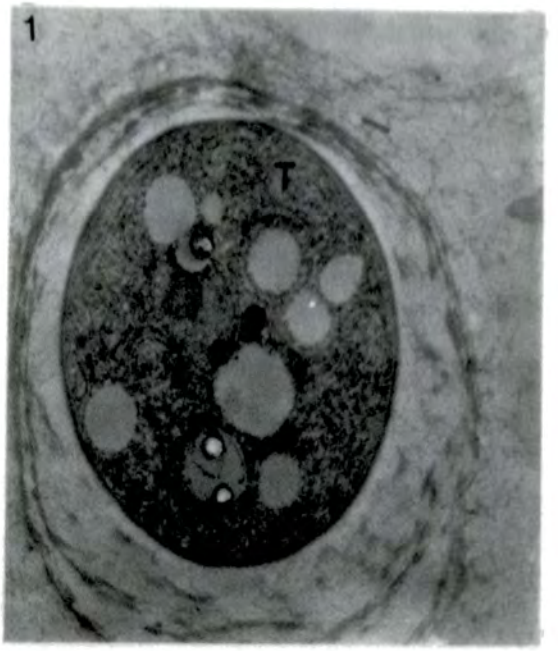
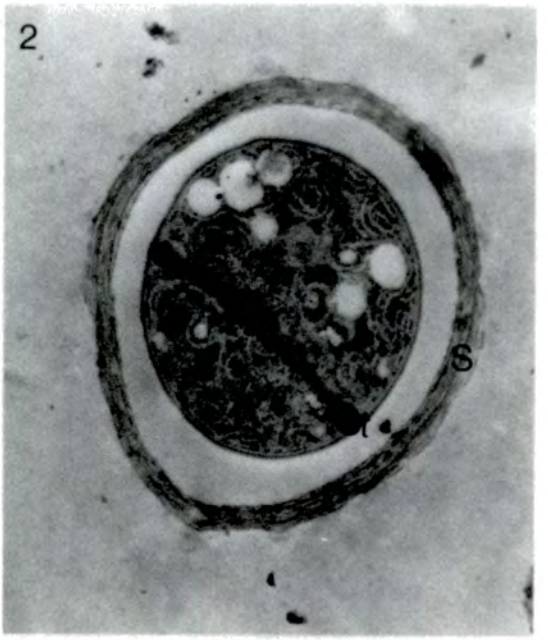

7

Fig. 4.7 Electron micrograph of Calothrix D550 24 h after addition of Fe to Fe-limited culture

1-3) Vegetative cells with thylakoid (T), polyphosphate granules (Pp), sheath (S), septa (st). 1-3 were all taken at x 7000


1
T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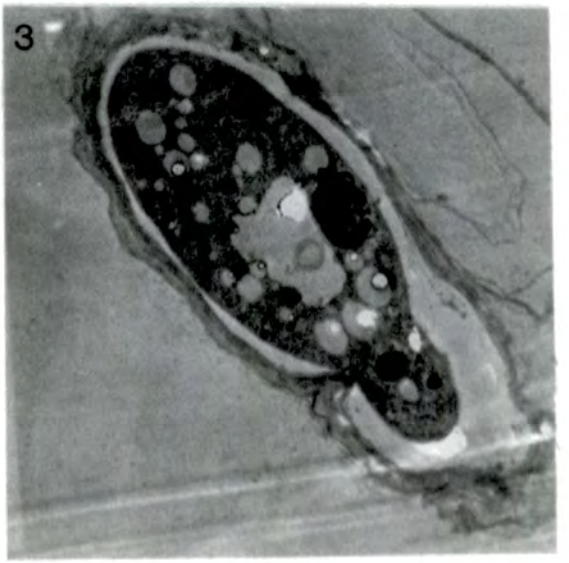

3

4.4 Morphological and cytological changes under P-limitation

4.41 Optical microscopy

Observation on P-limited cultures was made to confirm that the previous findings (4.3) were not an artifact of interaction between Fe- and P-limitation. The results for the various stages ere given in Tables 4.20-4.27, A1.7-1.8, Fig. 4.8-4.9.

Stages I and II

The first and second stages are combined togother since stage III was entered quickly and therefore there was no clear time for stage II.

Marked differences were obvious in the appearance of cultures from that in standard and - Fe medium. For instance none of the strains developed a secondary heterocyst and/or sheath similar to that in standard and - Fe medium.

Another marked difference also concerned the granular content of the vegetative cells during the initial days of growth. For instance polyphosphate granules were lost quickly from all strains, whilst cyanophycin increased slightly, especially in the basal area of trichomes.

Strains resembled each other by retaining their normal colour in contrast to - Fe medium. Production of hormogonia decreased more rapidly than with - Fe medium, and apical vegetative cells developed small vacuoles; this was regarded as the sign of hair formation and therefore the start of stage III.

Stage III

Marked differences were obvious in the appearance of cultures from that in standard and - Fe medium. For instance hair length was more pronounced and hairiness was more frequent. Although percentage of trichome as hair varied among the strains, the width of the hair was similar (2.5 μm) in all

strains, with the exception of Dichothrix D696 (4.0 µm). Another marked difference with Dichothrix D696 was that the hair cells were shorter. The transition region between the vegetative cells and mature hairs varied with the strain, as Calothrix D550 had a distinctive zone, while other strains had either a short zone or none. All strains resembled each other in that the polyphosphate granules were absent, while cyanophycin granules increased markedly in both basal and apical areas of the trichomes. There were no secondary or intercalary heterocysts in any strain.

Table 4.20 Dimensions (µm) of morphological characters of Calothrix D253 in low P saline medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	60.5	10.5	5.5	-	-	<1
2	70.5	10.5	5.5	-	-	<1
4	75.5	10.5	5.5	-	-	<1
6	112.5	10.5	5.5	35.5	2.5	<1
8	130.5	10.5	5.5	35.5	2.5	<1
10	175.5	10.5	5.5	35.5	2.5	<1
12	215.5	10.5	5.5	75.5	2.5	<1
14	250.5	10.5	5.5	75.5	2.5	<1
16	250.5	10.5	5.5	75.5	2.5	<1

Table 4.21 Dimensions (µm) of morphological characters of Calothrix D253 in low P freshwater medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	65.5	10.5	5.5	-	-	<1
2	70.5	10.5	5.5	45.5	2.5	<1
4	95.5	10.5	5.5	125.5	2.5	<1
6	180.5	10.5	5.5	252.5	2.5	<1
8	180.5	10.5	5.5	305.5	2.5	<1
10	175.5	10.5	5.5	350.5	2.5	<1
14	175.5	10.5	5.5	475.5	2.5	<1

Table 4.22 Dimensions (µm) of morphological characters of Calothrix D550 in low P freshwater medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	62.5	7.5	5.5	-	-	<1
2	125.5	7.5	5.5	25.5	2.5	<1
4	110.5	8.5	5.5	225.5	2.5	<1
6	100.5	10.5	5.5	505.5	2.5	<1
8	100.5	10.5	5.5	910.5	2.5	<1
10	80.5	10.5	5.5	950.5	2.5	<1
14	75.5	10.5	5.5	1052.5	2.5	<1

Table 4.23 Dimensions (µm) of morphological characters of *Dichothrix* D696 in low P saline medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	75.5	10.5	6.5	-	-	<1
2	80.5	10.5	6.5	-	-	<1
4	100.5	10.5	6.5	-	-	<1
6	120.5	11.5	6.5	50.5	4.5	<1
8	119.5	11.5	5.5	120.5	4.5	<1
10	149.5	12.5	5.5	190.5	4.5	<1
22	375.5	12.5	5.5	790.5	4.5	<1

Table 4.24 Granulation (% of cell profile) and frequency (%) of morphological changes in Calothrix D253 in low P saline medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	20	10	20	10	80	-	-	-
2	10	-	30	10	50	-	-	-
4	10	-	30	10	20	-	-	-
6	-	-	50	10	10	-	+	24
8	-	-	60	30	10	-	<0.5	24
10	-	-	60	30	-	-	<0.5	24
12	-	-	90	30	-	-	<0.5	24
14	-	-	90	30	-	-	<0.5	24

Table 4.25 Granulation (% of cell profile) and frequency (%) of morphological changes in Calothrix D253 in low P freshwater medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	20	10	10	10	80	-	-	-
2	10	-	20	10	40	-	+	39
4	10	-	40	20	20	-	20	57
6	-	-	80	30	<5	-	50	58
8	-	-	90	60	<5	-	90	62
10	-	-	90	60	-	-	>90	63
12	-	-	90	60	-	-	>90	67
14	-	-	90	60	-	-	>90	73

Table 4.26 Granulation (% of cell profile) and frequency (%) of morphological changes in Calothrix D550 in low P freshwater medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	10	0	30	-	80	-	-	-
2	10	10	30	-	50	-	<5	17
4	10	10	20	10	30	-	80	69
6	-	-	80	30	-	-	80	83
8	-	-	80	30	-	-	90	90
10	-	-	80	30	-	-	90	90
12	-	-	80	30	-	-	90	90
14	-	-	80	30	-	-	90	90
16	-	-	80	30	-	-	90	90

Table 4.27 Granulation (% of cell profile) and frequency (%) of morphological changes in Dichothrix D696 in low P saline medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	30	10	10	-	80	-	-	-
2	20	-	30	-	40	-	-	-
4	10	-	30	10	40	-	<5	-
6	10	-	50	20	20	-	10	29
8	-	-	60	40	10	-	20	50
10	-	-	60	40	-	-	40	56
12	-	-	80	40	-	-	-	68
14	-	-	80	40	-	-	-	67
16	-	-	80	40	-	-	-	68

Fig. 4.8 Morphological changes during growth in low P medium in batch culture: Calothrix D253 and Dichothrix D696 in saline medium. Calothrix D253 and Calothrix D550 in freshwater medium

a) Hormogonium

b) Young trichome with heterocyst

c) Hormogonial release from mature trichome with necridium

d) Mature trichome after ceasing of hormogonial release

e) Hormogonial release after addition of P to P-limited culture

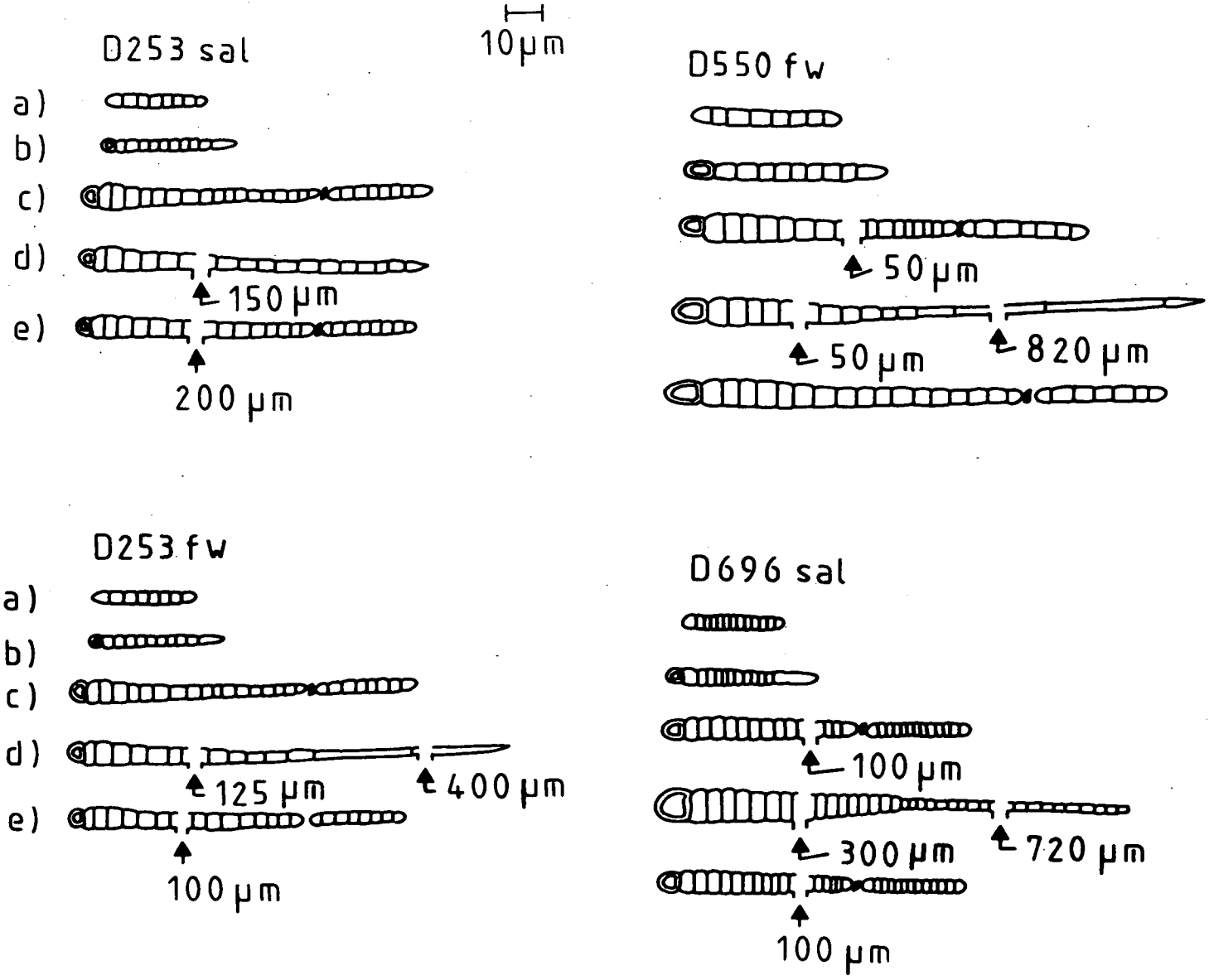

10 µm
D253 sal
a)
b)
c)
d)
150 µm
e)
200 µm
D550 fw
50 µm
50 µm
820 µm
D253 fw
a)
b)
c)
d)
125 µm
400 µm
e)
100 µm
D696 sal
100 µm
300 µm
720 µm
100 µm

Fig. 4.9 Optical micrograph of strains grown in low P medium

Heterocyst (Het), hair (Hr), akinete (Ak) and gas vacuoles (GV) in Anabaena D697. Scale bar = 10 µm

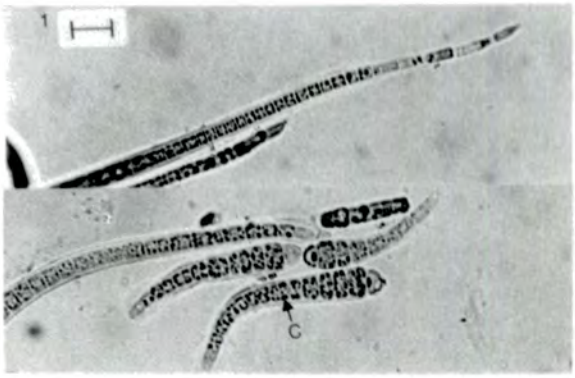
1) Calothrix D253 in saline medium

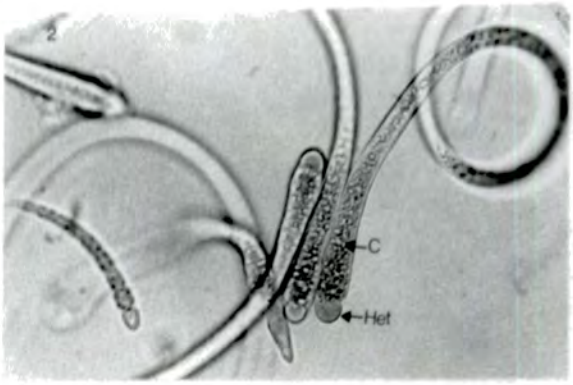
2-4) Calothrix D253 in freshwater

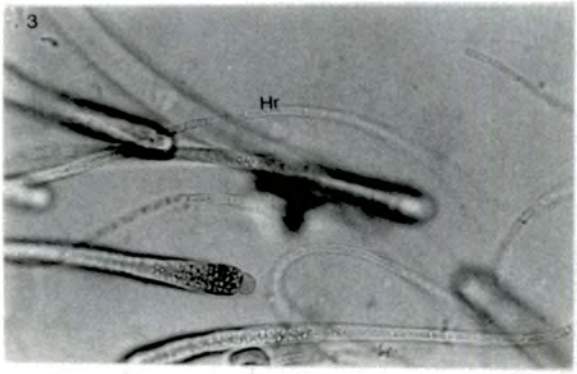
5-6) Dichothrix D696 in saline medium

7) Calothrix D550 in freshwater

8) Anabaena D697 in saline medium


1
C


2
C
Het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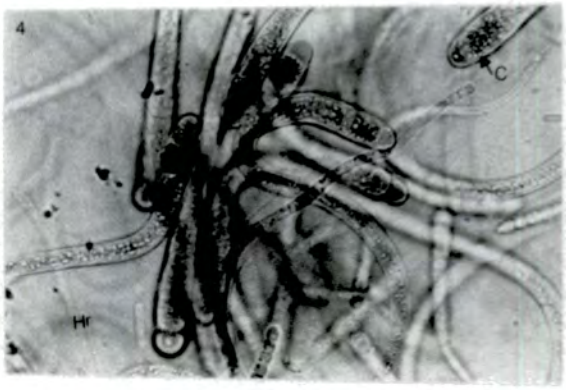
Hr


4
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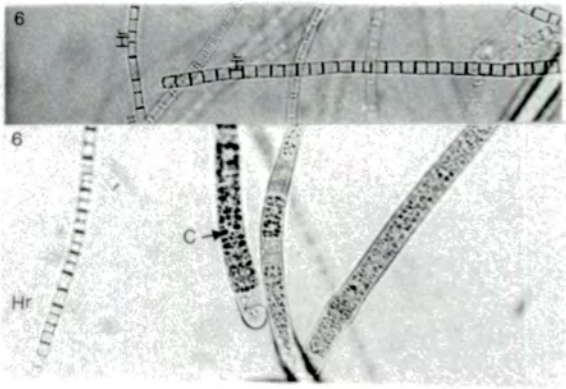
5
Ho
Hr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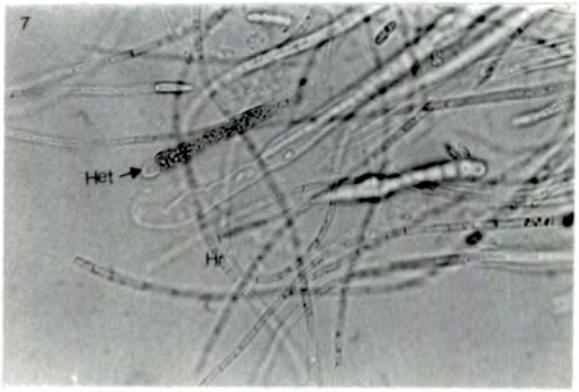
6
C
Hr


7
Het
Hr


8
Het
Ak
Ak
8
Het
Ak
GV

4.42 Recovery from P-limitation

Addition of P (1 mg L^{-1}) to P-limited cultures resulted in marked changes in morphology and cytology (Table 4.28-4.35, A1.9-1.10). Hairs formed as a result of P-limitation were shed within the first 24 h. All strains behaved similarly in that polyphosphate granules increased markedly, particularly in the basal area, while cyanophycin granules decreased slightly.

Strains resembled each other in that hormogonia were released between 48 - 72 h. with Calothrix D550 showing the quickest response. Consequently, as growth resumed, cyanophycin granules continued to decrease until lost from all strains. There was no obvious morphological response in P-limited cultures of any strain to either the addition of Fe or trace elements.

Table 4.28 Dimensions (µm) of morphological characters of Calothrix D253 after addition (day 8) of P to P-limited culture in saline medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0 (day 8)	75.5	10.5	5.5	-	-	<1
1	74.5	10.5	5.5	-	-	<1
2	75.5	10.5	5.5	-	-	<1
3	80.5	10.5	5.5	-	-	<1
4	85.5	10.5	5.5	-	-	<1

Table 4.29 Dimensions (µm) of morphological characters of Calothrix D253 after addition (day 8) of P to P-limited culture in freshwater medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0 (day 8)	75.5	10.5	5.5	455.5	2.5	<1
1	76.5	10.5	5.5	-	-	<1
2	79.5	10.5	5.5	-	-	<1
3	82.5	10.5	5.5	-	-	<1
4	95.5	10.5	5.5	-	-	<1

Table 4.30 Dimensions (µm) of morphological characters of Calothrix D550 after addition (day 8) of P to P-limited culture in freshwater medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0 (day 8)	98.5	10.5	5.5	910.5	2.5	<1
1	100.5	10.5	5.5	-	-	<1
2	110.5	10.5	5.5	-	-	<1
3	119.5	10.5	5.5	-	-	<1
4	120.5	10.5	5.5	-	-	<1

Table 4.31 Dimensions (µm) of morphological characters of Dichothrix D696 after addition (day 8) of P to P-limited culture in saline medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0 (day 8)	119.5	11.5	5.5	121.5	4.5	<1
1	119.5	11.5	5.5	-	-	<1
2	121.5	11.5	5.5	-	-	<1
3	129.5	11.5	5.5	-	-	<1
4	130.5	11.5	5.5	-	-	<1

Table 4.32 Granulation (% of cell profile) and frequency (%) of morphological characters of Calothrix D253 in saline after addition (day 8) of P to P-limited cultures

day	polyphosphate		cyanophycin		hormogonia	hairiness
	basal	apical	basal	apical	production	
0 (day 8)	-	-	60	30	-	<0.5
1	80	40	40	10	-	-
2	80	40	20	-	20	-
3	70	20	-	-	30	-
4	70	20	-	-	60	-

Table 4.33 Granulation (% of cell profile) and frequency (%) of morphological characters of Calothrix D253 in freshwater after addition (day 8) of P to P-limited cultures

day	polyphosphate		cyanophycin		hormogonia	hairiness
	basal	apical	basal	apical	production	
0 (day 8)	-	-	90	60	-	90
1	80	50	80	50	-	<5
2	80	50	60	30	10	-
3	80	40	20	10	20	-
4	80	40	-	-	40	-

Table 4.34 Granulation (% of cell profile) and frequency (%) of morphological characters of Calothrix D550 in freshwater after addition (day 8) of P to P-limited cultures

day	polyphosphate		cyanophycin		hormogonia	hairiness
	basal	apical	basal	apical	production	
0 (day 8)	-	-	80	30	-	90
1	80	50	80	10	-	<5
2	80	30	60	-	50	-
3	80	30	20	-	80	-
4	80	20	-	-	80	-

Table 4.35 Granulation (% of cell profile) and frequency (%) of morphological characters of Dichothrix D696 in saline after addition (day 8) of P to P-limited cultures

day	polyphosphate		cyanophycin		hormogonia	hairiness
	basal	apical	basal	apical	production	
0 (day 8)	-	-	60	40	-	60
1	70	20	50	20	-	-
2	70	20	30	10	20	-
3	70	20	20	-	30	-
4	70	20	-	-	40	-

Morphological and cytological changes in Anabaena D697 (Fig. A2.1-2.10)

Anabaena D697 was included for comparison with the strains of Rivulariaceae, during growth in standard, - Fe and low P media. In all media the first indication of akinete formation was considered as the start of stage III for Anabaena D697. Although there were many similarities between the changes in Anabaena D697 with those in Rivulariaceae in all media, marked differences were also present. For instance the changes in polyphosphate granules, intrathylakoidal vacuolation and colour were similar to those of all Rivulariaceae. Cyanophycin granules increased in - Fe medium (i.e. opposite to Rivulariaceae). Comparison between the responses of Anabaena D697 in three media revealed that akinetes formed on one or either side of heterocyst. However the overall frequency of akinetes was higher under P- than Fe-limitation. Heterocyst frequency (with no account for the fallen heterocysts) showed a marked increase (12.5 %) in - Fe medium, but not with low P medium (i.e. akinetes and heterocysts show an opposite response to nutrient limitation). The addition of Fe and P to their respective element-limited cultures led to recovery of growth, but with a marked difference in that a few heterocysts (1 %) became blue in response to Fe addition.

Blue heterocyst formation in response to addition of Fe

In Anabaena D697 examples of heterocysts turning blue (Fig. A2.3-2.4) started to appear 24 h after Fe addition and reached 1 % within the first 72 h, after which they were diluted as growth resumed. No change in the colour occurred when a similar experiment was carried out on Anabaena D697, Calothrix D253, Calothrix D550, Calothrix D603 (known to form blue heterocyst under other circumstances), Dichothrix D696 and Calothrix D704.

Ultrastructure of Anabaena D697 (Fig. A2.2-2.3)

Ultrastructure of heterocyst of D697 was examined before and after Fe addition. The addition of Fe le d to a rapid change in the thylakoid membranes, particularly in the heterocyst, where they became re-arranged in a uniform compact manner, and the intra-thylakoidal vacuolation was lost. Neither carboxysomes nor phycobilisomes were found in any heterocyst.

4.5 Influence of NaCl on hair formation by Calothrix D253

Calothrix D253 does not form hairs under Fe-limitation in saline medium, but forms long hairs in freshwater medium (4.3). Furthermore, under P-limitation the same strain forms only a few (0.5 %) short hairs in saline medium, whilst in freshwater medium hairiness reaches 90 % and most of these are long hairs (4.4). A detailed study was made therefore on hair formation in saline and freshwater media under Fe- and P-limitation to determine the degree of hairiness at a range of NaCl concentrations. Investigations were mainly restricted to P-limitation (Tables 4.36-4.39).

Calothrix D253 was subcultured in - Fe and low P media, including AD medium as a comparison with previous studies (Sinclair & Whitton, 1977). Under Fe-limitation there was no hair formation at 2.85 g L^{-1} Na, whilst 10 % hairiness was found at 0.045 g L^{-1} Na. In contrast with P-limitation, less than 0.5 % hairiness was found at 2.85 g L^{-1} Na, whilst 90 % hairiness was observed at 0.045 g L^{-1} Na. There was an inverse relationship between NaCl concentration and hairiness and hair length in saline and freshwater medium. Addition of Fe and P to the respective element-limited cultures led to recovery from limitation, with release of hormogonia (see 4.32, 4.42). Hair formed in response to the respective element-limitation, but only at low NaCl concentrations.

Influence of seawater on hair formation by Calothrix D253

This experiment was carried out under P-limitation. The alga was subcultured into 30 % seawater (enriched with Fe and trace elements). Seawater had a similar effect to NaCl with formation of less than 0.5 % hairiness and short hairs.

Influence of upshift and downshift of NaCl on hair formation by P-limited Calothrix D253

The influence of up- and downshift of NaCl concentration on hair formation was tested in filtrates of old media on two growth stages (days 4. 14). Algal material which had recently formed hairs was chosen from the freshwater medium and transferred to saline medium. Non-hairy material from saline medium was transferred to freshwater medium.

In the case of the downshift, 90 % hairiness was found after 24 h, whereas with the upshift the only obvious change was that a few hairs were shed and floated in the medium. After four days, there was no change with downshift, whereas in the upshift further changes had occurred, including loss of hairs. This provides further evidence of the influence of NaCl on hair formation under P-limitation.

In the 14-day experiment 60 % hairiness was found after downshift for 24 h, whereas after the upshift the only obvious change was that a few hairs were shed and floated in the medium. After four days, there was no change in the downshift, whereas after the upshift further changes had occurred, including shedding of all the hairs. No obvious change occurred when non-hairy material was transferred to fresh saline medium with no added P.

Table 4.36 Hair formation by Calothrix D253 under Fe- and P-limitation in saline and freshwater media.

medium	Na	Fe-limitation		P-limitation	
	($g\ L^{-1}$)	hair length	hairiness	hair length	hairiness
30 % ASP_6M(-N)	2.850	-	-	50 - 75	<0.5
30 % ASP_6M(-N)-NaCl	0.045	56 - 100	10	150 - 300	90
Chu-10D(-N)+NaCl	2.858	-	-	25 - 50	<0.5
Chu-10D(-N)	0.008	30 - 75	10	175 - 300	90
AD	0.111	30 - 75	5	150 - 300	90

Table 4.37 Influence of Na : K ratio (by altering Na concentration) on hair formation by Calothrix D253 in low-P saline medium.

	element concentration ($g\ L^{-1}$)					
Na ($\%\ ASP_6$)	Na	K	Cl	Na : K	hairiness %	hair length µm
0.5	0.05	0.113	0.35	0.68	90	150 - 300
6.0	0.57	0.113	1.23	8.56	90	150 - 250
12.0	1.14	0.113	2.10	17.13	90	150 - 200
18.0	1.71	0.113	2.98	25.68	70	50 - 200
24.0	2.28	0.113	3.86	34.25	15	50 - 75
30.0	2.85	0.113	4.73	42.82	<0.5	50 - 75

Table 4.38 Influence of Na : K ratio (by altering the concentration of Na) on hair formation by Calothrix D253 in low-P freshwater medium.

	element concentration ($g\ L^{-1}$)					
Na ($\%\ ASP_6$)	Na	K	Cl	Na : K	hairiness %	hair length µm
0.08	0.008	0.00224	0.02	6.4	90	75 - 300
6.08	0.578	0.00224	0.88	438.3	80	75 - 175
12.08	1.148	0.00224	1.75	870.7	20	75 - 175
18.08	1.718	0.00224	2.63	1303.0	5	50 - 130
24.08	2.288	0.00224	3.50	1735.3	<0.5	25 - 50
30.08	2.858	0.00224	4.73	2167.8	<0.5	25 - 50

Table 4.39 Influence of Na : K ratio (by altering the concentration of K) on hair formation by *Calothrix* D253 in low-P saline medium.

element concentration ($g\ L^{-1}$)						
K ($\%\ ASP_6$)	Na	K	Cl	Na : K	hairiness %	hair length µm
0.6	2.85	0.002	4.63	2105.2	<0.5	25 - 50
6.0	2.85	0.023	4.65	214.2	<0.5	30 - 75
12.0	2.85	0.045	4.67	107.1	<0.5	20 - 50
18.0	2.85	0.068	4.69	71.4	<0.5	20 - 75
24.0	2.85	0.090	4.71	53.6	<0.5	30 - 100
30.0	2.85	0.113	4.73	42.8	<0.5	30 - 50
60.0	2.85	0.226	4.83	21.4	<0.5	30 - 50
90.0	2.85	0.339	4.93	14.2	alga became unhealthy	
120.0	2.85	0.452	5.03	10.7	alga became unhealthy	
150.0	2.85	0.565	5.13	8.6	alga became unhealthy	
300.0	2.85	1.130	5.63	4.3	alga became unhealthy	
600.0	2.85	2.260	11.26	2.1	alga became unhealthy	

Is the influence of NaCl due to Na^+ or Cl^- ?

In order to test this possibility, normal Cl concentration was maintained by replacing Na with K in saline medium. The residual Na associated with other chemicals added as stock solution was 0.045 g L^{-1}. Only a few hairs started to form, but the alga became unhealthy and died, indicating the toxicity of K.

Does an increase in K^+ modify the effect of NaCl ?

It became clear that NaCl has a negative influence on hair formation by Calothrix D253, but it is not clear yet whether it is due to an osmotic effect, toxicity of Na, or other factors (example; interaction of Na with Fe- and P-limitation). Three experiments were carried out:

(a) The influence of an increase (by altering Na concentration) and decrease (by altering K concentration) in Na : K molar ratio on hair formation was tested in low P saline and freshwater media.

The results revealed a strong negative correlation between increase in Na concentration (Na : K ratio) and hairiness and hair length in saline (r = - 0.91 and - 0.97) and freshwater media (r = - 0.91 and - 0.97). In contrast there was no correlation between hairiness and hair length and increase in K concentration (decrease in Na : K), but the alga became unhealthy and died at more than 0.339 g L^{-1} K, indicating that possibly Na is the key factor involved in suppressing hair formation. There was neither an obvious increase in cell size nor plasmolysis.

(b) An experiment was carried out in low P saline medium with equimolar Na (2.85 g L^{-1}) and K concentrations. There was no hair formation, but the alga started to became unhealthy and died, indicating that K was toxic and did not antagonize Na. This suggests that the negative influence of Na on hair formation is not due to toxicity.

(c) Another experiment was carried out on alkaline phosphatase activity by non hairy (grown in saline medium) and hairy (grown in freshwater medium) P-limited cultures. No detectable activity by the non hairy, but activity induced when assayed in freshwater medium. In contrast with hairy, activity was high, but reduced (30 %) when assayed in saline medium.

Influence of upshift and downshift of NaCl on hair formation by Fe-limited Calothrix D253

Downshift of old culture of - Fe version of saline medium to similar version of freshwater medium led to synchronized development of hairs in few (5 - 10 %) of trichomes. A reciprocal upshift from freshwater to saline meidum led to loss of hairs.

4.6 Summary

Comparison of growth under Fe- and P-limitation has shown that in general all Rivulariaceae responded similarly to a particular limitation (Tables 4.40-4.43).

Table 4.40 Comparison of morphological and cytological response to addition of Fe or P to respective limited cultures of Calothrix D253 in saline medium. Character present (+), absent (-).

character	Fe-limitation		P-limitation	
	Fe addition		P addition	
	before	after	before	after
hairiness	-	-	<0.5	-
length of hair	-	-	75.5	-
trichome as hair	-	-	24	-
secondary heterocyst	+	-	-	-
polyphosphate granules	+	+	-	+
cyanophycin granules	-	+	+	-
thick sheath	+	-	-	-
hormogonium production	-	+	-	+
colour	pale	normal	normal	normal

Table 4.41 Comparison of the morphological and cytological responses before and after additions of Fe or P to respective limited culture of Calothrix D253 in freshwater medium. Character present (+), absent (-).

character	Fe-limitation		P-limitation	
	before	after	before	after
hairiness	10	-	90	-
length of hair	75.5	-	475.5	-
trichome as hair	34	-	73	-
secondary heterocyst	+	-	-	-
polyphosphate granules	+	+	-	+
cyanophycin granules	-	+	+	-
thick sheath	+	-	-	-
hormogonia production	-	+	-	+
colour	pale	normal	normal	normal

Table 4.42 Comparison of the morphological and cytological changes in responses before and after additions of Fe and P to respective limited culture of Calothrix D550 in freshwater medium. Character present (+), absent (-).

character	Fe-limitation		P-limitation	
	Fe addition		P addition	
	before	after	before	after
hairiness	20	-	90	-
length of hair	35.5	-	910.5	-
trichome as hair	17	-	90	-
secondary heterocyst	+	-	-	-
polyphosphate granules	+	+	-	+
cyanophycin granules	-	+	+	-
thick sheath	-	-	-	-
hormogonia production	-	+	-	+
colour	colourless	normal	normal	normal

Table 4.43 Comparison of the morphological changes in responses before and after additions of Fe and P to respective limited culture of Dichothrix D696 in saline medium. Character present (+), absent (-).

character	Fe-limitation		P-limitation	
	Fe addition		P addition	
	before	after	before	after
hairiness	20	-	80	-
length of hair	60.5	-	790.5	-
trichome as hair	17	-	69	-
secondary heterocyst	+	-	-	-
polyphosphate granules	+	+	-	+
cyanophycin granules	-	+	+	-
thick sheath	+	-	-	-
hormogonia production	-	+	-	+
colour	yellowish	normal	normal	normal

5 CHANGES IN Fe CONCENTRATION DURING GROWTH IN BATCH CULTURE

5.1 Changes during growth in saline medium

(i) Use of normal Fe : EDTA ratio

Once morphological and cytological changes in response to Fe-limitation had been identified (4.2), the next step was to investigate the range of Fe contents. This would enable physiological, morphological and cytological resposes to be related to particular stages of Fe-limitation.

As it was not clear whether growth in standard medium was limited by Fe or P (4.1), two concentrations of P, high (10 mg L^{-1}) and low (0.05 mg L^{-1}). were tested (in saline medium). The normal concentration of Fe (0.4 mg L^{-1}) was used. It seemed probable that with the high P concentration growth would ultimately to become Fe-limited; this was subsequently confirmed (see below). The inoculum was prepared as in 2.561 and experiment was conducted under standard conditions (2.44). Calothrix D253. Dichothrix D696 and Anabaena D697 were tested. The experiments were concluded before yields had reached a plateau (Fig. 5.1).

The Fe associated with the alga had reached a maximum value by 24 h in high and low P medium (Fig. 5.1). The values decreased subsequently. approaching a constant level after about three weeks of growth:

strain Fe associated with alga (% dry weight) at various growth stages

	high P			low P			% Fe removed
	inoculum	max.	min.	inoculum	max.	min.	
D253	0.14	1.62	0.13	0.34	1.94	0.17	90 - 95
D696	0.16	1.72	0.14	0.38	2.05	0.20	90 - 95
D697	0.09	1.65	0.06	0.26	1.99	0.10	90 - 95

Figure 5.1 Changes in Fe concentration during growth of three strains in high (left column) and low (right column) P saline medium at normal ratio of Fe : EDTA. Arrows (a-e) indicate time at which the key morphological changes were first observed:

a) apical vacuolation

b) hair

c) hormogonia production ceases

d) secondary heterocyst

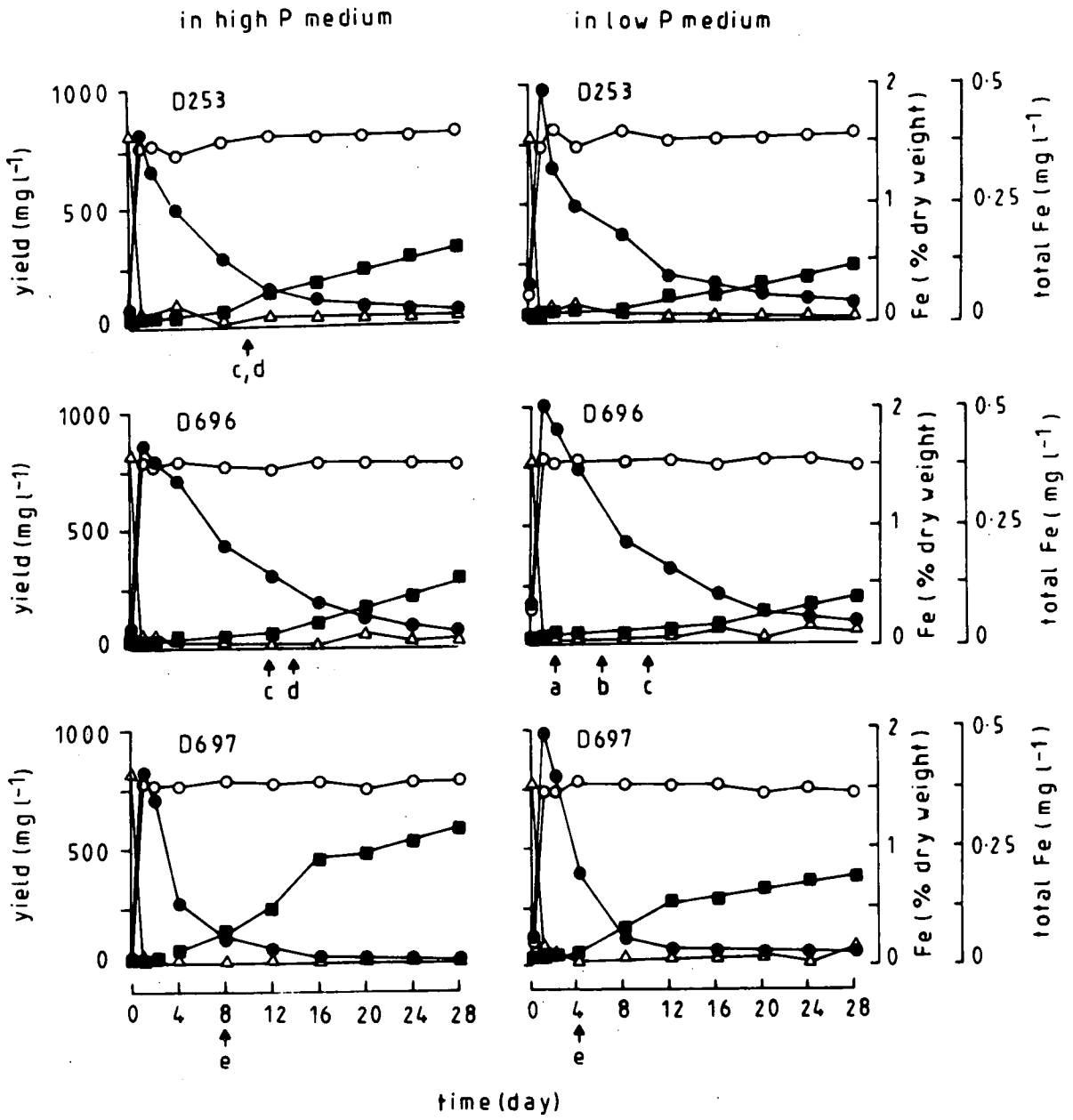
e) akinete

(■) yield ($mg\ L^{-1}$)

(●) algal Fe composition (% dry weight)

(○) total Fe in algae ($mg\ L^{-1}$)

(△) Fe concentration in medium ($mg\ L^{-1}$)


in high P medium
in low P medium
D253
D696
D697
yield (mg l⁻¹)
Fe (% dry weight)
total Fe (mg l⁻¹)
1000
500
0
2
1
0·5
0·25
c,d
c d
a b c
e
0 4 8 12 16 20 24 28
time (day)

Virtually all the Fe originally present in the medium was associated with the alga by 24 h (Fig. 5.1); total algal Fe thus showed no change throughout the remaining period of growth. In practice analysis of the medium showed values of about 0.02 mg L^{-1} Fe (5 % total Fe) throughout the remaining period of growth (Fig. 5.1).

Morphological changes (Fig. 5.1) were recorded when character first appeared, which took place before any degenerative events were apparent. In Calothrix D253 hairs did not appear during growth in high P medium but a few (< 0.5 %) short (35 - 75 µm) hairs formed at low P (see also Chapter 4). In Dichothrix D696 hairs started to form at day 6 and increased in number almost linearly at low P medium. In Anabaena D697 akinetes began to form from day 8 and 4 and increased in number linearly in high and low P medium respectively throughout the growth period. Some lysis was noticed in Anabaena D697 by the fourth week, but not in Calothrix D253 or Dichothrix D696 under either condition. Secondary heterocysts were formed in Calothrix D253 and Dichothrix D696 by the third week of growth in high P, but not in low P medium. A brown sheath was also formed in Calothrix D253, but not in other strains.

Two further treatments were carried out:

(a) Part of each culture was inoculated into fresh medium (complete, - Fe, - P, - trace elements);

(b) Addition of elements (Fe, P, trace elements) to the rest of culture.

Possible changes were monitored for 6 days. Rapid response in morphology and growth after being transferred to normal medium was taken as evidence that the culture had been limited by the element in question. Moreover, where recovery resulted from the addition of one element, this confirmed that the culture had ultimately been limited by that element. The results showed that the presumed Fe-limited cultures

recovered and resumed normal growth after being transfered to complete, - P and - trace elements media, but failed to recover or grow after being transferred to - Fe medium. Thus, according to the standard criteria for limitation (2.562), it was concluded that growth in the high P medium had led eventually to Fe-limitation.

Old, but relatively healthy cultures of all three strains grown in high P medium responded rapidly to the addition of Fe, whereas neither P nor trace elements caused any obvious response. Cultures from the low P medium resumed normal growth after being transferred to normal, - Fe and - trace elements media, but neither recovery did not occur after transfer to - P medium. In other words, growth in low P medium had led eventually to P-limitation. This was re-confirmed by results showing that cultures of three strains, grown in low P medium , responded rapidly and recovered in the addition of P, whilst neither the addition of Fe nor trace elements had any effect.

(ii) Use of low Fe : EDTA ratio

The changes in Fe content are similar under both Fe- and P-limitation. indicated that increase in Fe precipitation may enhance Fe content in P-limited cultures (i). As the apparent solubility of Fe in culture medium decraesed with time (3.1), lower ratios of Fe : EDTA are necessary to keep the Fe in solution. Therefore, changes in Fe concentration at a lower ratio of Fe : EDTA were also measured as a comparison with (i). In addition to strains used in (i), a freshwater strain, Calothrix D550 was included. The experiment was conducted as described in (i) with the except that Fe : EDTA was used at a ratio of 1 : 2.88 (3.1).

Growth of Calothrix D253 and Calothrix D550 was similar (Fig. 5.2) and had not reached a stable plateau although they may have not reached the

maximum possible yield in neither high nor low P medium; whereas the growth curve of Dichothrix D696 and Anabaena D697 had reached plateau at the end of the period. Under both limitation the yield was higher than that at normal Fe : EDTA ratio (i).

The Fe content of the algae rapidly increased (Fig. 5.2) and reached its maximum at 24 h of growth in high P medium. The maximum then decreased throughout the period and attained a constant level after about three weeks of growth:

strain	Fe content (% dry weight) at various growth stages			
	inoculum	max.	min.	% of Fe removed
Calothrix D253	0.18	1.43	0.084	75
Calothrix D550	0.085	1.12	0.070	65
Dichothrix D696	0.085	1.11	0.094	63
Anabaena D697	0.10	1.20	0.068	63

Conversely, in low P medium there was no rapid increase (Fig. 5.2) in the content of Fe which remained almost constant for the first few days and then started to decrease towards the end of the period ranging from:

strain	Fe content (% dry weight) at various growth stages			
	inoculum	max.	min.	% Fe removed
Calothrix D253	0.28	0.38	0.11	51
Calothrix D550	0.23	0.27	0.10	18
Dichothrix D696	0.36	0.37	0.14	50
Anabaena D697	0.2	0.35	0.10	37

In contrast with (i), it appears that the peak of Fe at low Fe : EDTA ratio in high P medium is lower (13 - 47 %) than that at normal ratio. Whereas in low P medium, there is no peak of Fe at low Fe : EDTA ratio similar to that at normal ratio.

Figure 5.2 Changes in Fe concentration during growth of four strains in high (left column) and low (right column) P saline medium at low ratio of Fe : EDTA. Arrows (a-e) indicate time at which the key morphological changes were first observed:

a) apical vacuolation

b) hair

c) hormogonia production ceases

d) secondary heterocyst

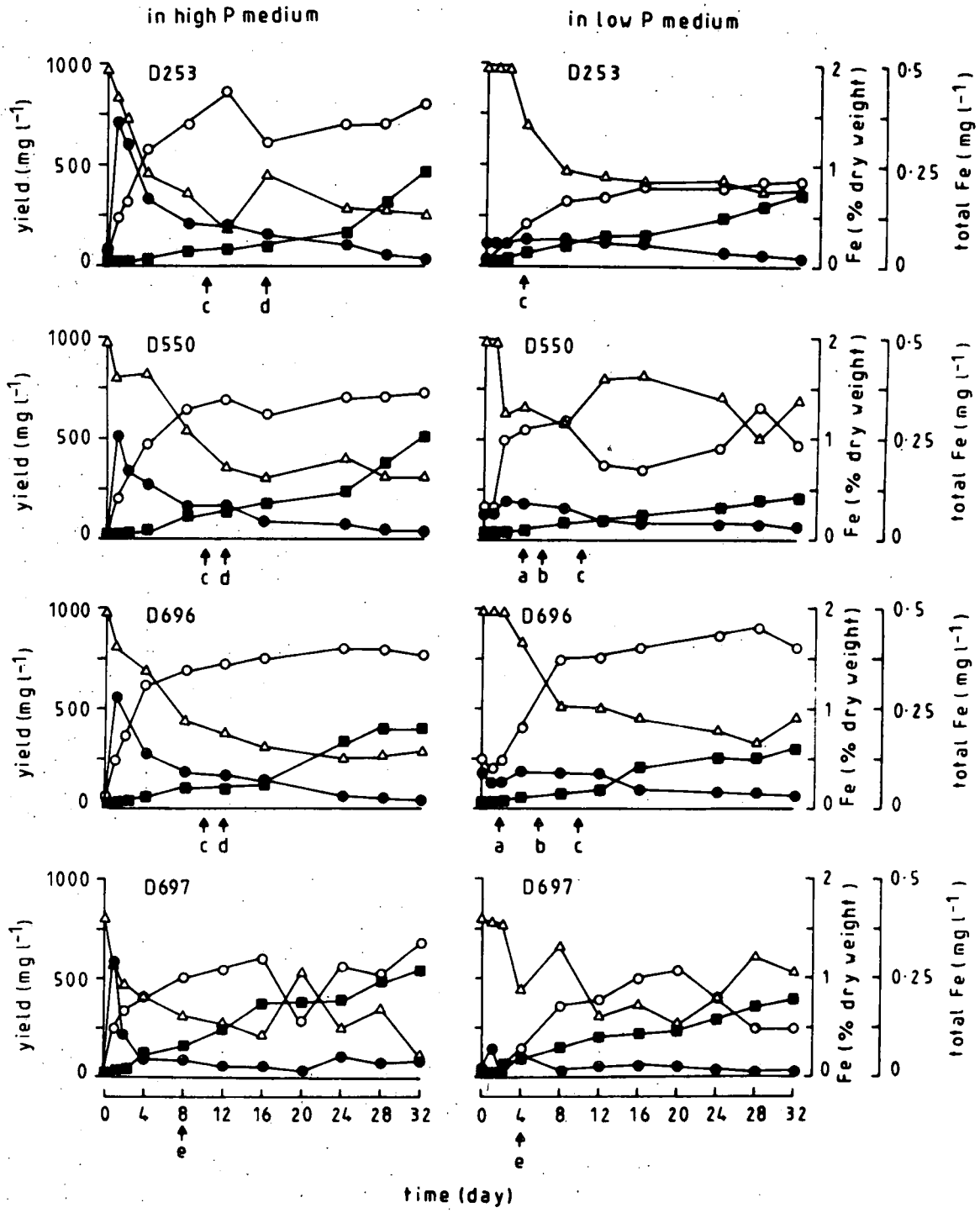
e) akinete

(■) yield (mg L^{-1})

(●) algal Fe composition (% dry weight)

(○) total Fe in algae (mg L^{-1})

(△) Fe concentration in medium (mg L^{-1})


in high P medium
in low P medium
D253
D550
D696
D697
yield (mg l⁻¹)
Fe (% dry weight)
total Fe (mg l⁻¹)
1000
500
0
2
1
0·5
0·25
c
d
a
b
e
0 4 8 12 16 20 24 28 32
time (day)

In Calothrix D550 and Anabaena D697 in high P medium the concentration of Fe in the medium decreased markedly (Fig. 5.2) at 24 h and continued to decrease until about the third week, when it started to fluctuate; there was no similar fluctuation in the Fe concentration with Calothrix D253 and Dichothrix D696. Conversely, in low P medium, the concentration of Fe with all four strains decreased gradually until the third week when, in the case of Calothrix D550 and Anabaena D697, it started to fluctuate (Fig. 5.2). Following the increase in Fe content in high P medium the total Fe in algae increased to form a plateau although, towards the end of the period, it began to decline again (Fig. 5.2). In high P medium the percentage of the Fe removed by algae increased sharply at the beginning and declined later, whereas in low P medium, it increased gradually and then fluctuated up and down towards the end of growth. The Fe in the medium under both conditions remained fully soluble (Fig. 5.2) for the first week and thereafter about 80 % soluble.

The onset of morphological features is indicated in Fig. 5.2. The results with low P medium were similar to those with saline medium in 4.4, in spite of the different Fe : EDTA ratio. The use of high P medium led to similarities with - Fe medium (4.3) in that secondary heterocysts were formed in Rivulariaceae, but no hairs were formed and in Calothrix D253, the sheaths were thicker. Lysis was obvious by day 12 in Calothrix D550 and Anabaena D697, being more severe in high P medium.

Additions were carried out as in (i). Cultures of Calothrix D253 and Dichothrix D696 grown in high P medium resumed normal growth after supplying (by transfer of inoculum or addition of missing component) the normal, - P and - trace elements media, but not after being transferred to - Fe medium. In Calothrix D550 and Anabaena D697 renewed growth took place only in normal medium, but only slight growth in - Fe, - P and - trace elements media.

Thus, according to the criteria for determining limitation (2.562), growth of Calothrix D253 and Dichothrix D696 in high P medium led eventually to Fe-limitation, but the results were not clear-cut for Calothrix D550 and Anabaena D697.

In low P medium all four strains recovered normal growth similar to that in (i). These results therefore fulfil the criteria for limitation (2.562) and demonstrate that growth of the four strains in low P medium had led eventually to P-limitation.

5.2 Changes during growth in freshwater medium

The brackish alga Calothrix D253 showed a markedly different morphology in freshwater, compared with saline medium (4.2, 4.3, 4.5). Therefore studies were extended to investigate its physiological behaviour, with respect to Fe, in freshwater medium during growth in batch culture. Calothrix D550 was also used as an example of a freshwater alga. The experiment was conducted as described in (i), with the exception that low (1 : 1.92) Fe : EDTA ratio was used (3.1). Fe remained fully soluble in the control (uninoculated medium) for the first week and continued to be greater than 90 % for the remainder of the period (Fig. 5.3). Other results are shown in Fig. 5.3 and summarized below:

High P medium

strain	Fe content (% dry weight) at various growth stages			
	inoculum	max.	min.	% of Fe removed
Calothrix D253	0.16	1.3	0.08	63
Calothrix D550	0.082	1.8	0.06	70

Figure 5.3 Changes in Fe concentration during growth of two strains in high (left column) and low (right column) P freshwater medium at low ratio of Fe : EDTA. Arrows (a-e) indicate time at which the key morphological changes were observed:

a) apical vacuolation

b) hair

c) hormogonia production ceases

d) secondary heterocyst

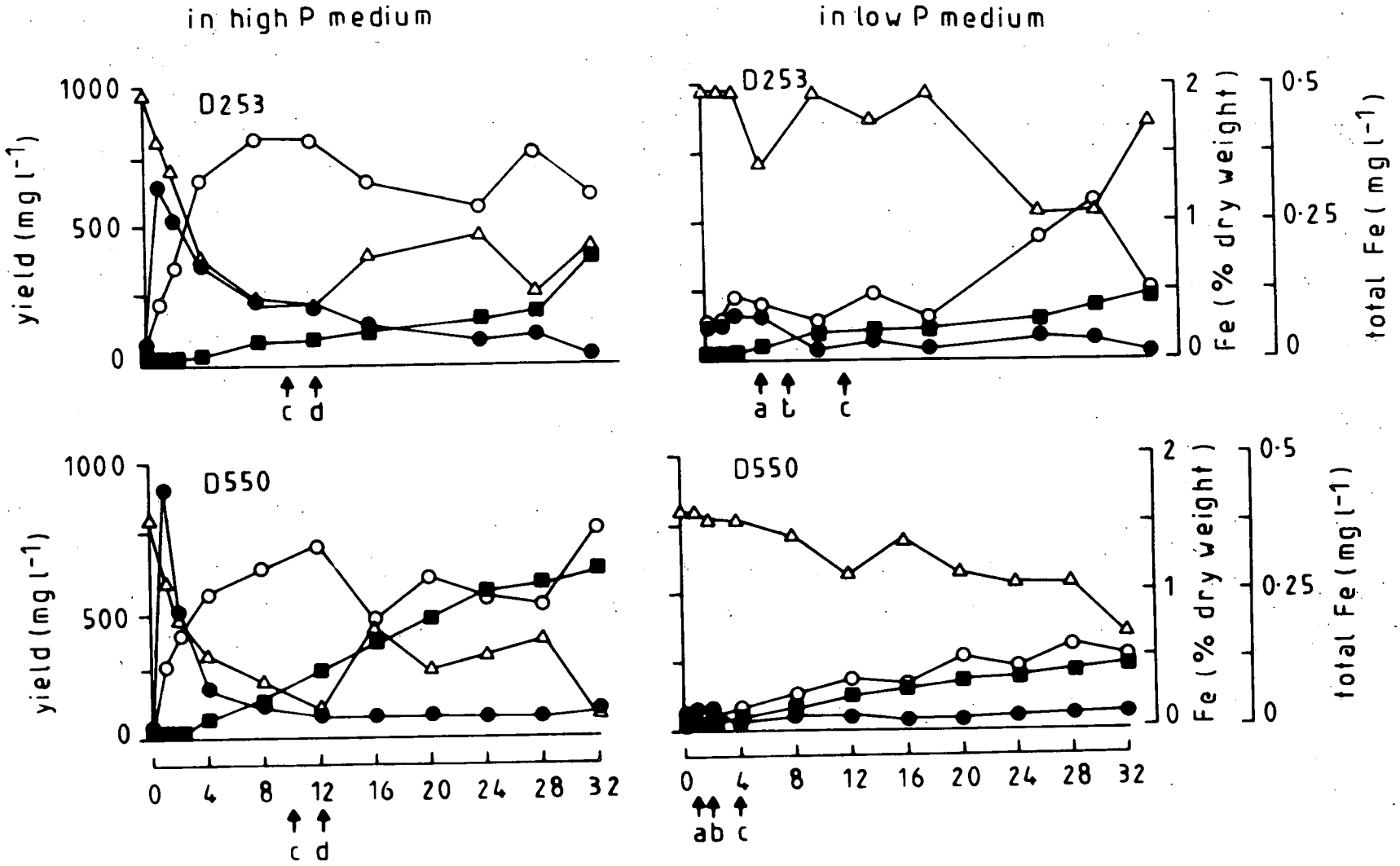
e) akinete

(■) yield $(mg\ L^{-1})$

(●) algal Fe composition (% dry weight)

(○) total Fe in algae $(mg\ L^{-1})$

(△) Fe concentration in medium $(mg\ L^{-1})$


in high P medium
in low P medium
D253
D550
yield (mg l⁻¹)
1000
500
0
Fe (% dry weight)
2
1
0
total Fe (mg l⁻¹)
0·5
0·25
0
a
b
c
d
0
4
8
12
16
20
24
28
32
time (day)

Low P medium

strain	Fe content (% dry weight) at various growth stages			
	inoculum	max.	min.	% of Fe removed
Calothrix D253	0.24	0.32	0.10	13
Calothrix D550	0.13	0.25	0.12	27

These results may be compared with those in 5.1. The reasons for the fluctuations in total Fe incorporated at various stages during culture (Fig. 5.3) may be due to lysis, at least in older cultures (see Chapter 9). Morphological changes were similar to those described in 5.2, with the exception that hairs were formed in Calothrix D253 in low P medium. Additions were again carried out as in 5.1 and the results were similar to those described in 5.2.

5.3 Minimum Fe content in algae during growth with no added Fe

Although the minimum Fe content had been determined in batch culture leading to marked Fe-limitation, the minimum content was also measured after an inoculum had been transferred to - Fe medium.

Inocula from exponential cultures were put in fresh high P (10 mg L^{-1}) medium including EDTA but not Fe, and then incubated as in 2.44. After about 10 - 12 days, when the cultures still look relatively healthy, samples were analyzed for Fe:

strain	Fe (% dry weight)
Calothrix D253	0.16
Calothrix D550	0.052
Calothrix D704	0.057
Dichothrix D696	0.12
Anabaena D697	0.02

6 ACCUMULATION OF Fe DURING FIRST 24 h AFTER SUBCULTURE

6.1 Influence of Fe and Fe : EDTA on Fe accumulation in saline medium

Study in batch culture, with a sampling programme at day intervals (Chapter 5) showed that the maximum value of Fe associated with algae was reached by day 1. More detailed studies of the first 24-h period were planned. The influence of Fe and EDTA on removal of Fe from the medium by Fe- and P-limited cultures was tested with four experiments in saline medium. Iron and P-limited inocula were prepared as in 2.561; the former was grown in medium modified from that used in Chapter 5 by the omission of Fe and reduction of EDTA (2.561). Low-P (0.05 mg L^{-1}) medium was used for P-limited material. The inoculum was homogenized, washed and suspended in medium minus FeEDTA. The alga was allowed to equilibrate for 30 min before Fe addition. The apparent solubility of Fe during the experiment was determined in a control (- alga) flask.

(i) Use of normal Fe : EDTA

Preliminary studies indicated that accumulation of Fe from a 0.4 mg L^{-1} medium was not detectable (using AAS) before 3 h, but an asymptotic value was reached by 10 min after increasing the Fe to 4.0 mg L^{-1}. Therefore, a comparative study of Fe accumulation by Fe- and P-limited cultures of Calothrix D253, Dichothrix D696 and Anabaena D697 was made at both Fe concentrations (0.4, 4.0 mg L^{-1}). Using 0.4 mg L^{-1} medium, the final Fe content was similar in Fe- and P-limited cultures for all three strains at 18-h period, but no asympote was approached. In contrast with 4.0 mg L^{-1} Fe, asympotes were approached after two min (Fig. 6.1 b-c). The apparent

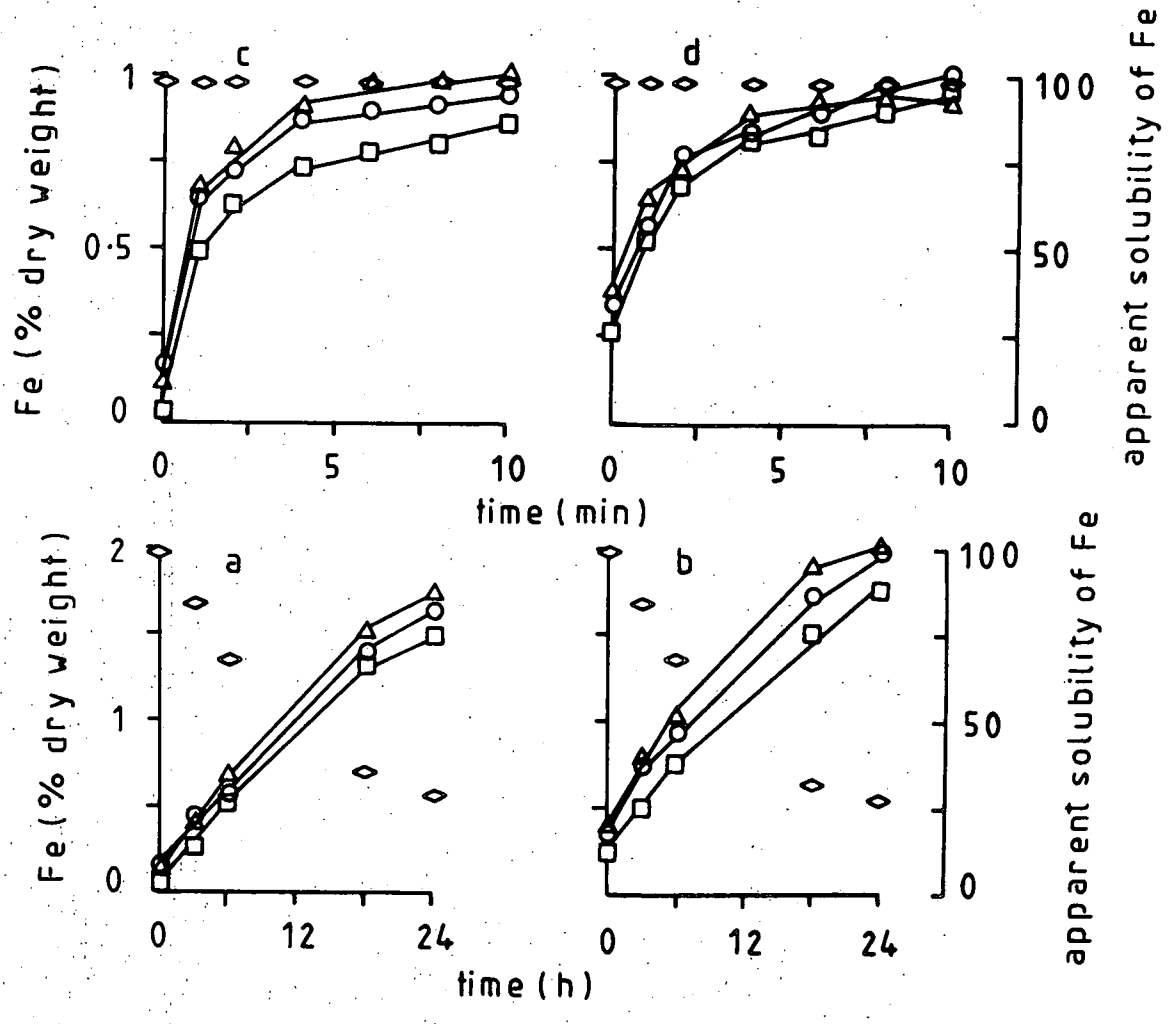
Fig. 6.1 Accumulation of Fe by Fe- and P-limited cultures of Calothrix D253 (○), Dichothrix D696 (△), Anabaena D697 (□) and apparent solubility of Fe (◇) using normal Fe : EDTA in saline medium

a) With 0.4 mg L^{-1} Fe by Fe-limited cultures over 24-h period

b) With 0.4 mg L^{-1} Fe by P-limited cultures over 24-h period

c) With 4.0 mg L^{-1} Fe by Fe-limited cultures over 10-min period

d) With 4.0 mg L^{-1} Fe by P-limited cultures over 10-min period


c
d
a
b
Fe (% dry weight)
apparent solubility of Fe
time (min)
time (h)
0
0·5
1
2
5
10
12
24
50
100

solubility of Fe as shown by the control (- alga) flasks was 25 % for Fe 0.4 mg L^{-1} Fe at 24 h and 97 % for 4.0 mg L^{-1} at 10 min.

A comparison of removal of Fe by the strains with the control showed that in 0.4 mg L^{-1} removal was similar to the control at 24 h, but was greater in 4.0 mg L^{-1} after 10 min. There was no difference in removal of Fe by Fe- and P-limited cultures with 0.4 mg L^{-1} by 24 h, but with 4.0 mg L^{-1} by 10 min.

(ii) Use of low Fe : EDTA

A further experiment was carried out with Calothrix D253 and Anabaena D697 using a reduced Fe : EDTA ratio to keep Fe in solution. In Fe-limited cultures there was no asymptote over 24 h using 0.4 mg L^{-1}, but asymptote was approached after 2 min using 4.0 mg L^{-1}. In contrast with P-limited cultures, there was about 60 % reduction in Fe accumulation. In contrast with Fe content at normal Fe : EDTA, the final Fe content of Fe-limited cultures was 50 % lower at the reduced ratio (using 0.4 mg L^{-1}) (Fig. 6.2 a), but the same for Fe-limited cultures and less than 60 % for P-limited cultures (using 4.0 mg L^{-1}) (Fig. 6.2 b-c). Using low Fe : EDTA ratio, Fe precipitate did not occur at normal (0.4 mg L^{-1}) or at high (4.0 mg L^{-1}) Fe; therefore it is assumed that Fe removed by the two strains is firmly attached and not precipitated around the algae.

(iii) Influence of Fe concentration on accumulation

In view of the differing results (i) with normal (0.4 mg L^{-1}) and high (4.0 mg L^{-1}) Fe, a more detailed experiment was performed on Calothrix D253 using Fe-limited culture. Accumulation by Fe-limited Calothrix D253 was tested in Fe concentrations over the range 0.4 - 4.0 mg L^{-1} with a constant EDTA concentration. An asympote was approched at about 1.7 mg L^{-1} Fe (Fig. 6.3).

Fig. 6.2 Accumulation of Fe by Fe- and P-limited cultures of Calothrix D253 (○). Anabaena D697 (□) and apparent solubility of Fe (◇) using low Fe : EDTA in saline medium

a) With 0.4 mg L^{-1} Fe by Fe-limited cultures over 24-h period

b) With 4.0 mg L^{-1} Fe by Fe-limited cultures over 10-min period

c) With 4.0 mg L^{-1} Fe by P-limited cultures over 10-min period

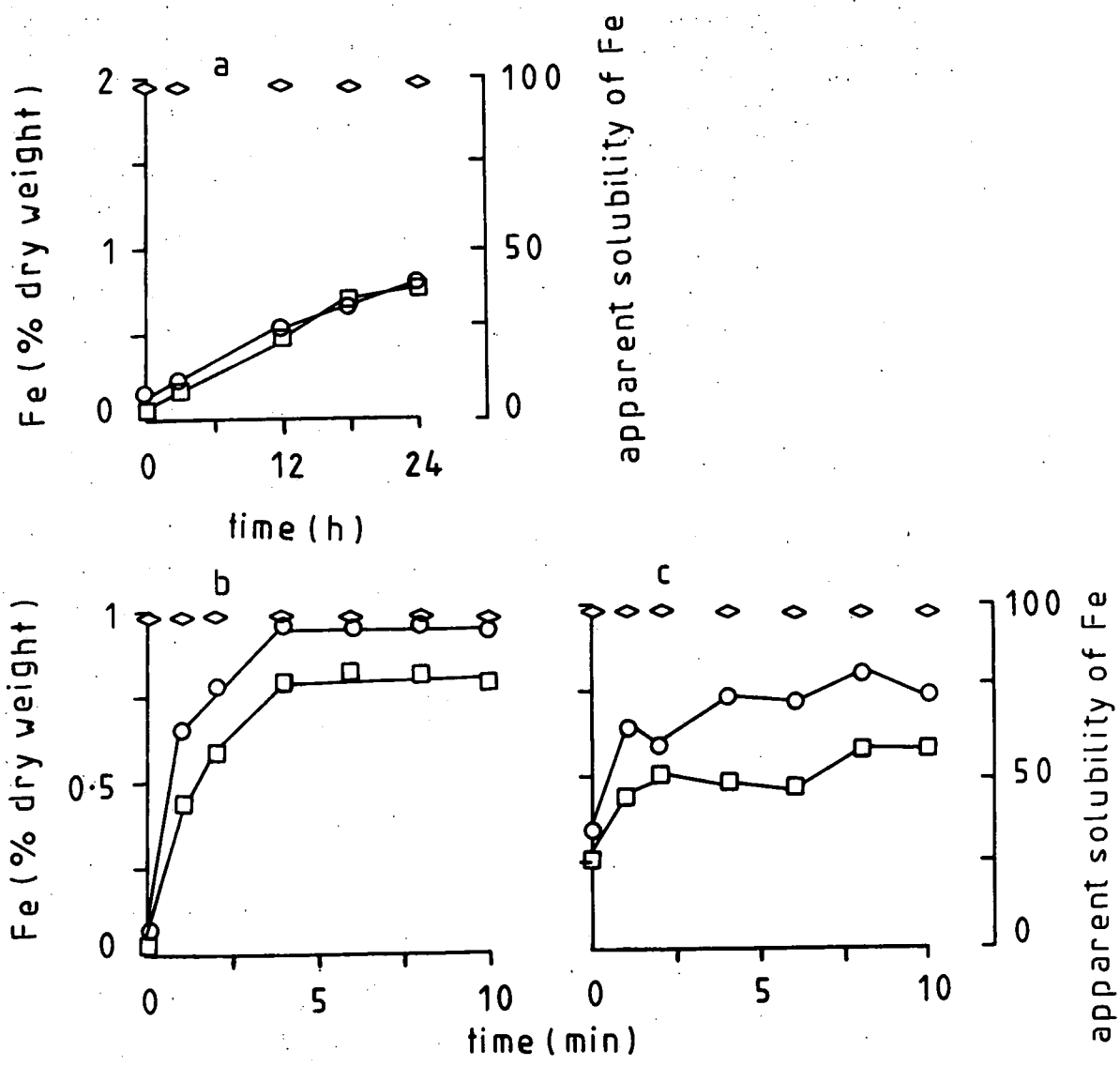
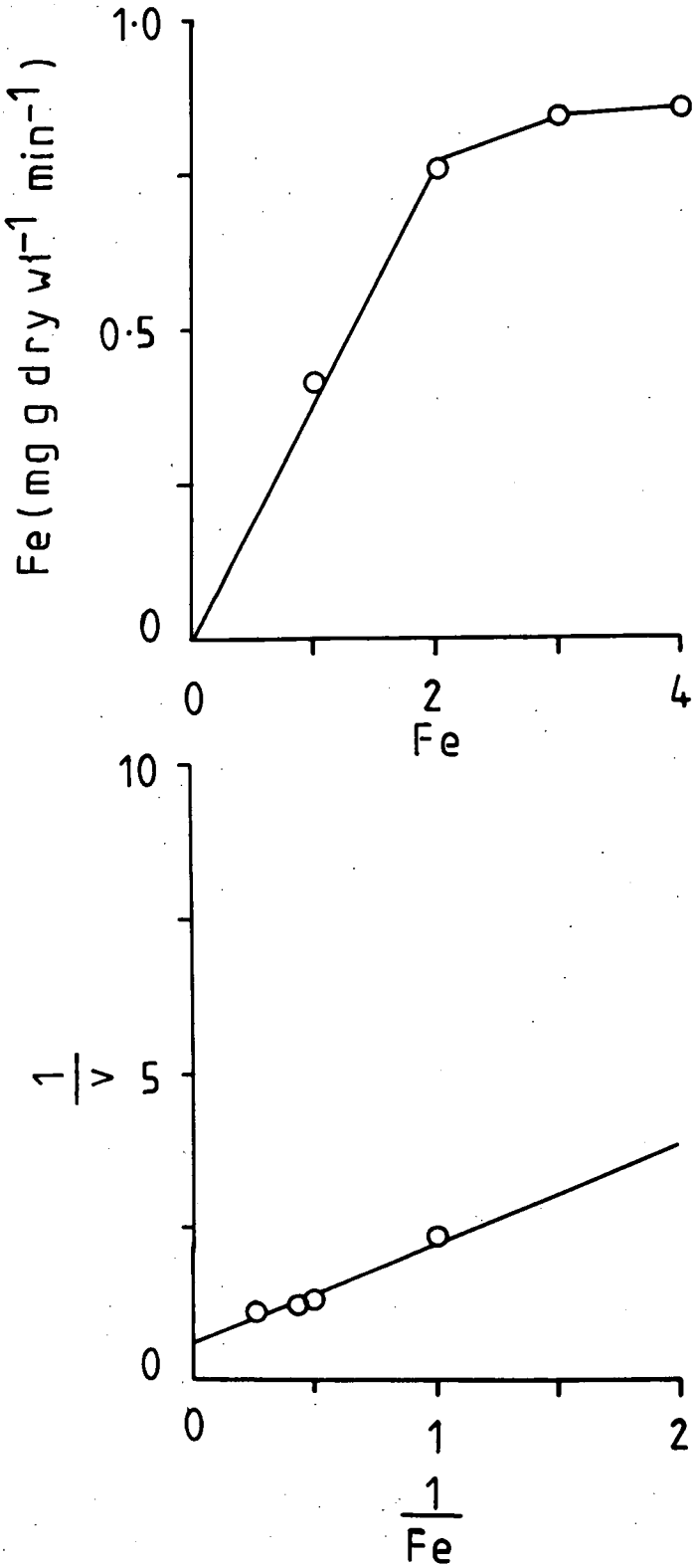

a
Fe (% dry weight)
2
1
0
0
12
24
time (h)
100
50
0
apparent solubility of Fe
b
Fe (% dry weight)
1
0·5
0
0
5
10
time (min)
c
0
5
10
100
50
0
apparent solubility of Fe

Fig. 6.3 (a) Influence of Fe concentration (0.4 - 4.0 mg L^{-1}) on Fe accumulation by Fe-limited culture of Calothrix D253 (○) in saline medium for 10 min, with constant EDTA (20.0 mg L^{-1})

(b) Double reciprocal plot of the data in (a), where V = rate of Fe accumulation (mg Fe g dry wt^{-1} min^{-1})


1·0
0·5
0
Fe (mg g dry wt^{-1} min^{-1})
0
2
4
Fe
10
5
0
$\frac{1}{v}$
0
1
2
$\frac{1}{Fe}$

(iv) Influence of EDTA concentration on Fe accumulation

Accumulation was the same (Table 6.1) at different EDTA concentrations. The apparent solubility of Fe in the control was 97 - 102 %, indicating that accumulation was independent of Fe precipitation and also that high EDTA concentrations had no influence on accumulation.

Table 6.1 Influence of EDTA concentration on Fe accumulation by Fe-limited Calothrix D253 using high Fe (4.0 mg L^{-1}).

EDTA (mg L^{-1})	algal Fe (% dry weight)
20	8.84
40	8.28
60	8.88
100	9.29

With normal Fe : EDTA ratio, the apparent solubility of Fe decreased with time; Fe associated with the alga with 0.4 mg L^{-1} Fe over 24 h may therefore include some precipitate. As no precipitate was observed with controls under the other conditions, it is assumed that the Fe associated with the alga did not include precipitate.

6.2 Influence of physical and chemical factors on Fe accumulation in saline medium

In view of the results in 6.1 it was not clear which factors were involved in the removal of Fe from the medium. The influence of physical and chemical factors on Fe accumulation by Fe-limited cultures was therefore investigated. The inoculum was prepared as described in 6.1, and accumulation was carried out using 4.0 mg L^{-1} Fe with the low Fe : EDTA

ratio over 10 min in saline medium. The apparent solubility of Fe during the experiment was determined in a control (- alga) flask.

(i) Influence of temperature

Accumulation of Fe by Calothrix D253 and Anabaena D697 was investigated at three temperatures (0, 10, 32° C). Accumulation increased with increased temperature (Fig. 6.4).

(ii) Influence of light

The rapid accumulation over the first 10 min (6.1) led to the question whether or not accumulation is largely passive during this period. The influence of light was therefore investigated for three strains (D253, D696, D697); no influence was found (Fig. 6.5).

A further experiment was carried out using Calothrix D253 to see whether pre-incubation in the dark may give different results. In this experiment the inoculum was incubated in dark for 24 h before the addition of Fe. The results (data not shown) were similar to those without dark pre-incubation, again suggesting that no role for energy on Fe accumulation during the first 10 min.

(iii) Influence of pH

The literature showed that pH plays a key role in Fe speciation and therefore may affect accumulation (1.22, 1.42). Accumulation of Fe by Calothrix D253 was investigated over the range of pH 6 - 10. The results (Table 6.2) showed accumulation was increased from pH 7.0 upwards. During

Fig. 6.4 Influence of temperature on accumulation of Fe by Fe-limited cultures of two strains in saline medium using 4.0 mg L^{-1} Fe over 10-min period: 0 °C (○), 10 °C (△), 32 °C (□)

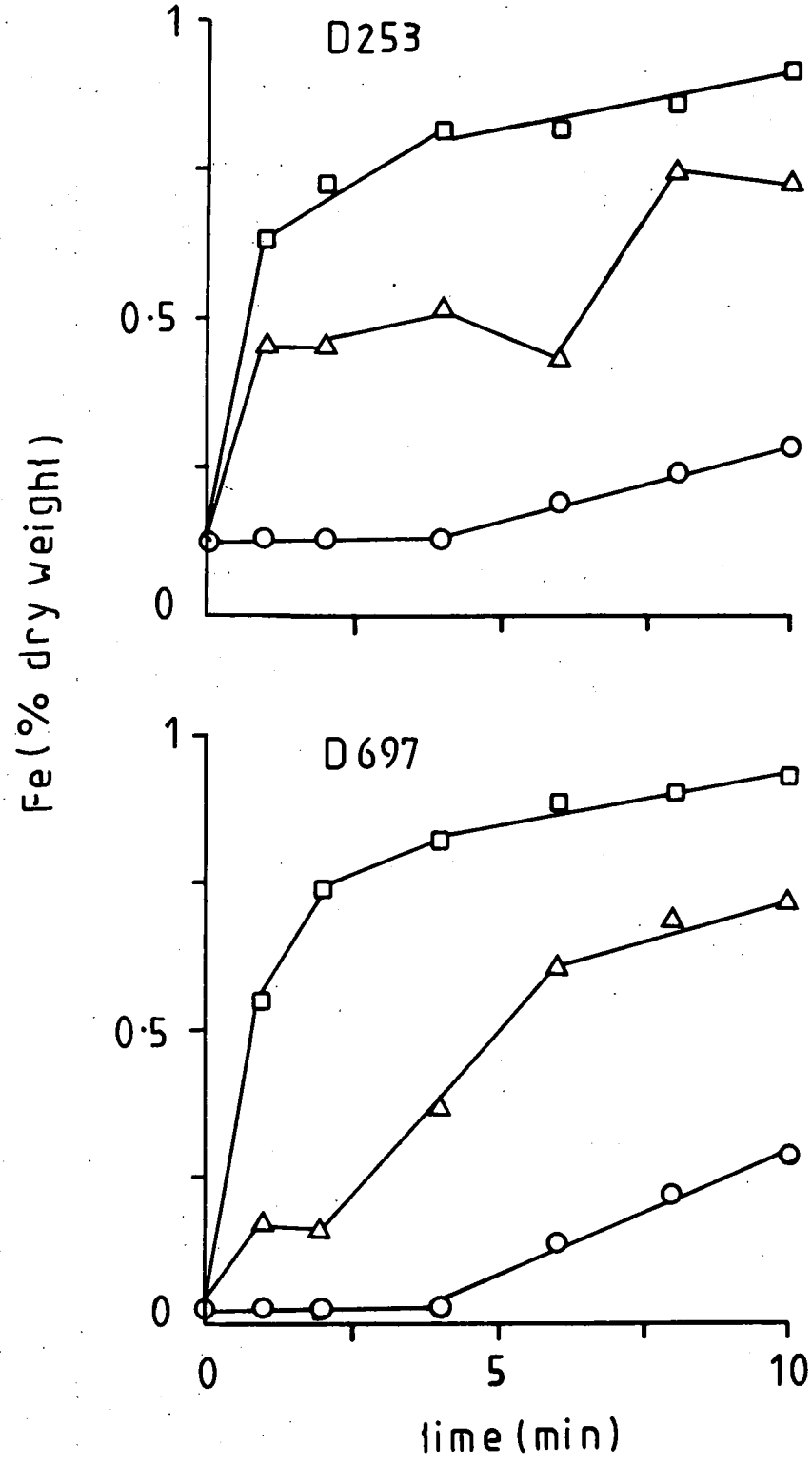
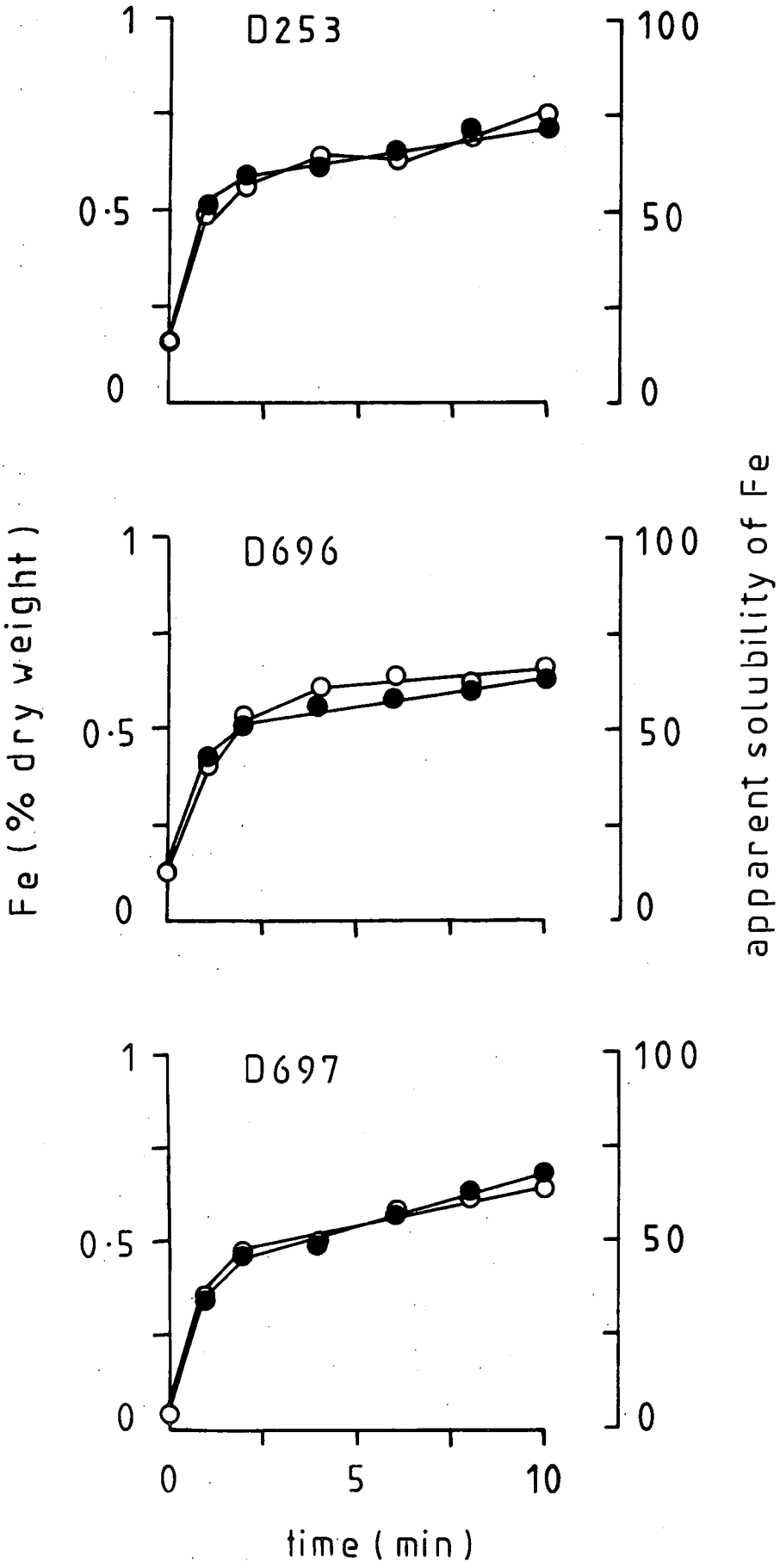

D253
D697
1
0·5
0
0
5
10
Fe (% dry weight)
time (min)

Fig. 6.5 Influence of light/dark incubation on accumulation of Fe by Fe-limited cultures of three strains in saline medium using 4.0 mg L^{-1} Fe over 10-min period: light (○), dark (●)


D253
D696
D697
1
0·5
0
100
50
0
0
5
10
Fe (% dry weight)
apparent solubility of Fe
time (min)

the experiment the apparent solubility of Fe was more than 97 % at pH 8 and below, but decreased above pH 8.

Table 6.2 Influence of pH on accumulation of Fe by Fe-limited Calothrix D253; using high Fe (4.0 mg L^{-1}).

pH	algal Fe (% dry weight)
6	6.6
7	6.4
8	8.0
9	10.0
10	17.1

(iv) Influence of salinity

Salinity affects the speciation and possibly Fe accumulation (1.41). Accumulation by Calothrix D253 was studied over the range 0.05 - 9.3 $^{o}/oo$ salinity (at pH 7.8). There was little, if any, effect (Table 6.3).

Table 6.3 Influence of salinity on accumulation of Fe by Fe-limited Calothrix D253: using high Fe (4.0 mg L^{-1})

$^{o}/oo$	algal Fe (% dry weight)
0.05	6.8
1.9	4.7
3.7	5.7
5.7	5.1
7.4	5.1
9.3	5.1

6.3 Influence of metabolic inhibitors on Fe accumulation in saline medium

The next step was to investigate the possible role of inhibitors: three strains (D253, D696, D697) were used for this purpose. CCCP (8 µM) and 2,4-DNP (50 µM) were chosen as inhibitors of photo- and oxidative

phosphorylation, respectively (9.62). The inhibitors did not cause any shift in pH. Fe-limited cultures were inoculated in medium containing one of the inhibitors and incubated for 60 min prior to the addition of Fe for 10 min. Both inhibitors were tested in dark and light. Accumulation was similar (Fig. 6.6 a-e) under all circumstances.

6.4 Production of siderophores in saline and freshwater media

6.41 Biological assay for chelating activity

A biological test for production of chelating agent(s) (2.7) was carried out on five strains (Calothrix D253, Calothrix D550, Calothrix D603, Dichothrix D696, Anabaena D697) in saline and freshwater media in the absence of both Fe and EDTA. None of the strains were able to grow (Table 6.4) in saline medium, but in freshwater medium three strains (D603, D550, D697) showed obvious initial growth and two (D253, D696) did not. The former three strains eventually ceased growth, presumably as a result of Fe-limitation.

The influence of EDTA on growth of three strains (D253, D696, D697) was also investigated in media in absence of Fe (Table 6.4). All three strains grew initially when sufficient EDTA was present. In addition, when EDTA was added to cultures previously subcultured to EDTA-free medium for six days, slow recovery occurred by about day four. The cultures, which were pale yellow and highly vacuolated before addition of EDTA, showed obvious recovery.

Fig. 6.6 Influence of 2,4-DNP (a-c) and CCCP (e-f) on accumulation of Fe by Fe-limited cultures of three strains in saline medium using 4.0 mg L^{-1} Fe over 10-min period: light (○), dark (●)

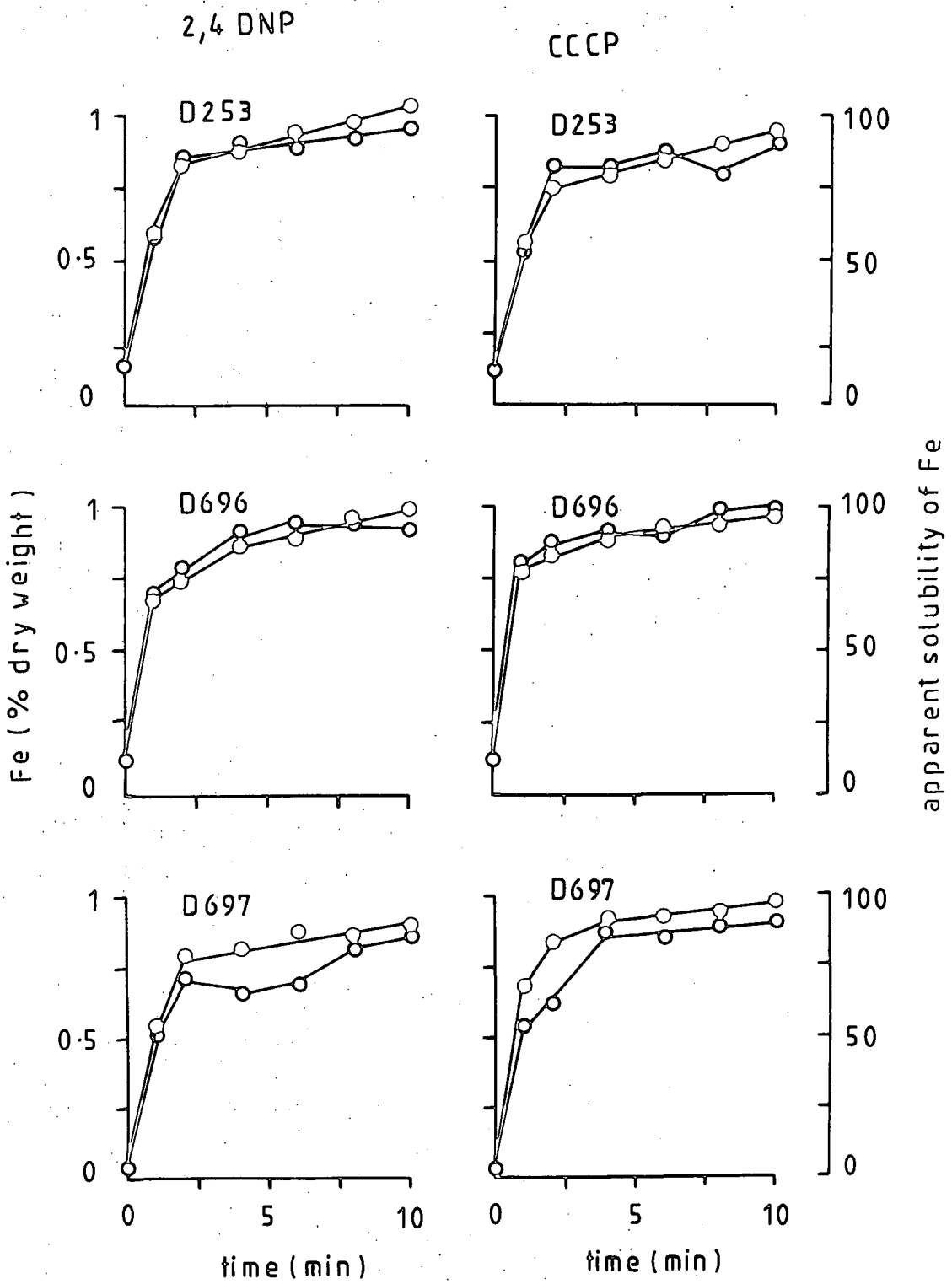

2,4 DNP
CCCP
D253
D253
D696
D696
D697
D697
1
0·5
0
100
50
0
Fe (% dry weight)
apparent solubility of Fe
0
5
10
time (min)
time (min)

Table 6.4 Influence of EDTA concentration on growth of algae in absence of Fe in saline and freashwater media. Growth (+), no growth (-).

	saline medium					freshwater medium				
EDTA(μM)	D253	D550	D603	D696	D697	D253	D550	D603	D696	D697
0.0	-	-	-	-	-	-	+	+	-	+
0.86	-	-	-	-	-	-	+	+	-	+
1.71	-	-	+	-	-	+	+	+	+	+
3.42	-	-	+	-	-	+	+	+	+	+
5.13	+	+	+	+	+	+	+	+	+	+
6.84	+	+	+	+	+	+	+	+	+	+

6.42 Chemical assay for siderophores

(i) Ferric perchlorate assay

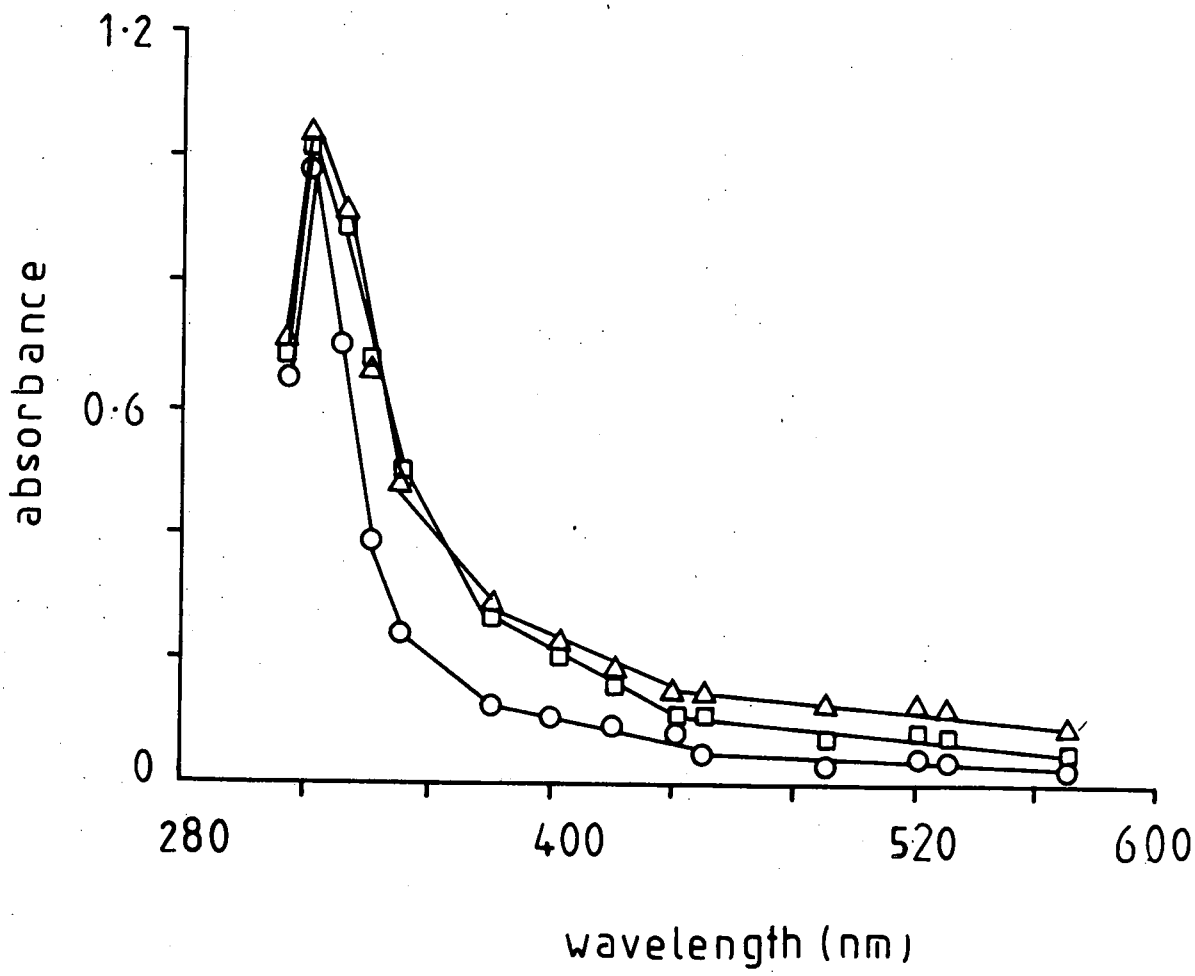
Following the above results the study was extended to look for chemical evidence for production of siderophore by the three strains. Absorbance of Fe-binding properties of siderophore were recorded using perchlorate assay at 490 nm (Goldman et al., 1983: Clarke et al., 1987) although no peak was found for culture filtrates in - Fe and + Fe medium:

strain	absorbance (490 nm)	
	- Fe	+ Fe medium
Calothrix D550	0.04	<0.001
Calothrix D603	0.08	<0.001
Anabaena D697	0.12	<0.001

(ii) Absorption spectrum assay

Followig the above results the next step was to charachterize the type of siderophore. The absorption spectrum for Fe-siderophore complex was determined as described in 2.7 and the results are given in Fig. 6.7. The Fe-siderophore complex of all three strains showed a similar pattern with the maximum peak at 320 nm. The spectrum does not fit that of the siderophore produced by the marine coccoid blue-green alga Agmenellum quadruplicatum strain PR-6 (Armstrong & Van Baalen, 1979) though it is fairly similar between 300 - 350 nm. The spectrum was completely different to that produced by Pseudomonas fluorescens (Meyer & Abdallah, 1978) and that of Fe(III)-ferribactin and Fe(III)-pyoverdine produced by P. fluorescens, but was very similar to the purple compound (un known chelating material) produced by the same species (Phislon & Llinas, 1981).

Fig. 6.7 Absorption spectra of Fe(III)-siderophore complex for Calothrix D550 (○). Calothrix D603 (□) and Anabaena D697 (△) grown in freshwater medium in absence of Fe and EDTA


1·2
0·6
0
absorbance
280
400
520
600
wavelength (nm)

(iii) Siderophore-mediated Fe transport system in tow *Calothrix* strains

An experiment was carried out on tow strains (D550, D603) to see whether the siderophore was used by algae in transport of Fe. *Calothrix* D550 and *Calothrix* D603 were grown in absence of EDTA with different concentrations of Fe in freshwater medium in batch culture. Both strains were grew normaly and healthy with growth being more rapid in *Calothrix* D603.

7 INTERACTION BETWEEN Fe- AND P-LIMITATION

7.1 Changes in P concentration during growth in saline medium

The possibility has been raised (1.34) that P availability may be a problem under conditions of Fe-limitation. The results presented earlier (Chapters 4, 5) did not indicate any such interaction. However a further experiment was planned to investigate changes in P in cultures grown to Fe- and P-limitation. The experiment was carried out in saline medium under conditions which led eventually to Fe- or P-limitation as in 5.1-ii, using four strains (D253, D550, D696, D697). The inoculum was prepared as in 2.561 and the experiment was carried out under standard conditions (2.44). The medium used with normal Fe (0.4 mg L^{-1}) contained high P (10 mg L^{-1}) in order to lead to Fe-limitation, but low P (0.05 mg L^{-1}) to led to P-limitation. In both cases low Fe : EDTA was used, as Fe precipitation does not occur (see 3.1, 5.1-ii). It is therefore assumed that the P removed by the algae was not due to precipitation associated around algae.

The P content increased rapidly and reached a maximum at 24 h in high P medium (Fig. 7.1). The maximum then decreased rapidly and attained a constant level by the end of the growth period:

strain		P content (% dry weight) at various stages			
		inoculum	max.	min.	% of P removed
Calothrix	D253	0.46	1.92	0.26	20
Calothrix	D550	1.1	2.67	0.96	66
Dichothrix	D696	0.84	1.23	0.76	32
Anabaena	D697	0.62	1.82	0.46	60

Figure 7.1 Changes in P concentration during growth of four strains in saline medium with high P and low P at low ratio of Fe : EDTA. Arrows (a-e) indicate time at which the key morphological changes were first observed:

a) apical vacuolation

b) hair

c) hormogonia production ceases

d) secondary heterocyst

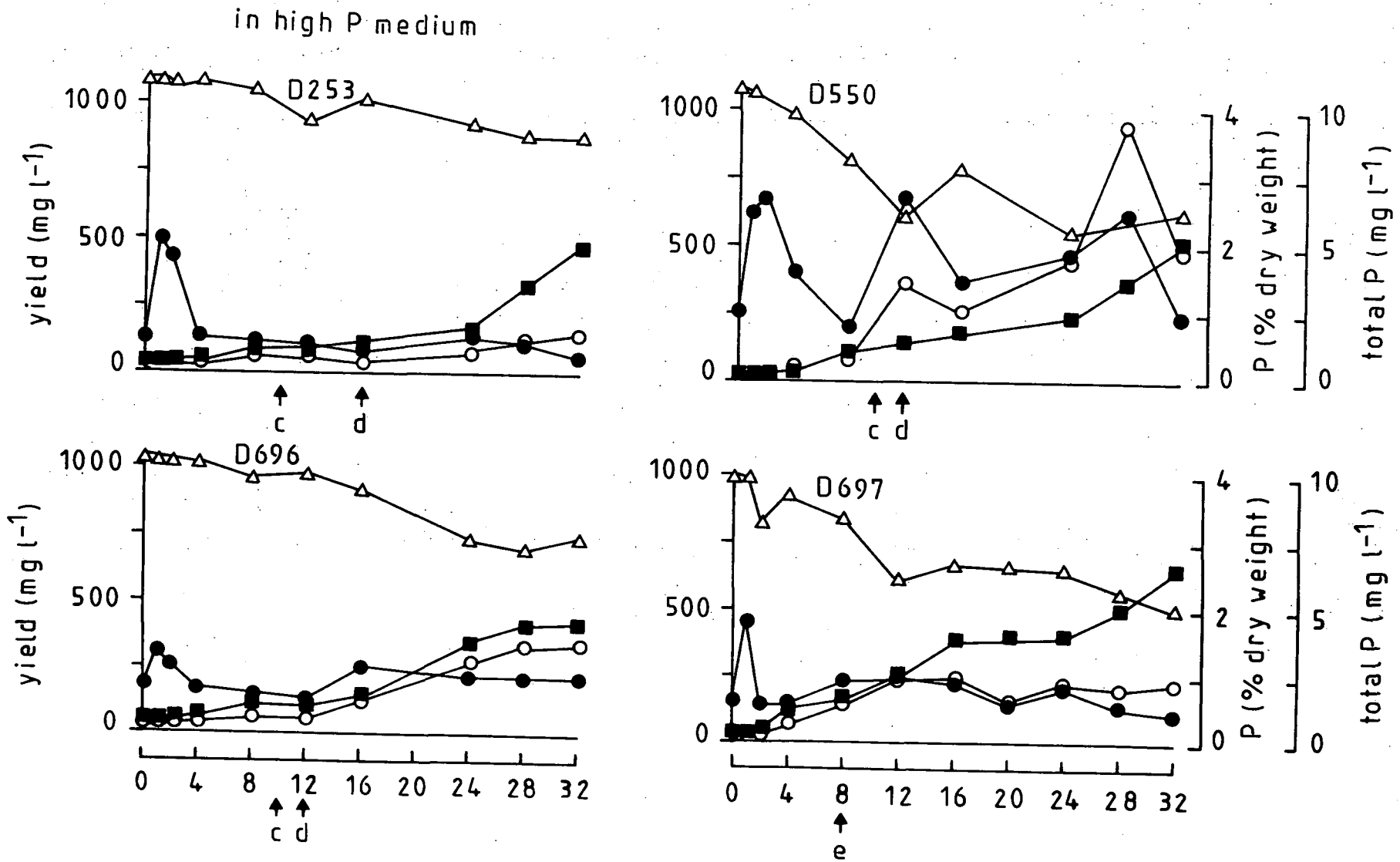
e) akinete

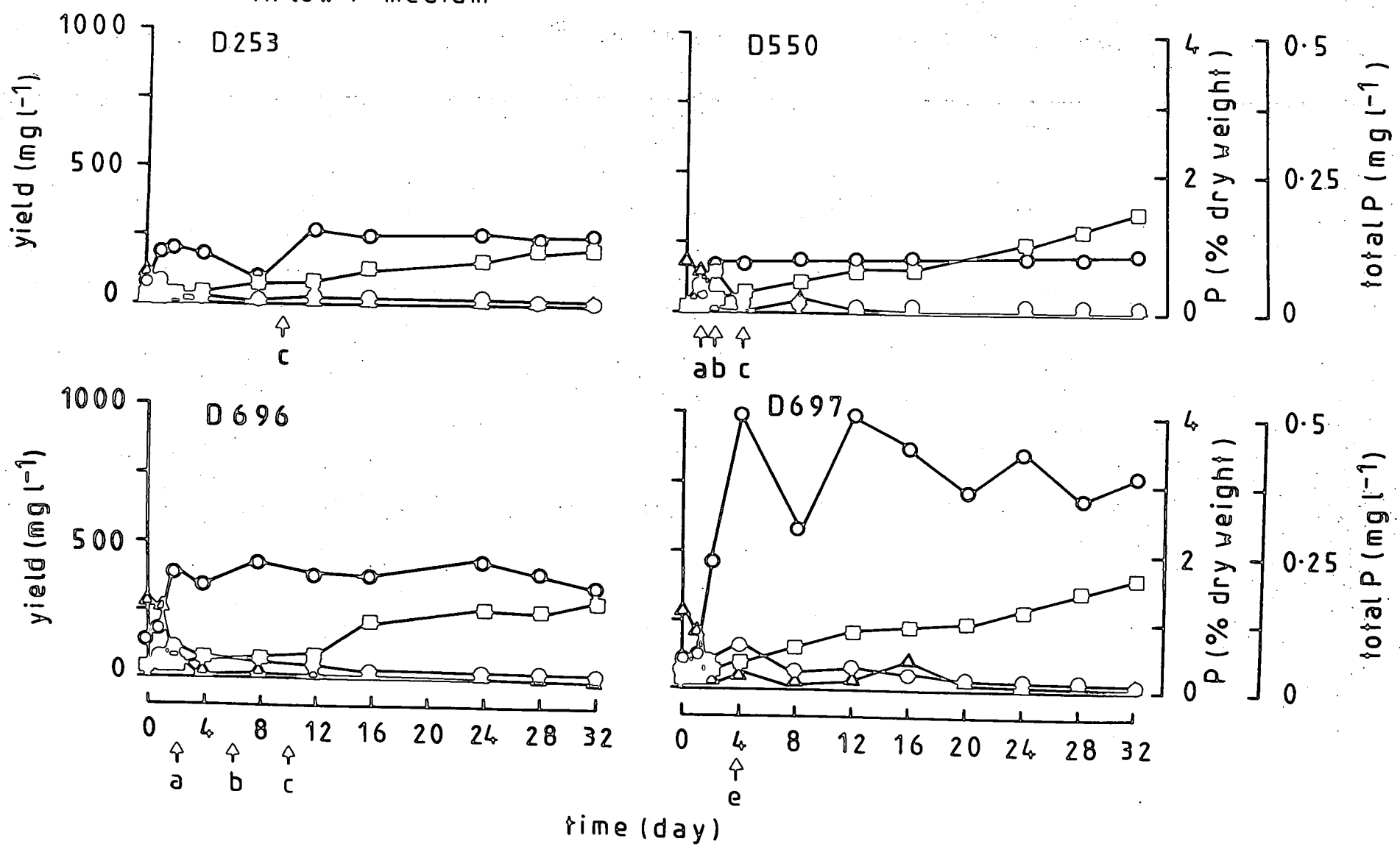
(■) yield (mg L^{-1})

(●) algal P composition (% dry weight)

(○) total P in algae (mg L^{-1})

(△) P concentration in medium (mg L^{-1})


in high P medium
D253
D550
D696
D697
yield (mg l⁻¹)
P (% dry weight)
total P (mg l⁻¹)
1000
500
0
4
2
10
5
c
d
e
0 4 8 12 16 20 24 28 32
time (day)


in low P medium
D253
D550
D696
D697
yield (mg l⁻¹)
P (% dry weight)
total P (mg l⁻¹)
1000
500
0
4
2
0·5
0·25
a
b
c
e
0 4 8 12 16 20 24 28 32
time (day)

Conversely, in low P medium there was only a small increase in P content (Fig. 7.1), which was followed by a rapid decrease:

strain		P content (% dry weight) at various stages			
		inoculum	max.	min.	% of P removed
Calothrix	D253	0.16	0.35	0.06	100
Calothrix	D550	0.16	0.59	0.03	96
Dichothrix	D696	0.19	0.47	0.06	100
Anabaena	D697	0.23	0.62	0.06	98

The total P removed by the algae in high P medium increased in all strains throughout the growth period, whereas in low P medium total P removed had reached a plateau by the middle of the first week.

7.2 Changes in P concentration during growth in freshwater medium

A comparative experiment was carried out in freshwater medium (see 5.2), but only with Calothrix D253 and Calothrix D550 as described in 7.1. In high P medium the P content increased rapidly (Fig. 7.2), with a maximum at 24 (D253) and 48 h (D550). The value then decreased rapidly and attained a constant level by the end of the growth period:

strain		P content (% dry weight) at various growth stages			
		inoculum	max.	min.	% of P removed
Calothrix	D253	1.4	2.3	0.39	16
Calothrix	D550	1.3	4.1	1.00	68

Figure 7.2 Changes in P concentration during growth of two strains in freshwater medium with high P and low P at low ratio of Fe : EDTA. Arrows (a-e) indicate time at which the key morphological changes were first observed:

a) apical vacuolation

b) hair

c) hormogonia production ceases

d) secondary heterocyst

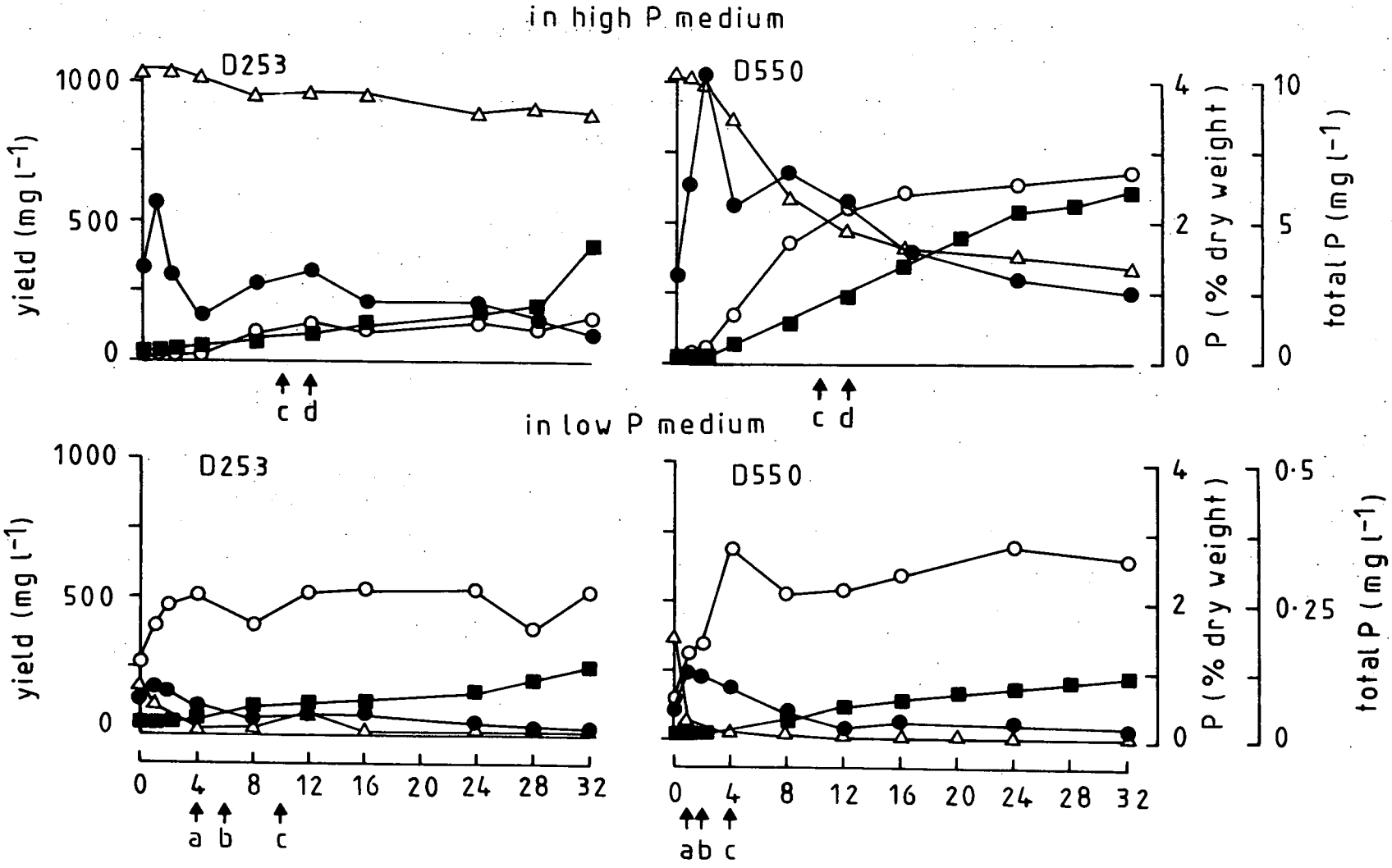
e) akinete

(■) yield (mg L^{-1})

(●) algal P composition (% dry weight)

(△) total P in algae (mg L^{-1})

(○) P concentration in medium (mg L^{-1})


in high P medium
D253
D550
1000
500
0
yield (mg l⁻¹)
4
2
0
P (% dry weight)
10
5
0
total P (mg l⁻¹)
c d
c d
in low P medium
D253
D550
1000
500
0
yield (mg l⁻¹)
4
2
0
P (% dry weight)
0·5
0·25
0
total P (mg l⁻¹)
0 4 8 12 16 20 24 28 32
a b c
0 4 8 12 16 20 24 28 32
a b c
time (day)

Conversely, in low P medium there was only a small increase in P content (Fig. 7.2), which was followed by a rapid decrease:

strain		P content (% dry weight) at various stages			
		inoculum	max.	min.	% of P removed
Calothrix	D253	0.49	0.71	0.10	100
Calothrix	D550	0.45	0.98	0.16	97

The total P removed by the algae in high P medium increased in all strains throughout the growth period, whereas in low P medium total P removed reached a plateau by the middle of the first week.

Morphological changes were described in 5.2. The use of limitation criteria (5.2) showed that growth in the high P medium had led eventually to Fe-limitation, whilst low P medium led to P-limitation. The results in Chapter 5 and 7.1 suggest that the Fe : P ratio may be used as an indicator for Fe- or P-limitation:

strain		Fe : P ratio at end of growth period			
		saline medium		freshwater medium	
		high P	low P	high P	low P
Calothrix	D253	0.25	2.5	0.2	1.5
Calothrix	D550	0.1	3.5	0.025	0.75
Dichothrix	D696	0.1	2.4	-	-
Anabaena	D697	0.08	0.75	-	-

7.3 Possible role of polyphosphate granules in Fe accumulation in saline and freshwater media

Formation of polyphosphate granules was chosen as a visual indicator of P status. The aim of the first experiment was to see whether Fe is required in polyphosphate formation. Two saline medium were used for Calothrix

D253, Dichothrix D696, Anabaena D697 (30 % $ASP_6M(-N)$) and Calothrix D704 (100 % $ASP_6M(-N)$), whilst freshwater medium was used for Calothrix D550 (Chu-10D(-N)). Cultures from late exponential growth were subcultured into - Fe and - P medium for two days before high P (10 mg L^{-1}) was added. The granules were obvious in all strains within five min and increased at 120 min, suggesting no need for Fe in uptake of P or polyphosphate formation.

A further experiment was carried out to see if Fe uptake increased as polyphosphate granules formed. 10 mg L^{-1} P was added to the P-limited cultures of Calothrix D253, Calothrix D550, Dichothrix D696 and Anabaena D697 in saline and to Calothrix D253 and Calothrix D550 in freshwater medium. There was no change in Fe concentration despite polyphosphate formation.

The experiment was repeated for the P-limited cultures of Calothrix D253 and Anabaena D697, but in freshly prepared medium with high P (10 mg L^{-1}). As before, there was no rapid accumulation, suggesting that polyphosphate granules did not enhance accumulation of Fe. These results suggest that neither is Fe essential for polyphosphate formation nor is the latter essential for uptake of Fe.

8 INFLUENCE OF Fe-LIMITATION ON NITROGENASE ACTIVITY

8.1 Changes in nitrogenase activity during growth in batch culture in saline and freshwater media

The formation of secondary heterocysts in Rivulariaceae (4.21) in response to Fe-limitation suggests this change may reflect a change in ability to fix nitrogen. As Fe is a major component of nitrogenase, an experiment was planned, therefore, to investigate changes in nitrogenase activity during growth under conditions which eventually lead to Fe-limitation. The experiment was carried out in medium with normal (0.4 mg L^{-1}) and a low Fe : EDTA ratio, high-P (10 mg L^{-1}) (see 5.2). The inoculum was prepared as in 2.561 and experiment was carried out under standard conditions (2.44). Calothrix D253 was grown in saline and freshwater media, Anabaena D697 in saline medium and Calothrix D550 in freshwater medium.

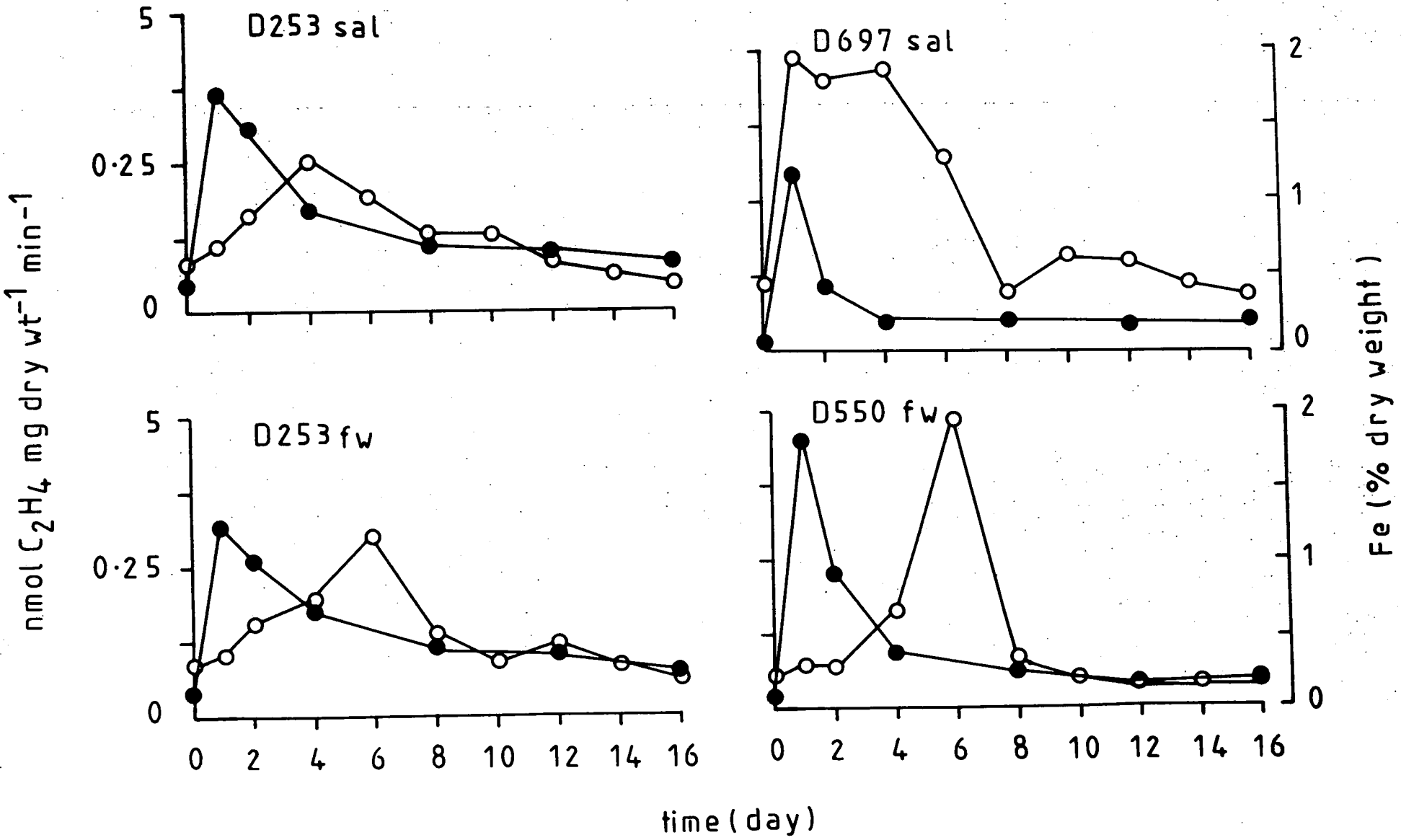
In all cases nitrogenase activity increased gradually (Fig. 8.1) to reach maximum values and then decreased subsequently. The peak of nitrogenase activity came three to five days after the peak of Fe in the tow Rivulariaceae, but was reached at the same time as the Fe peak in Anabaena D697. The maximum and minimum values during the experiments were:

strain		medium	nitrogenase activity (nmol C_2H_4 mg d wt^{-1} min^{-1})	
			max.	min.
Calothrix	D253	saline	2.5	0.47
Calothrix	D253	freshwater	3.0	0.61
Calothrix	D550	freshwater	4.8	0.26
Anabaena	D697	saline	4.7	0.92

Figure 8.1 Influence of Fe status on nitrogenase activity by algae during growth in medium leading to Fe-limitation: Calothrix D253, Anabaena D697 in saline, Calothrix D253 and Calothrix D550 in freshwater

(○) nitrogenase activity

(●) algal Fe composition


D253 sal
D697 sal
D253 fw
D550 fw
5
0·25
0
2
1
0
0 2 4 6 8 10 12 14 16
nmol C_2H_4 mg dry wt^{-1} min^{-1}
Fe (% dry weight)
time (day)

8.2 Influence of Fe addition on nitrogenase activity by Fe-limited cultures in saline and freshwater media

This was tested on Fe-limited cultures of Calothrix D253 and Anabaena D697 in saline medium and Calothrix D550 in freshwater medium as described in 2.561 (omission of Fe and reduction of EDTA). Before Fe addition nitrogenase activity increased gradually (Fig. 8.2) to reach the maximum on day 4 with all cultures (Fig. 8.2), and then initially decreased sharply, subsequently approaching a constant rate by day 10. Iron addition on day 10 led to a rapid increase (detectable within one h) in nitrogenase activity (Fig. 8.2), which reached a maximum within 24 h and then decreased again:

strain		medium	nitrogenase activity (nmol C_2H_4 mg d wt^{-1} min^{-1})			
			before Fe addition		after Fe addition	
			max.	min.	max.	min.
Calothrix	D253	saline	2.10	0.92	3.43	0.53
Calothrix	D550	freshwater	3.00	0.49	3.10	0.37
Anabaena	D697	saline	3.20	1.30	3.40	1.80

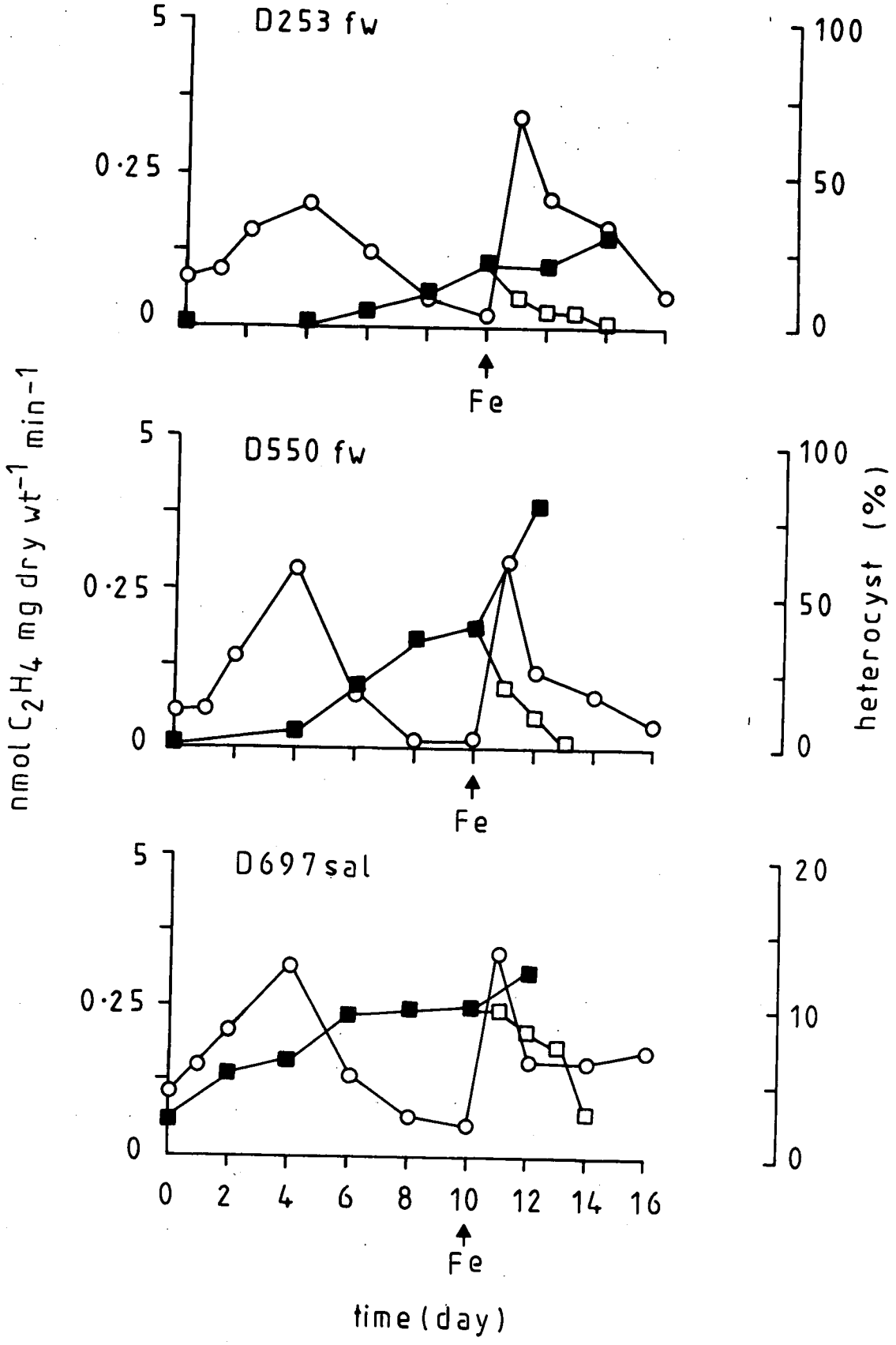
The experiment was repeated by resuspending the culture of Calothrix D253 in freshly prepared medium rather than adding Fe to the old culture. A Fe-limited culture (prepared as above) was homogenized, washed and aliquots were inoculated into saline medium minus FeEDTA. The alga was allowed to equilibrate for 30 min before Fe addition. A linear increase ($y = 0.169x - 0.31$; $r = 0.997$) in nitrogenase activity with time was found (Fig. 8.3) and no decline after 24 h as before, suggesting that limitation of some other element might have been developing in the previous experiment.

Figure 8.2 Influence of addition of Fe on nitrogenase activity by algae during growth in - Fe saline (D697) and freshwater (D253, D550) medium

(○) nitrogenase activity

(■) frequency of heterocyst without Fe being added

(□) frequency of heterocyst after Fe being added


D253 fw
D550 fw
D697 sal
Fe
Fe
Fe
5
0·25
0
100
50
0
20
10
0 2 4 6 8 10 12 14 16
nmol C_2H_4 mg dry wt^{-1} min^{-1}
heterocyst (%)
time (day)

8.3 Influence of inhibitor of protein synthesis on nitrogenase activity in saline and freshwater media

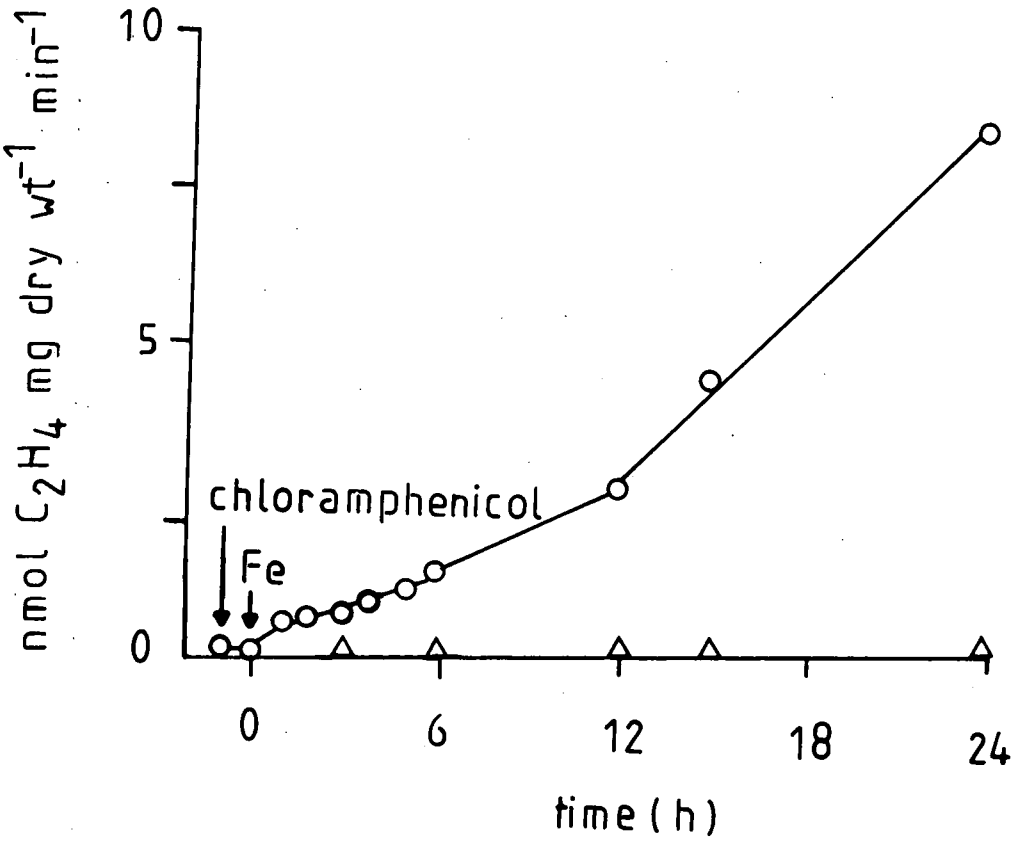
It was not clear whether the rapid increase in nitrogenase activity (Fig. 8.2) was due to activation of the process by Fe or to synthesis of certain proteins. Therefore, the influence of an inhibitor (chloramphenicol, 25 mg L^{-1}) for protein synthesis was tested on nitrogenase activity. A Fe-limited culture of Calothrix D253 was prepared as in 8.2. and the experiment was carried out in saline medium.

Chloramphenicol addition (30 min before Fe addition) prevented any increase in nitrogenase activity (Fig. 8.3), presumably by inhibiting the synthesis of nitrogenase.

Figure 8.3 Influence of of chloramphenicol addition (prior to Fe) on nitrogenase activity by Fe-limited culture of Calothrix D253 grown in saline medium. Chloramphenicol was added to a final concentration of 25 mg L^{-1} where indicated by the arrow

(○) nitrogenase activity in absence of inhibitor

(△) nitrogenase activity in presence of inhibitor


nmol C_2H_4 mg dry wt^{-1} min^{-1}
10
5
0
chloramphenicol
Fe
0
6
12
18
24
time (h)

9 DISCUSSION

9.1 Morphological and cytological response to Fe-limitation

All Rivulariaceae strains responded markedly to increasing Fe deficiency (4.3). Hormogonia production, which occurred vigorously during early stages of growth, started to decrease as nutrient depletion led to Fe-limitation (4.3). A similar response was shown to P-deficiency (4.4), as has been reported previously for Calothrix D184 (Wood, 1984) and Calothrix D550 (Livingstone & Whitton, 1983).

Another morphological feature as a response to increasing Fe-limitation is the development of intra-thylakoidal vacuoles in otherwise quite typical vegetative cells, features easy to see with the light microscope (4.3), though requiring the electron microscope for confirmation (4.3). This is similar to previous results (Sinclair & Whitton, 1977; Douglas et al., 1986) and has been proposed as to be a pathological response since cells were able to regain their original appearance upon restoration of the element in question (Ueda, 1971). Such vacuolation varied among strains, but was most pronounced in Calothrix D550. Possible explanations for the vacuolation may be to condense vegetative material, decrease the demand for Fe, or due to a reduction in membrane synthesis.

An early effect of Fe-limitation was a decrease in content of cyanophycin granules, perhaps because Fe-limitation led to a decrease in nitrogenase activity (Chapter 8, 9.8). However Anabaena D697 showed the opposite response, with increased formation of cyanophycin granules. This may be associated with the fact that increasing Fe-limitation leads to increased heterocyst frequency in Anabaena, in contrast to the response shown by Calothrix (see below). This fits with results in Chapter 8 where Anabaena

D697 maintained a relatively high rate of nitrogenase activity for longer than Rivulariaceae strains.

Another marked response to Fe-limitation was the formation of a new heterocyst apical to the original one (4.3), similar to that reported by Sinclair & Whitton (1977), Wood (1984) and Douglas et al. (1986) for various Calothrix strains. Increasing Fe deficiency led to repeated replacement of the heterocyst, with a series of collapsed heterocysts basal to it. Douglas et al. suggested that this successive formation of heterocysts may be associated with a switch to molecular components with reduced demand of Fe. The previous heterocysts presumably become non-functional, possibly by loss of the ability to synthesize nitrogenase or its destruction.

Ultrastructure of the heterocyst was also markedly affected by Fe-limitation. Comparison of the collapsed and secondary heterocysts in Calothrix D550 or the normal vs Fe-limited in Anabaena D697 (4.22) revealed a marked decrease in the granulation and membrane content of the heterocyst. One possible explanation is that Fe acts as a structural component of membranes or plays a key role in their synthesis.

All Rivulariaceae strains produced hairs under conditions of P-limitation and Fe-limitation (4.3, 4.4), although Calothrix D253 did so only in freshwater medium (see below). However other strains have been reported by Sinclair & Whitton (1977), which form hairs as a response to P-limitation, but not Fe-limitation. Hairs formed as a response to Fe-limitation were less frequent and much shorter than those formed in response to P-limitation. Similar data were recorded for Calothrix D253 (Whitton et al., 1973) and Calothrix D550 (Douglas et al., 1986) and for the marine C. crustacea (Guglielmi, 1975).

Increased hair formation under nutrient limitation suggests a role in the absorption of particular nutrient(s) in question (Palla, 1893; Sinclair &

Whitton, 1977), either by facilitating the passage of nutrients through the large surface of hairs, or by carrying the necessary binding sites along their length. Whatever the physiological role of hairs under Fe- or P-limitation, it is clear they are not required when the element in question is unlimited.

One of the most striking observations on hair formation was the influence of salinity in Calothrix D253. Hairs did not form in saline medium with either Fe- or P-limitation. Downshift of cultures from saline to freshwater medium led to development of synchronized hairs from the apical region of most trichomes. This phenomenon may be accounted for one of the following:

(a) switch of materials from hair-formation to osmoregulatory product;

(b) interaction of Na with Fe and/or P metabolism.

Fe-deficiency led to the development of a brown pigmented sheath by Calothrix D253 (4.3), a morphological effect similar to that reported for four (D184, D251, D253, D266) of the 13 Calothrix strains studied by Sinclair & Whitton (1977). All the strains studied by the latter authors showed sheaths which became thicker as a response to Fe-deficiency, but only two strains (D253, D696) did so in the present study. Sheath material of Calothrix D550 has been reported (Weckesser et al., 1988) to bind Fe the most effectively of any heavy metal tested.

A pale appearance of all strains was another marked response to Fe-limitation, whilst normal colour was observed under P-limitation (4.3, 4.4). This chlorotic effect is likely to be due to a reduction in chlorophyll synthesis (1.3); see also Douglas et al. (1986). A striking response during recovery of Fe-limited cultures as a result of Fe addition (but not other nutrients) is the rapid formation of a few blue heterocysts in Anabaena D697 (4.24); these heterocysts are much bluer than normal

vegetative cells. There are reports which refer to a similar colour change in response to combined N by Tiwari (1978) in Nostochopsis lobatus and Whitton et al. (1986) for heterocysts of Calothrix D603. In the present study heterocysts at an early stage of differentiation may begin to dedifferentiate to normal vegetative cells.

9.2 Comparison of Fe content in algae grown in batch culture

The algal strains responded to nutrient limitations not only by morphological and cytological changes, but also by physiological responses. The similarities between the changes in Fe content by all strains suggest a parallel physiological response for storage of Fe. All strains reach a maximum Fe content by 24 h during growth in batch culture in medium designed eventually to lead to Fe-limitation (Chapter 5).

The maximum Fe content for any strain (1.43 % and 1.80 % dry weight in saline and freshwater media, respectively) in the present study is less than that (4.59 % dry weight) reported by Hayward (1969) for the diatom Phaeodactylum tricornutum and that (3.45 % dry weight) reported by Knauss & Porter (1954) for the green alga Chlorella. The minimum value (0.07 % and 0.06 % dry weight in saline and freshwater media, respectively) in relatively healthy culture found in the present study agrees with that (0.06 % dry weight) for Synechoccoccus sp. (Entsch et al., 1983).

Overall the results provide evidence which clearly illustrates the great flexibility in capacity of blue-green algae during growth in saline and freshwater media to accumulate Fe. Accordingly, based on the definition of "overplus" by Voelz et al. (1966) and Weinberg (1975), it is suggested that the results (6.1) indicate that an "iron overplus" phenomenon similar to that for P occurs in the strains studied here. The physiological and ecological importance of Fe in blue-green algae underlines the usefulness of

"iron overplus" by storage of Fe during periods of excess and utilization when it scarce. This gives the blue-green algae the ability to compete successfully with other groups of algae in the natural environment.

Iron limitation was achieved in two different ways, by using either - Fe medium (4.3), or a medium with low Fe (Chapter 5) and allowing the alga to remove this Fe progressively during growth. The strains were grown in low Fe (0.4 mg l^{-1}), high EDTA (X 3 in saline, X 2 in freshwater) and high P (10 mg l^{-1}) and growth continued in batch culture. This led to an important difference in the morphological changes to those observed when the alga was subcultured directly to - Fe medium. No hairs were formed with any strain, although the other morphological features noted in - Fe medium (4.3) still occurred. A possible explanation is the higher EDTA concentration in the batch culture experiment than in the - Fe medium.

Secondary heterocysts were formed long before the Fe concentration in cultures had reached the mimimum value. During growth in batch culture leading to Fe-limitation, the Fe contents when secondary heterocysts were first observed were: 0.31 % (D253), 0.32 % (D696) dry weight in saline medium and 0.40 % (D253), 0.18 % (D550) dry weight in freshwater medium.

9.3 Is there any interaction between Fe- and P-limitation ?

It has been suggested (Douglas et al., 1986; Whitton, 1987) that hair formation as a response to Fe-deficiency may result from an interaction of Fe and P in some cell process leading to P deficiency, despite a high P concentration in medium. Differences in morphological, cytological and physiological responses to either Fe- or P-limitation, which may help to consider possible interaction, may be summarized as follows:

(a) In Fe-limitation (- Fe medium), hairs are present at high cellular P (e.g. 4.7 % dry weight for D550) and external P (8.79 mg L^{-1}), whilst they

were present with P at 0.97 % dry weight under P-limitation (low P medium). This indicates that neither cellular nor external P stopped hair formation in response to Fe-limitation and therefore the hairs were a clear response to Fe-limitation. This might underline the possible functional siginficance of hairs in response to Fe-limitation.

(b) The formation and presence of polyphosphate granules in all strains under Fe-limitation suggests that Fe is not needed in uptake of P. The formation of polyphosphate granules by P-limited cultures had no influence on Fe accumulation, suggesting that cellular P is not needed in Fe uptake. The gradual decrease (with time) of polyphosphate granules in Fe-limited culture indicates that P is being supplied to the cell. If interaction does occur, it might occur at the level of DNA synthesis, as Fe-deficiency reduces DNA synthesis in Neurospora crassa (Landner, 1972).

(c) Recovery of cultures from each limitation was a clear response to the addition of the element in question (Chapters 4 and 5). Nonetheless, a possibility is that addition of Fe to Fe-limited cultures led to uptake of Fe and subsequently released the "lock" on availability of cellular P, which would then lead to hairs being shed.

(d) Phosphorus content in Fe-limited cultures is much higher (420 %) than that in P-limited culture, suggesting a negligible chance that the Fe-limited cultures were also P-limited.

(e) The Fe : P ratio may be used as indicator of the type of limitation, decreasing under Fe-limitation, but increasing under P-limitation. This suggests that under the latter demand was for P whilst under the former it was for Fe.

(f) The lack of formation of hairs by Calothrix D253 in saline medium, but not in freshwater medium, in response to either Fe- or P-limitation, conclusively shows that neither low cellular Fe nor P is a unique "trigger"

for hair formation but, in fact, other external factor (NaCl) is involved. The fast rate at which hairs were induced once NaCl is removed suggests the possibility that there is a specific signal for initiation of hair formation.

According to the above, it appears superficially that there is no interaction between Fe- and P-limitation, but neither biochemical nor molecular interaction can be excluded.

9.4 Siderophore production

None of the five strains tested were able to grow in saline medium in the absence of FeEDTA; however in freshwater media three strains (Calothrix D550, Calothrix D603, Anabaena D697) were able to grow in absence of FeEDTA and produced a siderophore (6.4). In the case of Calothrix D550 and D603, the presence of a siderophore was shown clearly by the experiment (6.42) on prolonged growth in the presence of Fe, but absence of EDTA. Inhibition of growth of Calothrix D253 and Dichothrix D696 is presumably due to these strains possessing little, if any, ability to form a siderophore. This cannot explain the absence of growth of some strains in freshwater, but not marine medium. The inability of the two strains to grow in either medium may perhaps be due to mutation through prolonged subculturing in the presence of EDTA. It must be stressed, however, that growth in the laboratory, particularly in batch culture, is different to that in the natural environment, in which alga might obtain Fe by other ways than use of siderophore.

Excretion of siderophore under Fe stress indicates a high degree of cellular regulation over Fe-assimilatory mechanisms and Fe-dependent pathways. Indeed, regulation of the aerobactin operon has been characterized on plasmid ColV-K30 of E. coli (Bagg & Neilands, 1987).

9.5 Influence of algal Fe status on nitrogenase activity

Wurtsbaugh and Horne (1983) reported that rates of nitrogenase activity in blue-green algal populations were stimulated by up to 500 % above control levels by Fe additions in laboratory and in situ. In the present study, addition of Fe at day 10 (8.2) led to a rapid increase in nitrogenase activity of Calothrix D253 (X 19), Calothrix D550 (X 21), Anabaena D697 (X 7). This may be explained by Fe addition leading to rapid synthesis of either nitrogenase or other Fe-containing molecules required in the fixation process. Chloramphenicol prevented any stimulation of nitrogenase activity in response to Fe addition to Fe-limited culture, suggesting inhibition of protein synthesis.

During growth in batch culture in medium designed to lead eventually to Fe-limitation, there was first a rapid increase in nitrogenase activity, followed by a sharp decrease in activity (8.1). The peaks of nitrogenase activity (in Rivulariaceae) came approximately three to five days after the peak of Fe content had dropped to 0.6 % (D253) or 0.3 % (D550) dry weight. The rate of nitrogenase activity has been reported to be positively correlated with Fe availability (Elder & Horne, 1977). This correlation could be expressed, in the present study, using the ratio between the rate

of increase in activity and the rate of decrease in Fe content, which was approximately constant (1.67) between days one and four.

In Anabaena D697 there were two marked differences from Rivulariaceae strains:

(a) Peak of nitrogenase activity reached soon after the Fe peak and continued for three days, by which time the Fe content had dropped to 0.2 % dry weight; this may be associated with the fact that increasing Fe-limitation leads to increased heterocyst frequency in Anabaena, in contrast to the response shown by Calothrix.

(b) Although the minimum nitrogenase activity was similar to that in Rivulariaceae strains the minimum Fe content in the latter was lower; apparently the latter may be more efficient in using Fe.

The peak of activity in Calothrix D253 in saline medium extended longer and came two days earlier than in freshwater medium, but both ended at the same day, coresponding to a similar Fe content. This suggests that nitrogenase activity became Fe-limited at the same time, although accelerated differently at different salinities. Conversely, Niven et al. (1987) showed that nitrogenase activity by freshwater Anabaena variabilis was inhibited within 30 min of exposure to NaCl (50, 100, 200 mM), but recovered within 24 h, possibly because the rapid need for ATP in Na efflux led to a shortage of ATP and, subsequently, to a reduction in nitrogenase activity.

9.6 Concluding remarks

The overall observations in this study show that Fe-limitation has a marked influence on morphology, cytology and physiology of Rivulariaceae strains, though differing in several important ways from that in Anabaena D697. The successive replacement of heterocysts in Rivulariaceae strains

contrasts with the increase in frequency of heterocysts in Anabaena D697, though both are strategies to survive Fe-limitation.

The lack of formation of hairs by Calothrix D253 in saline medium in contrast to freshwater medium in response to Fe- or P-limitation shows that neither low cellular Fe nor P is an unique "trigger" for hair formation, but another external factor (NaCl) is involved. The fast rate at which hairs were induced once NaCl is removed suggests the probability that there is a specific signal for initiation of hair formation.

The increase in Fe content of algae during growth in batch culture in medium designed eventually to lead to Fe-limitation provides evidence which clearly illustrates the great flexibility in capacity of blue-green algae during growth in saline and freshwater media to accumulate Fe. The increase in Fe content greatly exceeded that found during exponential growth, indicating that an "iron overplus" phenomenon similar to that for P occurs in the strains studied here.

The comparison of all responses under Fe- and P-limitation indicates superficially that the two limitations do not interact, but neither biochemical nor molecular interaction can be excluded. The changes in Fe : P ratio in algae during growth in batch culture may be used as an indicator for the type of limitation.

Production of siderophore in response to Fe-limitation by three different strains from three different environments suggests that siderophore secretion may be widespread and that there is a high degree of cellular regulation over Fe-assimilatory mechanisms and Fe-dependent pathways.

The rapid increase in nitrogenase activity as a result of addition of Fe to Fe-limited culture, underlining the importance of Fe in N_2-fixation.

SUMMARY

1. Five Rivulariaceae strains (Calothrix D253, Calothrix D550, Calothrix D603, Dichothrix D696, Calothrix D704) were used for various types of study:

	Calothrix	Calothrix	Calothrix	Dichothrix	Calothrix
culture no	D253	D550	D603	D696	D704
source					
marine	-	-	-	-	+
brackish	+	-	-	+	-
freshwater	-	+	+	-	-
whether study conducted on strain					
morphology	+	+	+	+	+
cytology	+	+	-	+	+
E.M.	-	+	-	-	-
Fe content	+	+	-	+	-
siderophore	+	+	+	+	-
nitrogenase	+	+	-	-	-

Calothrix D253 was the key strain used in all experiments. Anabaena D697 was also included for various types of comparison with the Rivulariaceace.

2. A study was made on morphological and cytological features after subculture into batch culture using three versions of saline and freshwater media (standard, - Fe, low P). The - Fe and low P media were designed specifically to ensure that growth towards the end of batch culture was limited by Fe and P, respectively. In general the response of all the Rivulariaceae was similar for any one particular medium, but there were marked differences between media.

3. In - Fe medium trichomes were characterized (on moving from base to apex) by: formation of a series of new heterocysts apical to the original heterocyst, intra-thylakoidal vacuolation in vegetative cells, pale appearance, absence of cyanophycin granules, presence of polyphosphate

granules and a high frequency of trichomes with hairs (except Calothrix D253 in saline medium: see below), but with these hairs only short. A brown sheath was formed in one strain (Calothrix D253) (4.3). In terms of a time sequence the first morphological indication of Fe-deficiency was the development of intra-thylakoidal vacuoles in vegetative cells, followed by formation of hairs.

4. Iron limitation was also achieved in a different manner, by using a medium with low Fe and allowing the alga to remove this Fe progressively during growth: the strains were grown in low Fe (0.4 mg l^{-1}), high EDTA (X 3 in saline, X 2 in freshwater) and high P (10 mg l^{-1}) and growth continued in batch culture. This led to an important difference in the morphological changes to those observed when the alga was subcultured directly to - Fe medium. No hairs were formed with any strain, although the other morphological features noted in (3) still occurred.

5. In low P medium, the following features were noted: no extra heterocysts, presence of cyanophycin granules and absence of polyphosphate granules, no obvious colour change and both a high frequency of hairiness and with long hairs (4.4).

6. In Calothrix D253 (but not other strains) hairs did not form in saline medium in either - Fe or low P medium; this effect was demonstrated using seawater or additional NaCl as the source of salinity. Downshift from old cultures in a - Fe, low P version of saline medium to a similar version of freshwater medium led to synchronized development of hairs in the apical region of most trichomes. This is in marked contrast to hair formation under normal circumstances, where many stages are present at the same time.

A reciprocal upshift from freshwater to saline medium led to loss of hairs, whether formed as a response to Fe- or P-limitation (4.5).

7. The addition of Fe and P (but not other nutrients) led to recovery of growth in the respective limited cultures. Recovery from Fe-limitation included (in temporal sequence): loss of the collapsed heterocysts, colour restoration, loss of vacuolation, loss of hairs, appearance of cyanophycin granules and, finally, differentiation and release of hormogonia. Electron microscopy of Calothrix D550 confirmed that the vacuoles in Fe-limited vegetative cells are intra-thylakoidal and that addition of Fe leads to the thylakoids recovering their normal shape within 24 h.

8. Recovery from P-limitation included (in temporal sequence): rapid increase in polyphosphate granules, decrease in cyanophycin granules, loss of hairs and differentiation and release of hormogonia.

9. Changes in Fe content were studied during growth in batch culture in three strains (Calothrix D253, Calothrix D550, Dichothrix D696) using saline and freshwater versions of a medium designed to avoid precipitation of Fe: low Fe (0.4 mg l^{-1}) and high EDTA (X 3 in saline, X 2 in freshwater); it was shown that less than 2 % of Fe was removed by a GF/C filter. Use of this medium led eventually to Fe limitation. The maximum Fe content was reached by 24 h (1.42 % and 1.80 % dry weight in saline and freshwater medium, respectively); at the end of batch culture growth, it had dropped to 0.07 and 0.06 % dry weight in saline and freshwater medium, respectively (5.2).

10. In low P medium, the Fe content ranged within narrow limits (0.38 - 0.10 % dry weight for saline and 0.38 - 0.10 % dry weight for freshwater) throughout the growth period (5.1, 5.2).

11. During growth in batch culture leading to Fe-limitation, Fe contents when secondary heterocysts were first observed were: 0.31 % (D253), 0.32 % (D550), 0.32 % (D696) dry weight in saline and 0.40 % (D253), 0.18 % (D550) dry weight in freshwater medium.

12. The rapid formation of polyphosphate granules by P-limited cultures had no influence on accumulation of Fe.

13. The P content increased in all strains on subculture to low Fe medium reaching a maximum by 24 h, but subsequently decreased markedly. The P contents when hairs were first observed during growth leading to P-limitation (in low P medium) were: 0.60 (D550), 0.30 (D696) % dry weight in saline and 0.30 (D253), 0.97 (D550) % dry weight in freshwater medium.

14. The Fe : P ratio (by weight) decreased with time under conditions leading to Fe deficiency, whilst it increased in conditions leading to P deficiency (low P medium). The mean ratio for the strains was 0.11 at the time hairs were starting to form in - Fe medium. In the case of secondary heterocysts, it was 0.075 when they started to form in - Fe medium and 0.79 and 0.25 in saline and freshwater media, respectively, when they started to form in low Fe medium.

When hairs were formed in low P medium as a result of P-limitation, the mean Fe : P ratio was 1.6 and 1.5 in saline and freshwater media, respectively.

15. The main differences from the Rivulariaceae shown by Anabaena D697 in both (- Fe, low Fe) media leading to Fe limitation were the absence of secondary heterocysts adjacent to the original heterocyst, increase in cyanophycin granules and development of akinetes. Similarities were increase in intra-thylakoidal vacuolation, presence of polyphosphate granules and pale colour. Addition of Fe to Fe-deficient material led to the development of a blue colour in c 1 % of heterocysts, a feature not observed with any Rivulariaceae tested; none of the strains showed blue heterocysts on addition of P to P-deficient culture. Electron microscopy confirmed the loss of intra-thylakoidal vacuolation on addition of Fe to Fe-deficient Anabaena.

16. The production of siderophores by five strains (Calothrix D253, Calothrix D550, Calothrix D603, Dichothrix D696, Anabaena D697) was tested by a spectrophometric method in saline and freshwater media. Three strains (Calothrix D550, Calothrix D603, Anabaena D697) produced a siderophore, but only in freshwater.

17. Influence of Fe status on nitrogenase activity was tested for three strains (Calothrix D253, Calothrix D550, Anabaena D697) in saline and freshwater media in batch culture. In Rivulariaceae strains, peaks of nitrogenase activity came approximately three to five days after the peak of Fe content, by which stage the Fe content had dropped to: 0.6 % (D253), 0.3 % (D550) dry weight. In contrast, with Anabaena peak of nitrogenase activity was reached soon after the Fe peak and continued for three days, by which stage the Fe content had dropped to 0.2 % (D697) dry weight. Addition of Fe to Fe-limited cultures of D253 led to a rapid increase in nitrogenase activity, detectable within one hour and reaching a maximum within 24 h. However nitrogenase activity showed no change when

chloramphenicol was added 30 min prior to the addition of Fe to Fe-limited cultures.

APPENDIX 1. DATA ON Calothrix D704 DURING GROWTH IN STANDARD, - Fe, LOW P MEDIUM.

This appendix presents the data on Calothrix D704, which refers to the text in 4.2, 4.3, 4.4. Definition of the characters are given in 2.9.

Table A1.1 Dimensions (µm) of morphological characters of Calothrix D704 in standard 100 % $ASP_6M(-N)$ medium (4.2).

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	30.5	12.5	2.5	-	-	<1
2	45.5	12.5	2.5	-	-	<1
4	98.5	12.5	2.5	-	-	<1
6	98.5	12.5	2.5	50.5	1.5	<1
8	102.5	12.5	2.5	152.5	2.5	<1
10	110.5	12.5	2.5	349.5	2.5	<1
14	125.5	12.5	2.5	380.5	2.5	<1

Table A1.2 Granulation (% of cell profile) and frequency (%) of morphological changes in D704 in standard 100 % ASP6M(-N) medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	20	10	20	10	60	-	-	-
2	80	30	10	10	60	-	-	-
4	60	20	10	10	60	-	-	-
6	40	20	10	60	60	-	-	33
8	20	10	20	10	20	-	5	60
10	10	-	50	20	10	-	30	77
12	-	-	80	20	-	-	60	76

Table A1.3 Dimensions (µm) of morphological characters of Calothrix D704 in - Fe medium (100 % $ASP_6M(-N)$) (4.3).

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	30.5	12.5	4.5	-	-	<1
2	45.5	12.5	4.5	-	-	<1
4	52.5	12.5	4.5	75.5	1.5	<1
6	50.5	12.5	4.5	120.5	1.5	<1
8	51.5	12.5	4.5	170.5	1.5	<1
10	53.5	12.5	4.5	172.5	1.5	<1
14	50.5	12.5	4.5	175.5	1.5	<1

Table A1.4 Granulation (% of cell profile) and frequency (%) of morphological changes in Calothrix D704 in - Fe 100 % $ASP_6M(-N)$ medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	80	10	10	-	80	-	-	-
2	80	30	-	-	60	-	-	-
4	80	30	-	-	20	+	<1	52
6	80	10	-	-	10	+	10	78
8	80	10	-	-	10	+	20	79
10	80	10	-	-	10	+	40	77
12	60	-	-	-	-	+	40	78
14	30	-	-	-	-	+	60	78

Table A1.5 Dimensions (µm) of morphological characters of Calothrix D704 after addition (day 11) of Fe to Fe-limited cultures in 100 % $ASP_6M(-N)$ medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0 (day 11)	50.5	12.5	2.5	175.5	2.5	<1
1	45.5	12.5	2.5	-	-	<1
2	55.5	12.5	2.5	-	-	<1
3	52.5	12.5	2.5	-	-	<1
5	55.5	12.5	2.5	-	-	<1

Table A1.6 Granulation (% of cell profile) and frequency (%) of morphological characters of Calothrix D704 in 100 % $ASP_6M(-N)$ after addition (day 11) of Fe to Fe-limited cultures

day	polyphosphate		cyanophycin		hormogonia	hairiness
	basal	apical	basal	apical	production	
0 (day 11)	60	10	-	-	-	40
1	60	10	-	-	10	<5
2	60	10	-	5	10	-
3	50	10	-	10	20	-
4	50	10	-	10	50	-

Table A1.7 Dimensions (µm) of morphological characters of Calothrix D704 in low P medium (100 % $ASP_6M(-N)$) (4.4).

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0	30.5	12.5	4.5	-	-	<1
2	35.5	12.5	4.5	-	-	<1
4	80.5	12.5	3.5	30.5	2.5	<1
6	80.5	12.5	3.5	250.5	2.5	<1
8	80.5	12.5	3.5	320.5	2.5	<1
10	100.5	12.5	3.5	370.5	2.5	<1
14	100.5	12.5	3.5	450.5	2.5	<1

Table A1.8 Granulation (% of cell profile) and frequency (%) of morphological changes in D704 in low P 100 % ASP_6M(-N) medium.

day	polyphosphate		cyanophycin		hormogonia	vacuolation	hairiness	trichome
	basal	apical	basal	apical	production			as hair
0	80	10	10	-	80	-	-	-
2	40	10	20	10	60	-	+	-
4	20	-	20	10	20	-	10	27
6	10	-	40	10	10	-	20	76
8	-	-	60	10	10	-	40	80
10	-	-	60	10	<5	-	80	79
12	-	-	60	10	-	-	90	79
14	-	-	60	10	-	-	90	79
16	-	-	60	10	-	-	90	79

Table A1.9 Dimensions (µm) of morphological characters of Calothrix D704 after addition (day 8) of P to P-limited cultures in 100 % $ASP_6M(-N)$ medium.

day	vegetative part of trichome			hair		sheath
	length	basal	apical	length	width	
0 (day 8)	80.5	12.5	2.5	320.5	2.5	<1
1	80.5	12.5	2.5	-	-	<1
2	80.5	12.5	2.5	-	-	<1
3	80.5	12.5	2.5	-	-	<1
4	85.5	12.5	2.5	-	-	<1

Table A1.10 Granulation (% of cell profile) and frequency (%) of morphological characters of Calothrix D704 in 100 % $ASP_6M(-N)$ after addition (day 8) of P to P-limited cultures

day	polyphosphate		cyanophycin		hormogonia	hairiness
	basal	apical	basal	apical	production	
0 (day 8)	-	-	60	10	-	60
1	80	40	40	-	-	-
2	80	40	20	-	20	-
3	80	40	-	-	40	-
4	80	40	-	-	40	-

Table A1.11 Comparison of the morphological changes in responses before and after addition of Fe and P to respective Fe- and P- limited cultures of Calothrix D704 in saline (100 % $ASP_6M(-N)$) medium.

	Fe-limitation		P-limitation	
	Fe addition		P addition	
character	before	after	before	after
hairiness	60	-	80	-
length of hair	175.5	-	370.5	-
trichome as hair	79	-	79	-
secondary heterocyst	60	-	-	-
polyphosphate granules	+	+	-	+
cyanophycin granules	-	-	+	-
thick sheath	-	-	-	-
hormogonia production	-	+	-	+
colour	pale	normal	normal	normal

Fig. A1.1 Morphological changes of Calothrix D704 grown in saline medium (100 % $ASP_6M(-N)$)

(i) standard medium

a) Hormogonium

b) Young trichome with heterocyst

c) Hormogonial release from mature trichome with necridium

d) Mature trichome after ceasing hormogonial release

(ii) - Fe medium

a) Hormogonium

b) Young trichome with heterocyst

c) Hormogonial release from mature trichome with necridium

d) Mature trichome after ceasing hormogonial release

e) Hormogonial release after addition of Fe to Fe-limited culture

(iii) low P medium

a) Hormogonium

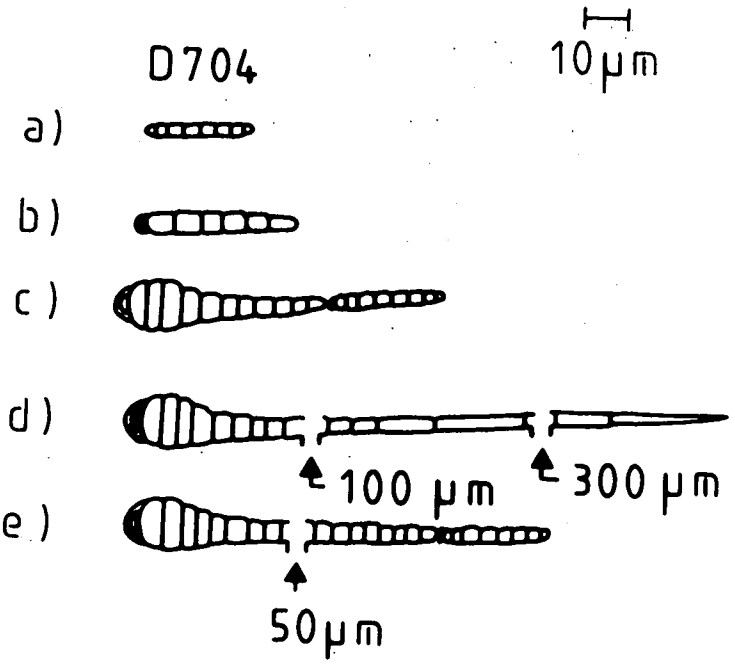
b) Young trichome with heterocyst

c) Hormogonial release from mature trichome with necridium

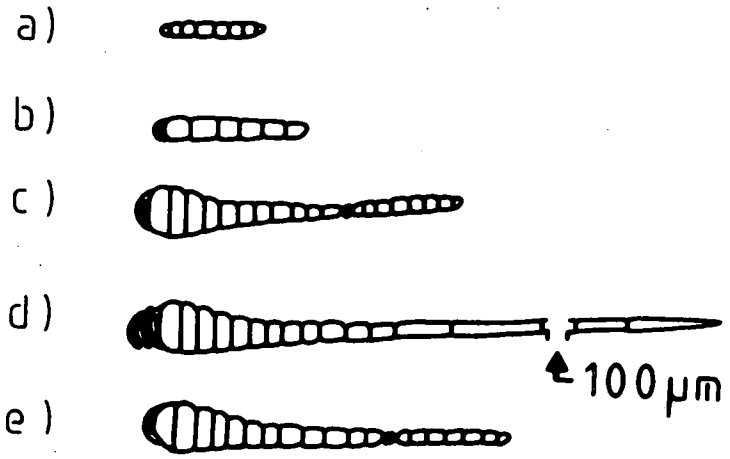
d) Mature trichome after ceasing hormogonial release

e) Hormogonial release after addition of P to P-limited culture

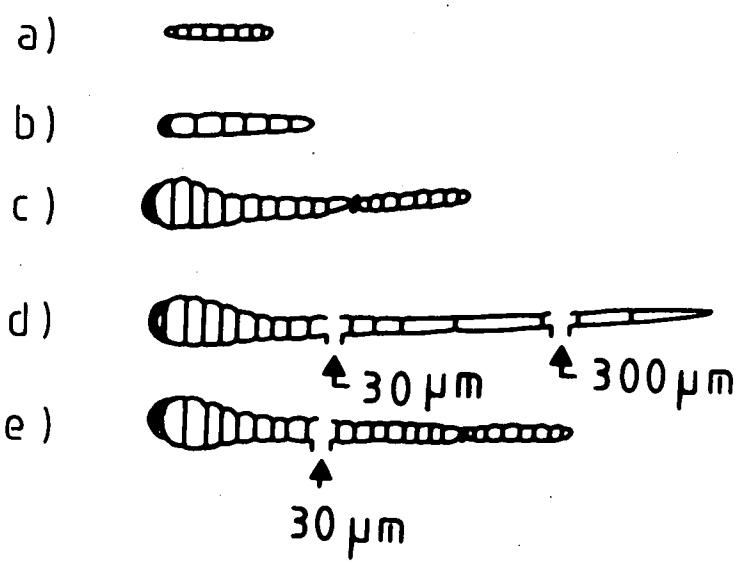
standard medium



-Fe medium



low P medium



APPENDIX 2. DATA ON Anabaena D697 DURING GROWTH IN STANDARD, - Fe, LOW P MEDIUM.

This appendix presents the data on Anabaena D697, which refers to the text in 4.2, 4.3, 4.4. Definition of the characters are given in 2.9.

Table A2.1 Dimensions (µm) of morphological characters of Anabaena D697 in standard saline medium (4.2).

day	vegetative cell		heterocyst		akinete		sheath
	length	width	length	width	length	width	
0	5.5	7.5	10.5	10.5	-	-	
2	5.5	7.5	12.5	12.5	-	-	-
4	5.5	8.5	12.5	12.5	-	-	-
6	5.5	8.5	12.5	12.5	15.5	12.5	-
8	5.5	8.5	12.5	12.5	15.5	12.5	-
10	5.5	8.5	12.5	12.5	15.5	12.5	-
12	5.5	8.5	12.5	12.5	15.5	12.5	3.5
16	5.5	8.5	12.5	12.5	20.5	19.5	7.5
20	5.5	8.5	12.5	12.5	20.5	20.5	7.5

Table A2.2 Granulation (% of cell profile) and frequency (%) of morphological changes in D697 in standard saline medium.

day	polyphosphate	cyanophycin	vacuolation	gas vacuole	heterocyst frquency	akinete frequency
0	25	-	-	80	2.6	-
2	55	-	-	80	2.2	-
4	35	-	-	80	2.7	-
6	15	-	-	35	2.7	+
8	5	5	-	15	2.7	1.0
10	-	5	-	5	2.8	1.5
12	-	25	-	5	2.8	1.5
14	-	65	-	-	2.8	1.5
16	-	65	-	-	2.0	1.5
20	-	65	-	-	4.8	6.5

Table A2.3 Dimensions (µm) of morphological characters of Anabaena D697 in - Fe saline medium (4.3).

day	vegetative cell		heterocyst		akinete		sheath
	length	width	length	width	length	width	
0	6.5	7.5	9.5	10.5	-	-	-
2	6.5	8.5	9.5	10.5	-	-	-
4	6.5	8.5	9.5	10.5	-	-	-
6	7.5	8.5	10.5	12.5	15.5	12.5	-
8	7.5	8.5	10.5	18.5	15.5	12.5	-
10	7.5	8.5	10.5	13.5	22.5	16.5	2.5
14	8.5	10.5	10.5	13.5	25.5	17.5	6.5

Table A2.4 Granulation (% of cell profile) and frequency (%) of morphological changes in D697 in - Fe saline medium.

day	polyphosphate	cyanophycin	vacuolation	gas vacuole	heterocyst frequency	akinete frequency
0	35	0	-	80	2.6	-
2	45	10	35	35	5.6	-
4	35	10	35	15	6.6	-
6	35	20	10	35	9.3	10
8	15	50	60	-	10	9.1
10	10	80	80	-	10	9.5
12	<10	80	80	-	12.5	10.5

Table A2.5 Dimensions (μm) of morphological characters of Anabaena D697 in low P saline medium (4.4).

day	vegetative cell		heterocyst		akinete		sheath
	length	width	length	width	length	width	
0	6.5	7.5	8.5	9.5	-	-	-
2	6.5	7.5	10.5	12.5	-	-	-
4	7.5	9.5	10.5	12.5	-	-	-
6	7.5	9.5	10.5	12.5	15.5	12.5	-
8	7.5	9.5	10.5	12.5	15.5	12.5	-
10	8.5	9.5	10.5	12.5	18.5	12.5	<1
12	8.5	9.5	10.5	12.5	17.5	12.5	2.5
14	8.5	9.5	10.5	12.5	17.5	15.5	5.5
20	8.5	9.5	10.5	12.5	30.5	15.5	5.5

Table A2.6 Granulation (% of cell profile) and frequency (%) of morphological characters of Anabaena D697 in saline after addition (day 12) of Fe to Fe-limited cultures.

day	polyphosphate	cyanophycin	heterocysts frequency	akinete frequency
0 (day 12)	10	80	12.5	9.1
1	10	70	12.5	10.0
2	10	40	9.3	6.3
3	10	20	3.2	2.7
4	10	10	3.2	2.7

Table A2.7 Dimensions (µm) of morphological characters of Anabaena D697 after addition (day 12) of Fe to Fe-limited cultures in saline medium.

day	vegetative cell		heterocyst		akinete		sheath
	length	width	length	width	length	width	
0 (day 12)	6.5	7.5	12.5	12.5	15.5	12.5	4.5
1	6.5	7.5	12.5	12.5	15.5	12.5	4.5
2	6.5	7.5	7.5	10.5	12.5	12.5	4.5
3	6.5	7.5	7.5	10.5	10.5	9.5	-
4	6.5	7.5	7.5	10.5	10.5	9.5	-

Table A2.8 Granulation (% of cell profile) and frequency (%) of morphological changes in Anabaena D697 in low P saline medium.

day	polyphosphate	cyanophycin	vacuolation	gas vacuole	heterocyst frequency	akinete frequency
0	5	-	-	70	2.6	-
2	5	-	-	70	2.2	-
4	-	-	-	70	2.3	2.3
6	-	15	-	35	2.5	2.5
8	-	25	-	20	2.5	3.0
10	-	30	-	20	3.0	4.0
12	-	40	-	7	2.7	4.0
16	-	60	-	7	2.9	5.0

Table A2.9 Granulation (% of cell profile) and frequency (%) of morphological characters of D697 in saline after addition (day 8) of P to P-limited cultures

day	polyphosphate	cyanophycin	heterocysts	akinete
			frequency	frequency
0 (day 8)	-	30	3	6
1	70	20	3	6
2	70	10	3	3
3	70	-	3	3

Table A2.10 Comparison of the morphological changes in responses before and after addition of Fe and P to respective Fe- and P- limited cultures of Anabaena D697 in saline medium.

	Fe-limitation		P-limitation	
	Fe addition		P addition	
character	before	after	before	after
polyphosphate granules	+	+	-	+
cyanophycin granules	+	-	+	-
sheath	+	-	+	-
colour	pale	normal	normal	normal
akinetes	+	+	+	+
blue heterocyst	-	+	-	-

Fig. A2.1 Morphological changes of Anabaena D697 grown in saline medium

(i) standard medium

a) Young filament

b) Mature filament

c) Mature filament with akinete

(ii) - Fe medium

a) Young filament

b) Mature filament with vacuolation

c) Mature filament with akinete

(iii) low P medium

a) Young filament

b) Mature filament

c) Mature filament with akinete

standard medium

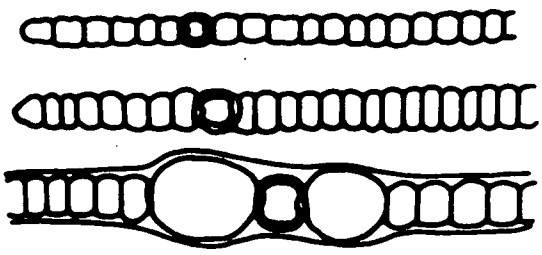
10 µm

D697

a)

b)

c)

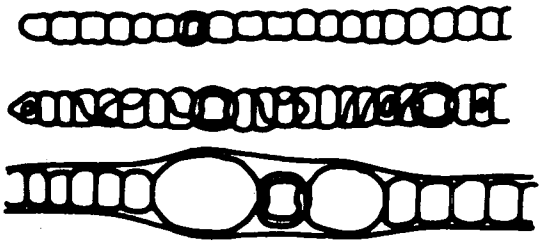


-Fe medium

a)

b)

c)



low P medium

a)

b)

c)

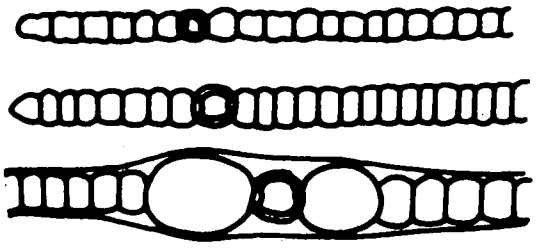


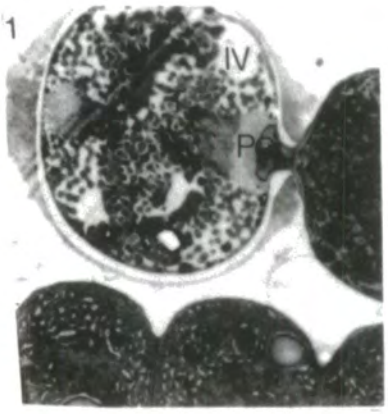
Fig. A2.2 Electron micrograph of Anabaena D697 grown in - Fe saline medium

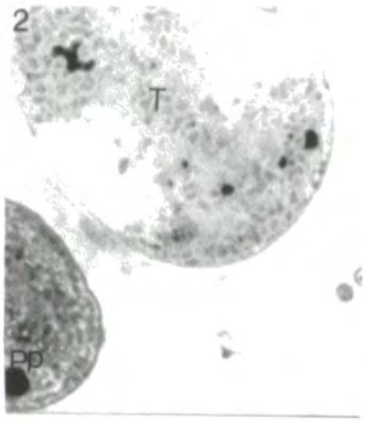
1-2) Heterocyst with thylakoid (T), intra-thylakoid vacuoles (IV), pore channel (PC)

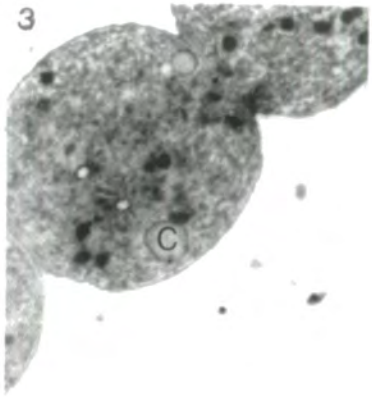
3,5, 7-10) Vegetative cell with thylakoid (T), intra-thylakoid vacuoles (IV), polyphosphate granules (Pp), carboxysomes (Ca), gas vacuole (GV), cyanophycin (C)

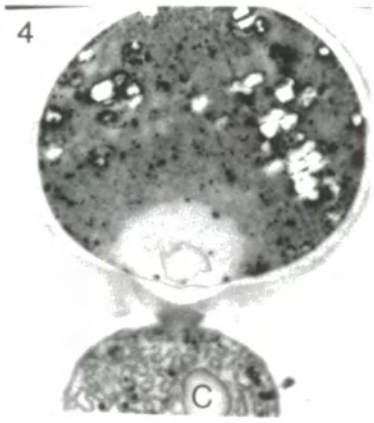
6) Akinete with thick sheath (S), cyanophycin (C)

1-10) were taken at x 5500; 9000; 7000; 7000; 7000; 7000; 11700; 19500; 9000; and 33000 respectively


1
IV
Pp


2
T
Pp


3
C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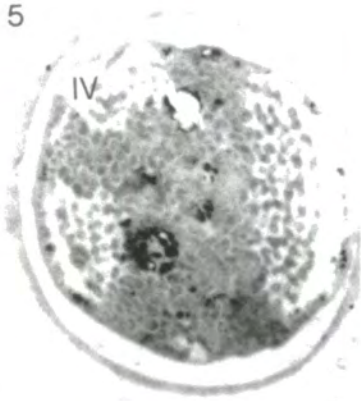
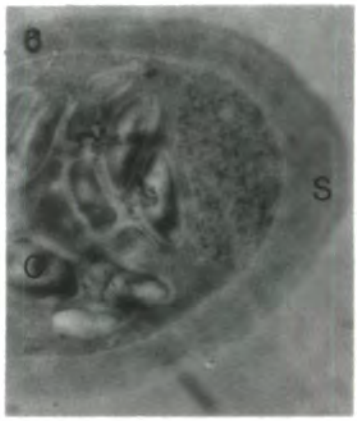
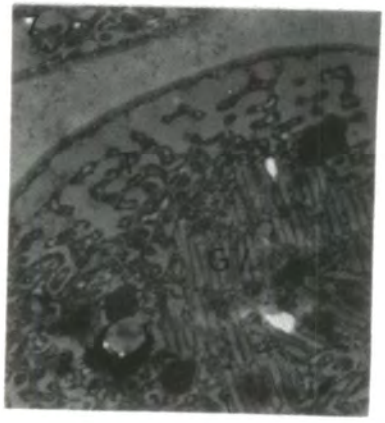
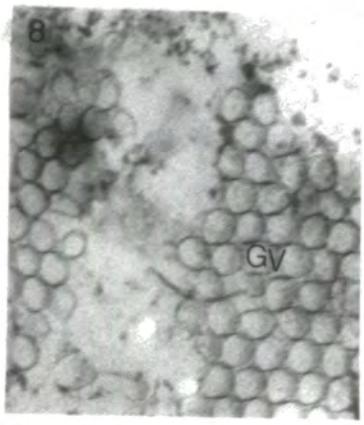
C


5
IV


6
S


7


8
GV


9

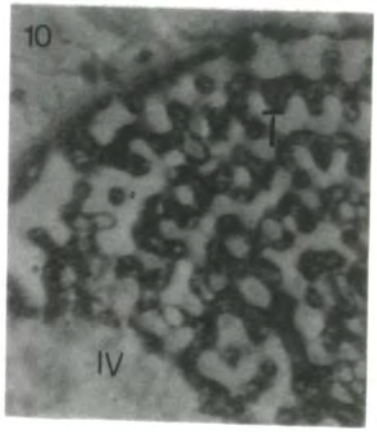

10
T
IV

Fig. A2.3 Optical micrograph of blue heteroccyst (1-8) of Anabaena D697 after addition of Fe to Fe-limited culture in saline medium Blue heterocyst (B Het), vegetative cell (VC), gas vacuoles (GV) and the unrecognized granules (UG)

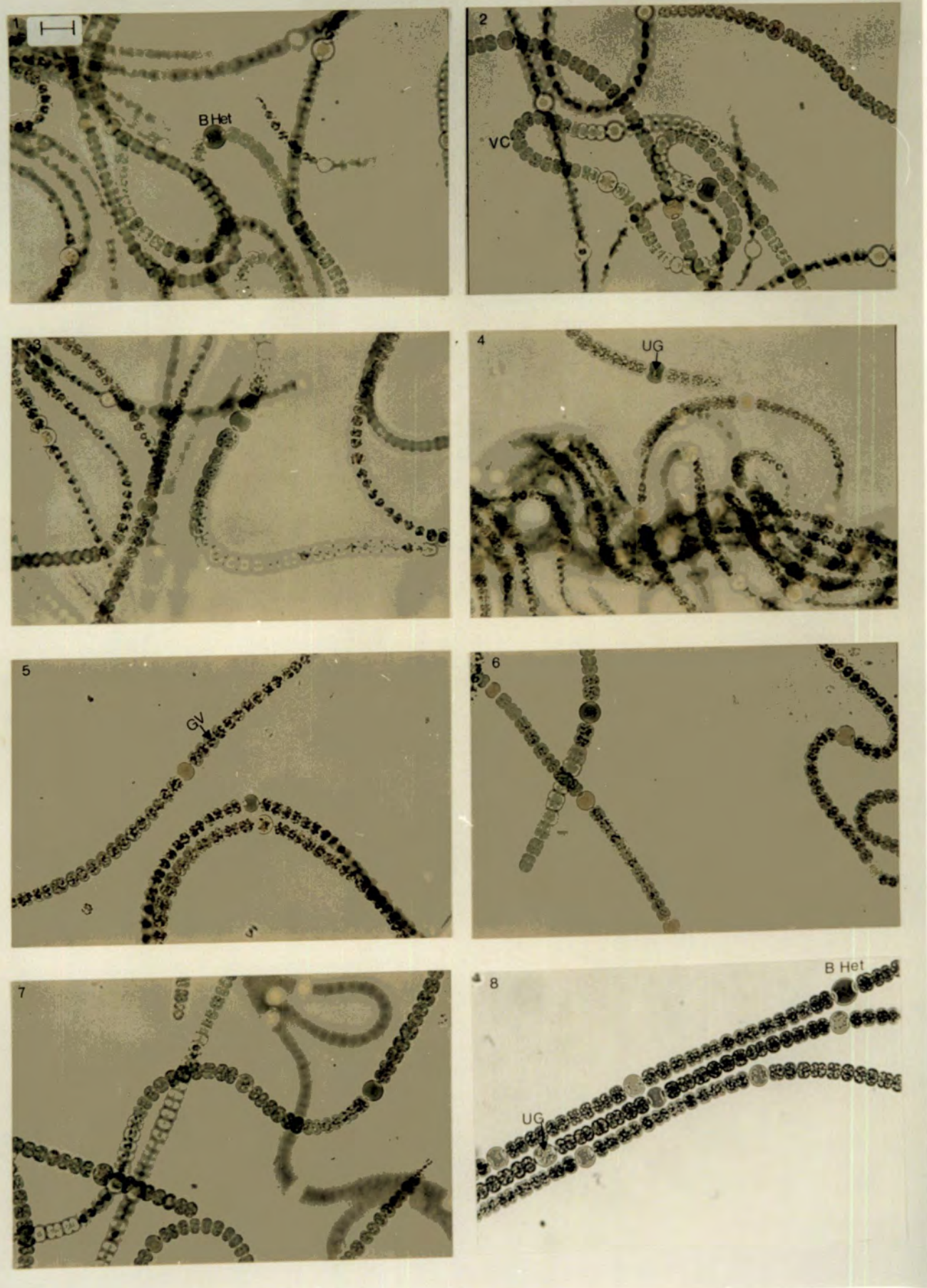
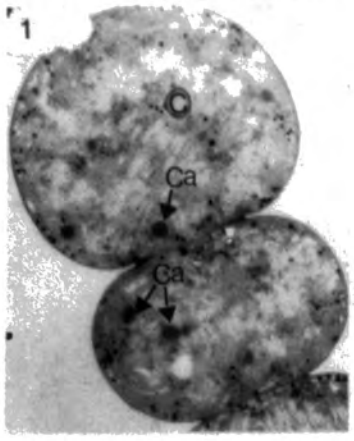
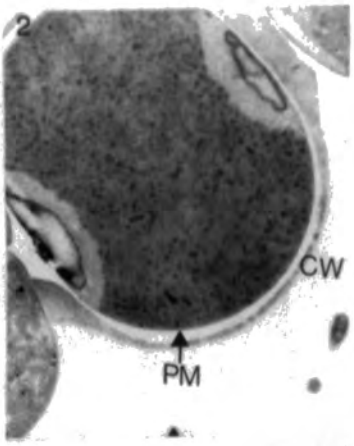

1
B Het
2
VC
3
4
UG
5
GV
6
7
8
B Het
UG

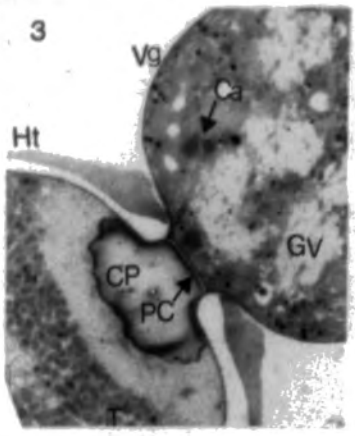
Fig. A2.4 Electron micrograph of Anabaena D697 after addition of Fe to Fe-culture in saline medium

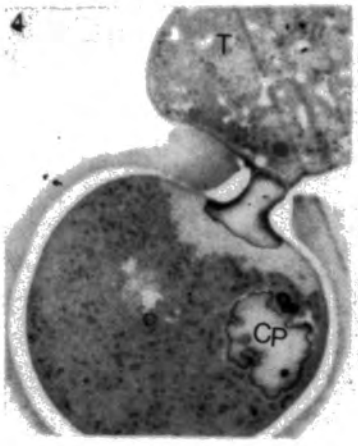
1-12) Vegetative cell (VG), thylakoid (T), polyphosphate (Pp), carboxysomes (Ca), gas vacuole (GV), cyanophycin pool (CP), lipid bodies (Lb)

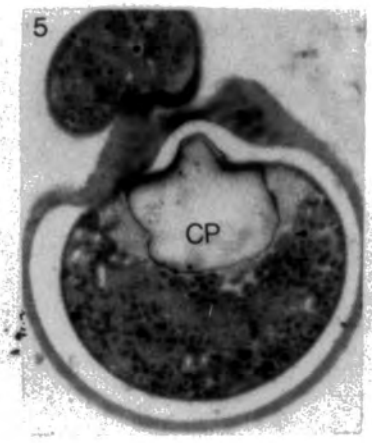
Heterocyst (Het), cyanophycin pool (CP), pore channel (PC), plasma membrane (PM), cell wall (CW). no 1-12 were taken at x 9000; 11700; 11700; 9000; 7000; 11700; 15200; 9000; 5500; 9000; 9000; and 9000 respectively

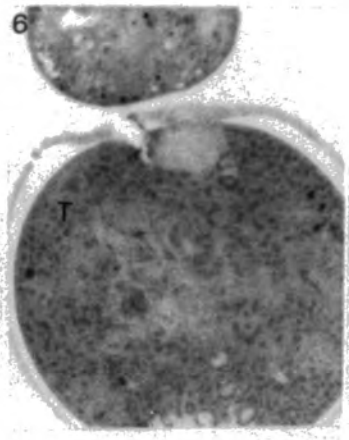

1
C
Ca
Ca

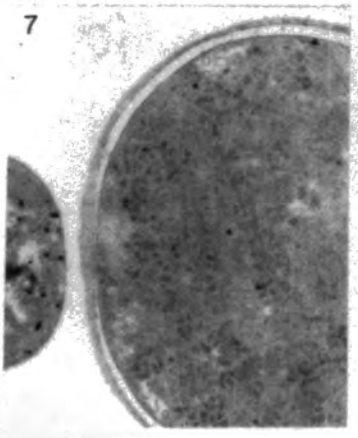

2
CW
PM

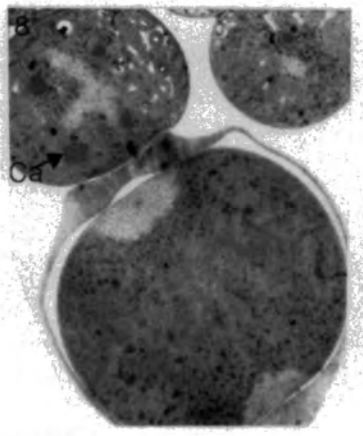

3
Vg
Ca
Ht
Gv
CP
PC
T

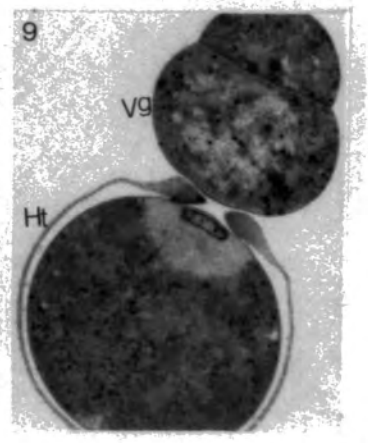

4
T
CP


5
CP

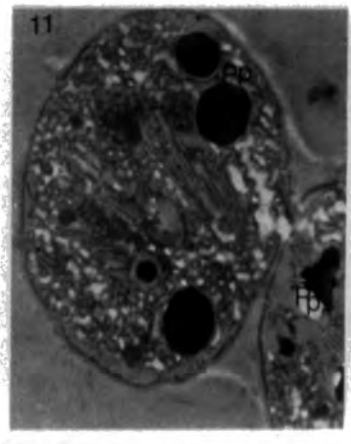

6
T


7


8
Ca


9
Vg
Ht


10
T


11
CP
Hb


12

APPENDIX 3. CULTURE COLLECTION RECORDS

This appendix presents the data recorded for each strain, used in the present study, on the Durham Culture Collection database. The record are presented in the standard format for output from the database.

```
D0253   CALOTHRIX          VIGUIERI                FREMY
Strain                       Repeat isolates:
Country found:   Date:      Finder:                 Details of site:
 CUBA
Culture source:  Date:      Sender:                      Isolated by:
 CCAP 1410/6      30/05/74
Axenic on 02/05/84  and clonal on 00/06/74 by
Growth: temp 25 light            medium AD P(1) Fe(0.4)
```

```
D0550   CALOTHRIX          PARIETINA               THURET
Strain                       Repeat isolates:
Country found:   Date:      Finder:                 Details of site:
 ENGLAND          00/11/79  D.LIVINGSTONE           0020-45
Culture source:  Date:      Sender:                      Isolated by:
                                                      D.LIVINGSTONE
Axenic on 22/08/80  and clonal on 22/08/80 by D.LIVUNGSTONE
Growth: temp 25 light 100        medium AD P(1) Fe(0.4)
```

```
[D0603 ][CALOTHRIX          ][SP.                   ][
 Strain[                      ] Repeat isolates[D0604
 Country found:     Date:     Finder:              Details of site:
[BANGLADESH        ][         ][H.D.CATLING       ][AGRAKHOLA DWR stem
 Culture source:    Date:     Sender:                   Isolated by:
[                  ][00/00/80][                        ][J.W.SIMON
 Axenic on[00/05/82] and clonal on [00/05/82] by[J.W.SIMON
 Growth:  temp[32] light[100     ] medium[CHU 10D -N pH 7.0 + HEPES
 Liquid nitrogen  [06B1][16/07/86 ][CRYOPRES. 10% DMSO.
                  [06B2][16/07/86 ][CRYOPRES. 10% DMSO.
                  [06B3][10/06/83 ][CRYOPRES. NO,
```

```
D0696   DICHOTHRIX         SP.
Strain                       Repeat isolates:
Country found:   Date:      Finder:                 Details of site:
 SEYCHELLES       05/08/84  B.A.WHITTON              ALDABRA
Culture source:  Date:      Sender:                      Isolated by:
                                                      I.A.M.MAHASNEH
Axenic on 28/10/84  and clonal on 28/10/84 by I.A.M.MAHASNEH
Growth: temp 32 light 100        medium CHU 10D / 70% SEAWATER
```

D0697 ANABAENA SP.
Strain
Repeat isolates:
Country found: Date: Finder: Details of site:
SEYCHELLES 05/08/84 B.A.WHITTON ALDABRA Cinq Cases pool
Culture source: Date: Sender: Isolated by:
I.A.M.MAHASNEH

Axenic on not and clonal on 22/11/84 by
Growth: temp 32 light 100 medium ASP6 modified / 30% distilled water

D0704 CALOTHRIX SP.
Strain
Repeat isolates:
Country found: Date: Finder: Details of site:
JORDAN 10/01/85 AMINE SAMMOUR AQABA BAY
Culture source: Date: Sender: Isolated by:
I.A.M.MAHASNEH

Axenic on 04/03/85 and clonal on 04/03/85 by I.A.M.MAHASNEH
Growth: temp 32 light 100 medium ASP6 modified

REFERENCES

Akers H.A. (1983) Isolation of the siderophores schizokinen from soil of rice fields. Applied. environ. Microbiol. 45, 1704-1706.

Allen M.B. and Arnon D.I. (1955) Studies on nitrogen-fixing blue-green algae. Growth and nitrogen-fixation by Anabaena cylindrica Lemm. Pl. Physiol. 30, 366-372.

Almon H. and Böger P. (1984) Nickel-dependent uptake-hydrogenase activity in the blue-green alga Anabaena variabilis. Z. Naturforsch. 39, 90-92.

Anderson L.M. (1984) Iron reduction by juvenile Macrocystis pyrifera (L.) Agardh. Hydrobiologia 116, 493-497.

Anderson M.A. and Morel F.M.M. (1978) Copper sensitivity of Gonyaulax tamarensis. Limnol. Oceanogr. 23, 283-295.

Anderson M.A. and Morel F.M.M. (1980) Uptake of Fe(III) by a diatom in oxic culture medium. Mar. biol. Lett. 1, 263-268.

Anderson M.A. and Morel F.M.M. (1982) The influence of aqueous iron chemistry on the uptake of iron by the coastal diatom Thalassiosira weissflogii. Limnol. Oceanogr. 27, 789-813.

Armstrong J.E. and Van Baalen C. (1979) Iron transport in microalgae: The isolation and biological activity of a hydroxamate siderophore from the blue-green algae Agmenellum quadruplicatum. J. gen. Microbiol. 3, 253-262.

Aston S.R. and Chester R. (1973) The influence of suspended particles on the precipitation of iron in natural waters. Est. Coast. Mar. Sci. 1, 225-231.

Bagg A. and Neilands J.B. (1987) Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. 51, 509-518.

Bailey K. M. and Taub F.B. (1980) Effects of hydroxamate siderophores (strong Fe(III) chelators) on the growth of algae. J. Phycol. 16, 334-339.

Barber R.T., Dugdale R.C., MacIsaac J.J. and Simth R.L. (1971) Variations in phytoplankton growth associated with the source and conditioning of upwelling water. Invest. Pesq. 35, 171-194.

Bates S.S, Tessier A., Campbell P.G.C. and Letourneau M. (1985) Zinc-phosphorus interaction and variation in zinc accumulation during

growth of Chlamydomonas variabilis (Chlorophyceae) in batch culture. Can. J. Fish. Aquat. Sci. 42, 86-94.

Bengis C. and Nelson N. (1977) Subunit structure of chloroplast photosystem I reaction center. J. Biol. Chem 252, 4564-4569.

Bowyer J.W and Skerman V.B.D. (1968) Production of axenic cultures of soil borne and endophytic blue-green algae. J. gen. Microbiol. 54, 299-306.

Brand L.E., Sunda W.G. and Guillard R.R. (1983) Limitation of marine phytoplankton productivity rates by zinc, manganese, and iron. Limnol. Oceanogr. 28, 1182-1198.

Brown J.C. (1965) Iron in chlorosis. A. Rev. Pl. Physiol. 7, 171-189.

Brooks R.R., Presley J. and Kaplan.I.R. (1967) APDC-MIBK extraction system for the determination of trace elements in saline waters by atomic-absorption spectrophotometry. Talanta 14, 809-816.

Byrne R.H. and Kester R.D. (1976) Solubility of hydrous ferric oxide and iron speciation in seawater. Mar. Cehm. 4, 225-274.

Cain J.R., Paschal D.C. and Hayden.C.M. (1980) Toxicity and bioaccumulation of cadmium in the colonial green algae Scenedesmus obliquus. Arch. Environ. Contam. Toxicol. 9, 9-19.

Clarke S.E., Stuart J. and Sanders-Loehr J. (1987) Induction of siderophore activity in Anabaena spp. and its moderation of copper toxicity. Appl. environ. Microbiol. 53 (5), 917-922.

Chester R. and Stoner J.H. (1974) The distribution of zinc, nickel, manganese, cadmium, copper and iron in some surface waters from the world ocean. Mar. Chem. 2, 17-32.

Cohn C.L ,Alam J. and Krogmann, W. (1985) Multiple ferredoxins from cyanobacteria. Physiol. Veg. 23, 659-667.

Cooper L.H.N. (1937) Some conditions governing the solubility of iron. Proc. Roy. Soc. B 124, 299-307.

Davies A. G. (1970) Iron chelation and growth of marine phytoplankton. I. Growth kinetics and chlorophyll production in cultures of the euryhaline flagellate Dunaliella tertiolecta under iron limiting conditions. J. Mar. biol. Ass. U.K. 50, 65-86.

Douglas D, Peat A., Whitton B.A. and Wood P. (1986) Influence of iron status on structure of the cyanobacterium (blue-green alga) Calothrix parietina. Cytobios 47, 155-165.

Eady R.R. and Smith B.E. (1979) Physical-chemical properties of nitrogenase and its components. In: Hardy R.W.F., Bottomley F. and

Burns R.C. (Eds) A treatise on dinitrogen fixation pp 399-490 John and Wiley sons, New York.

Ebel J.P., Colas J. and Muller S. (1958) Recherches cytochimiques sur les polyphosphates inorganiques contenus dans les organisms vivants. II. Mise au Point de methodes de detection cytochimiques specifiques des polyphosphates. Exp. Cell Res. 15, 28-36.

Eisenreich S.J., Bannerman R.T. and Armstrong D.E. (1975) A simplified phosphorus analysis technique. Environ. Letters 9, 43-53.

Elder J.F. and Horne A.J. (1977) Biostimulatory capacity of dissolved iron for Cyanophyceae blooms in nitrogen-rich reservoir. Chemosphere. 9. 525-530.

Entsch B., Sim R.G. and Hatcher B.G. (1983) Indications from photosynthetic components that iron is a limiting nutrient in primary procedurs on coral reefs. Mar. biol. 73, 17-30.

Fan K.C. (1956) Revision of the genus Calothrix. A. Rev. algol. 2, 154-178.

Finden D.A.S., Tipping E., Tawarski G.H.M., Reynolds C.S. (1984) Light-induced reduction of natural iron(III)oxide and its relevance to phytoplankton. Nature, Lond. 309, 783-784.

Fisher N.S., Bjerregaard P. and Fowler S.W. (1983) Interaction of marine plankton with transuranic elements. 1. Biokinetics of neptunimum, plutonium, americium and californium in phytoplankton. Limnol. Oceanogr. 28 432-447.

Fisher N.S., Bohe.M. and Teyssie J-L. (1984) Accumulation and toxicity of Cd, Zn, Ag, and Hg in four marine phytoplankters. Mar. Ecol. Prog. Ser. 18, 201-213.

Fogg G.E., Stewrt W.D.P. and Fay P., Walsby A.E. (1973) The blue-green algae pp. 315 Academic Press, New York.

Fuhs G.W. (1973) Cytochemical examination. In: Carr N.G. and Whitton B.A. (Eds) The Biology of Blue-green algae. 676 pp., pp. 117-143. Blackwell, Oxford.

Giesy J.P. (1976) Stimulation of growth in Scenedesmus obliquus (Chlorophyceae) by humic acids under iron limiting conditions. J. Phycol. 12, 172-179.

Glover H. (1977) Effects of iron deficiency on Isochrysis galbana (Chrysophyceae) and Phaeodactylum tricornutum (Bacillariophyceae). J. Phycol. 13, 208-212.

Goldberg E.D. (1952) Iron assimilation by marine diatoms. Biol. Bull. 102, 243-248.

Goldman C.R. (1972) The role of minor nutrients in limiting the primary productivity of aquatic ecosystems. pp 21-33. In: Likens G.E. (Eds) Nutrients and Eutrophication: the limiting nutrient controversy. 328 pp., pp 21-33. Spec. Sympo. Am. Soc. Limnol. Oceanogr.

Goldman S.J., Lammers P.J., Berman M.S. and Sanders-Loehr J. (1983) Siderophore-mediated iron uptake in different strains of Anabaena sp. J. Bact. 156, 1144-1150.

Guglielmi G. (1975) Etude de quelques espèces marines de Rivularicées en microscopie electronique. Vie et Milieu 25. 189-213

Guikema J.A. and Sherman L. A. (1984) Influence of iron deprivation on the membrane composition of Anacystis nidulans. Pl. Physiol. 74, 90-95.

Haibach M., Pearl H.W. and William G.S. (1985) Relative iron requirements for the growth of freshwater phytoplankton. Abstract in 24th. Northeast algal symposium.

Haraguchi H. and Fuwa.K (1982) Marine analysis by atomic absorption spectrometry. In: Cantle J.E. (Eds) Atomic Absorption Spectroscopy. 448 pp., pp. 95-122. Elsevier, Amstrdam.

Hardie L.P., Balkwill D.L. and Stevens S. E., Jr (1983) Effect of iron starvation on the physiology of the cyanobacterium Agmenellum quadruplicatum. Appl. environ. Microbiol. 45, 999-1006.

Hardy R.F., Burns R.C. and Holsten R.D. (1973) Application of acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. 5, 47-81.

Harrison G.I. and Morel F.M.M. (1983) Antagonism between cadmium and iron in the marine diatom Thalassiosira weissflogii. J. Phycol. 19, 495-507.

Harrison G.I. and Morel F.M.M. (1986) Response of the marine diatom Thalassiosira weissflogii to iron stress. Limnol. Oceanogr. 31, 989-997.

Hartman H. (1984) The evolution of photosynthesis and microbial mats: a speculation on the banded iron formations. In: Cohen.Y., Castenholz R. W. and Halvorson H.O. (Eds) Microbial Mats: Stromatolites. 498 pp., pp. 449-453. Alan R. Liss Inc., N.Y.

Hayward J. (1968) Studies on the growth of Phaeodactylum tricornutum III. The effect of iron on growth. J. Mar. biol. Ass. U.K. 48,295-302.

Healey F.P. and Hendzel C.C. (1980) Physiological indicators of nutrient deficiency in lake phytoplankton. Can. J. Aquat. Sci. 37, 442-453.

Hill-Cottingham D.G. (1955) Photosensitivity of iron chelates. Nature. Lond. 175, 347-348.

Hiyama T., Murakami A., Itoh S., Fujita Y. and Sakurai H. (1985) A low potential iron-sulfur cluster bound to membranes in blue-green algae. J. Biochem., Tokoy 97, 89-95.

Hoshaw R.W. and Rosowaski J.R. (1973) Methods for microscopic algae. In: Stein J.R.(Eds) Handbook of phycological methods. pp. 53-67 Cambridge Univ. Press.

Huntsman S. A. and Sunda W. G. (1980) The role of trace metals in regulation of phytoplankton growth. In: I. Morris (Eds.) The Physiological Ecology of Phytoplankton. 625 pp., pp. 285-328 Blackwell. Oxford.

Hutber G.N., Hutson K.G. and Rogers L.J. (1977) Effect of iron deficiency on levels of two ferredoxins and flavodoxin in a cyanobacterium. FEMS Microbiol. Lett. 1. 193-196.

Hutchinson G.E. (19570 A Treatise on Limnology. Vol.1, Geography. Physics and Chemistry. John Wiley and sons, New York.

Jackson G.A and Morgan J.J. (1978) Trace metal-chelator interactions and phytoplankton growth in seawater media: theoretical analysis and comparison with reported observations. Limnol. Oceanogr. 23, 268-282.

James D.E. (1974) Culturing Algae. Carolina Biological Supply Company. pp. 20.

Johnston R. (1964) Seawater, the natural medium for phytoplankton. 2. Trace metals and chelation and general discussion. J. Mar. biol. Ass. U.K. 44, 87-109.

Kelly J. C.O. (1974) Inorganic nutrients: In Stewart W.D.P. (Eds) Algal Physiology and Biochemistry. pp., pp. 610-635. Blackwell Oxford.

Kester D.R., Oconnor T.P. and Byrne R.H. Jr (1975) Solution chemistry, solubility and adsorption equilibria of iron, cobalt and copper in marine systems. Thallassia Jugosl. 11, 121-134.

Knauss H.J and Porter J.W. (1954) The absorption of inorganic ions by Chlorella pyrenoidosa. Pl. Physiol. Lancaster 29, 229-234.

Konopka A., Kshnur M. (1980) Effect of light intensity upon macromolecular synthesis in cyanobacteria. Microb. Ecol. 6, 291-301.

Landner L. (1972) A possible causal relationship between iron deficiency, inhibition of DNA synthesis and reduction of meiotic recombination frequency in Neurospora crassa. Molec. gen. Genet. 119, 103-117.

Lange W. (1970) Blue-green algae and humic substances. Proc. Conf. Great Lakes Res. 13, 58-70.

Lange W. (1974) Chelating agents and blue-green algae. Can. J. Microbiol. 20, 1311-1321.

Lammers P.J. and Sanders-Loehr J. (1982) Active transport of ferric schizokinen in Anabaena sp. J. Bact. 151, 288-294.

Lewin J. and Chen C.-H (1971) Availability of iron: a limiting factor for marine phytoplankton. Limnol. Oceanogr. 16, 670-675.

Lingle J.C., Tiffin L.O. and Brown J.C. (1963) Iron uptake-transport of Soybeans as influenced by other cations. Pl. Physiol. 38, 71-76.

Livingstone D. and Whitton B.A. (1983) Influence of phosphorus on morphology of Calothrix parietina (Cyanophyta) in culture. Br. phycol. J. 18, 29-38.

Maciasr F.M. (1965) Effect of pH of the medium on the availability of chelated iron Chlamydomonas mundana. J. Protozool. 12, 500-504.

Malmeström B. (1972). The genus Calothrix in the black zone. Bot. mar. 15, 87-90.

Manley S.L. (1981) Iron uptake and translocation by Macrocystis pyrifera. Pl. Physiol. 68, 914-918.

Mantoura R. F.C., Dickson A. and Riley J. P. (1978) The complexation of metals with humic materials in natural waters. Estuar. Coastal mar. Sci. 6, 387-408.

McKnight D.M. and Morel F.M.M. (1979) Release of weak and strong copper complexing agents by algae. Limnol. Oceanogr. 24, 823-837.

McKnight D.M. and Morel F.M.M. (1980) Copper complexation by siderophores from filamentous blue-green algae. Limnol. Oceanogr. 25, 62-71.

McLachlan J. (1964) Some considerations of the growth of marine algae in artificial media. Can. J. Microbiol. 10, 769-782.

McMahon J.W. (1969) The annual and diurnal variation in the vertical distribution of acid-soluble ferrous and total iron in a small dimictic lake. Limnol. Oceanogr. 14, 357-367.

Menzel D.W. and Ryther J.H. (1961) Nutrients limiting the production of phytoplankton in the Sargasso Sea with special reference to iron. Deep Sea Res. 7, 276-281.

Meyer J.M. and Abdullah M.A. (1978) The fluorescent pigment of Pseudomonas fluorescens: biosynthesis, purification and physical chemical properties. J. gen. Microbiol. 107, 319-328.

Miller K.L (1985) Effects of temperature and sodium chloride concentration on the phospholipid and faty acid compositions of a halotolerant Planococcus sp. J. Bact. 162, 263-270.

Miller W.E., Maloney T.E. and Green J.C. (1974) Algal productivity in 49 lake waters as determined by algal assays. Water Res. 8, 667-680.

Moody M.D. and Dailey H.A. (1984) Siderophore utilization and iron uptake by Rhodopseudomonas sphaeroides. Arch. Biochem. 234, 178-186.

Moody M.D. and Dailey H.A. (1985) Ferric iron reductase of Rhodopseudomonas sphaeroides. J. Bact. 163, 1120-1125.

Morel F.M.M. and Hudson.R.T.M. (1985) The Geobiological Cycle of Trace Elements in Aquatic Systems: Redfield Revisited. In : Stumm W. (Eds) Chemical Processes in Lakes. 435 pp., pp. 251-283. John Wiley and Sons, New York.

Morel F.M.M. and Morel-Laurens N.M.L. (1983) Trace metals and plankton in the ocean: Facts and speculations. In: Wong, C.S et al (Eds) Trace Metal in Seawater, Plenum. pp. 841-869.

Morel F.M.M. and Morgan J. (1972) A numerical model for computing equilibria in aqueous chemical system . Environ. Sci. Technol. 6, 58-67.

Morgan J.J. and Stumm W. (1965) The role of multivalent oxides in limnological transformations, as exemplified by iron and manganese. In: Jaag O., Baars J.K. and Pearson E.A. (Eds) Advances in Water Pollution research. 103-118 pp. Pergamon, New York.

Mortenson L.E. and Thorneley R.N.F. (1979) Structure and function of nitrogenase. A. Rev. Biochem. 48, 387-418.

Murphy T.P. Lean D.R.S. and Nalewajko C. (1976) Blue-green algae: their excretion of iron-selective chelators enables them to dominate other algae. Science, N.Y. 192, 900-902.

Nalewajko C. and Lean D.R.L. (1980) Phosphorus, p 235-258. In: I. Morris (Eds). The Physiological Ecological Phytoplankton. Blackwell, Oxford.

Neilands J.B. (1973) Microbial iron transport compounds. In: Eichhorn G.L. (Eds) Inorganic Biochemistry, Vol. 1. 167-202 pp. Elsevier, New York..

Neilands J.B. (1974) Iron and its role in microbial physiology. In Neilands J.B. (Eds) Microbial Iron Transport. pp. 3-34. Academic press, New York.

Neilands J.B. (1977) Siderophores: Biochemical ecology and mechanisms of iron transport in enterobacteria. In: Raymond K.N. (Eds) Advances in Chemistry, no 162, Bioinorganic Chemistry II. pp. 3-32 American Chemical Society, Washington.

Neilands J.B. (1981) Microbial iron compounds. A. Rev. Biochem. 50, 715-731.

Neilands J. B. (1982) Microbial envelope proteins related to iron. A. Rev. Microbiol. 36, 285-309.

Niven G.W., Kerby N.W., Rowell P., Reed R.H. and Stewart W.D.P. (1987) The effects of salt on nitrogen-fixation and ammonium assimilation in Anabaena variabilis. Br. Phycol. J. 22, 411-416.

Öquist G.(1971) Changes in pigment composition and photosynthesis induced by iron deficiency in the blue-green alga Anacystis nidulans. Physiol. plant 12, 183-191.

Öquist G.(1974) Iron deficiency in the blue-green alga Anacystis nidulans: Changes in pigmentation composition and photosynthesis. Physiol. plant. 30, 30-37.

Owens L.D. and Chaney R.L (1971) Iron uptake by Chlorella requires reduction of ferric chelates. Pl. Physiol. 47, (suppl.) 46.

Page W.J. and Huyer M. (1984) Derepression of the Azotobacter vinelandii siderophoresystem using iron-containing minerals to limit iron repletion. J. Bact. 158, 496-502.

Palla E. (1893) Beitrag zur Kentniss des Baues des Cyanophyceen protoplasts. Jb. wiss. Bot. 25, 511-562.

Pakrasi H.B., Goldenberg A. and Sherman L.A. (1985) Membrane development in the Cyanobacterium Anacystis nidulans recovery from iron starvation.

Pl. physiol. 79, 290-295.

Patterson A., Hallbom L. and Bergman B. (1986) Aluminium uptake by Anabaena cylindrica. J. gen. Microbiol. 132, 1771-1774.

Philson S.B. and Llinas M. (1982) Siderochromes from Pseudomonas fluorescens. I. Isolation and characterization. J. biol. Chem. 257, 8081-8085.

Prakash A. and Rashid M.A. (1968) Influence of humic substances on marine phytoplankton dinoflagellates (Gonyaulax). Limnol. Oceanogr. 13, 598-606.

Price C.A. (1968) Iron compounds and plant nutrition. A. Rev. Pl. Physiol. 19, 239-248.

Provasoli L. McLaughlin J.J.A. and Droop M.P. (1957) The development of artificial media for marine algae. Arch. Microbiol. 25, 392-428.

Rhee G-Y. and Gotham I.J. (1980) Optimum N : P ratios and the coexistence of planktonic algae. J. Phycol. 16, 486-489.

Raymond K.N., Muller G. and Matzanke B.F. (1984) Complexation of iron by siderophores: A review of their solution and structural chemistry and biological function. Top. Curr. Chem. 123, 49-1023

Ryther J.H. and Kramer D.D. (1961) Relative iron requirement of some coastal and off-shore planktonic algae. Ecology 42, 444-446.

Sakamoto M. (1971) Chemical factors involved in the control of phytoplankton production in the Experimental Lakes area, northwestern Ontario. J. Fish. Res. Board Can 28, 203-213.

Sauchelli V. (1969) Trace Elements in Agriculture. Van Nostrand Reinhold, New York, 248 pp.

Schelske C.L., Hooper.F.F. and Haertl E.J. (1962) Responses of a marl lake to chelated iron and fertilizer. Ecology 43, 646-653.

Sedlăcek J., Kallquist T. and Gjessing E.T. (1983) Effect of aqueous humus on uptake and toxicity of cadmium to Selenastrum capricornutum. In: Christman R.F. and Gjessing E.T. (Eds) Aquatic and Terrestrial humic materials. pp. 495-516 Ann Arbor, Mich.

Shapiro J. (1957) Chemical and biological studies on the yellow organic acids of lake waters. Limnol. Oceanogr. 2, 161-179.

Sherman D.M and Sherman L.A. (1983) Effect of iron-deficiency and iron restoration on the ultrastructure of Anacystis nidulans. J. Bact. 156, 393-410.

Simpson F.B. and Neilands J.B. (1976) Siderochromes in cyanophyceae:

isolation and characterization of schizokinen from Anabaena sp. J. Phycol. 12, 44-48.

Sinclair C. (1977) Physiological and morphological studies on tapering blue-green algae. Ph.D Thesis, University of Durham, England.

Sinclair C. and Whitton B.A. (1977) Influence of nutrient deficiency on hair formation in the Rivulariaceae. Br. phycol. J. 12, 297-313.

Skyring G.W. and I.A. Johns (1980) Iron in Cyanobacterial mats. Micron. II, 407-408.

Spurr A.R. (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31-43.

Stewart W.D.P. (1964) Nitrogen fixation by Myxophyceae from marine environments. J. gen. Microbiol. 36, 415-422.

Stumm W. and Lee G.F. (1960) The chemistry of aqueous iron. Hydrobiologia 22, 295-319.

Sunda W.G., Engel D.W. and Thoutte R.M. (1978) Effect of chemical speciation on toxicity of cadimum to the grass shrimp, Palaemonetes Pugio: Importance of free cadmium ions. Environ Sci. Technol. 12, 409-413.

Theis T.L and Singer P.C. (1974) Complexation of iron(II) by organic matter and its effect on iron(II) oxygenation. ES&T 8, 569-573.

Ueda K. (1971) Licht-und elektronmikroskopische Untersuchungen über die Keritomie bei Oscillatoria borneti Zukal und Oscillatoria pseudogeminata Schmidle. Biochem. Physiol. Pflanz. 162, 225-233.

Van Baalen C. (1962) Studies on marine blue-green algae. Bot. mar. 4, 129-139.

Verstreate D.R., Storch T.A. and Dunham V.L. (1980) A comparison of the influence of iron on the growth and nitrate metabolism of Anabaena and Scenedesmus. Physiol. plant. 50,47-51.

Voelz H., Voels U. and Ortigoza R.O. (1966) The "polyphosphate overplus" phenomenon in Myxococcus xanthus and its influence on the architecture of the cell. Arch. Microbiol. 53, 371-388.

Waris H. (1958) The significance for algae of chelating substances in the nutrient solutions. Physiol. Plant. 6, 538-543.

Weckesser J., Hofmann.K., Jurgens. U.J. ,Whitton B.A. and Rafflsberger. B. (1988) Isolation and chemical analysis of the sheaths of the filamentous Cyanobacteria Calothrix Parietina and C. Scopulorum. J. gen. Microbiol. 134, 629-634.

Weimberg R. (1975) Polyphosphate level in non-growing cells of Saccharomyces mellis as determined by magnesium ions and the phenomenon of "Uberkompensation". J. Bact. 121, 1122-1130. Whitton B.A. (1987) The biology of Rivulariaceae. In: Fay.P. and Van Baalen.C. (Eds) The Cyanobacteria. 543 pp., pp. 513-534. Elsevier, Amsterdam.

Whitton B.A., Grainger S.L.J. and Harris N. (1986) Blue heterocysts in the Rivulariaceae. Br. phycol. J. 21, 338.

Whitton B.A., Kirkby S.M., Peat A. and Sinclair C. (1973) Environmental effects on the morphology of Rivulariaceae trichomes. In: Abstracts of symposium on prokaryotic photosynthetic organisms, Freiburg I. Br., Germany, Sept. 19-23, 1973. pp 180-181.

Whitton B.A. and Potts M. (1982) Marine Littoral. In: Carr N.G. and Whitton B.A (Eds), The Biology of Cyanobacteria. 688 pp., pp. 515-542 Blackwell, Oxford.

Wiebe W.J., Johannes R.E. and Webb K.L. (1975) Nitrogen fixation in a coral reef community. Science, Lond. 188, 257-259.

Wood P. (1984) Structural and physiological studies on the effects of mineral deficiencies on the Rivulariaceae (Cyanobacteria). volumes I and II. Ph.D Thesis, Sunderland Polytechnic.

Wood P., Peat A. and Whitton B.A. (1986) Influence of phosphorus status on fine structure of cyanobacterium (blue-green alga) Calothrix parietina. Cytobios. 47, 89-99.

Wurtsbough W.A. and Horne A.J. (1983) Iron in eutrophic Clear Lake, California: its importance for algal nitrogen fixation and growth. Can. J. Fish. Aquat. Sci. 40, 1419-1429.

Zevenboom W., DeVaate A.B. and Mur L.R. (1982) Assessment of factors limiting growth rate of Oscillatoria aghardii in hypertrophic Lake Wolderwijd 1978, by the use of physiological indicators. Limnol. Oceanogr. 27, 39-52.