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BIOLOGICAL STUDIES ON ALGAE IN RICE-FIELD SOIL FROM THE IRAQI MARSHES

By

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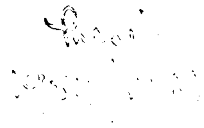
A thesis submitted for the degree
of Doctor of Philosophy
in the University of Durham, England
Department of Botany, September, 1984

-5. NOV. 1984



This thesis results entirely from my own work and has not previously been offered in candidature for any other degree.

Abdullah H. A. Al-Mousawi

A. Al-Mousawi

September, 1984

To my family

ABSTRACT

The algae in one soil sample from a rice-field in the southern marshes of Iraq were studied in detail. 11 taxa were found by direct microscopic inspection, with *Microcoleus chthonoplastes* and *Nostoc muscorum* the dominants. A total of 49 taxa were noted after various enrichment culture techniques.

The influence of temperature, light, salinity, reducing conditions, nitrogen sources and phosphorus (phosphate) were tested on mixed populations. Blue-green algae dominated all cultures incubated at temperatures between 25-45 ^{o}C, while eukaryotic algae dominated cultures at lower temperatures (10-20 ^{o}C). Six taxa showed good growth in a medium enriched with 0.5 M NaCl and three also grew at 1 M NaCl, though growth was very slow. Most of heterocystous blue-green algae were sensitive to reducing conditions (0.1 mM Na_2S). As a consequence, in mixed populations there was an increase in the relative abundance of non-heterocystous forms.

Fifteen strains were brought into clonal culture, nine of them also being axenic: *Anabaena cylindrica*, three *Calothrix* spp., *Cylindrospermum muscicola*, *Gloeotrichia* sp., *Hapalosiphon welwitschii*, *Microcoleus chthonoplastes*, *Nostoc muscorum*. Detailed morphological and physiological studies were made for 14 strains under different nitrogen and phosphorus conditions.

All axenic cultures showed marked alkaline phosphatase activity under phosphorus-deficient conditions. They were capable of utilizing a wide range of organic phosphorus compounds as a sole source of phosphorus.

Axenic cultures of heterocystous strains were capable of fixing nitrogen, judged by positive results obtained with acetylene reduction assays. Nitrogen fixation and heterocyst differentiation of these strains was almost completely suppressed when the strains were grown with high concentrations of combined nitrogen (initial = 140 mg l^{-1} NO_3-N or 50 mg l^{-1} NH_4-N).

A discussion is included of how field research on these algae might indicate ways of modifying cultivation practices to increase the phosphorus and nitrogen status of the soil and also how algal morphology might be used to help indicate the nutrient status of the soil.

Abbreviations

^{o}C	degrees Celcius
s	second
min	minute
h	hour
nm	nannometre
μm	micrometre
mm	millimetre
cm	centimetre
m	metre
km	kilometre
μg	microgramme
mg	milligramme
g	gramme
ml	millilitre
l	litre
μM	micromolar
mM	millimolar
M	molar
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
HEPES	N-2-hydroxypiperazine-N-2-ethane-sulphonic acid
FRP	filtrable reactive phosphorus
TFP	total filtrable phosphorus
$\bar{x}$	mean
SD	standard deviation
n	number of measurements
CV	coefficient of variation
PAR	photosynthetic active radiation between 400-700nm
w/v	weight to volume
v/v	volume to volume

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CHAPTER 1. INTRODUCTION

1.1 General Introduction

In the southern part of Iraq large marshes fed by water from the Tigris and Euphrates occur. The area of these southern marshes is very extensive, though the exact size given by various authors differs, presumably due to the difficulty of defining a "marsh". According to Buringh (1960), more than 35000 km^2 is covered by water at the time of peak flood. Thesiger (1964) describes the marshes as occupying an area of more than 1500 km^2. However, permanent marshes cover only about 10000 km^2 (Buringh, 1960). Most of the area is covered by *Phragmites* and *Typha*, but there is rice cultivation of considerable economic importance. It is unclear just what percentage of the marshes which could be used for rice cultivation is in fact cultivated. The importance of the crop is however evident from the fact that about 80% of Iraqi rice production comes from the south, especially the marshes (Chakravarty, 1976).

It has long been recognized that algae can play an important role in the maintenance of soil fertility in rice-fields. This is especially true of blue-green algae, since many of the widespread rice-field species are capable of fixing atmospheric nitrogen (Singh, 1961; Roger &

Kulasooriya, 1980). In view of known features of blue-green algae, it would seem likely that they play an important role in soil fertility in these rice-fields of the southern marshes. For instance the marshes have a calcareous and alkaline soil and water (Buringh, 1960; Guest, 1966), no nitrogenous fertilizers are applied (Chakravarty, 1976), the oxygen tension is low due to salinity, high temperature and the presence of sulphide (Al-Kaisi, 1976) and the weather is hot for most of the year (Section 3.2). However, apart from two published reports based on short-term collections of algal and/or soil samples (Al-Kaisi, 1976; Hamdi et al., 1978), there are no floristic, ecological or physiological studies of the algae in these rice- fields. In addition, in spite of many studies eleswhere, it is still difficult to comment on the importance of different environmental factors and any selective effect these may have on the occurrence or abundance of particular species in the field (Roger & Kulasooriya, 1980). In contrast to the broad floristic survey of Al-Kaisi (1976) the present study was planned to be an intensive account of algae in a soil sample from one small area in the rice-fields.

1.2 Algal communities in rice-fields

Rice-fields constitute an artificial biotope of a particular character, which can involve communities more typical of water and ones more typical of soil. The "life-cycle" of organisms in the water of rice-field is , however, of a short duration, extending over three to six months. After harvest the majority of the aquatic microorganisms either die or form dormant structures (Venkataraman, 1972). According to Heckman (1979) in his account of the rice-field ecology of northeast Thailand the rice field environment, and particularly its shallow water does not allow the development of pure planktonic communities.

The algal flora of rice-fields has attracted the attention of many workers in temperate and tropical regions (Singh, 1961; Venkataraman, 1972; Roger and Kulasooriya, 1980). In temperate regions, filamentous Chlorophyta are generally dominant and constitute the "pioneer" algal communities. They are developed throughout the vegetative period of the rice plant; they are followed quantitatively and seasonally by Xanthophyta and Cyanophyta (Tsangridis, 1982). For example, in the rice-field of south Yugoslavia the development of algae is poor in April - May with few representatives of dinofagellate and non-filamentous Chlorophyta (Petrovska and Stojanov, 1972) later on,

Bacillariophyta and Cyanophyta follow. The Cyanophyta are more abundant with Anabaena cylindrica and Stratonostoc (Nostoc) linckia as dominants. A rich development of algae is observed in June and July with Chlorophyta dominant. Greek rice-fields showed a seasonal succession of communities from April till September; Spirogyra spp. and Sphaeroplea annulia at the beginning followed by Hydrodictyon reticulatum, Aulosira prolifica, Chara vulgaris and finally Pithophora polymorpha (Anagnostidis et al., 1981).

In the tropics , in contrast to the temperate region, Cyanophyta dominate over other algae (Gupta, 1966; Pandey, 1965a, b; Sharma and Gaur, 1981). The "pioneer" microphyte community consists of species of Anabaena and Cylindrospermum, followed by Chara spp. (Gupta, 1966; Khan and Mathur, 1976). In Australia the general succession of algal communities from October till March was found by Happey-Wood (1982) to be first diatoms, then green algae and finally blue-green algae. The most abundant forms were Oedogonium spp. Blue-green algae were found to form only a minor part of the rice-field algae. In deep water rice-fields of Bangladesh Catling et al. (1981) found that blue-green algae comprise 25% of all algal forms identified; these were common up to October, green algae comprise 35% of

the algal species and as with blue-green algae they decline in October. Diatoms were well representated throughout the flooding period but they become completely dominant and comprised 68% when the receding flood water became turbid. Species of Euglenophyta and Pyrrophyta were rare. In the rice-fields of the southeast marshes of Iraq, blue-green algae dominated overwhelmingly the algal flora in July and August and formed up to 86% of the taxa (Al-Kaisi, 1976).

1.21 Blue-green algal communities

Rice-fields provide an environment favourable for the growth of blue-green algae with respect to their requirement of light, water, high temperature, low oxygen tension and nutrient availability (Singh, 1961; Venkataraman, 1972; Fogg et al., 1973; Stewart, 1977; Roger and Kulasooriya, 1980). In addition the photoautotrophic nutrition of blue-green algae precludes their need for a large amount of organic materials as is the case with heterotrophic bacteria (Sankaram et al., 1966). This may, therefore, account for the higher abundance of blue-green algae in the rice-field than in other cultivated area (Watanabe and Yamamoto, 1971).

In the rice-fields blue-green algae grow as free-living forms or in symbiotic association with the aquatic fern Azolla (Stewart, 1982). Free-living blue green algae may

grow in the rice-field water, as planktones, epiphytic on the rice-plants or other aquatic plants or on the soil surface.

Among symbiotic associations the one between Anabaena and the Azolla can serve as an important source of biologically fixed nitrogen for the growth of the rice plant (Stewart, 1980).

In wetland rice-fields planktonic and epiphytic blue-green algae are of limited importance due to the short duration of flood water (Venkataraman, 1972; Kulasooriya et al., 1980). However, free-living blue-green algae are common and often abundant in rice-field soil (Singh, 1961; Roger and Kulasooriya, 1980); they are more abundant in tropical and subtropical soils than in the temperate soils (Watanabe and Yamamoto, 1971).

Different forms of blue-green algae have been recorded from the soil of rice-fields at different localities. However, the relative occurrence of each group varies considerably according to the nature of the soil and flood water, stage of growth of rice plant and other environmental factors. In addition, the species recorded depends upon the methodology used i. e. , direct observation or culturing of a soil sample. For example, Gupta (1966) found that certain blue-green algae (e.g. Gloeotrichia and Aphanothece) were

observed only <u>in situ</u> and others (like <u>Fischerella</u>) grow only in soil cultures. In enrichment cultures of mud or soil Pandey (1965a) and Tsangridis (1982) found that non-heterocystous forms of blue-green algae were dominant, while Tiwari (1972) found heterocystous forms were dominant. In the rice-field heterocystous forms are usually abundant toward the end of the growth cycle of the rice plants when light is greatly reduced due to the dense growth of rice plants (Roger and Reynaud, 1976, 1977). However, the growth of nitrogen - fixing forms has been reported in the rice-field of Mali at the earliest - stage of rice plant growth (Traore <u>et al.</u>, 1978). Observations carried out in Sri Lanka indicated that nitrogen - fixing forms were present throughout the cultivated cycle, with <u>Nostoc</u> and <u>Anabaena</u> appearing during the early stages and continuing throughout the cycle, but <u>Gloeotrichia</u> and <u>Rivularia</u> appeared during the later part (Thirukkanasan <u>et al.</u>, 1977).

In the rice-fields of Italy where the dry season is relatively short, nitrogen - fixing blue-green algae comprise only about 30% of the algal flora (Materasi and Baloni, 1965). In Senegal, on the other hand where the dry season lasts about 8 months, spores of heterocystous blue-green algae constitute more than 95% of the potential flora at the end of the dry period and non-heterocystous

flora at the end of the dry period and non-heterocystous forms are present, primarily because of their introduction by irrigation water (Roger and Reynaud, 1976). Singh (1961) observed that in soils of Uttar Pradesh (India) a general mixture of nitrogen-fixing blue green algae appears initially, which is soon followed by a huge growth of Aulosira fertilissima. As the soil dries out, species of Cylindrospermum become dominant. Some blue-green algae are found to be associated with saline soil (Shtina, 1972; Ali and Sandhu, 1972; Al-Kaisi, 1976).

1.22 Factors affecting algal distribution in rice-fields

Algal communities in rice-fields appear to be highly susceptible to changes in environmental factors and exihibit rapid qualitative and quantitative variations during the cultivation cycle (Roger and Kulasooriya, 1980). Major factors affecting the growth and composition of algal communities in the rice-fields are mentioned below.

(i) Temperature

Temperature is an important factor determining the general distribution of certain algae (Soeder and Stengel, 1974). In general, low temperature inhibits the growth of blue-green algae and favours the growth of eukaryotic algae

(Roger and Reynaud, 1977), while high temperature favours the growth of the blue-green algae and inhibits the growth of eukaryotic algae (Sorokin, 1959) with the growth of most eukaryotic forms in liquid medium being inhibited in the 30 - 50 °C range (Miller and Fogg, 1957). In the laboratory this has become a convenient method for obtaining cultures of blue-green algae from almost any environment (Allen and Stanier, 1968). The high temperature requirement of the rice plant, besides other factors in the tropical and subtropical areas favoured the growth of blue-green algae (Watanabe and Yamamoto, 1971; Fogg et al., 1973; Whitton and Sinclair, 1975; Stewart, 1977). As mentioned above in temperate regions growth of algal groups other than the blue-green algae is usually common (Section 1.2).

The optimum temperature for growth of blue-green algae is more or less similar to that of the rice plants. However, in uncultivated areas or in the rice-fields after harvesting soil temperature may increase up to the inhibitory level for the majority of the blue-green algal species. The ability of certain blue-green algae to form akinetes enables them to tolerate such high temperature (Chapman and Chapman, 1973). In addition, several workers have found that certain blue-green algae grow and survive at elevated temperatures (Castenholz, 1973).

(ii)Light

Of the environmental factors which affect the growth and composition of algal communities in the rice-fields, the most important is the light (Ichimura, 1954; Roger and Reynaud, 1979). The tolerance of algal groups to light is different and may be roughly correlated with the taxonomic group. Thus, many green algae are adapted to high light, red algae to low light while diatoms seem less sensitive to light conditions (Whitford, 1960). Blue-green algae are generally sensitive to the high light conditions and may be regarded as low light species (Brown and Richardson, 1968). However, certain blue-green algae appear to be more resistant to high light conditions. Thus *Cylindrospermum* was found to develop a large biomass in a harvested rice-field in Mali where high light (more than 10000 lux) occured (Traore *et al.*, 1978). *Oscillatoria princeps* was also found to dominate in soil exposed to full sunlight (Roger and Reynaud, 1978). Light deficiency, however, may be a factor limiting algal growth in the rice-field. For example, in Japan phytoplankton productivity increased in the early summer but decreased in late July when the rice canopy decreased the light quantity to below the compensation point (Ichimura, 1954).

(iii) Salinity

Salinity is an important agricultural problem especially in the arid and semiarid regions. It results from the accumulation of an excess of soluble salts on the soil surface as a result of high evaporation and accompanied by the reduction of physiologically available water (Bernstein and Haywad, 1958). Soil algae, especially the blue-green algae, are well adapted for existence in climatic zones in which available water is the primary limiting factor (Metting, 1981). However, there are several algal groups which are less tolerant to the low water potential. For example, diatoms and Xanthophyta are generally most susceptible to severe or prolonged drought (Lund, 1962). In contrast, several blue-green algae were found to grow in conditions of extremely low water or high salt content (Singh, 1950; Ali and Sandhu, 1972; Potts and Friedmann, 1981). Several authors have found a definite requirement by some blue-green algae for salinity (Stacey et al., 1977; Apte and Thomas, 1980; Nordin and Stein, 1980; Rao and Talapasyi, 1982; Anantani and Vaidya, 1983). In Iraq, the characteristic feature of soil in the southern parts is high salinity (Buringh, 1960), yet blue-green algae are more abundant in the rice-fields than in any other cultivated area (Hamdi et al., 1978).

(iv) Reducing conditions

In the rice-fields, water-logged soil results in the formation of deoxygenated conditions (low oxidation-reduction potentials) (Fogg et al., 1973); low redox levels have been found by many workers to have a stimulatory effect on the growth of blue-green algae (Stewart and Pearson, 1970; Singh, 1978), while it has an inhibitory effect on the activity of eukaryotic algae (Tchan, 1953). Sulphide is known to create reducing conditions (Kashyap et al., 1983) and hydrogen sulphide has been detected in many ecosystems including rice-fields where blue-green algae are dominant (Stewart and Pearson, 1970; Al-Kaisi, 1976).

(v) Combined nitrogen

The selective action of nitrogenous compounds on algal communities has been documented in laboratory and field experiments. Rinaudo (1974) found that nitrogenous fertilizers had an inhibitory effect on nitrogen-fixing blue-green algae. A survey of Australian rice-field soils showed that although nitrogen-fixing blue-green algae were isolated from almost all the soil samples, their presence was not apparent (Bunt, 1961). This has been attributed partially to the application of heavy dressings of ammonium

sulphate (Venkataraman, 1972). Okuda and Yamaguchi (1952) found that only green algae appeared dominant in soils treated with ammonium sulphate and that the nitrogen-fixing blue-green alga Anabaena sp. became abundant only in unfertilized soil. In India, Subrahmanyan et al. (1965) observed that after ammonium sulphate treatments Spirogyra sp. and Euglena sp. were so abundant that rice farmers had to intertill their crop to prevent the algae from smothering the rice plants. In pot experiments, Yoshida et al., (1973) showed that nitrogen fertilizer increased algal growth but that generally there were more blue-green algae in pots without nitrogen fertilizer.

(vi) Phosphorus

Kuhl (1974) concluded that the phosphorus requirement for optimal algal growth differs considerably among species and no conclusion could be made about the relationships between the phosphorus requirement and taxonomic groups. It is generally believed that algal growth is often limited by phosphorus availablity (Kuhl, 1962). This is especially true for nitrogen-fixing blue-green algae (Fogg et al., 1973); presumably nitrogen fixation prevents them from becoming nitrogen deficient, thus allowing phosphorus deficiency to develop further (Healey, 1982). In

rice-fields phosphorus supply has been found to enhance algal growth and nitrogen fixation (De and Mandal, 1958; Srinivasan, 1978). Thus Okuda and Yamaguchi (1952) found that the growth of blue-green algae in submerged soil is closely related to the available phosphorus, they also found that algal growth was poor in soils containing 0 to 5 mg P kg^{-1} soil, but vigorous growth occurred above 6 mg P kg^{-1} soil. However, many workers have found examples where high phosphorus concentrations in laboratory cultures have an inhibitory effect on the growth of blue-green algae (Section 1.31)

(vii) Biotic factors

Biotic factors capable of limiting algal growth in the rice-fields are pathogens, antagonisms, and grazers. Of these, only grazers have been well documented . The common grazers of the algae in the rice-fields are invertebrates. The development of such populations may prevent the establishment of algal inocula (Watanabe et al., 1955) and cause the disappearence of algal blooms within one or two weeks (Venkataraman, 1961). Evidence of preferential grazing among algae has been presented. Thus Watanabe et al. (1955) showed that unicellular green algae are excellent food for daphnids, while filamentous

nitrogen-fixing blue-green algae also served as a nutrient source, although less effectively. Certain bacteria, fungi and viruses have been shown to be pathogenic to blue-green algae. However their role in rice-field has not yet been reported (Roger and Reynaud, 1979). Of the other limiting biotic factors antagonisms and competition have been cited (Venkataraman, 1961 & 1975).

1.3 Influence of phosphorus on algae

1.31 Phosphorus nutrition of algae

Phosphorus is fundamental to life, being a structural and functional component of all living organisms (Ehrlich, 1981), including algae (Myers, 1951; Ketchum, 1954; Provasoli, 1958). Its availability has been implicated as a factor limiting the growth of algal species (Kuhl, 1962). Compounds containing phosphorus play an important role in many aspects of metabolism. In photosynthetic organisms this element receives additional attention because phosphorylated compounds participate in photosynthesis (O'Kelley, 1973).

As mentioned before (Section 1.22) the phosphorus requirements for optimal algal growth differ considerably among species. Thus, Rodhe (1948) distinguished three main groups of freshwater algae according to their ability to

tolerate phosphate within the ranges below, around and above 20 $\mu g\ l^{-1}$ P. Many worker have found optimal growth at low phosphorus levels. Batterton and Van Baalen (1968) found that the limiting growth of blue-green algae is below 3.7 $\mu g\ l^{-1}$ P . Allen (1963) grew a marine Calothrix in a range of phosphorus concentrations and observed appreciable growth with 0.03 mg l^{-1} P and the yield increased with increasing phosporus concentration up to 10 mg l^{-1} P, but 31 mg l^{-1} had an inhibitory effect . Kantz and Bold (1969) found that several strains of Nostoc and Anabaena could not grow in Bold s Basal medium because of its high phosphate concentration and Fogg (1969) commented that even low concentrations of inorganic phosphate in artificial media were inhibitory to Gloeotrichia.

A widespread feature of the phosphorus metabolism in algae, including blue-green algae, is their capacity to make the best use of available phosphorus by uptake and accumulation in excess of immediate need (Kuhl, 1962; Stewart et al., 1978). The utilization of stored phosphorus may enable algae to continue growth in the absence of detectable phosphorus in the culture medium (Mackereth, 1953; Al-Kholy, 1956; Rhee, 1973; Healey, 1973). Generally no clear relationship was found between the growth rate of blue-green algae or eukaryotic algae and the external

phosphorus concentration, but a linear or hyperbolic rerlationship was found between the growth rate and the internal phosphorus concentration of Anabaena variabilis (Healey and Hendzel, 1975) and Oscillatoria agardhii (Ahlgren, 1978).

Many workers found that the yeild of several blue-green algae increased as the external phosphorus concentration increased (Allen, 1963; Sinclair and Whitton, 1977a; Lam, 1973; Livingstone et al., 1983). Although only orthophosphate can be absorbed directly by algal cells (Fogg, 1973; Healey, 1982), it is now clear that many other forms of phosphorus may serve as a phosphorus sources (Section 1.32).

Several blue-green algae have been reported to release into the external medium large amounts of phosphorus in organic forms which can be utilized by the organisms themselves or other organisms (Kuenzler, 1970; Lean, 1973; Lean and Nalewajko, 1976). However, Grillow and Gibson (1979) found that Synechococcus sp. released little or no phosphorus into the medium with or without excess phosphorus in solution. In agreement with the later findings others have found evidence for only limited release of recently taken up phosphorus by both prokaryotic and eukaryotic microbes (Harold and Spitz, 1975; Robertson and Button,

1979).

The process of phosphorus uptake requires energy (Fogg, 1973; Healey, 1982). This energy can be supplied by photosynthesis and respiration or combination of both processess. If photosynthesis alone is involved, then light quantity plays an important role . However, at high concentrations of phosphorus passive uptake may become dominant (Simonis *et al.*, 1974) and Whitton (1967) found that some *Nostoc* colonies could accumulate phosphorus from the environment by non-active means.

1.32 Phosphorus nutrition sources

Phosphorus may be present in a variety of forms in natural waters and soils (Fogg, 1973; Cosgrove, 1967; Golachowsk, 1979). It is almost invariably found in the fully oxidized state but this may be in organic and inorganic combinations (Fogg, 1973). In freshwater, sea water and several soils, organic phosphorus is generally present in concentrations several times higher than those of inorganic forms (Hutchinson, 1957; Armstrong, 1965; Cosgrove, 1967). Organic phosphorus is represented by a large group of compounds including phosphoprotein, phospholipid, phosphoglicosides and nucleic acids (Cosgrove, 1967; Cheshire and Anderson, 1975; Langowska, 1982). These

compounds may result from the secretions and excretions of living bacteria, plants, animals or from the decomposition of dead organisms (Johannes, 1964, 1965; Cosgrove, 1976; Langowska, 1982). Residuals of synthetic organic phosphorus compounds may also participate in the phosphorus budget of certain ecosystems.

Although only the orthophosphate ion can be absorbed directly by algal cells, phosphate from other forms may be used after hydrolysis by enzymes produced by the alga itself (Section 1.33). As a result of such enzymic activity most algae can utilize a wide range of phosphorus compounds. Thus, Fitzgerald (1970) has shown that a variety of relatively insoluble forms of phosphorus, including animal teeth can serve as a phosphorus sources for algal growth and Bose _et al_. (1971) found that many species of the blue-green algae are able to solubilize bound phosphate from tricalcium phosphate compounds. It has been demonstrated that several green and blue-green algae as well as various microorganisms are able to hydrolyse and assimilate several forms of condensed phosphate (Davis and Wilcomb, 1967; 1968; Clesceri and Lee, 1965; Rivkin and Swift, 1980). Stewart and Alexander (1971) found that a variety of detergents could act as a phosphorus source for the bloom-forming blue-green alga _Anabaena flos-aquae_. Doonan and Jensen

(1979) found that *Plectonema boryanum* could hydrolyse 15 organic phosphorus compounds at different rates, but could not hydrolyse some other forms (DNA, bis-nitrophenyl phosphate, cyclic UMP) . Recently Livingstone *et al*. (1983) found that the blue-green alga *Calothrix parietina* D550 grew on various organic phosphorus compounds as a sole phosphorus source.

1.33 Physiological and morphological variations associated with phosphorus deficiency

Various physiological and morphological changes have been observed in phosphorus deficient cultures of algae. The most notable is the great variation in the internal phosphorus concentration of the algae under phosphorus sufficient and deficient conditions which can exceed ten fold (Healey, 1982). According to Al-Kholy (1956), for example, the minimum internal phosphorus concentration for *Chlorella pyrenoidosa* was 10^{-7} µg P per cell and the maximum was 1.5×10^{-6} µg P per cell. In *Plectonema boryanum* the minimum and maximum values were 4 and 52 ug P mg dry weight, respectively (Jensen and Sicko-Coad, 1976). In *Calothrix parietina* D550 the minimum phosphorus level was found by Livingstone *et al*. (1983) to be 2.7 µg P mg^{-1} dry weight. Several other investigators have found that the minimum

internal phosphorus concentrations of blue-green algae are similar to those of eukaryotic algae but lower than those of heterotrophic bacteria (Fuhs et al., 1972; Chen, 1974; Healey; 1975). In addition to the decrease in the internal phosphorus content of the algae, algal dry weight, chlorophyll a and protein content were found to decrease under phosphorus deficient conditions (Healey, 1978).

The effect of phosphorus deficiency on physiological rates has been studied by several workers, who have found that the rates of growth, photosynthesis, respiration and nitrogen fixation fell with phosphorus deficiency (Stewart et al., 1970; Stewart and Alexander, 1971; Senft, 1978). Under conditions of phosphorus deficiency cell-surface phosphatase activity in many organisms increases greatly (Section 1.4)

In addition to the variation in the composition and metabolism of algae under phosphorus deficiency mentioned above, several morphological changes have been observed in many blue-green algae under phosphorus deficient conditions. These include formation by Plectonema boryanum of very long and very short vegetative cells (Jensen and Sicko, 1974), increase in hair length and frequency and sheath thickness in several Rivulariaceae (Sinclair and Whitton, 1977a; Livingstone and Whitton, 1983, Livingstone et al., 1983),

increase in cyanophycin granules (Sinclair and Whitton, 1977a; Sutherland _et al._, 1979; Stevens and Paone, 1981). Akinete formation and frequency were found to be increased under phosphorus deficiency in many species of blue-green algae. For example, in _Anabaena cylindrica_ and _Gloeotrichia ghosei_ (Wolk, 1965; Sinclair and Whitton, 1977a), _Aphanizomenon flos-aquae_ (Gentile and Maloney, 1969), _Cylindrospermum licheniforme_ (Fisher and Wolk, 1976), _Nodularia spumigena_ (Pandey and Talpasayi, 1980)and _Anabaena torulosa_ (Fernandes and Thomas, 1982). In contrast to akinete formation, heterocyst frequency is found to be reduced by phosphorus deficiency in some but not all of the blue-green algae (Ogawa and Carr, 1969; Healey, 1973; Healey and Hendzel, 1975; Guert van Kessel _et al._, 1977).

1.4 Phosphatase activity in the blue-green algae

Phosphatases are a large and complex group of enzymes functioning over a wide range of substrates and hydrogen ion concentrations. They catalyse the hydrolysis of both esters and anhydrides of phosphoric acid (Schmidt and Laskowiski, 1961; Feder, 1973). Phosphatases have also been known to act as transferases by catalysing the transfer of phosphate groups from one substrate to another (Stadtman, 1961). They are divided into acid phosphatases and alkaline phosphatases

according to their pH optima. They have been found to occur in all taxonomic groups (Schmidt and Laskowiski, 1961; Feder, 1973) however, because of the growth of the blue-green algae in neutral to alkaline conditions alkaline phosphatase is predominant.

Phosphatase activity is usually associated with phosphorus limitation (Fogg, 1973; Healey, 1982). Fitzgerald and Nelson (1966) reported that when several algae grown in a medium containing high phosphorus (7.2 mg l^{-1} PO_4-P) alkaline phosphatase activity was very low . However, when grown in a medium with only 0.4 mg l^{-1} PO_4-P the activity was increased up to 25 times. Similar observation have been made for Anabaena flos-aquae (Bone, 1971a), Anabaena variabilis (Healey, 1973), Anacystis nidulans (Ihlenfeld and Gibson, 1975)and Calothrix parietina D550 (Livingstone et al., 1983). These observations indicated that the enzymes of the organisms studied are inducible. Constitutive enzymes have also been reported in several other organisms (Keunzler, 1965; Healey and Hendzel, 1975; Doonan and Jensen, 1980). In the field phosphatase activity has been found to be associated with a very low or undetectable orthophosphate and/or high organic phosphorus concentration (Perry, 1972; Khoja et al., 1984).

Alkaline phosphatase from different sources shares several common features. The pH optima, for example, of the enzyme of most organisms studied falls within the range of 8 to 11 (Torriani, 1960; Kuenzler and Perras, 1965; Heath and Cooke, 1975; Rivkin and Swift, 1980). It was also found that the enzyme from a variety of sources has the ability to hydrolyse a large number of phoshate bonds (Selitrennikoff and Sonneborn, 1977; Livingstone et al., 1983) however, the relative rates of hydrolysis of the various compounds were different (Aaronson and Patni, 1976; Ihlenfelds and Gibson, 1975; Doonan and Jensen, 1980; Fernandez et al., 1981). Healey (1982) mentioned that some organic phosphorus compounds are not or only slightly hydrolysed by microbial phosphatases. Lean (1973) found that colloidal phosphorus produced by the phosphorus metabolism of microflora in lakes is hydrolysed at a very slow rate.

The ionic requirements of phosphatase activity are quite variable for microorganisms as a whole but probably much more uniform when only blue-green algae are considered. Thus, Patni and Aaronson (1974) found that addition of K+, Mg++, Ca++ or Mn++ had no significant effect on the acid phosphatase activity of the chrysophyte Ochromonas danica, while Healey (1973) and Healey and Hendzel (1975) reported that for maximum activity of the alkaline phosphatase of

Anabaena variabilis and Pseudanabaena catenula calcium should be added externally.

Doonan and Jensen (1979) studied the effect of different concentrations of several ions on the alkaline phosphatase activity of Plectonema boryanum and found that there was an increase in the activity with increasing calcium concentration from 0.02 to 20 mM. Of the other ions studied Mg^{++} was found to have different effects on alkaline phosphatase of certain organisms. This cation, for example, has a low stimulatory effect on the cell-bound and cell-free enzyme of P. boryanum (Doonan and Jensen, 1979). Healey (1973) found that magnesium could not substitute for calcium for maximum activity of the alkaline phosphatase of Anabaena variabilis.

Several organisms have been found to release alkaline phosphatase into the growth medium. For example, Escherichia coli, Torriani (1960) found only 10-15% of the total enzyme activity in the growth medium while Glew and Heath (1971) found that most of the alkaline phosphatase of Micrococcus sodonensis is secreted into the growth medium. In the blue-green algae studied small amounts of the enzyme were found in the growth medium of A. variabilis by Healey (1973) and Kuenzler and Perras (1965) observed a very small amount of the enzyme in the growth medium of Oscillatoria

Oscillatoria woronichinii. Among 18 strains of blue-green algae tested by Doonan and Jensen (1980) *Plectonema boryanum* (UTEX 581) , *Nostoc commune* (UTEX 584) and *Gloeocapsa* sp. (UTEX, 1938) did not release any quantity of the enzyme.

1.5 Nitrogen fixation in the rice-fields

A number of microorganisms have been identified as agents responsible for nitrogen fixation in rice-fields (Balandreau *et al.*, 1975). The major groups appear to be heterotrophic bacteria (Watanabe *et al.*, 1978; Barraquio *et al.*, 1982), free-living or in symbiotic association with the aquatic fern *Azolla*, blue-green algae (Watanabe, 1959; Moore, 1969; Peters, 1978; Stewart, 1982) and photosynthetic bacteria (Kobayashi and Hague, 1971; Habte and Alexander, 1980).

Heterotrophic bacteria are generally confined to habitats rich in organic carbon and low combined nitrogen (Spernt, 1979) and their nitrogen-fixing efficiency is low in terms of nitrogen fixed per unit of carbon consumed (Stewart, 1977). On the other hand, most of the photosynthetic bacteria when grown on nitrogen gas as a sole nitrogen source generally live strictly anaerobically (Sprent, 1979). Anaerobic conditions in the rice-fields are restricted to the subsurface layer of soil in flooded

rice-fields. However, because of the activity of soil fauna and excretion of oxygen by rice plant roots strictly anaerobic conditions are not likely to be widespread in the rice-fields during the cultivation cycle (Kikuchi et al., 1977). On the other hand, because of the similar growth requirements of rice plants and blue-green algae, the latter grow abundantly in the rice-fields. Several workers have attributed the maintenance of the soil fertility in rice-fields to which chemical fetilizers are not added to the nitrogen fixation activity of the blue-green algae (Singh, 1961; Venkataraman, 1972; Roger and Kulasooriya, 1980).

1.51 Nitrogen fixation by blue-green algae

Nitrogen fixation (nitrogenase activity) is widely distributed among blue-green algae, including representatives of each of the major groups (Stewart, 1973a,b; Both, 1982). Aerobic nitrogen fixation among the unicellular forms is rare (Stewart, 1982). There are very few reports in which aerobic nitrogen fixation by unicellular forms has been confirmed (Wyatt and Silvey, 1969; Rippka et al., 1971; Singh, 1973). However, about 50% of the forms tested showed nitrogenase activity under microaerobic or anaerobic conditions (Rippka and Waterbury,

1977; Rippka and Stanier, 1978). Among the non-heterocystous filamentous forms, pure cultures of *Plectonema boryanum*, *Phormidium foveolarum*, *Oscillatoria* sp., *Lyngbya* sp. were found to reduce acetylene under microaerobic and/or anaerobic conditions (Stewart and Lex, 1970; Kenyon *et al.*, 1972; Weisshaar and Böger, 1983). However, a relatively long (72-120 h) incubation period is required for the development of active nitrogenase activity under these conditions(Stewart and Lex, 1970, Kenyon *et al.*, 1972). There are also few reports of aerobic nitrogenase activity in some members of the non-heterocystous filamentous forms including natural populations of *Trichodesmium erythraeum* and pure cultures of *Microcoleus chthonoplastes* (Dugdale *et al.*, 1961; Bunt *et al.*, 1970, Carpenter and Price, 1976; Pearson *et al.*, 1981). Ecologically the most important group of the blue-green algae are the filamentous heterocystous forms which invariably fix nitrogen under aerobic and anaerobic conditions (Stewart, 1977).

In natural ecosystems nitrogen-fixing blue-green algae are subjected to a variety of environmental factors which affect and/or control their nitrogenase activity. Among these factors are:

(i) Temperature Nitrogen fixation by blue-green algae has been observed near freezing point (Fogg and Stewart, 1968; Horne, 1972; Davey, 1982) and in hot springs (Stewart, 1970; Wickstrom, 1980). The highest temperature at which nitrogenase activity was detected is about 60 °C (Stewart, 1974). However, the range and the optimum temperature for nitrogenase activity varied considerably between the species. for example, Whitton *et al.*, (1979b) found that the maximum temperature at which there is a detectable nitrogenase activity for two *Nostoc* species from Aldabra Atoll in the Indian Ocean was 46 °C, while the maximum temperature at which there is a detectable activity for *N. commune* from a temperate region (England) was 38 °C. Similarly Wickstrom (1980) found that *Calothrix coriacea* (growing at 30 °C) and *C. thermalis* (growing at 40 °C) in a thermogradient stream had maximum nitrogenase activities at 30 and 40 °C, respectivily. Stewart *et al.* (1977) found in comparative studies of nitrogen fixation by soil algae from temperate, subtemperate and tropical regions, that the temperate soil algae (dominated by *Nostoc* and *Cylindrospermum*) reduce acetylene at 0 °C and have high activity at 40 °C. However, the optimum temperature was 15-25 °C. In contrast, tropical soil algae (dominated by *Scytonema*) showed little activity at 5 °C and the activity

increased with increases in temperature to 40 ^{o}C. On the other hand, subtemperate soil algae (dominated by *Stigonema panniformis*) had an optimum temperature at 30 ^{o}C. The greatest responses to temperature increase were found from 0 to 10 ^{o}C, 15 to 20 ^{o}C and 20 to 25 ^{o}C for temperate, subtemperate and tropical soil algae, respectivily. These results led the authors to state that in general there is a direct correlation between the temperature responses of the algae and the temperature of the habitat from which they were collected.

(ii) Light

Nitrogen fixation by the blue-green algae investigated is light stimulated (Bothe, 1982). The rate of nitrogen fixation showed much the same relationship as photosynthesis to light conditions (Fogg, 1974). Most of the data related to the effect of light on nitrogen fixation are for aquatic habitats and the results show that there is a dependance on light for nitrogen fixation and that a high photon flux density may be inhibitory (Fogg, 1974; Peterson *et al.*, 1977). Stewart (1974) summarize the information for the effect of light on terrestrial blue-green algae and states that there is a rapid decrease in acetylene reduction at depths below 1.0 cm and that the light at the soil surface

may be excessive. Reynaud and Roger (1979) suggest that in general blue-green algal nitrogen fixation is inhibited at high photon flux densities. Jones (1977) found that both unialgal cultures and mats of *Nostoc* sp. in subtropical grassland showed linear relationships between photon flux density and acetylene-reducing activity up to 323 lux, but no significant increase in the activity for photon flux densities above that. He also found a slight decrease in the activity at high photon flux densities and a sharp reduction in the activity after 3 hours incubation in the dark. Wickstrom (1980) found a linear response in the acetylene-reducing acivity of a thermal *Calothrix* sp. up to about 2000 µmol pphoton $m^{-2}s^{-1}$. Coxson and Kershaw (1983) found that the saturation level for nitrogenase activity of *Nostoc muscorum* was about 900 µmol photon $m^{-2}s^{-1}$. The saturation level of nitrogenase activity of free-living blue-green algae seems to be higher than that of symbiotic forms which range between 75-150 µmol photon $m^{-2}s^{-1}$ (Cox and Fay, 1969). Dark treatments caused different effects on the nitrogenase activity of the blue-green algae. For example, Wiebe *et al.* (1975) and Khoja *et al.* (1984) found that dark nitrogenase activity of *Calothrix crustacea* and a marine *Rivularia* population was only 2-5% of the light activity. Potts and Whitton (1977) on the other hand, found

only about 50% reduction in the nitrogenase activity of lagoon intertidal population of blue-green algae on Aldabra Atoll after 100 minutes dark incubation. Nitrogenase activity of the Gloeocapsa (Gloeothece) sp. 1430/3 was found by Gallon et al. (1973) to be 40% of the activity in the light after 4 hours incubation in the dark. The same alga showed an immediate increase in the activity after dark incubation for 2-5 hours followed by a gradual decrease to a negligable activity after 12 hours incubation in the dark (Mullineanx et al., 1981). In this respect Gloeocapsa differs from other heterocystous blue-green algae tested by Fay (1976) and Bottomley and Stewart (1977), which showed exponential decline after transfer to darkness and almost complete loss of activity after 4 hours incubation in the dark. Blue-green algae in symbiotic associations have been found to be able to use green, red and blue light with the highest nitrogenase activity under green light (Holst and Yopp, 1979).

(iii) Salinity

Nitrogenase activities of several blue green algae showed differances in response to salt treatment. For example, Stacey et al. (1977) and Gotto et al. (1979) reported active nitrogen fixation by Anabaena sp. under

marine conditions. Jones and Stewart (1969) found that nitrogen fixation by *Calothrix scopularum* was maximum at 5% NaCl (0.09 M) and was reduced to about half at 45% NaCl (0.8 M). Antarikanonda (1982) found that the *Anabaena* sp. (TA1) isolated from rice-field of Thailand is more sensitive than *Anabaena cylindrica* to salt treatment for both growth and nitrogenase activity. Tel-Or (1980) found that nitrogenase activity of *Nostoc muscorum* is more sensitive to salinity treatment than that of *Calothrix scopulorum*. This was attributed to the natural habitat of the two species being freshwater and marine, respectively. Several workers have found that growth and/or other metabolic activities of blue-green algae are more resistant to salt treatment than their nitrogenase activity (El-Nawawy *et al.*, 1968; Tel-Or, 1980). These findings are in agreement with the statment made by Whitton and Sinclair in 1975 that "nitrogen fixation as a process is more sensitive to variations and extremes of environmetal conditions than photosynthesis or overall growth".

(iv) Combined nitrogen

The effect of combined nitrogen on nitrogenase activity and biosynthesis has been examined in many nitrogen-fixing organisms. It is often found that the addition of combined

nitrogen suppress nitrogenase biosynthesis rather than the activity of the existing enzyme (Stewart *et al.*, 1968; Brill, 1975; Rippka and Stanier, 1978). Thus several workers have found that there is no immediate effect of combined nitrogen on nitrogenase activity (Ohmori and Hattori, 1972; Stewart *et al.*, 1975; Murry *et al.*, 1983). It was found that the noticeable inhibitory effect is due to the interruption of reductants and/or ATP supply (Ohmori and Hattori, 1974, 1978; Upchurch and Mortenson, 1980; Murry *et al.*, 1983). However, on long-term treatment the inhibitory effect is the result of inhibition of heterocyst differentiation and dilution of the enzyme due to growth (Stewart *et al.*, 1975; Ramos and Guerrero, 1983). The model of effect of combined nitrogen on nitrogen fixation of the blue-green algae appears to differ according to the organism tested and source of combined nitrogen used (Bothe, 1982). For instance, addition of ammonia to *Anabaena cylindrica* abolished its nitrogenase activity within 48 h, where as this effect was only gradual when the organism was grown with nitrate as nitrogen source (Stewart *et al.*, 1975; Bothe and Eisbrenner, 1977). In contrast to *Anabaena cylindrica* Bottomley *et al.* (1979) found that addition of 10 mM KNO_3 or NH_4NO_3 to *Anabaena* sp. (CA) completely repressd nitrogenase activity while the same concentration of NH_4Cl

reduced the activity by about 70%. Chen (1983) found that the nitrogenase activity of three Anabaena strains isolated from rice-fields in Taiwan showed different responses to the addition of combined nitrogen. For example, addition of 1 mM nitrite or ammonia completely repressed the activity of the two strains while the third one maintained activity at 25% of the control.

In heterocystous blue-green algae detectable nitrogenase activity is usually concomitant with the appearance of fully differentiated heterocysts (Bradely and Carr, 1976). Combined nitrogen inhibits heterocyst differentiation in many strains. There are also differences between the strains in their response to the effect of combined nitrogen on heterocyst differentiation. Ogawa and Carr (1969) noticed that Anabaena variabilis still has few heterocysts at 28 mg l^{-1} NO_3-N while the same concentration of NH_4-N stopped heterocyst formation completely. Thomas and David (1971) found that heterocyst formation was totally inhibited when nitrate was supplied in batch culture, but not in continuous culture at high dilution rate. In contrast to other heterocystous blue-green algae, heterocyst differentiation in Anabaena sp. (CA) is much more strongly inhibited by nitrate than by ammonia (Bottomley et al., 1979). Singh and Viswanathan (1972) found that 0.5 mM of

either $NaNO_3$, NH_4Cl or urea completely repressed heterocyst formation by Camptylonema lahorense while 20 mM KNO_3 is required to achieve the same effect; the experiment was carried out on solid medium.

In the case of tapered heterocystous blue-green algae, several workers have reported not only the repression of nitrogenase activity and heterocyst differentiation in the presence of combined nitrogen but also loss of typical trichome polarity (Fay et al., 1968; Kirkby, 1975; Wyatt et al., 1973). However, combined nitrogen can suppress heterocyst formation but leave polarity in several tapered blue-green algae (Sinclair and Whitton, 1977a; Rai et al., 1978)

Organic nitrogen is also likely to exert an inhibitory effect on nitrogenase activity provided that it is first reduced to ammonia (Stewart, 1973a). Thus, in Plectonema boryanum glutamine and arginine were found to repress the an anaerobic induction of nitrogenase, but aspartate, asparagine and lysine were ineffective (Nagatani and Haselkom, 1978).

(v) Phosphorus

Nitrogenase activities of natural populations of blue-green algae were found to vary markedly and rapidly,

depending on the levels of phosphorus which were available for their metabolism (Stewart et al., 1970). Stewart (1964) found that addition of about 9 mg l^{-1} PO_4-P to natural sea water increased nitrogen fixation of a natural population of blue-green algae (Calothrix scopulorum and Nostoc entophytum) by 17% and 12%, respectively. The response in nitrogen fixation activity of phosphorus starved blue-green algae to the addition of inorganic phosphorus or phosphorus containing detergent was found to be very rapid and at very low concentrations (5 ug l^{-1} P) (Stewart and Alexander, 1971). The same authors found that the saturation level of phosphorus for nitrogenase activity was found to be very low (<50 ug l^{-1} P). Bone (1971b) found that there is only little effect on the nitrogenase activity of Anabaena flos-aquae when the phosphate concentration was increased from 0.022 to 0.066 mM.

1.6 Aims

The literature indicated that blue-green algae are widespread and abundant in the rice-fields of Iraq (Section 1.2). All of the studies carried out so far are, however, floristic accounts. In spite of many studies on blue-green algae in rice-fields elsewhere, it is still difficult to comment on the importance of different environmental factors

on the occurrence or abundance of particular species.

The present study was planned initially to survey nitrogen fixation both in the field and laboratory. Unfortunately, because of the war between Iraq and Iran during the last three years, it was changed to become entirely laboratory based. The aim was to establishe some key features of the biology of the various blue-green algal species present in the soil from one small area. It was hoped that such study might suggest factors likely to be particularly important in nature. In particular it was hoped to establish the importance of different nitrogen and phosphorus sources.

CHAPTER 2. MATERIALS AND METHODS

2.1 Soil

2.11 Collection

A sample of soil was collected by Dr B. A. Whitton on 21 September 1979 by pooling materials from microhabitats ranging from wet to dry within an area of 2 m^2. Soil was included to a depth of 3 mm.

2.12 Analysis

The sample was dried for 48 hours at 105 ^{o}C, lightly ground and put through an 80 mesh (210 µm) sieve; the material passing through the mesh was used for analysis. Part was muffled at 550 ^{o}C to give the ash weight (neglecting loss of CO_2 from carbonate). Part was digested with atomic absorption spectroscopy grade HNO_3 and boiled for 30 min, 5 mg of the soil sample was also added to 50 ml of either deionized distilled water or AD-N medium and after 12 hours of continuous shaking at 32 ^{o}C, filtrates (passed through GF/C glass fibre filter) were collected. Metal analysis of acid digest and filtrates was carried out on a Perkin-Elmer model 403 atomic absorption spectrophotometer (Holmes and Whitton, 1981).

Chloride and sulphate, in the filtrates only, were determined as follows:

(i) Chloride.

Use of silver nitrate-potassium chromate titration method (American Public Health Association, 1981) checked by a chloride specific ion electrode (Orion, combination chloride electrode model 961700) as described by the manufacturer.

(ii) Sulphate. Use of a gravimetric barium sulphate method on the dried residue (American Public Health Association, 1981).

Total phosphorus was determined after digestion with a persulphate-sulphuric acid digestion (Eisenreich *et al.*, 1975).

Total nitrogen was determined according to the method of Smart *et al.* (1983) using a nitrate electrode (Orion nitrate specific ion electrode model 93-07).

2.13 Floristic records

The algal flora of the soil sample was determined by direct inspection, incubation of a soil sample wetted with distilled water and after soil culturing (Section 4.2).

Direct inspection was carried out by rewetting about 1g of soil sample with distilled water in a petri dish and incubating the sample at room temperature (c. 20 °C) for at least 30 min. Slides were then prepared and examined carefully. Petri dish with wet soil was then incubated in a growth room at 32 °C; 30-40 µmol photon $m^{-2}s^{-1}$ for 2 weeks (soil rewetted when necessary). Algal taxa developed in the wet soil sample were identified every week as described above. For a mixed community, algal populations in culture flasks were homogenized lightly before aliquots were removed for microscopy. In all cases several slides were prepared using a drop from culture medium to prevent any osmotic effect. Slides were then examined carefully using a light microscope (Nikon fluophot Microphot V seies, Nippon Kogak K.K. Tokyo, Japan) equipped with Nikon M-35D 92850 camera for photograhy. The conventions used here are the same as those used by Potts and Whitton (1980); a justification for using width categories for certain genera rather than binomials is given by Whitton et al. (1979a). Binomials were allocated after consultation of taxonomic literature. Thses included floras of Geitler (1932), Frémy (1933), and Desikachary (1959). In some cases, however, generic names followed Rippka et al. (1979). Detailed descriptions of the main organisms isolated into clonal and/or axenic

cultures are given in Section 4.3.

2.2 Culture techniques

2.21 Cleaning of glassware

These were usually soaked in hot tap water and detergent for at least 30 min. Subsequently they were rinsed thoroughly with hot tap water and then soaked for a minimum of 30 min in 10% Analar HCl solution prior to being rinsed at least ten times in glass distilled water and dried at 105 °C.

Pipettes and Pipettman tips used for preparation of media (Section 2.4), subculturing of algae (Section 2.64) and phosphatase assays (Section 2.81), were washed with 10% HCl. 10% Analar H_2SO_4 solution was used originally but because of lack of algal growth in some experiments and a clear deterioration of the silicon bungs used to close culture flasks, it was replaced by 10% HCl.

The glassware used for analysis of phosphate was never washed with detergent; it was rinsed three times with tap water after each run, then soaked in 10% Analar H_2SO_4 solution for a minimum of 30 min before being rinsed ten times in distilled water followed by three times in deionized distilled water and then dried at 105 °C.

2.22 Cleaning of silicon bungs

These were cleaned occasionally by soaking them in a 2% solution of phosphate-free detergent (Decon 90, Decon Laboratories Ltd, England) for at least 2 hours, then rinsed five times in distilled water and dried at room temperature.

2.23 Culture vessels

All experiments were carried out in 100 ml Erlenmeyer flasks plugged with high grade non-absorbant cotton bungs or silicon bungs (Type S28, manufactured by Sanko Plastic Ltd, Osaka Japan). All flasks were of Pyrex glass and acid washed (Section 2.21). For solid media pre-sterilized plastic petri dishes (manufactured by Northern Media Supplies, Hall, England or Sterilin Ltd, Teddington, Middlesex, England).

2.3 Chemicals and Gases

(i) Chemicals

All chemicals used in the preparation of media were Analar grade, except for those listed in Table 2.1.

(ii) Gases

In the experiments of Section 7.3 , oxygen-free nitrogen and carbon dioxide were used. Acetylene and pure

Table 2.1 Specification and suppliers of the chemicals other than Analar grade used in the study.

chemicals	specification	supplier
HEPES	Sigma grade	Sigma Chemical Co.
nutrient agar		Oxoid, U.K.
agar		Difco, U.S.A
tryptone		Oxoid, U.K.
yeast extract		Oxoid, U.K.
peptone		Difco, U.S.A.
Casamino acid	technical grade	Difco, U.S.A.
inositol hexaphosphate sodium salt	Sigma grade	Sigma Chemical Co.
β-glycerophosphate sodium salt	general purpose reagent	BDH
glucose-1-phosphate dipotassium salt	Sigma grade	Sigma Chemical Co.
DNA, sodium salt from Herring sperm Type VII	Sigma grade	Sigma Chemical Co.
phosphatidycholine (Lecithin) from egg	general purpose reagent	BDH
$Na_2H_2P_2O_7$ (Pyrophosphate)	practical grade	Sigma Chemical
$Na_3P_3O_9$ (Metaphosphate)	practical grade	Sigma Chemical Co.
$Na_5P_3O_{10}$ (Polyphosphate)	practical grade	Sigma Chemical Co.
p-nitroheny1 phosphate	Sigma grade	Sigma Chemical Co.
bis-nitrophenyl phosphate	Sigma grade	Sigma Chemical Co.
Na_2SiO_3 5H2O	technical grade	BDH

ethylene standards were used in the experiments of section 2.82 . All but the pure ethylene standards were supplied by British Oxygen Company Ltd. The pure ethylene standards were obtained from BDH Laboratory Gas Service (Poole, Dorset, England).

2.4 Media

2.41 Mineral liquid media

According to soil chemical composition (Table 3.1). the closest medium to the chemical composition was found to be a modified version of Allen and Arnon (1955) called 'AD' by Sinclair and Whitton (1977a). AD medium was used for initial experiments on soil culturing and isolation and purification of strains. It was further modified by reduction of the phosphorus concentration to 2.5 mg l^{-1}P, when it was found that number of taxa of mixed populations were higher at this concentration in comparison with the normal concentration (44.5 mg l^{-1}P) (Al-Mousawi and Whitton, 1983). At this phosphorus concentration the medium was buffered with 5 mM HEPES. It was noticed that normal iron concentration in AD medium (4 mg l^{-1}Fe) caused precipitation after autoclaving; it was therefore reduced to 1 mg l^{-1}.

For consistency, however, the iron concentration was kept at the normal level (4 mg l^{-1}) for the experiments of Chapter 5. The potassium concentration was reduced to 25 mg l^{-1}. AD medium was the basal medium used for almost all of the experiments. It is free from combined nitrogen (Tables 2.2, 2.3). In some experiments, however, it was enriched with combined nitrogen either as NO_3 or NH_4 (Section 2.45). For simplicity AD medium free from combined nitrogen is referred to here as AD-N and AD medium enriched with combined nitrogen as AD+N. Chu 10D medium a modified version of Gerloff et al., (1950) , as described by Sinclair and Whitton (1977a) and its nitrogen-free version were also used for initial soil culturing as well as isolaton and purification. Growth in all cases was rather poor and neither version was used in subsequent experiments. ACM medium was used for culturing of Anacystis nidulans D33 (Section 6.4); it was derived from medium "C" of Kratz and Myers (1955) as described by Shehata and Whitton (1981) with the exception of including Zn in the microelement stock solution.

The composition of AD, ACM and Chu 10D, together with the original media upon which they were based are shown in Tables 2.2 and 2.3. In many experiments, however, the concentrations of components were varied; details are given

Table 2.2 Composition of media (mg l^{-1} of salts)

	AD	Allen and Arnon (1955)	Chu 10D	Gerloff *et al.* (1950)	ACM	Kratz and Myers (1955)
KNO_3	-	-	-	-	500.00	1000.00
$Ca(NO_3)_2.4H_2O$	-	-	57.60	40.00	-	25.00
K_2HPO_4	250.00	348.37	-	10.00	10.00	1000.00
KH_2PO_4	-	-	7.80	-	-	-
$MgSO_4.7H_2O$	200.00	246.48	25.00	25.00	250.00	250.00
$Na_2SiO_3.5H_2O$	-	-	10.88	25.00	-	-
NaCl	230.00	233.76	-	-	23.00	-
$NaHCO_3$	-	-	15.85	-	-	-
Na_2CO_3	-	-	-	20.00	-	-
$CaCl_2.2H_2O$	66.20	73.50	-	-	19.86	-
$FeCl_3.6H_2O$	19.40	?	2.42	-	1.21	-
Na $EDTA.2H_2O$	25.40	?	3.18	-	0.5	-
$Na_3C_6H_5O_7.2H_2O$	-	-	-	-	-	165.00
$C_6H_5O_7Fe.5H_2O$	-	-	-	3.00	-	-
Citric Acid	-	-	-	3.00	-	-
$Fe_2(SO_4)_3.6H_2O$	-	-	-	-	-	4.00
$MnCl_2.4H_2O$	-	-	0.045	-	1.81	1.81
$MnSO_4.4H_2O$	0.50	2.03	-	-	-	-
$NaMoO_4.4H_2O$	0.06	-	0.007	-	0.027	-
MoO_3	-	0.15	-	-	-	0.018
$ZnSO_4.7H_2O$	0.055	0.22	0.056	-		
$CuSO_4.5H_2O$	0.02	0.079	0.02	-	0.079	0.079
$CoCl_2.6H_2O$	0.01	-	-	-	-	-
$Co(NO_3)_2.6H_2O$	-	0.049	-	-	-	-
$CoSO_4.7H_2O$	-	-	0.01	-	0.04	-
H_3BO_4	0.70	2.86	.72	-	2.86	2.86
NH_4VO_3	-	0.02	-	-	-	-
$Na_2WO_4.2H_2O$	-	0.018	-	-	-	-
$NiSO_4.7H_2O$	0.01	0.045	-	-	-	-
$Cr_2(SO_4)_3K_2SO_4.2H_2O$	-	0.19	-	-	-	-
$TiO(C_2O_4)X.YH_2O$	-	?	-	-	-	-
pH after autoclaving	7.3-7.5	?	7.5-7.8	8.0-9.5	7.3-7.5	7.5-7.8

Table 2.3 Composition of media (mg l^{-1} of elements)

Element	AD	Allen and Arnon (1955)	Chu 10D	Gerloff *et al.* (1950)	ACM	Kratz and Myers+ (1955)
N	-	-	6.83	6.79	69.27	140.02
P	44.46	61.95	1.78	1.78	1.78	177.83
S	26.10	32.41	3.26	3.25	32.55	33.31
Na	92.05	92.00	6.63	14.10	9.17	-
K	112.23	156.40	2.24	4.49	195.58	835.63
Ca	18.05	20.20	9.78	6.97	5.41	-
Mg	19.72	24.30	2.47	2.47	24.65	24.65
Fe	4.00	4.00	0.50	0.50	0.88	-
EDTA	19.80	?	2.49	-	0.50	-
Cl	179.10	177.26	-	-	24.18	-
Si	-	-	1.44	3.31	-	-
Mo	0.02	0.10	0.0028	-	0.011	0.012
Zn	0.01	0.05	0.013	-	0.05	0.05
Cu	0.005	0.02	0.005	-	0.02	0.02
Co	0.002	0.01	0.002	-	0.01	0.008
Mn	0.12	0.50	0.012	-	0.50	0.50
B	0.12	0.50	0.125	-	0.50	0.50
V	-	0.01	-	-	-	-
W	-	0.01	-	-	-	-
Ni	0.002	0.01	-	-	-	-
Cr	-	0.02	-	-	-	-
Ti	-	0.01	-	-	-	-

with indivdual experiments.

Media were made up freshly as required, from stock solutions prepared in double distilled deionized water. Stocks were stored at 4 °C in the dark. The microelements were also added as a single solution. Glass distilled water was used for all media.

For subculturing, aliquots of 25 ml (Chapter 6,7) or 50 ml (Chapter 5) of the medium were used in 100 ml in Erlenmeyer flasks. The pH of the media was adjusted to 7.6 using 0.2 M NaOH before autoclaving and it was measured after autoclaving using an EIL model 7050 direct reading pH meter fitted with a combination electrode (Electronic Instruments Ltd, Chertsey, Surrey, England).

2.42 Solid media

Solid media were prepared by mixing mineral liquid media with 1% agar(Allen, 1968). The agar was added to the mineral medium before autoclaving. After autoclaving and cooling to about 40-45 °C, media were poured into 9 mm pre-sterilized petri dishes in a laminar flow cabinet. After solidification they were kept in a refrigerator (4 °C) in an inverse position. Solid media were used mainly for isolation and purification (Section 2.62) and routine test of bacterial or fungal contamination (Section 2.63).

2.43 Buffers

Some experiments (earlier parts of Chapter 5) were performed in unbuffered media relying on the buffering capacity of phosphorus (PO_4-P) in the medium. It was found that the buffering capacity of AD medium was insufficient when the medium was supplemented with a high level of nitrogen sources (Sections 4.4, 5.6). Furthermore, reduction of the phosphorus concentration of the medium to 2.5 mg l^{-1}P reduced its buffering capacity and made it desirable to have an additional buffer for adequate pH control. Smith and Foy (1974) chose HEPES as a buffer for freshwater algal media because of its favourable pKa of 7.55 and the negligible metal binding capacity reported by Good et al. (1966). Three HEPES conentrations (2.5, 5.0, 10 mM) were tested for their buffering capacity as well as their effect on the growth (expressed as chl a) of *Cylindrospermum muscicola* D579 grown in AD medium over a period of 30 days. All concentrations restricted the pH variations within 0.1 and 0.22 units. However, growth was slightly lower at the highest concentration. In addition the experiment was carried out in AD medium with a very high phosphorus concentration (44.5 mg l^{-1}P) to compare its buffering capacity. In subsequent experiments the phosphorus concentration was reduced to 2.5 or 1 mg l^{-1}P, and 2.5

mM HEPES may have confered inadequate buffering to the medium therefore 5 mM HEPES was chosen. HEPES was added before autoclaving and the pH was adjusted to 7.6 using 0.2 M NaOH.

The pH optima of cellular and filtrate alkaline phosphatase of Iraqi strains and Anabaena cylindrica D2 (Section 6.5) were determined using a wide range of buffers (Table 2.4). Buffers were prepared as described by Dawson and Elliot (1959).

2.44 Chelating agent

Experiments were carried out in media with a pH 7.6 or greater (Table 2.2). Under these conditions iron would be expected to precipitate as a phosphate salt if no chelating agent was used. Iron was therefore added to the media as Fe.EDTA. The molar ratio of EDTA:Fe is 0.94:1. EDTA was first used for algae by Waris (1953) and is now recommended for algal growth by many authors (Stein, 1973; Fogg, 1975).

2.45 Addition of other selected chemicals

Most of the experimental work is concerned with the effect of nitrogen and phosphorus sources (Table 2.5). Details are given below.

Table 2.4 Buffers used in the study of the effect of pH on alkaline phosphatase activity of Iraqi isolate and <u>Anabaena</u> <u>cylindrica</u> D2 (Section 6.5)

pH	Buffer	Molarity (M)
7	3,3 dimethylglutaric acid - NaOH	0.05
7	HEPES (N-2-hydroxyethylpiperazinc N-2-ethane sulfonic acid) -NaoH	0.01
8	glycine-NaOH	0.05
8	tris(hydroxymethyl) methylamine (2-amino-2-(hydroxymethyl) propane-1, 3 diol(tris)-HCl	0.05
8	HEPES-NaOH	0.01
8	borax (sodium tetraborate) -HCl	0.05
9	glycine-NaOH	0.05
9	tris-HCl	0.05
9	borax-HCl	0.05
10	glycine-NaOH	0.05
10	borax-NaOH	0.05
11	glycine-NaOH	0.05
11	borax-NaOH	0.05
12	glycine-NaOH	0.05
12	KCl- NaOH	0.1
13	KCl-NaOH	0.1

Table 2.5 Nitrogen, phosphorus and sulphide sources whose influence were tested together with the complementary salt added to bring the required changes

Chemical tested	salt in AD medium	salt used as a source	complementary salt
NO_3-N	-	$NaNO_3$	-
NH_4-N	-	NH_4Cl	NaCl
PO_4-P	$K_2HPO_4 \cdot 3H_2O$	β-glycerophosphate	KCl
"	"	DNA	"
"	"	inositol hexaphosphate	"
"	"	glucose-1-phosphate	"
"	"	phosphatidylcholine	"
"	"	$Na_2H_2P_2O_7$	"
"	"	$Na_3P_3O_7$	"
"	"	$Na_5P_3O_{10}$	"
H_2S	-	Na_2S9H_2O	-

(A) Nitrogen

(i) Ammonium-nitrogen

This was added as ammonium chloride solution. As a precaution against loss of ammonia during autoclaving; a stock solution of NH_4Cl was sterilized by filtration. Difficulties were found in buffering media with ammonia in long-term experiments (Sections 4.4, 5.6); as a result nitrate was used as a nitrogen source. However, in short-term experiments (Section 7.2) the changes in pH values was found to be within 0.2 units. For experiments using NH_4-N, the +N medium contained 50 mg l^{-1}N.

(ii) Nitrate-nitrogen

Sodium nitrate was used in preference to potassium nitrate because of the high concentration of potassium (112.2 mg l^{-1}) in the original AD medium. The maximum level of nitrogen in the medium was designed to exceed the theoretical algal requiements. It was assumed that the dry weight of the algal was not likely to exceed 2 mg l^{-1} and that 7% of the dry weight was nitrogen (Fay, 1969); therefore the alga would contain a maximum of 140 mg l^{-1}N. For experiments using NO_3-N, the +N medium contained 140 mg l^{-1}N.

(B) Phosphorus

Dipotassium hydrogen phosphate is the phosphorus source in the original medium (Table 2.1). This was omitted when organic and anhydrous phosphorus were used (Section 6.4). Specification and suppliers of all the phosphorus sources were given in Table 2.1. All were sterilized by filtration (Section 2.46).

(C) Reducing chemicals

Sulphide, sulphite and thiosulphate were added as sodium salt which had been sterilized by filtration.

2.46 Sterilization

(i) Autoclaving

Liquid and solid media were sterilized by autoclaving at 121 °C (10.35 kN m^{-2}) for 15 min. In the case of liquid media containing a high concentration of phosphorus or iron, these were autoclaved separately and added to each vessel after the medium had cooled and re-equilibrated with the atmosphere to avoid precipitation during autoclaving.

(ii) Filtration

All the organic additives were sterilized by passing the solution through a pre-sterilized 0.2 µm Nuclepore

membrane filter under aseptic conditions. The filter was fitted in a Swinnex filter holder, which was wrapped in aluminium foil and autoclaved as in (i). The filter sterilized sample was collected in a sterile empty flask. After sterilization the sample was added aseptically to the medium.

2.5 Environmental conditions

2.51 Incubation chambers

Most of the experiments in Chapter 5 were carried out on a temperature gradient apparatus (Fig. 2.1); it is similar to that described by Van Baalen and Edwards (1973). Some experiments, however, were carried out in an illuminated incubator (Gallenkamp 1H-270, England). Flasks were shaken by hand twice a day, but otherwise incubated standing. Most cultures used for isolation and purification (Section 2.62) as well as maintenance of stock cultures were grown in thermostatically controlled growth rooms. Some experiments were carried out here too.

Inoculum pre-incubation and all experiments of Chapters 6 and 7 were carried out in thermostatically controlled tanks of distilled water. A shaking mechanism moved the flasks through a horizontal distance of 30 mm approximately 65 times a minute.

A

B

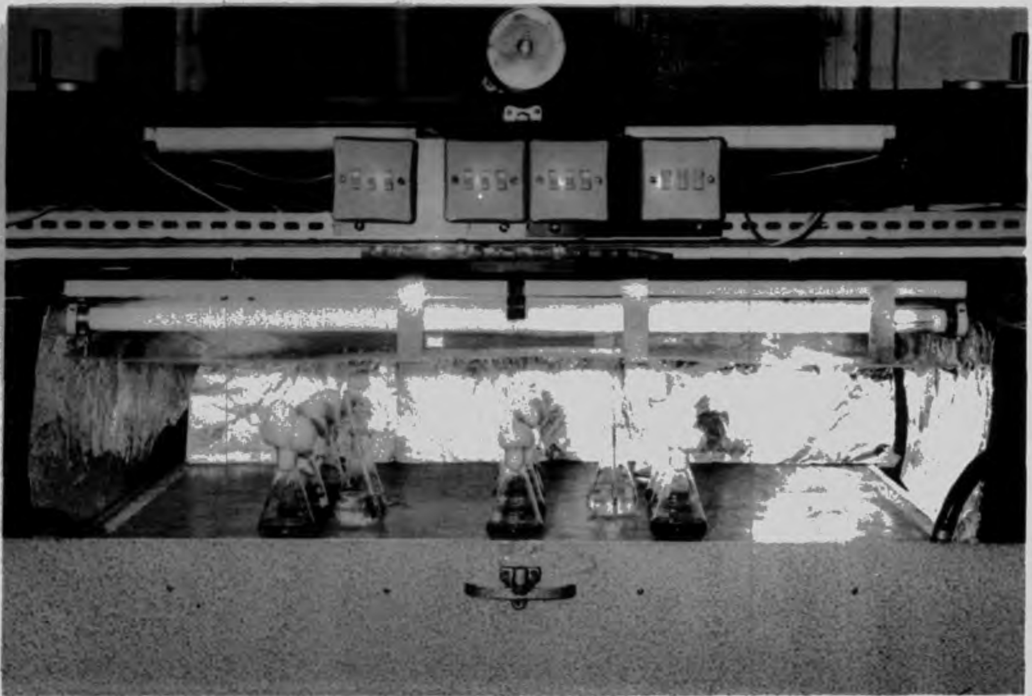


Fig. 2.1 Temperature gradient apparatus used in soil culturing
A. general view B. internal view

2.52 Light and temperature

(i) Light

In the temperature gradient, illuminated incubator and growth rooms continuous illmination was supplied from above by a bank of white flourescent tubes. In the shaking tanks of distilled water illumination was also continuous and supplied from beneath by a series of warm white flourescent tubes.

For experiments on the effect of light quantity and quality on mixed populations (Section 5.3) and on nitrogenase activity of Calothrix sp. and Nostoc muscorum (Section 7.2) aluminium foil, neutral density filters (No209 and 211) and green filter (No124) were used.Filters were manufactured by Lee Filters Ltd, Andover, U.K.). For dark/light experiment (Section 5.3) flasks were first wrapped with black polythene sheets and then covered over with aluminium foil during the dark period. The level of radiation at the surface of the vessel was found to vary spatially in all growth chambers (Table 2.6). To avoid these variations flasks were moved once or twice a day around the growth chamber.

Light measurement was made using a Macam Quantum/Radiometer/Photometer Model Q101 (Macam Photometric

Table 2.6 Light conditions in various growth chambers

growth chamber	type of fluorescent tubes	position of fluorescent tubes	photon flux density µmol photon $m^{-2} s^{-1}$
growth room	white	above	40-60
shaking tanks			
(i) all tubes on	warm white	below	120-300
(ii) 1/2 tubes on	warm white	below	80-120
Temperature gradient			
(i) all tubes on	white	above	150-300
(ii) 1/2 tubes on	white	above	80-120
illuminated incubator	white	above	40-60

Ltd, Livingston, Scotland)

(ii) Temperature

Experiments on the effect of temperature on mixed populations were carried out in a temperature gradient over a range of temperatures between 20-50 $^{o}C \pm 1$. For 5 and 10 ^{o}C experiments an illuminated incubator was used. In all experiments using the temperature gradient, 35 $^{o}C \pm 1$ was used.

For the experiments of Section 7.2 a shaking tank was used. The temperature range used was between 10-40 ^{o}C. Ice was used to reduce the temperature gradually from 40 to 10 ^{o}C. For 50 ^{o}C a water bath (Griffin student water bath, Gallenkamp & Co. Ltd, U.K.) was used and flasks were shaken more or less continuously by hand.

Thermostatically controlled growth rooms were available at temperatures 15, 25 and 32 ^{o}C. Stock cultures were kept at 25 ^{o}C. Isolation and purification and some experiments were carried out at 32 ^{o}C. Unless otherwise indicated the standard growth conditions for the experiments of Chapter 5 were 35 $^{o}C \pm 1$ and a photon flux density (PAR) 90-120 μmol photon $m^{-2}s^{-1}$. For the experiments of Chapters 6 and 7 the standard growth conditions were 32 ^{o}C and 90-120 μmol photon $m^{-2}s^{-1}$ and continuous shaking.

2.53 Gaseous atmosphere

In the experiments on nitrogenase activity of Microcoleus chthonoplastes (Section 7.3), 100 ml Erlenmeyer flasks with side arms, for the introduction of the required gas, were used. The gas inlet was close to the flask base to ensure distribution of the injected gas throughout the medium. Before connection to the gas supplier via a manifold of teflon tubing and needle (0.63 mm diameter) the flask and sidearm openings were fitted with gas tight suba-seal stoppers (W.Freeman & Co. Barnsley, U.K.). Media were deaerated for 15 min by bubbling with nitrogen gas (oxygen-free). In some experiments carbon dioxide (oxygen-free) was injected to give a concentration of about 1% v/v.

2.6 Algal cultures

2.61 Origin

Apart from the three control organism, all strains used for experimental work were isolated from the soil sample collected from rice-field in the southern marshes of Iraq Section 2.1. Details of the strains used in the study are given in Table 2.7.

Table 2.7 Algal cultures and their origin

Algae	Durham culture No.	Origin	whether axenic	clonal
Iraqi isolates heterocystous forms				
Anabaena cylindrica	582	Iraqi rice field	+	+
Anabaena oscillarioides	693	"		+
Calothrix sp.	580	"	+	+
Calothrix sp.	583	"	+	+
Calothrix sp.	585	"	+	+
Cylindrospermum muscicola	579	"	+	+
*Cylindrospermum muscicola**	678	"		+
Hapalosiphon welwitschii	581	"	+	+
Gloeotrichia sp.	636	"	+	+
Nodularia harveyana	635	"		+
Nostoc muscorum	584	"	+	+
Nostoc linckia	586	"		+
non-heterocystous forms				
Lyngbya aestuarii	638	"		+
Lyngbya $> 1 \leqslant 2\ \mu m$	639	"		+
Microcoleus chthonoplastes	634	"	+	+
control strains				
Anabaena cylindrica	2	Cambridge 1403/2a	+	+
Ancystis nidulans	33	" 1405/1	+	+
Calothrix parietina	550	Sand sike England (Holmes and Whitton 1981)	+	+

* spore forming strains

2.62 Isolation and purification

The air-dried soil sample was added to liquid medium. AD medium Tables 2.2 and 2.3 and a wide range of its modifications were used initially. Healthy growth of the majority of blue-green algae occurred only in AD medium and its low phosphorus (0.45 mg l^{-1}P) version. Cultures were incubated in a temperature gradient Fig. 2.1 over a wide range of temperatures and photon flux densities. Soil culturing resulted in the growth of a mixture of algae. Cultures in which heterocystous blue-green algae dominated were selected for further subculturing into liquid media or onto solid media. Repeated subculturing resulted in unialgal cultures.

Clonal and bacteria-free cultures of hormognia-producing strains were obtained according to the method described by Boyer and Skerman (1968) with some modifications as follows. Small pieces of a young vigorously growing culture were placed onto the middle of agar plates of AD or AD low phosphorus (0.45 mg l^{-1}P) media. Plates were then incubated in a growth room at 32 ^{o}C and photon flux density of 30-40 µmol photon $m^{-2}s^{-1}$. After 2-5 days there was usually a zone of hormogonia around the inoculum, some sufficiently well separated for individuals to be picked off. Clean hormogonia were located using a stereoscopic binocular microscope and a single hormogonium,

together with a block of the surrounding agar, was transferred onto a fresh agar plate. After successive transfers on agar plates, a hormogonium was transferred into fresh liquid medium. Flasks were incubated at 32 ^{o}C. After 2-3 weeks algal growth was seen in some of the flasks. The cultures were then tested for bacterial and fungal contamination (Section 2.63).

For strains which do not form hormogonia, clonal and bacteria free cultures were established according to the following procedure, which is a combination of the methods of Brown and Bischoff (1962) and Wiedeman *et al.* (1964). Algal material from young cultures was transferred into fresh liquid medium in a sterile sonicator tube. The culture was sonicated in a Soniprep 150 for 5-10 seconds. It was then centrifuged at about 500xg for 2 min. The supernatant was discarded and pellet resuspended in fresh sterile medium. This sonication/centrifugation process was repeated three times. The sonification process fulfilled two purposes; firstly bacterial cells adhering to the surface of algal cells were likely to be dislodged and secondly algal filaments were broken into short segments which facilitated their spreading on the agar plates (Patterson, 1983). Continuous discarding of centrifuged supernatant increased the overall algae to bacteria ratio,

since the smaller bacterial cells sediment more slowly than the algal segments under the influence of gravity. After the final sonication algal material was sprayed onto sterile agar plates using a sterile air current. Plates were then incubated for a short period of 2-3 days in a 32 °C growth room. Following incubation the plates were examined under a stereoscopic binocular microscope contained in a laminar flow cabinet. Clean algal filaments were removed from the agar surface using a very fine sterile needle and inoculated onto fresh agar plates. After successive transfers onto agar plates, a filament was transferred into fresh liquid medium. Flasks were then incubated in a 32 °C growth room. When algal growth became obvious cultures were tested for any bacterial and/or fungal contamination (Section 2.63)

2.63 Test for purity

The strains isolated were examined after the initial purification and periodically before each subculturing and before use as experimental inocula. Three different tests were carried out after the initial purification and at least two were used routinely. The tests were as follows.

(i) Algal mate^r^ial was examined under the microscope using both normally transmitted light and phase contrast

microscopy; if no contaminants could be seen the second test followed.

(ii) Small pieces of algal material with few drops of growth medium were sprayed on the surface of agar plates of the following bacterial and fungal test media as described by Hoshaw and Rosowski (1973).

peptone-glucose

malt extract

yeast extract

nutrient broth

SST

Usual agal medium +0.1% casamino acid

Plates were incubated in the dark at 32 ^{o}C for 3 weeks, if no growth of either bacteria or fungi was seen the third test followed.

(iii) Usual liquid growth medium enriched with either 0.05% sucrose or 0.1% casamino acid was inoculated with algal material and flasks were incubated in the dark and in the light in the 32 ^{o}C growth room. If the appearence of the cultures did not change the cultures were re-examined under the microscope as described in (i).

Sometimes algal cultures under the microscope showed particles which exhibited brownian movement similar to that

of non-motile bacteria, in this case cultures were autoclaved and re-examined under the microscope.

If the presence of contaminants could not be shown it was concluded that an algal strain was axenic.

2.64 Maintenance and subculturing

(i) Maintenance

Stock cultures for use as an inoculum were maintained in 50 ml liquid medium contained in 100 ml straight neck conical flasks closed with silicon bungs (Type C 20, manufactured by Sanko Plastic Ltd , Osaka, Japan); cultures were incubated on glass shelves in a thermostatically controlled growth room at 25 ^{o}C and with a photon flux density 20-30 μmol photon $m^{-2}s^{-1}$. Transfers to fresh medium were made every 3-4 months. Cultures were also preserved cryogenically in liquid nitrogen soon after their isolation into a clonal or axenic state.

(ii) Subculturing

Subculturing was carried out with standard aseptic techniques, using a horizontal laminar flow cabinet (Microflow Pathfinder, conforming to B.S. 5295 Class 1). This takes in air through a filter which removes the large

particles and then passes the air through a highly efficient particulate air filter and out horizontally across the work surface in a laminar flow pattern. Before subculturing the cabinet was sprayed with absolute alcohol and the fan switched on and left for about 5 minutes. The cabinet was checked every 6 months, to see if the filters in the inoculating cabinet were functioning properly, by placing bacterial test plates in different locations in the cabinet and incubating them in the dark at 32 ^{o}C.

The subculturing of algae for isolation and purification (Section 2.62) and routine subculturing of stock cultures, was perfomed by transferring algal material with a sterile wire loop. For experimental work a Gilson Adjustable Volume Pipettman with sterile plastic tips was used. Before each subculturing algal material was tested for any contamination by both microscopic inspection and by subculturing onto a range of bacterial and fungal test media (Section 2.63).

2.65 Preparation of alga for assay

2.651 Inoculum

Inoculum materials were always taken from logarithmic growth phase cultures which were acclimated to the growth conditions by subculturing 2-3 times into a fresh medium

under aseptic conditions (Section 2.64). After 5-7 days from the second subculture the contents of many flasks were pooled and lightly homogenized aseptically by sequential syringing with a sterile disposable plastic syringe (B-D plastipak, Becton, Dickinson and Co. Ltd, Ireland) fitted with a sterile disposable hypodermic needle 0.63 mm or 1.10 mm in diameter (Sabre Gilette Surgical Ltd, U.K.). Algal homogenate was mixed continuously using a magnetic stirrer. A known volume of the homogenate was then inoculated into fresh medium using a Gilson Adjustable Volume Pipettman and sterile plastic tips. Uniform inocula for experimental work, for each alga, were maintained using chl a as a biomass criterion. Chlorophyll a measurements were made just prior to inoculation in order to allow calculation of the inoculum volume. The inoculum volume was always $\leq 2\%$ of the total experimental volume.

2.67 Estimation of growth

The following standard procedure was used in harvesting algal cultures for chl a and dry weight determinations. Algal material was removed from the flask by a wire loop or glass rod fitted with a rubber tube and homogenized lightly using a syringe and needle (Section 2.651), it was then transferred to a measuring cylinder, and the volume made up

to the original volume using distilled water and/or fresh medium minus phosphorus (Section 2.81). A known volume was then used for chl a and dry weight determinations.

2.671 Chlorophyll a

The chl a content of algal material was determined according to the following procedure based on the recommendations of Marker et al. (1980). A known volume of algal homogenate (Section 2.67) was harvested either by vacuum filtration through Whatman GF/C glass fibre filter or by centrifugation (3000xg for 10 min). The pigment was then extracted with 90% methanol for 10 min at 70 ^{o}C. The extraction method was sometimes repeated two or three times to ensure complete extraction of the pigment. The extract was then filtered through GF/C filters and the volume made up in a volumetric flask using 90% methanol. Absorbance of the extract was then measured at 665 nm and 750 nm; against a solvent blank in both cases, using a Shimadzu (model UV-150-02) double beam spectrophotometer. A known volume of 0.1 M HCl was then added to the extract to give a final concentration of 10^{-3} M HCl. After 60 min, absorbance at 665 nm and 750 nm was measured, and the chl a content calculated according to the following formula.

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

where A_b = absorbance of extract at 665 nm before acidification minus absorbance at 750 nm

A_a = absorbance of extract at 665 nm after acidification minus absorbance at 750 nm

R = maximum acid ratio (A_b/ A_a)

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

V = volume of solvent used to extract the sample in ml

L = path length of the cuvette in cm

Marker et al. (1980) recommended a specific absorption coefficient of chl a in 90% methanol of 77, and a maximum acid ratio of 1.59 for 90% methanol in 10^{-3} M HCl, therefore,

μg chl a = 34.94(Ab-Aa)xV/L

The extraction and measurements were carried out in the dark as much as possible to prevent photodecompostion of the pigments.

2.672 Dry weight

An aliquot of algal homogenate (Section 2.67) was spun at 3000xg for 10 min , and washed three times with distilled water before being transferred into pre-dried, acid washed (Section 2.21) snap-top glass vials; these had been weighed previously. The vials and algal material were then dried at 105 ^{o}C for 48 hours. On removal from the oven they were placed immediately into a desiccator to prevent absorption of water. The vials were then re-weighed in order to determine algal dry weight.

2.673 Equations

The growth rates of isolated strains and Calothrix parietina D550, as a control , were determined in batch culture under the standards growth conditions (Section 2.671). Chlorophyll a was used as a measure of growth. Growth rates were expressed in terms of the relative growth constant, K, in $\log_{10}$ day units (Fogg, 1975) as follows.

$$\bar{K} = \frac{\log_{10} N_t - \log_{10} N_o}{t}$$

where t = time in days after inoculation

N_t = yield after t days

N_o = amount of algal material in the culture at the beginning

The mean doubling time, G, can be derived from $\bar{K}$:

$$G = \frac{0.301}{\bar{K}}$$

2.7 Chemical analytical procedures

2.71 Atomic absorption spectrophotometer

The analysis of metals in soil digests (Section 2.12), AD medium, filtrates of AD + 5 mg soil and filtrates of distilled water +5 mg soil were carried out on a Perkin-Elmer model 403 atomic absorption spectrophotometer, using matrix matched standards.

2.72 Phosphorus

(A) In growth medium

Analysis of phosphorus in growth medium was carried out after separation of algal material by filtration (GF/C filters). Two forms of filtrable phosphorus were measured: "filtrable reactive phosphorus" (FRP) and "filtrable total phosphorus" (FTP) (=organic + orthophosphate) (Mackereth et al., 1978). Both involve the reaction of phosphorus(phosphate) with molybdate in an acid solution. The organic forms were broken down with the addition of sulphuric acid and potassium persulphate before autoclaving (Eisenreich et al. 1975). A single reagent solution was used for both determinations; it is a modification of the reagents of the method of Eisenreich et al., (1975) (Livingstone, pers. comm.) The reagents used were:

1. Acid - antimony-molybdate reagent

0.57 g of potassium antimonyl tartrate was dissolved in 500 ml of distilled water and 45 ml of concentrated sulphuric acid (S.G. 1.84) was added with continuous mixing and cooling. 8.52 g of sodium molybdate was dissolved in 400 ml of distilled water. The two solutions were then mixed and volume made up to 1000 ml in volumetric flask. After cooling to room temperature, the reagent was transferred

into a dark glass bottle and kept in a refrigerator at 4 °C.

2. Sulphuric acid (approx. 1.0 N)

28 ml of concentrated sulphuric acid (S.G. 1.84) was added to 800 ml of distilled water with continuous shaking and cooling. After cooling the volume was made up to 1000 ml with distilled water.

3. Potassium persulphate

Analoid compressed tablets (Ridsdale and Co. Ltd, Middlesborough, England) containing 0.7 g of potassium persulphate were used.

4. Phosphorus standard solution

4.39 g of potassium dihydrogen phosphate K_2HPO_4 was dissolved in 500 ml of distilled water and the volume made up to 1000 ml in a volumetric flask. This solution contained 1000 mg l^{-1}P.

5. Mixed reagent

0.62 g of ascorbic acid was dissolved in 100 ml of reagent(1). This reagent does not keep and should be freshly prepared.

The measurements for FRP and TFP were as follows.

(i) Filtrable reactive phosphate 1 ml of 1 N H_2SO_4 solution was added to an aliquot of sample diluted to 25 ml with distilled water (or to 25 ml of diluted sample) in a 125 ml conical flask and mixed thoroughly. Mixed reagent (5 ml) was then added to the sample and mixed thouroughly. After at least 10 min of colour development absorbance was taken at 880 nm on a Shimadzu (model UV-150-02) double beam spectrophotometer using 4 or 10 cm glass cells. Distilled water was used as a reference. In the case of coloured samples, a blank was included which consisted of sample to which mixed reagent minus ascorbic acid and antimonyl potassium tartrate was added (American Public Health Association, 1981). The detection limits achieved by this method were 1 $\mu g\ l^{-1}$ (10 cm cell) and 5 $\mu g\ l^{-1}$ (4 cm cell).

(ii) Total filtrable phosphorus

This was determined as follows. To an aliqout of sample diluted to 100 ml with distilled water (or 100 ml of diluted sample) in a 250 ml conical flask, 5 ml of 1 N H_2SO_4 was added and mixed thoroughly, this was followed by addition of

one potassium persulphate tablet. The flasks were closed with aluminium foil and autoclaved for 30 min at 121 ^{o}C and 10.35 kN m^{-2}. After cooling the samples to room temperature, 5 ml of mixed reagent was added and they were mixed thoroughly. Absorbance was read after 10 min as in the case of FRP .

Phosphorus was determined from a calibration curve, which was made once for each mixed reagent preparation. The mixed reagent was found to be stable for 3 weeks. The effectivness of the digestion mixture and the autoclaving time were checked using wide range of organic and anhydrous phosphorus compounds (Table 2.8) and by comparing the calibration curves for orthophosphate , inositol hexaphosphate (phytic acid) and β-glycerophosphate (Fig. 2.2)

The method was further checked against the method of Murphy and Riley (1962) as outlined in Standard Methods (American Public Health Association, 1981). A summary of the comparison is shown in Table 2.9.

(B) Total phosphorus analysis in algae

Total phosporus of algal material was determined according to Batterton and Van Baalen (1968) with some modifications as follows. 10 ml distilled water was added to

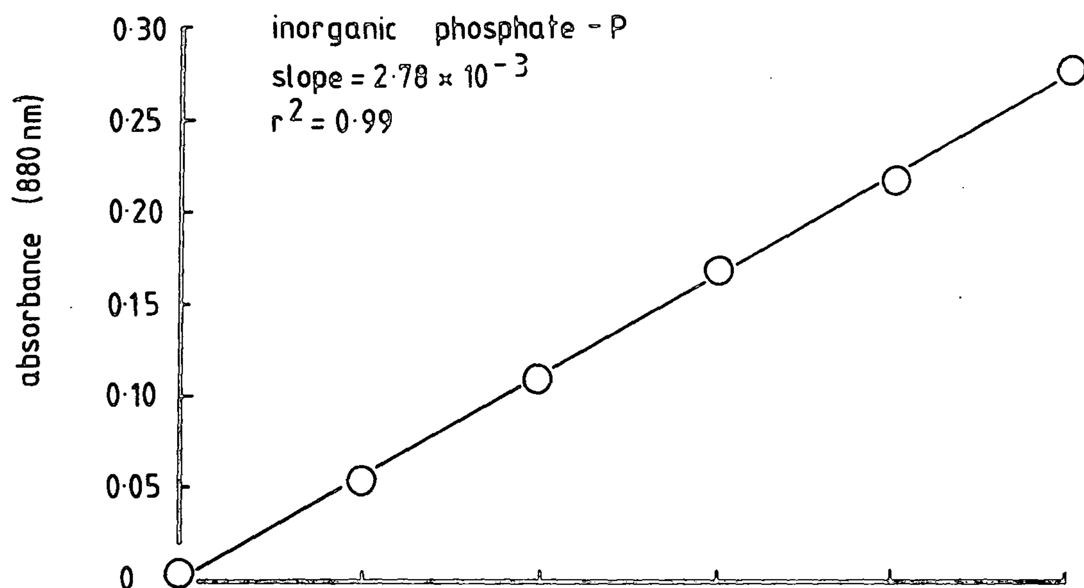
Table 2.8 Effect of digestion mixture of the method of Eisenreich *et al.* (1975) and autoclaving time on total phosphorus recovery from organic and anhydrous phosphorus compounds (1 mg l^{-1} P stock solutions were used, n=4)

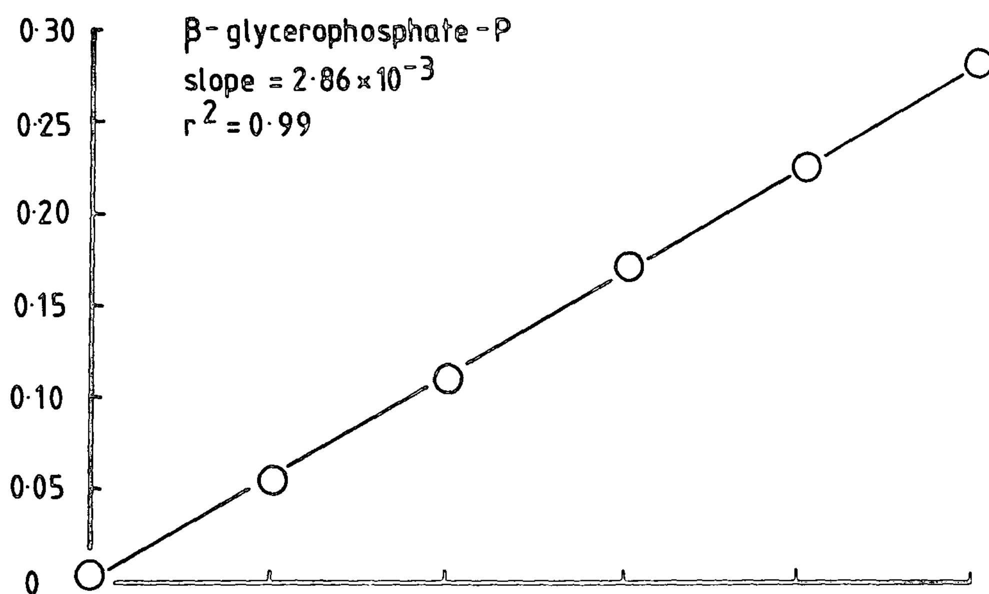
phosphorus compounds	P-found (mg l^{-1}) $\bar{x}$	± SD	%recovery
β-glycerophosphate	1.01	0.008	101%
DNA	0.95	0.01	95%
inositol hexaphosphate	1.08	0.01	108%
lecithin	0.87	0.02	87%
glucose-1 -phosphate	1.04	0.005	104%
pyrophosphate	0.93	0.17	93%
metaphosphate	0.89	0.06	89%
polyphosphate	1.03	0.13	103%

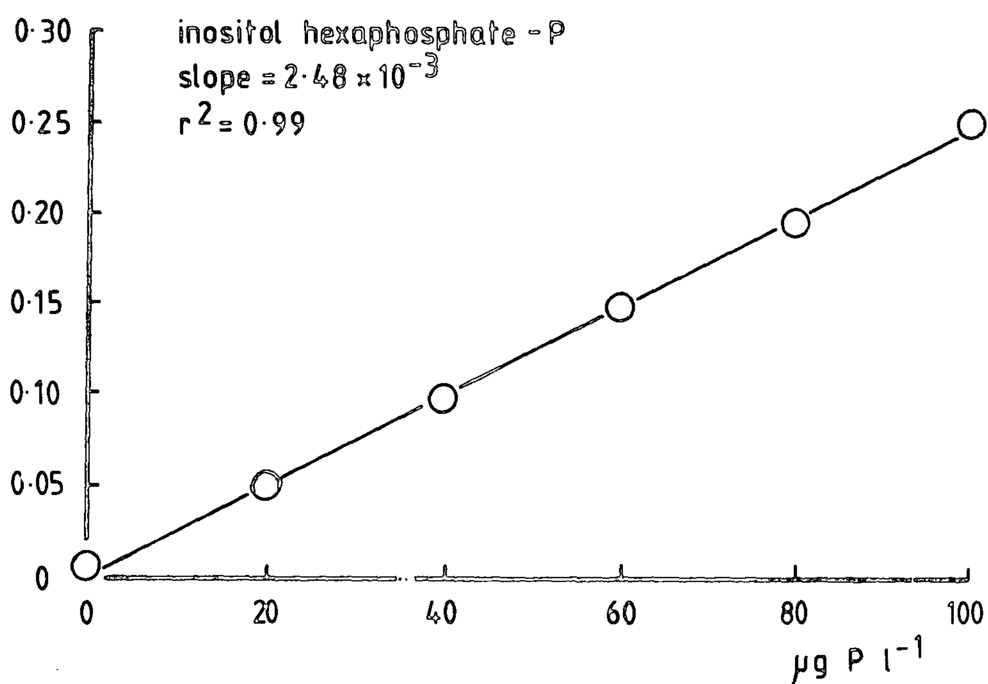
Table 2.9: Comparison of two methods used in phosphorus analysis in growth medium [modified method of Eisenreich et al. (1975) and method of Murphy and Riley (1962) as outlined in American Public Health Association (1981)]

Modified Eisenreich *et al.* (1975) method	Murphy and Riley (1962) method (American Public Health Association 1981)
1. Stability creeping in absorbance present; recording absorbance after a definite time (10 min) gives similar values always	no creeping after 30 min, and absorbance fairly stable up to 24 hours.
2. Accuracy A: Soluble reactive phosphorus Chu 10-D: 54.64 $\mu g\ l^{-1}$ P for 53.4 $\mu g\ l^{-1}$ P (actual conc.) β-glycerophosphate solution: 0.93 $\mu g\ l^{-1}$ P in 50.0 $\mu g\ l^{-1}$ P	Chu 10-D 55.51 $\mu g\ l^{-1}$ P for 53.40 $\mu g\ l^{-1}$ P β-glycerophosphate solution 1.9 $\mu g\ l^{-1}$ P in 50.00 $\mu g\ l^{-1}$ P
B: Total phosphorus Chu 10-D 55.19 $\mu g\ l^{-1}$ P for 53.4 $\mu g\ l^{-1}$ β-glycerophosphate solution 49.32 $\mu g\ l^{-1}$ for 50.00 $\mu g\ l^{-1}$	Chu 10-D: 59.23 $\mu g\ l^{-1}$ P for 53.4 $\mu g\ l^{-1}$ β-glycerophosphate solution 51.54 $\mu g\ l^{-1}$ for 50.00 $\mu g\ l^{-1}$
3. Detection limit 4.0 cm cell: 5.0 $\mu g\ l^{-1}$ 10.0 cm cell: 1.0 $\mu g\ l^{-1}$	4.0 cm cell: 5.0 $\mu g\ l^{-1}$ 10.0 cm cell: 1.0 $\mu g\ l^{-1}$
4. Time and labour Less than half the time and glassware are required for multiple samples.	more than double the time and glassware are required for neutralization, dilution etc.
5. Chemicals and error Less chemicals needed and less chances of contamination (e.g. no need for neutralization of treated samples).	more chemicals needed and high chances of contamination during neutralization dilution etc.

Fig. 2.2 Effect of digestion mixture of the method of Eisenreich *et al.*, (1975) and autoclaving time (Section 2.72) on total phosphorus recovery from three phosphorus sources.


inorganic phosphate - P
slope = 2·78 × 10⁻³
r² = 0·99
absorbance (880 nm)
0·30
0·25
0·20
0·15
0·10
0·05
0


β - glycerophosphate - P
slope = 2·86 × 10⁻³
r² = 0·99
0·30
0·25
0·20
0·15
0·10
0·05
0


inositol hexaphosphate - P
slope = 2·48 × 10⁻³
r² = 0·99
0·30
0·25
0·20
0·15
0·10
0·05
0
0
20
40
60
80
100
μg P l⁻¹

algal material subsequent to dry weight determination (Section 2.672), this was followed by the addition of potassium persulphate tablet (0.7 g). Vials were then covered with aluminium foil and autoclaved for 30 min at 121 °C and 10.35 kN m^{-2}. After cooling to room temperature the volume was made up to 25 ml with distilled water and phosphorus analysis was carried out as described for FRP and TFP . A blank of distilled water was always included.

The method of Batterton and Van Baalen, outlined above, was compared with five different methods to ensure complete recovery of the phosphorus of algal material and to check for any interference; the methods tested are listed below.

1. Sulphuric acid-persulphate digestion method

   As described by Livingstone *et al.* (1983)

2. The method of Harwood *et al.* (1969) modified by the addition of two amounts of 30% H_2O_2 solution (phosphate-free, BDH Laboratory reagent), the two amounts are (A) 0.25 ml and (B) 1.0 ml.

3. As in 2 plus addition of one potassium persulphate tablet (0.7 g).

4. Sulpuric acid-perchloric acid digestion method

The method was carried out as follows. To dry algal samples in glass snap-cap vials 0.5 ml of concentrated sulphuric acid was added. The vials were heated on a hot plate gently at 100 °C until the colour of the sample was changed to dark-brown. When cool 1 ml of a mixture of concentrated sulphuric acid and perchloric acid (70%) was added slowly with swirling, the vials were heated again until the colour changed to colourless or pale yellow then allowed to cool. After that volume was made up to 25 ml with distilled water and phosphorus analysis was carrried out as described above.

5. Persulphate-distilled water digestion method

This was run in two ways:

A. To dry algal sample, 10 ml distilled water was added followed by one persulphate tablet and the mixture was autoclaved for 30 min at 121 °C and 10.35 kN m^{-2} (Batterton and Van Baalen, 1968).

B. As in A but the content was boiled for 60 min on a hot plate at 100 °C.

The results of the comparison of these methods are shown in Tables 6.4 and 6.5. Phosphorus analysis was always carried

out in H_2SO_4 washed glassware (Section 2.21).

2.8 Biological analytical procedures

2.81 Phosphatase activity

Alkaline phosphatase activity for algal homogenate (total), algal material (cellular) and in the growth medium (filtrable) was monitored by the colorimetric technique described in Sigma Technical Bulletin number 104 (1974) with the following modifications.

1. Tris-HCl buffer 0.1 M, pH 8.5 at 32 ^{o}C was used instead of glycine-NaOH 0.1 M pH 10.3 at 20 ^{o}C with 0.1 mM $MgCl_2$ and choloroform as preservative.

2. The substrate (p-nitrophenyl phosphate) was prepared in the same buffer containing $CaCl_2$ to give a final concentration of 2 mM Ca, in a final volume of 2 ml of reaction mixture, except for pH optima where the substrate was dissolved in distilled water.

(i) Total

This was assayed as follows: 0.5 ml of algal homogenate (Section 2.67) was transferred into universal bottles (c 25 ml) follwed by the addition of 1.0 ml buffer, the mixture

was then allowed to equilibrate thermally to 32 °C for 10 min. The reaction was started by adding 0.5 ml of the substrate solution to give a final concentratin of 2.69 mM and terminated by adding 9.0 ml of 0.05 M NaOH. The entire content was then mixed thouroughly and filtered using GF/C filters. The absorbance of the filtrate was then read immediately, or after being kept in an ice bath (Healy, 1973), at 410 nm using 1 cm glass cells on a Shimadzu (model UV-150-02) double beam spectrophotometer. The amount of p-nitrophenol liberated was then determined using a calibration curve. The specific activity was expressed as μmol p-nitrophenol $ml^{-1}h^{-1}$. Appropriate dilutions were made of sample tested so that the spectrophotometric absorbance values fell within the range 0.1 - 0.7 wherever possible. A blank was always run for each set of assays using 0.5 ml of distilled water or buffer. All assays were always carried out under the same experimental conditions and in acid washed glassware (Section 2.21).

(ii) Cellular

Unless otherwise indicated, the following standard assay conditions were used. Algal material was collected on pre-washed GF/C filters and washed three times with distilled water (5 ml each). The filter and the algal material was

transferred into Universal bottles and 1.5 ml buffer was added. The subsequent steps were as for total.

(iii) Filtrable

This was referred to as the enzyme activity in the filtrate of algal homogenate. It was assayed as described in (i) using 0.5 ml of the filtrate.

(iv) Test for other phosphatases

This was made qualitatively by testing the ability of the strains to grow on different phosphorus substrates as a sole phosphorus source (Section 6.3) with the exception bis-nitrophenyl substrate for which a colorimetric assay was used.

2.82 Nitrogenase activity

Nitrogenase activity was assayed using the acetylene reduction technique discussed by Hardy *et al.* (1973). The algae were maintained in a nitrogen-free mdium in Erlenmyer flasks. The flasks were plugged with cotton bungs and contained 25 ml of the growth medium. The total volume of the flasks was 128 ml ± 1. All experiments were incubated under the standard growth conditions using shaking tanks (Section 2.52). The assay starts when cotton bungs were

replaced by gas-tight suba-seal bungs (W. Freeman and Co., Barnsley, U.K.) and 15 ml of acetylene (BOC product) was injected into each flask. After injecting the acetylene, the pressure inside the flasks was brought to the prevailing atmospheric pressure by venting through the suba-seal bungs. Flasks were then incubated under the same experimental conditions as was used to culture the algae (Section 2.52), except photon flux density which was maintained at 110 $\pm$ 10 μmol photon $m^{-2}s^{-1}$.

Four replicate flasks were used in all assays. An incubation period of 60-120 min was used, after which the gas phase in each flask was sampled using 5 ml draw pre-evacuated tubes (Vacutainer)(Ezee -draw, Rocket, London, U.K.), connected to the flask via a multiple sample Vacutainer needle (Becton and Dickinson Ltd , U.K.)

A gas sample (1 ml) from the pre-evacuated tube (Vacutainer) was then withdrawn using 1 ml diposable syringe (B-D Plastipak, Becton-Dickinson and Co. Ltd , Ireland) fitted with 0.5 mm diameter sterile hypodermic needle (Sabre, Gillete Surgical, U.K.) and injected into a Varian Aerograph 1400 series (2700 Mitchel Drive, Walnut Creek, California, U.S.A.) gas chromatograph equipped with a hydrogen flame ionization detector. The column (3.0 mm x 2.0 m stainless steel) was packed with "Porapak" R (Waters

Associates Inc, U.S.A.). The operating conditions were as follows: detector temperature, 150 ^{O}C; column temperature, 100 ^{O}C; hydrogen flow rate, 30 ml min^{-1};air, 300 ml min^{-1}; nitrogen, as a carrier gas, 45 ml min^{-1}. Ethylene peaks were identified on recorder traces by the retention time and quantified with standard curves. The chromatograph was calibrated using dilutions, in air, of high purity ethylene standards (BDH Poole, Dorset, England) on each day of use.

The following controls were employed at the beginning of the assay, but only the first one was used routinely.

(i) Distilled water, to give an estimate of ethylene present as a contaminant in the acetylene as well as a test for possible leakage of hydrocarbons from the suba-seal bungs (Postgate, 1972).

(ii) Autoclaved algal culture to see if it differs from (i).

(iii) Algal cultures incubated in the dark; a large differerence between light and dark rates of reduction was found. This could be taken as evidence that the activity was of algal origin and that contamination of cultures during the experiment was unlikely.

The results of the acetylene reduction assays were expressed as nmol C_2H_4 μg^{-1}chl a min^{-1}.

CHAPTER 3. STUDY AREA AND SOIL SAMPLE

3.1 Introduction

The present study was based on one soil sample collected from a rice-field in the southern marshes of Iraq (Section 2.11). These marshes lie in the delta of the Tigris and Euphrates (Fig. 3.1). The sample site (Fig. 3.2) lies near site D (Um al Schwaich) used in a study (Maulood *et al.*, 1981) of the algal ecology of the permanent marshes. The sample was taken from the side of a field about 200 m from the main navigation channel (Fig. 3.3).

Inside the marshes, rice is grown on land which is under water during the peak of the flood from the Tigris and Euphrates, but which requires earth dams to maintain the water level during summer (Fig. 3.4). The sample was taken from the edge of a rice-field with obvious cracks in the soil surface (Fig. 3.5). There was an almost continuous algal cover over the surface of the soil, but no evidence of dried algal fragments on the rice plants themselves (Fig. 3.6). Unfortunately, the author could not visit the area during the present study, but the site has been visited twice in the recent years (Maulood *et al.*, 1979 & 1981). There were no obvious changes in the site during these two visits.

The literature on the study area is sparse, reflecting the relative inaccessibilty of the region. Apart from the two

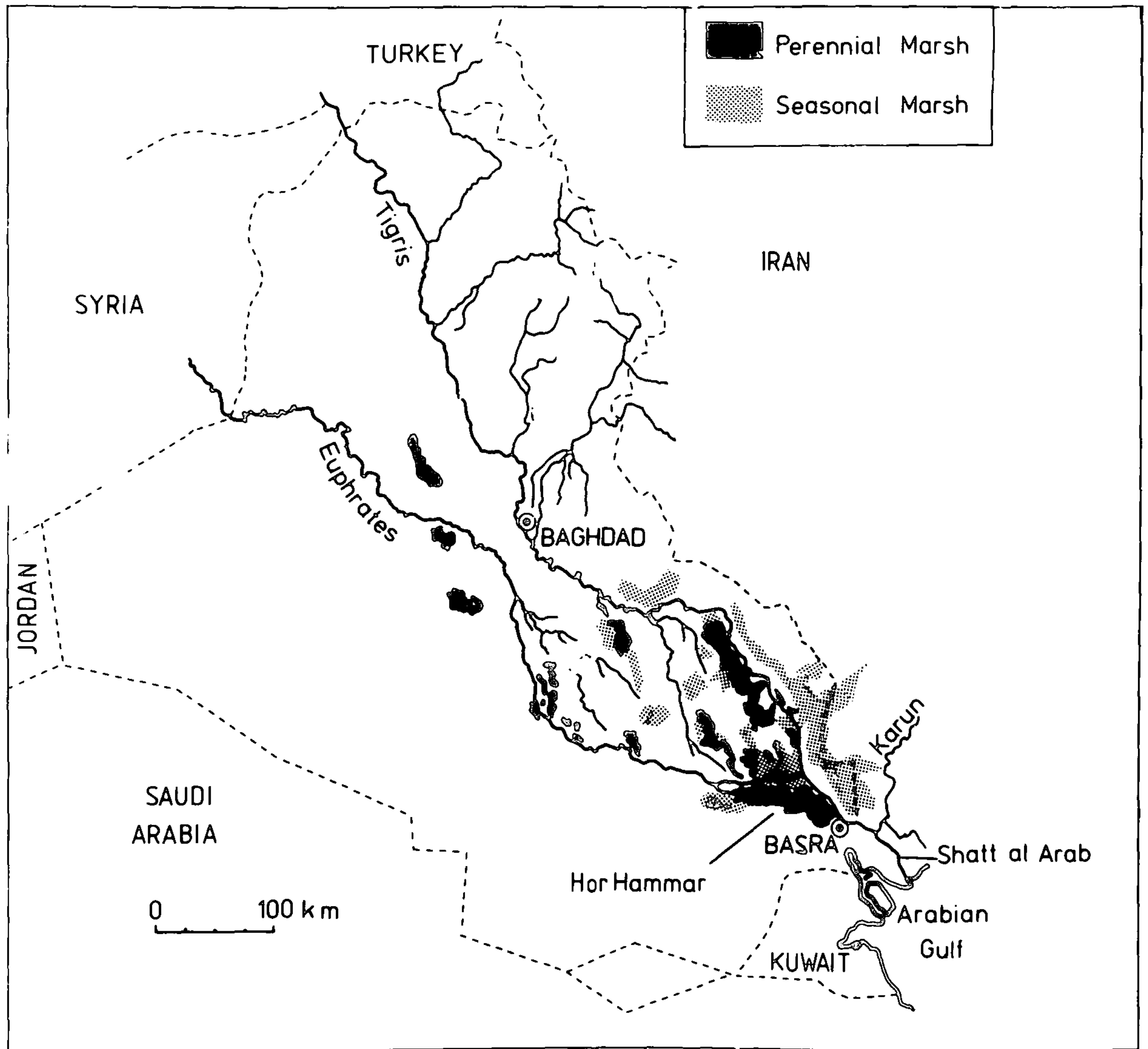


Fig. 3.1 Map of Iraq showing the southern marshes

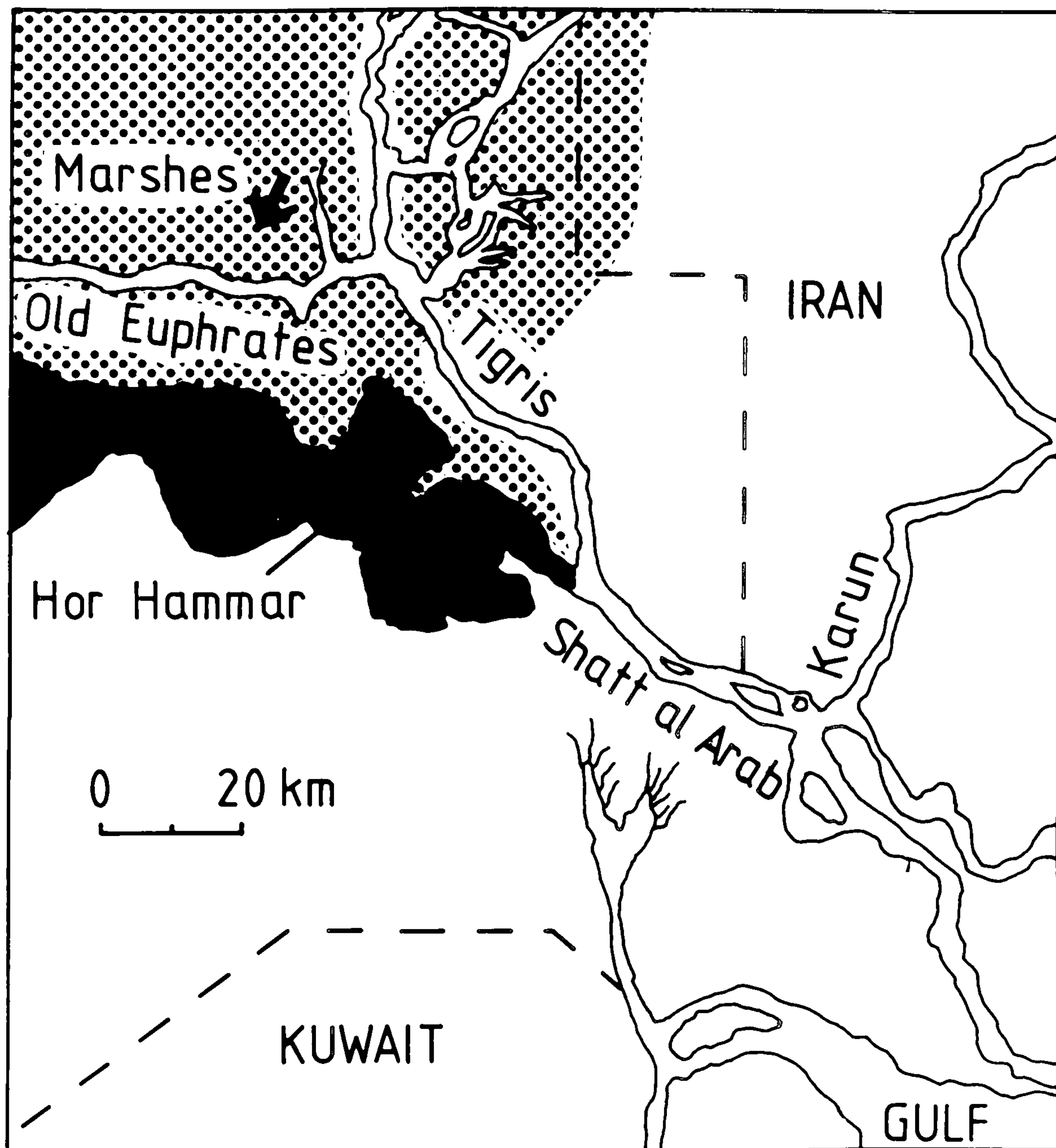


Fig. 3.2 The southern marshes showing location of site from which rice-field soil collected (arrow)

Fig. 3.3 Main navigation channel at the site studied
(Um al Schwaich)

Fig. 3.4 General view of rice-field in the southern marshes showing the earth dam built to maintain water level in the field during summer

Fig. 3.5 Edge of rice-field from which soil sample collected

Fig. 3.6 Algal crust on the soil surface in the rice-field

papers listed above, a paper describing some of the work reported in this thesis has been published (Al-Mousawi and Whitton, 1983). This describes the influence of environmental factors on algae from the soil sample. Almost all the data given there is reported also in the thesis.

3.2 Environmental background

The literature reviewed in Section 1.1, makes it clear that the study area (rice-field) is part of the southern marshes and that the environmental factors influencing the marshes as a whole are important in the rice-field.

Rice-fields are covered by water during the peak of the flood (March-May); standing water typically remains until late August or September. There are very few reports about the physico-chemical properties of the water in the marshes (Panko et al., 1979; Maulood et al., 1979; 1981; Al-Saadi et al., 1981). Most of these reports were based on one visit and measurement of the parameters in situ. The approximate annual range of temperature is between 15-33 ^{o}C, salinity ranged from 0.18 to 1.33‰, the water is highly calcareous and probably typically mesotrophic. The soil gradually dries out when the water recedes and reaches minimum levels in October. The soil in the rice-fields consists of very fine loamy sand, silt and

clay deposited by the rivers. These soils are extremely calcareous and have moderate to high salinity (Buringh, 1960). After the harvest of rice plants, the land is sometimes ploughed (Buringh, 1960). The same land may also be planted with other crops during the winter such as wheat, barely and oats (Al-Kaisi, 1976).

The characteristic feature of the climate in the region is a long dry summer, with an air temperature often exceeding 40 °C during July and August and probably much higher on the soil surface during September. There are some slight frosts in the winter (General Establishment for Studies and Design, 1979). The annual rainfall of only 200-300 mm is restricted to autumn, winter and early spring. The maximum relative humidity in the range of 46-48% in summer and 70-80% in winter. Evaporation is very high from free water surfaces and irrigated land, often exceeding 16 times the rainfall (General Establishment for Studies and Design, 1979). There are no recorded data on the photon flux density in the area. However, the prevailing clear, cloudless sky and long periods of sunshine during most of the spring, summer and early autumn suggest a very high photon flux density in the rice fields during most of the growth period, especially in open places where algae form a thin cover on the soil surface (Fig. 3.6).

However, algae grown in between the rice plants may be subjected to low photon flux density.

Inside the rice-fields, growths of other plants such as <u>Marsilea</u> were only moderate, presumably due to previous weeding.

3.3 Soil sample

The sample was taken when the soil in most places had started to dry out and standing water inside the rice-field was restricted to a few scattered pools (Fig. 3.5).

The sample was air-dried, ground lightly and kept in a polythene bag at room temperature. Most of the experiments in Chapter 5 were carried out during the first year, though no critical experiments were made to see if there were any changes in live algal flora during the study.

3.31 Chemical composition

The chemical composition of the acid digest of the soil sample (Section 2.11) is shown in Table 3.1. The soil is highly calcareous with a calcium content of 172300 $\mu g\ g^{-1}$ ash weight. The proportion of other metals differs slightly from that of the sediments from the floor of the main channel in the marsh reported by Maulood <u>et</u> <u>al</u>. (1981). The main difference is the high sodium content in the rice-field (1720 versus 966 $\mu g\ g^{-1}$ in ash material).

Table 3.1 Chemical composition of acid digest of soil sample (dried at 105 °C; ashed at 550 °C; n=4)

	$\mu g\ g^{-1}$ dry weight		$\mu g\ g^{-1}$ ash weight	
element	$\bar{x}$ ±	SD	$\bar{x}$ ±	SD
N	8575	699.4	9432.5	769.34
P	676.18	12.17	764.08	13.75
Na	1470	394	1720	438
K	6370	629	7460	574
Mg	26750	2320	31350	2069
Ca	146800	3060	172300	1990
Al	11100	1340	13000	1410
Mn	730	75	852	86
Fe	16200	1840	19000	1880
Co	42.5	8.6	49.2	10.4
Ni	120	8.1	138	8.6
Zn	315	251	365	290
Cd	1.5	0.5	1.7	0.5
Pb	24	8.7	27	9.9

The metal composition of an aqueous extract of soil (Section 2.11) is shown in Table 3.2. Calcium shows the highest content (2.8 mg l^{-1}) followed by potassium (1.4 mg l^{-1}); sodium and magnesium contents are similar and relativily low.

3.32 Influence of inoculation of soil on composition of the growth medium

Table 3.3 shows the elemental content of AD-N medium and AD-N+5 mg (5mg added to 50ml of the medium) soil (Section 2.12). Inoculation of the soil did not greatly change the composition of the medium. The concentration of most elements was not changed appreciably, except for iron which decreased by about 70% after inoculation. Phosphorus content decreased slightly. This might be due to adsorption by soil particles and removal by filtration.

Table 3.2 Chemical composition of aqueous extract of soil (5 mg soil) added to 50ml deionized distilled water, shaken continuously for 12 h at 32 °C and filtered through GF/C filter; n=2)

element	mg l^{-1}
Na	0.46
K	1.40
Mg	0.46
Ca	2.80
Al	0.02
Mn	0.028
Fe	0.048
Co	0.008
Zn	0.038
Cd	0.0004
Mo	<0.1
Cu	0.019
B	<1.0

Table 3.3 Chemical composition of AD-N medium and AD-N+ 5mg soil (soil added to 50 ml medium, shaked continuously for 12 h at 32 ^{o}C and filtered through GF/C filter; n=2)

	mg l^{-1}	
element	AD-N	AD-N+5 mg soil
P (FRP)	45	40.59
(TFP)	47.16	43.37
S	29.68	31.88
Cl	190	196.21
Na	99.8	97.3
K	118	122
Ca	16.5	22.81
Mg	18.32	17.87
Fe	4.2	2.93
Mn	0.25	0.21
Mo	<0.1	<0.1
Zn	0.025	0.075
Co	0.01	0.017
B	<1.0	<1.0
Cd	0.002	0.003
Cu	0.017	0.02

CHAPTER 4. ALGAL FLORA OF THE RICE-FIELD SOIL

4.1 Introduction

Little is known about the algal flora of the rice-fields in the southern marshes of Iraq (Section 3.1). Moreover, the available reports were floristic and based on short-term visits and collection of algal samples. However, reports indicated widespread and abundance of algae in the area. A study was therefore planned to investigate in detail the algal flora of the soil sample collected from the rice-field (Section 3.3). The study included inspection of the soil sample directly and after two weeks incubation of wet sample at 32 °C and 30-40 μmol photon $m^{-2}s^{-1}$. The flora was also recorded after various laboratory culture enrichments under different environmental conditions. A list of algal species seen by different techniques is given in Section 4.2, detailed description of strains isolated in Section 4.3.

4.2 List of species

Relatively few algae were seen by direct inspection of the rewetted sample shortly after collection and after about one year (Table 4.1); Microcoleus chthonoplastes and Nostoc muscorum were dominants. Almost the same taxa were seen when

Table 4.1 Algal species identified by direct inspection of soil sample (1 g of soil sample moistened with distilled water and kept at room temperature for at least 30 mins)

taxa	1st inspection	2nd inspection
Anabaena catenula	+	-
Aphanothece stagnina	-	+
Chroococcus > 4 ≤ 8 µm	+	-
Lyngbya aestuarii	+	+
Microchaete > 4 ≤ 8 µm	+	+
Microcoleus chthonoplastes	+	+
Nostoc muscorum Ag.	+	+
Plectonema ≤ 2 µm	+	+
Plectonema > 2 ≤ 4 µm	+	-
Schizothrix ≤ 2 µm	+	-
Schizothrix > 2 ≤ 4 µm	+	-

a wet soil sample was incubated for two weeks at 32 ^{o}C and 30-40 μmol photon $m^{-2}s^{-1}$ (Table 4.2). Laboratory cultures, however, led to a much greater list of species (Table 4.3). Almost every factor has a selective effect on the growth of various taxa (Table 4.4). Of the 49 taxa found, 35 were Cyanophyta , 12 Chlorophyta, one Euglenophyta and one Xanthophyta (Table 4.5); 18 of the Cyanophyta were heterocystous. Empty diatom frustules were seen in the soil sample, but no live cells. The relative abundances are shown in Table 4.3.

4.3 Description of the strains isolated

A standard method was followed in summarising data for each isolate. The method resembled that of Potts (1977). Observations were made initially on the strains in the mixed population under different environmental conditions. For consistancy and standardization (Whitton, 1969) observation was repeated for strains brought into clonal and colonal axenic cultures. The standard conditions were: the growth medium was the modified version of that of Allen and Arnon (1955) (Section 2.41), photon flux density (except for light experiments) was 90-120 μmol photon $m^{-2}s^{-1}$ for mixed populations and 40-60 μmol photon $m^{-2}s^{-1}$ for strains isolated. The temperature was 35 ^{o}C + 1 for mixed populations (except for the experiments on temperature

Table 4.2 Algal species identified in wet soil sample after two two weeks incubation at 32 ^{o}C and 30-40 umol photon m^{-2} s^{-1} (rewetted when necessary)

taxa	1st week	2nd week
Aphanothece stagnina	+	+
Lyngbya aestuarii	+	+
Microchaete > 4 ≤ 8 µm	-	+
Microcoleus chthonoplastes	+	+
Nostoc muscorum Ag.	-	+
Nostoc linckia	+	+
Plectonema > 2 ≤ 4 µm	+	+
Schizothrix ≤ 2 µm	+	+
Scytonema crispum	-	+

Table 4.3 List of algae identified from soil sample after various laboratory culture enrichments (abundance is scored on a scale of 1-5 based on subjective estimates (1, rare; 2, occasional; 3, frequent; 4, abudant; 5, very abundant)

Cyanophyta	relative abundance	notes on distribution	whether clonal	axenic
Anabeana catenula	1			
A. cylindrica	4	Section 4.3	+	+
A. oscillarioides	5	" "	+	
Aphanocapsa $> 2 \leqslant 4$ µm	1			
Aphanothece stagnina (Spreng) A. Br.	5			
Calothrix fusca (Kütz.) Bornet et Flah.	3	Section 4.3		
Calothrix parietina (Näg.) Thuret	2	" "		
Calothrix sp. $> 2 \leqslant 4$ µm	2	" "	+	+
Calothrix sp. $> 4 \leqslant 8$ µm	1	" "	+	+
Calothrix sp.	3	" "	+	+
Chroococcus $> 4 \leqslant 8$ µm	2			
Cylindrospermum muscicola (Kütz.)	5	Table 4.4 Section 4.3	+	+
Gloeothece $> 2 \leqslant 4$ µm	1			
Gloeotrichia sp. (Ag.)	3	Section 4.3	+	+
Hapalosiphon welwitschii	3	Section 4.3 Section 4.3	+	+
Lyngbya aestuarii (Mert.) Liebm	4	" "	+	

Table 4.3 continue

Lyngbya ≤ 1 µm	5			
Lyngbya > 1 ≤ 2 µm	3	Section 4.3	+	
Lyngbya > 2 ≤ 4 µm	3			
Lyngbya > 4 ≤ 8 µm	2			
Microchaete > 4 ≤ 8 µm	1			
Microcoleus chthonoplastes Thuret	4	Table 4.4	+	+
Nodularia harveyana (Thw.) Thuret	5	Section 4.3 Table 4.4	+	
Nostoc linckia (Roth) Born et Thuret	4	Section 4.3 Table 4.4	+	
N. *muscorum*	5	Section 4.3 " "	+	+
N. *paludosum* (Kütz.)	2			
Oscillatoria redekei van Goor	1			
Oscillatoria > 1 ≤ 2 µm	2			
Oscillatoria > 2 ≤ 4 µm	1			
Phormidium > 2 ≤ 4 µm	2			
Plectonema > 1 ≤ 2 µm	3			
Schizothrix > 1 ≤ 2 µm	3			
Schizothrix > 2 ≤ 4 µm	2			
Scytonema crispum (Ag.) Born.	3			
Tolypothrix distorta Kütz.	2			
Euglenophyta				
Euglena sp.	4			

Table 4.3 continue

Xanthophyta		
Tribonema minus (Wille) Hazen	4	
Chlorophyta		
Chlamydomonas sp.	2	
Chlorella vulgaris Beijerinck	2	
Chlorococcum humicola (Näg.) Raben.	5	Table 4.4
Draparnaldia sp.	1	
Gonium sociale (Duj.) Warming	1	
Hormidium flaccidum A. Br. (6 µm)	3	
Pandorina morum (Müll.) Bory	1	
Scenedesmus abundance (Kirch.) Chodat	1	
Scenedesmus arcuatus Lemm.	4	Table 4.4
Stichococcus bacillaris Näg	3	
Stigeoclonium nanum	1	
Ulothrix sp.	4	

Table 4.4 Selective effects of environmental factors on the dominant taxa of soil sample

factor		dominant 1	dominant 2
temperature	low	*Chlorococcum*	*Tribonema*
	high	*Cylindrospermum*	*Nostoc*
photon flux density	low	*Lyngbya*	*Nostoc*
	high	*Scenedesmus*	*Nostoc*
salinity	high	*Nodularia*	*A. oscillariodes*
sulphide	low	*Microcoleus*	*Nostoc linckia*
nitrogen	high	*Lyngbya* $>1 \leq 2$ µm	*L. aestuarii*
phosphorus	low	*Nostoc*	*Hapalosiphon*
	high	*Cylindrospermum*	*Anabaena*

Table 4.5 Summary of number of live taxa in various algal groups identified in the soil sample after various laboratory culture enrichments

phylum	total number of taxa	% of total number
Cyanophyta	35	71.5 %
Euglenophyta	1	2%
Xanthophyta	1	2%
Chlorophyta	12	24.5 %
total	49	100%

effect) and 32 ^{o}C for the strains isolated, macro and microscopic observations were made after 1-2 weeks usually or continuously for 28 or 30 days in case of growth rate experiments and selective enrichment experiments, respectively. The basic format of the standard method is outlined below.

1. Growth form, in mixed population and after being obtained in clonal or clonal axenic cultures both in liquid and on solid media, colour of the plant mass.

2. Morphology. This includes: range of cell width and length (without its boundary layer), shape, range of width and length of the specialized cells (akinetes, heterocysts, end cells) and their position to the other cells; arrangment of trichomes, shape, width of filaments, shape, width of sheath, colour, if lamellate.

3. Observation on its distribution under different environmental conditions and morphological changes found in each case if occurred.

4. Literature is mentioned when it was of particular relevance.

A. Heterocystous forms

Anabaena spp.

Three Anabaena spp. were identified in the soil sample

(Table 4.3).

(i) Anabaena catenula

This species was dominant in the field but not in the laboratory cultures. However, an organism with similar trichome width (> 4 ≤ 8 µm) was found in many cultures was probably the same, but akinetes were never seen. therefore no detailed description was made for this species.

(ii) Anabaena cylindrica D582

1. This alga formed a thin mat on the surface of the liquid medium and thin scum on the base of the culture flask in the mixed population. In clonal culture the same structure was seen, however, when brought into clonal axenic culture the alga forms ball-like structures (Fig. 4.1). On solid medium it forms a mat-like growth with a fembriate margin. The colour of the plant mass is green.

2. Vegetative cells are quadrate to cylindrical, 3-4 µm wide, 3-6 µm long; heterocysts intercalary, cylindrical, barrel or oval, 3-5 µm wide, 5-7 µm long, akinetes on both side of the heterocysts adjust to the heterocysts, single or in chain cylindrical (mostly) or barrel in shape, 4-8 µm wide, 7-20 µm long, green at the beginning becoming yellowish brown in old cultures; it lost the ability to form akinetes after purification; trichomes are straight or flexuous in mixed populations, irregularly contorted in clonal axenic culture; sheath very thin and colourless.

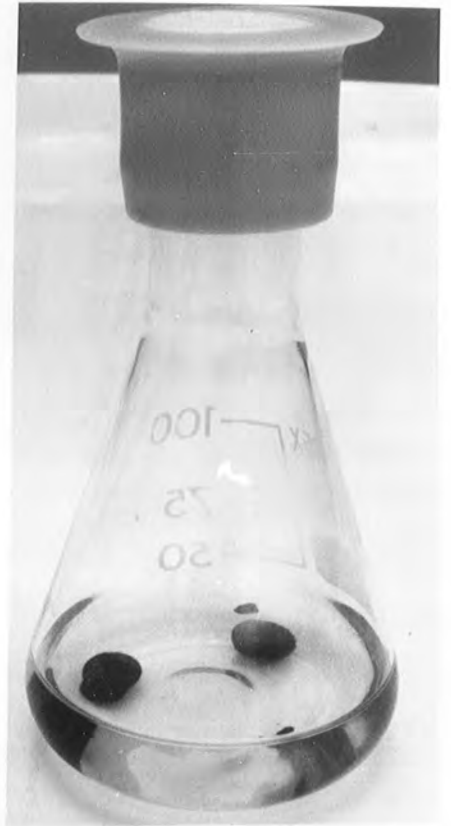
Fig. 4.1 Growth forms and morphology of
Anabaena cylindrica D582

1. Growth forms (a) on solid medium
   (b) in liquid medium

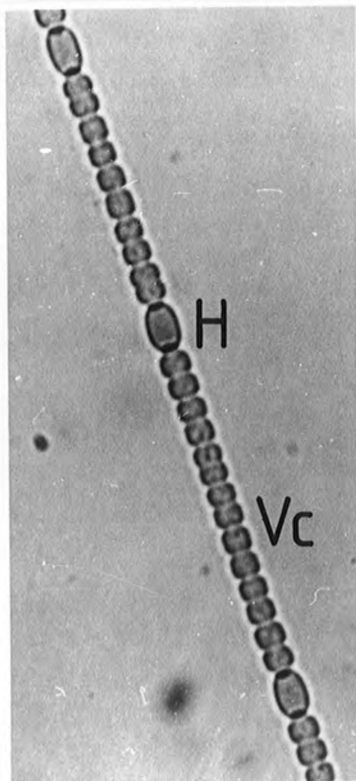
2. Morphology (i) in mixed populations
   (ii) in clonal axenic culture

(H heterocyst, VC vegetative cell,
A akinete)

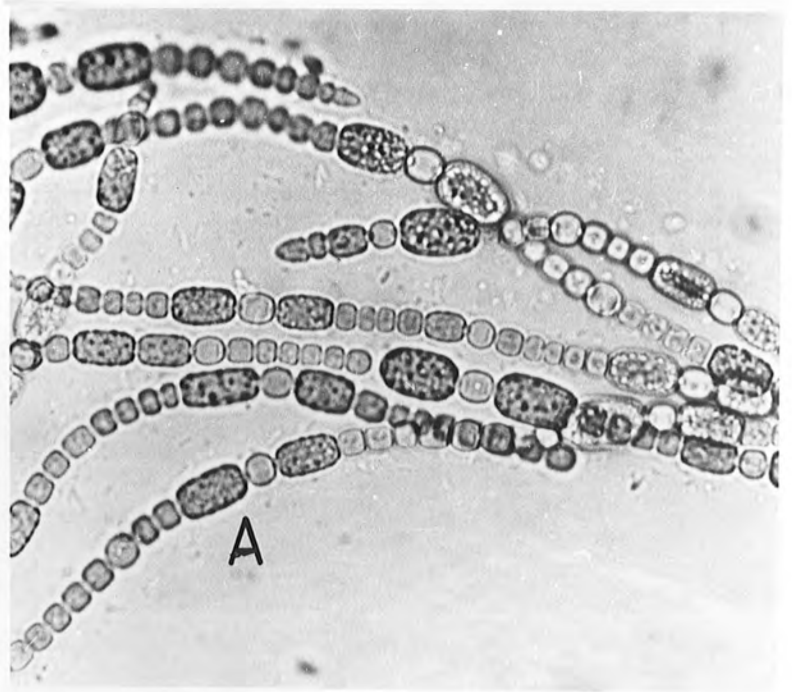
(a)



(b)



(i)



(ii)

3. It is one of the dominant algae in -N medium in most of the laboratory cultures particularly at 20-25 °C. It showed high sensitivity to both salinity and reducing conditions.

4. Similar observations were mentioned for strain Cambridge 1403/2a by Kantz and Bold (1969) and Stulp and Stam (1982) under laboratory conditions.

(iii) *Anabaena oscillarioides* D693

This species was found toward the end of the present study in -N medium enriched with 1 M NaCl. Therefore no detailed description was made. Vegetative cells quadrate (mostly), spherical or cylindrical, 2.5-4 µm wide, 2.5-5 µm long; heterocysts intercalary, spherical, barrel, oval or cylidrical, 4-5 µm wide, 4-7 µm long; akinetes cylindrical, barrel, oval or spherical, 5-6 µm wide, 5-12 µm long, on both sides of the heterocyst single or in chains, epispore smooth, yellowish-brown to orange; end cell usually conical but sometimes rounded; trichomes straight or spirally coilled; sheath thin and colourless.

Calothrix spp.

Five *Calothrix* spp. were identified in the soil sample after various enrichments (Table 4.3). *C. fusca* and *C. parietina* both formed hairs in enrichment cultures. Three spp. were obtained in clonal axenic cultures.

(i) <u>Calothrix</u> sp. D580

1. In mixed populations this alga formed small stellate structures of 5-7 filaments with heterocysts at the centre. After purification many trichomes cluster in these structures on the base of the culture flask (mainly) or on the surface of liquid medium (Fig. 4.2). On solid media it formed a mat with fimbriate margin. The colour of the plant mass was green to greenish.

2. Basal cells quadrate to cylindrical 3-10 µm wide, 3-5 µm long in mixed populations, 3-5 µm wide, 3-17 µm long in clonal axenic culture; heterocyst basal, single or in chains of 2-4, basal heterocysts spherical, oval or oblong, 4-5 µm wide, 5-12 µm long, sub-basal heterocysts quadrate or cylindrical, 4-5 µm wide, 5-10 µm long, trichomes in mixed populations short, slightly bent or strongly curved at the base grdually tapered from the base to the apex; after purification long cylinrical trichomes formed which were slightly bulbous at the base; sheath thin and colourless close to the trichomes.

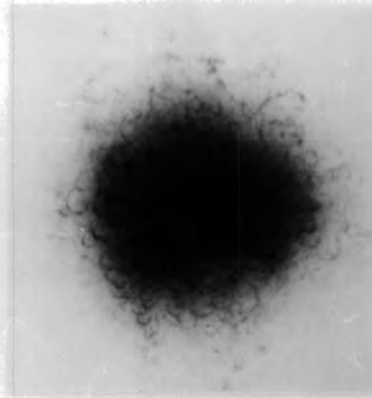
3. This alga only occasionally found in -N medium with realtive abundance of 2 (Table 4.3). It had the lowest growth rates and nitrogenase activity among the strains islolated (Tables 6.2, 7.1). Attempts to induce hair formation in this alga using low phosphorus (0.45 mg $l^{-1} PO_4$- P), green light, red light, dark/light cycle and

Fig. 4.2 Growth forms and morphology of *Calothrix* sp. D580

1. Growth forms (a) on solid medium
   (b) in liquid medium

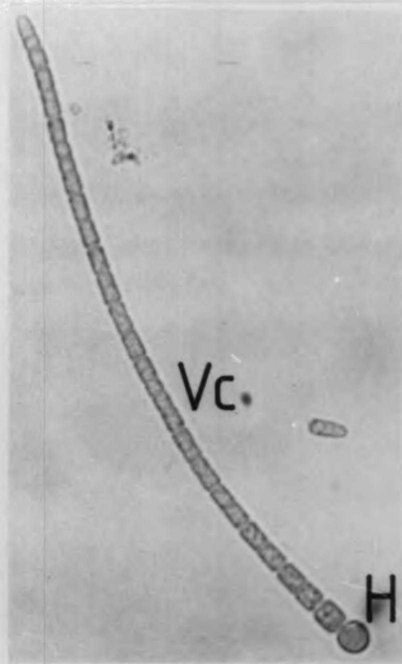
2. Morphology (i) filament
   (ii) cluster sturcture

(H heterocyst, VC vegetative cell)

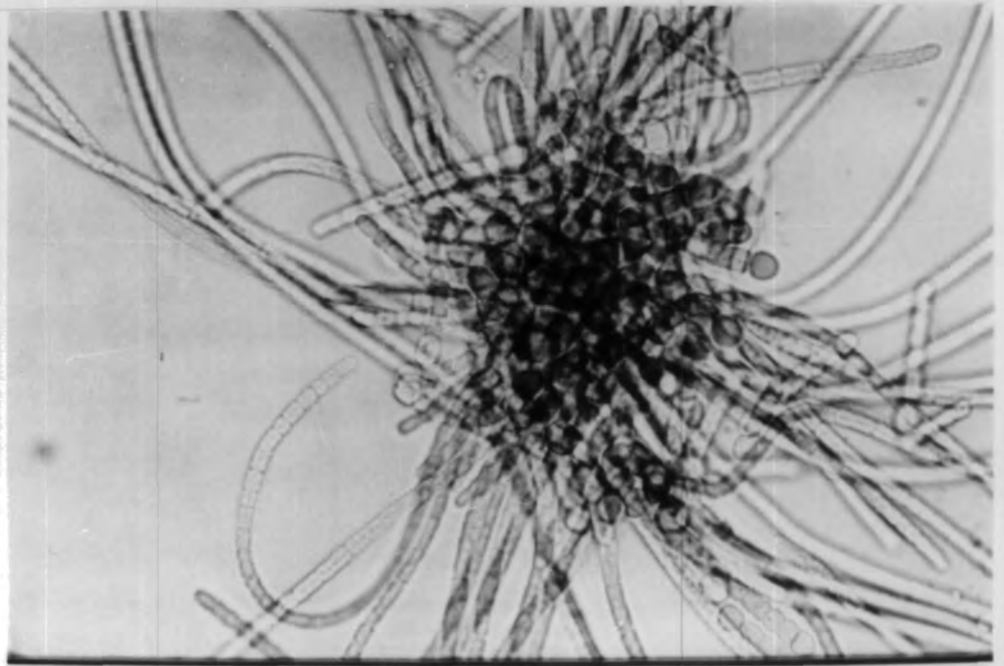


(a)

(b)



(i)



(ii)

combined nitrogen were unsuccessful.

Calothrix sp. D583

1. No observation was made for this alga in the mixed population because of its rare abundance (Table 4.3). In clonal axenic culture it formed cluster-like colonies in liquid medium and stellate colonies on solid medium (Fig. 4.3). The colour of the plant mass is brown to yellowish-brown.

2. Basal cells quadrate to cylindrical, 3-6 µm wide, 4-7 µm long, heterocysts basal or intercalary; basal heterocysts spherical, oval or oblong, single or in chains of 2-3, 5-6 µm wide, 6-15 µm long; intercalary heterocysts quadrate or cylindrical, 2.5-5 µm wide, 5-10 µm long; trichomes straight or slightly curved, slightly swallen at the base and gradually tapered toward the tips. The released hormogonia usually remained attached to the parent filaments at the heterocysts to form a structure similar to false branches, sometimes two of these structures formed. These two cases were formed in slightly old culture (15-20 days) giving an impression of a Tolypothrix-like or Scytonema-like organism, sheath thin, firm and colorless. Attemps to induce hair formation under the same conditions mentioned for strain D580 were unsuccessful also.

Fig. 4.3 Growth forms and morphology of *Calothrix* sp. D583

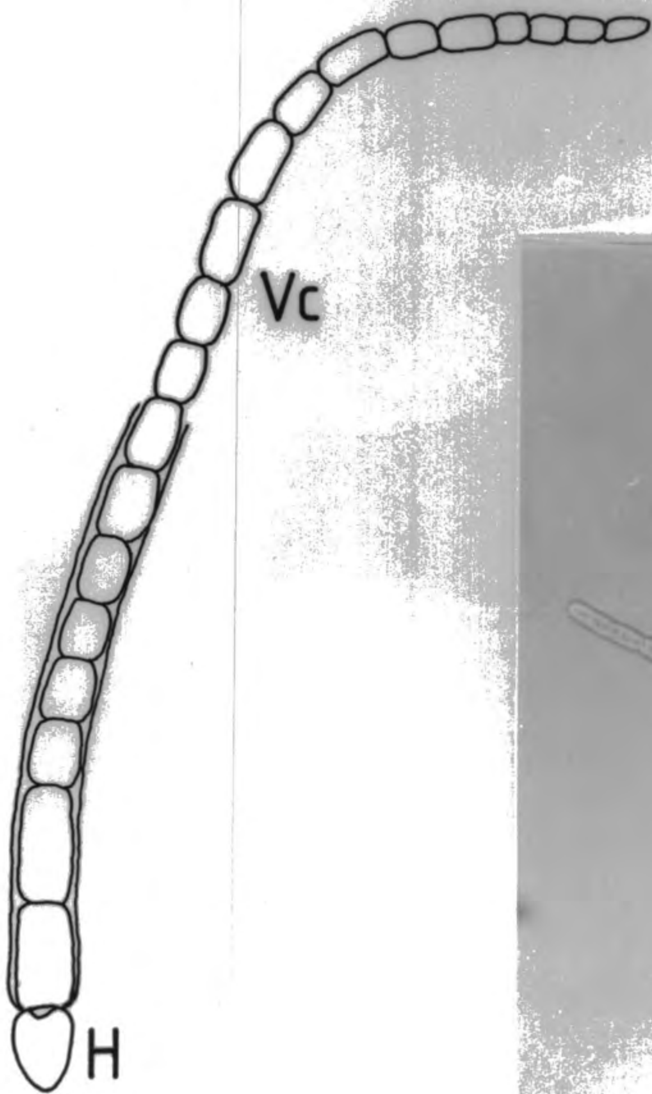
1. Growth forms (a) on solid medium
   (b) in liquid medium

2. Morphology (i) filament
   (ii) filament showing false branching

(H heterocyst, VC vegetative cell)

(a)

(b)



(i)

(ii)

(iii) Calothrix sp. D585

1. In mixed populations this species formed small stellate clusters with heterocysts at the base, the same structures formed in liquid medium after purification in the early stage of the growth, however, continuous release of hormogonia resulted in the formation of a mat-like structure on the surface of liquid medium and thin scum on the base of the culture flask with little suspended growth (Fig. 4.4). On solid medium mat-like growth occurred with fimbriate margin. The colour of the plant mass is brown to yellowish-brown.

2. Basal cells mostly quadrate or slightly cylindrical, 4-6 µm wide, 5-7.5 µm long; heterocysts spherical or oval, single or 2-3, 5-7 µm wide, 5-8 µm long; sub-basal and intercalary heterocysts are quadrate to cylidrical, 5-10 µm wide, 5-12 µm long; trichomes straight or slightly bent more or less uniform in width; sheath thick and colourless not lamellate extended beyond the trichomes; filaments 8-10 µm wide, false branches were very rare.

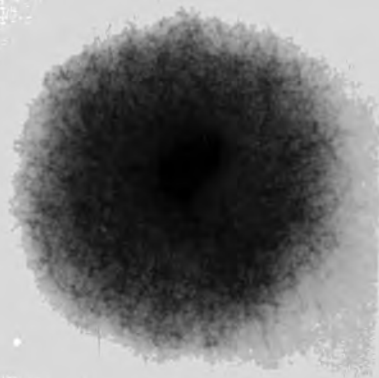
3. This species was found in old cultures of mixed populations with a relative abundace of 3 under most of the environmental conditions tested except salinity and reducing conditions.

Fig. 4.4 Growth forms and morphology of
Calothrix sp. D585

1. Growth forms (a) on solid medium
(b) in liquid medium

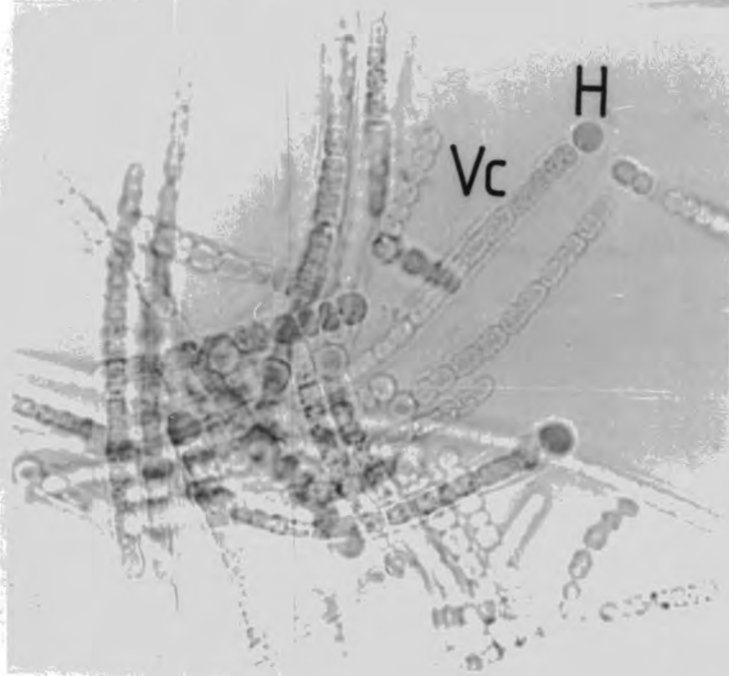
2. Morphology (i) young filaments
(ii) mature filament

(H heterocyst, VC vegetative cell)



(a)

(b)



(i)

(ii)

Cylindrospermum muscicola D579, D678

Two strains of C. muscicola were obtained in clonal (strain D678) and clonal axenic culture (strain D579). The two strains are similar to each other except that strain D579 had lost its ability to form akinetes after purification. The two strains therefore are described together. However, akinete description belongs to strain D678 only.

1. Both strains form soft gelatinous mat-like thalli on the surface of liquid medium and a thin scum on the base of the culture flask with little suspended growth in mixed population and in clonal and clonal axenic culture, however culture of strain D678 was very unhealthy and the mat stucture is easly lost upon shaking. On solid medium mat-like sturucture formed for both strains with fimbriate margin (Fig. 4.5). The colour of the plant mass is bright blue-green, green or greenish, however, upon aging strain D678 may become brown due to extensive formation of akinetes.

2. Vegetative cells cylindrical to isodimetric in shape, 2-4 µm wide, 2-7 µm long; heterocysts terminal at one or both ends of the filaments, oval or mostly oblong, 2-4 µm wide, 6-8 µm long; akinete oval or cylindrical in shape, 5-12 µm wide, 10-20 µm long, mostly at one end of the filaments very rarely at both ends, yellowish-brown becoming brown on aging, epispore smooth; trichomes uniformly broad,

mostly straight or slightly curved, constricted at the cross-wall, sheath delicate and imperceptile.

3. Cylidrospermum strains dominated both -N and +N medium under high temperature and phosphorus enrichments (Table 4.4). They showed high sensitivity for both salinity and reducing conditions. Under later enrichment and at the low concentrations trichomes lacking heterocysts are formed. At high temperatures (40-45 °C) some morphological changes took place including formation of very long bone-like thin cells, 1.5-3 µm wide, 7-10 µm long, as well as very short cells 1-2 µm wide, 1-3 µm long. After loss of akinete formation attempts to induce akinete formation in strain D579 under the same environmental conditions mentioned for Calothrix spp. togther with the addition of different amount of filtrate from akinete-forming strain (D678) were unsuccessful.

Gloeotrichia sp. D636

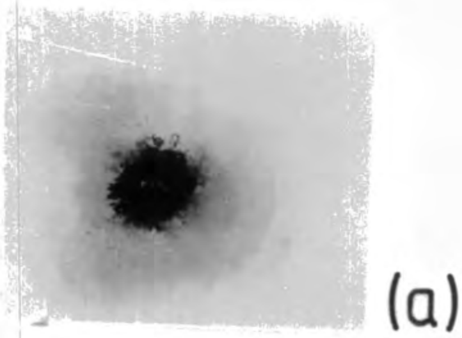
1. In mixed populations this alga forms small stellate colonies of 5-8 filaments on the base of the culture flask in liquid medium. In clonal axenic culture similar structures formed at the early stage of growth on the surface of liquid medium, however, continuous release of hormogonia led to the formation of a thin mat on the surface of liquid medium as well as base of the culture flask with

Fig. 4.5 Growth forms and morphology of *Cylindrospermum muscicola* D579, D678

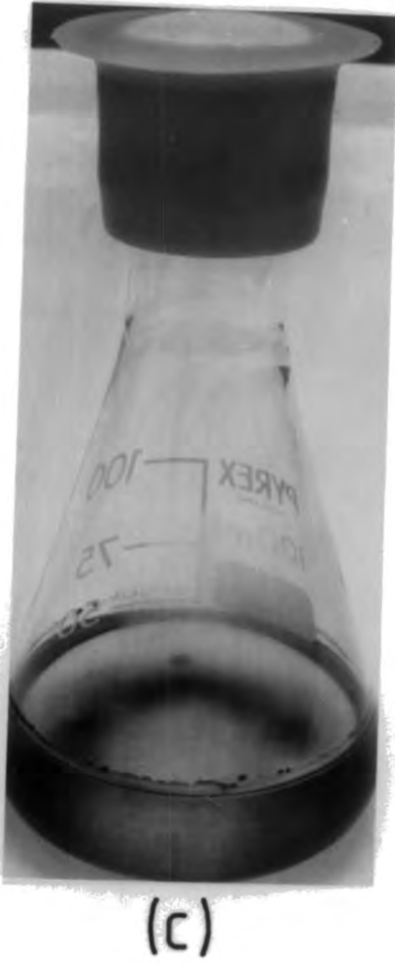
1. Growth forms
   (a) on solid medium
   (b) in liquid medium D579
   (c) in liquid medium D678

2. Morphology
   (i) filament with double heterocysts D579
   (ii) filament with akinete D678

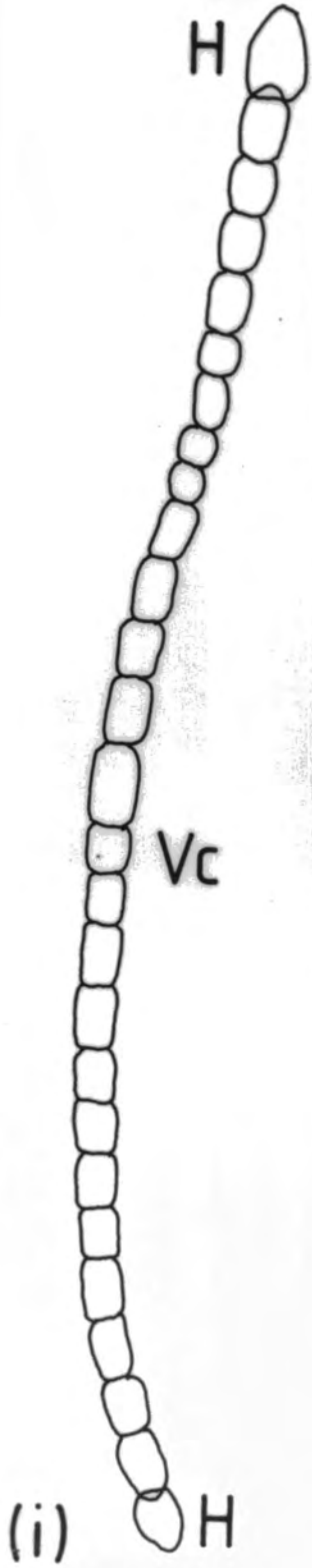
(H heterocyst, VC vegetative cell, A akinete)


(a)

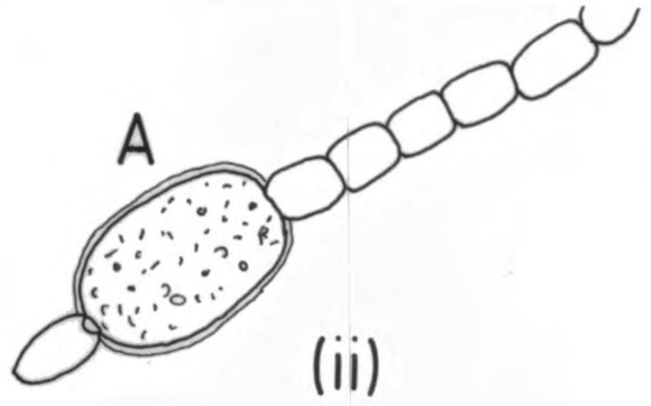
(b)



(c)



(i)



(ii)

little suspended growth. On solid medium the released hormogonia form minute stellate colonies around the inoculum (Fig. 4.6). The colour of the plant mass is brown to yellow-brown.

2. Basal cells mostly cylindrical or quadrate, 6-8 µm wide, 6-15 µm long; heterocysts basal, single or 2-5 some trichomes have intercalary heterocysts, basal heterocysts, spherical, subspherical or oval rarely oblong, 5-7.5 µm wide, 5-8 µm long; sub-basal heterocysts and intercalary heterocysts are quadrate to cylindrical, 10-15 µm wide, 10-17.5 µm long; akinete single or in chains of 2-3 separated by discs, akinetes are mostly long, cylindrical with rounded ends 10-14 µm wide, 20-38.5 µm long, epispore smooth and brown in colour; trichomes straight or curved shortly after straight base, attached centrally at the base; filaments up to 25 µm wide at the base; sheath thin hyaline close to the trichome in the early stage of growth becoming thick yellowish-brown in old filaments with funnel shape diversed at the base and the apex of the filaments, filaments ending with long narrow hairs, no false branching was seen in this alga.

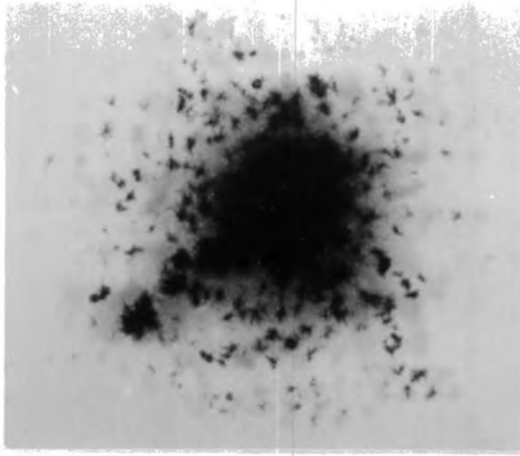
3. This alga differs from Calothrix spp. by the formation of akinetes and hairs, both were found to be suppressed at high phosphorus concentrations (Section 4.5). It seems possible that this alga might be included with Calothrix spp. in the

Fig. 4.6 Growth forms and morphology of *Gloeotrichia* sp. D636

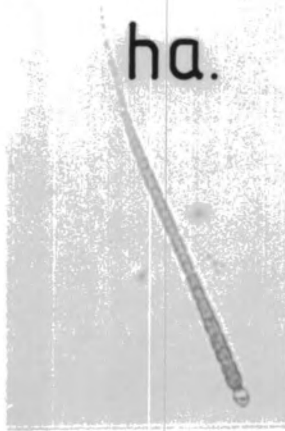
1. Growth forms (a) on solid medium
   (b) in liquid medium

2. Morphology (i) single filament with hair (ha.)
   (ii) mature filaments with akinete

(H heterocyst, VC vegetative cell
A akinete, Sp separating sheath)

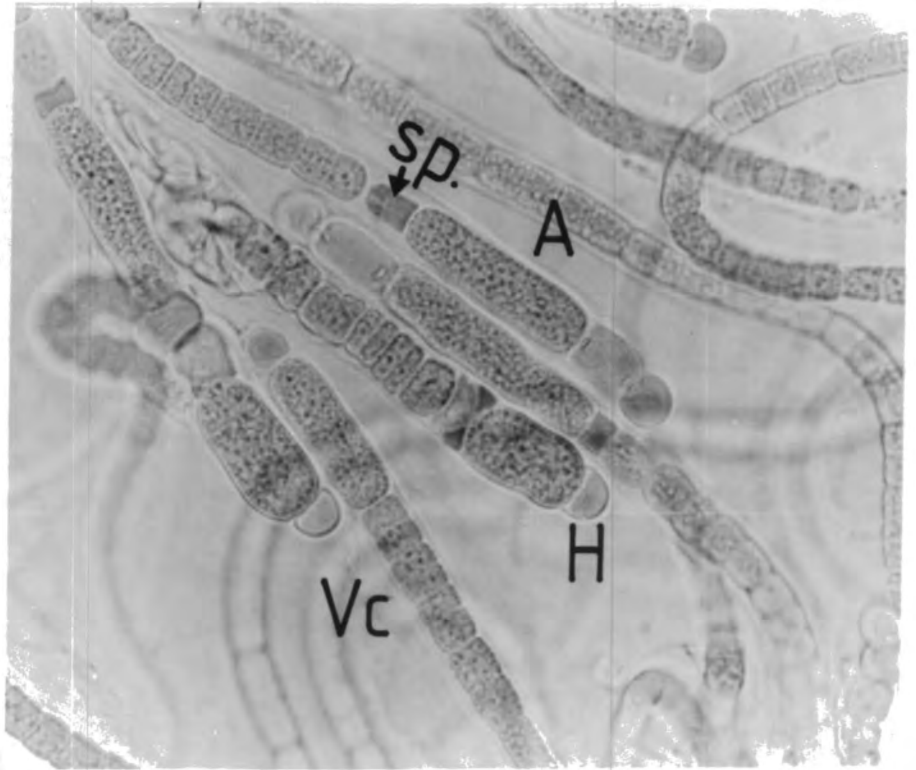


(a)



(i)

(b)



(ii)

earlier parts of the present study (Sections 5.2, 5.4). It was first recognized under phosphorus enrichments experiments (Section 5.?) as it showed its typical morphology under low phosphorus concentrations (0.5 and 2.5 mg l^{-1} P). It also showed high abundance in old cultures of the green light experiment (Section 5.32).

4. Chang (1979) mentioned that the typical globular colonies of Gloeotrichia echinulata disappeared under laboratory conditions with resulting of formation of two types of colonies, membrane and cluster-like colonies.

Hapalosiphon welwitschii D581

1. In liquid medium this alga formed cottony tufts floating in the medium or attached to the base of the culture flasks both in mixed populations and in clonal axenic culture (Fig. 4.7). On solid medium the alga form a mat with more or less smooth margins. The colour of the plant mass is bright blue-green in mixed populations and green or greenish in pure culture.

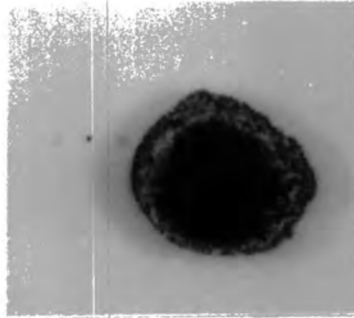
2. Vegetative cells: main filaments, subspherical, quadrate or cylidrical, 5-8 µm wide, 5-10 µm long; lateral branch, basal cells quadrate to cylindrical 3-4 µm wide, 4-20 µm long; heterocysts in main branch oval, barrel or cylindrical, 5-8 µm wide, 5-28.5 µm long; trichomes uniseriate closely entangled bearing true branches which

Fig. 4.7 Growth forms and morphology of
Hapalosiphos welwitschii D581

1. Growth forms (a) on solid medium
   (b) in liquid medium

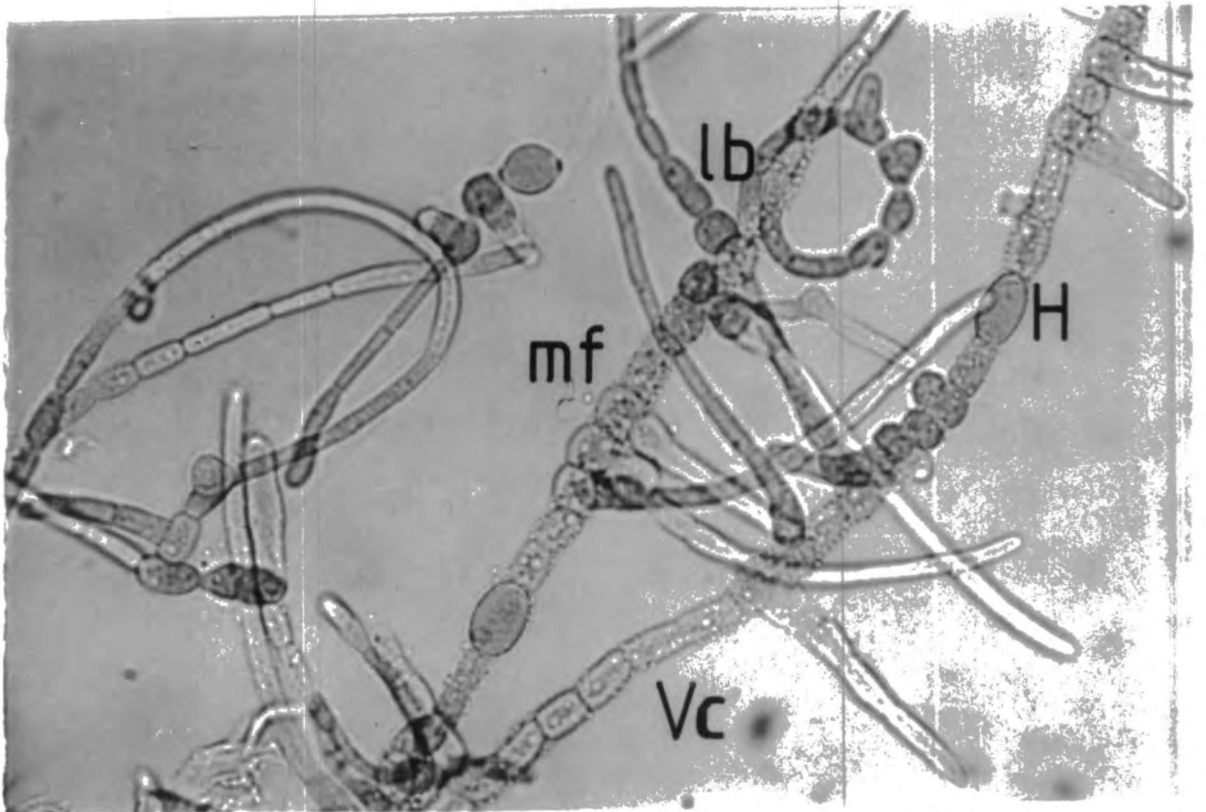
2. Morphology (young thallus)

(H heterocyst, VC vegetative cell,
MF main filament, Lb lateral branch)



(a)

(b)



have intercalary heterocysts cylindrical in shape, 2-4 µm wide, 3-15 µm long, branches sometimes re-branches, the lateral branches have same diameter as main filaments at the base, but gradually attenuated toward the apex; sheath thin colourless, yellowish to orange in old cultures.

3. This organism was found in most of the laboratory cultures, but was sensitive to both salinity and reducing conditions.

Nodularia harveyana D635

1. The alga form thin gelatinous mats on the surface of liquid medium, as well as a belt around the wall of culture flask at the liquid-air interface. Cultures of this alga are very unhealthy and the mat-like growth could be ver easily lost (Fig. 4.8). On solid medium it forms a mat with a rough to fimbriate margin. The colour of the plant mass is green to dull green.

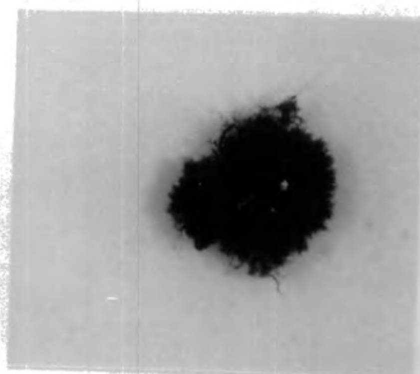
2. Vegetative cells short, depressed, discoid, 5-7 µm wide, 2-4 µm long; heterocysts intercalry compressed, oval, barrel, spherical or sub-spherical in shape, 5-8 µm wide, 2-6 µm long; akinetes single or in series, globular, sub-spherical or spherical in shape, 7-10 µm wide, 6-9 µm long, starts mid-way between two heterocysts and matures

Fig. 4.8 Growth forms and morphology of *Nodularia harveyana* D635

1. Growth forms (a) on solid medium
   (b) in liquid medium

2. Morphology (i) mature filament with akinetes
   (ii) young filaments showing heavy bacterial growth

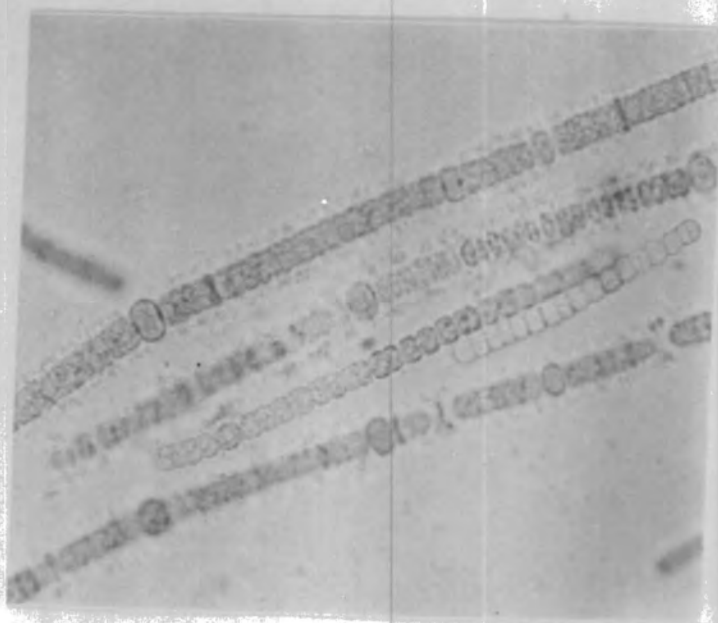
(H heterocyst, VC vegatative cell, A akinete)



(a)

(b)

(i)



(ii)

from the centre toward the heterocysts; epispore smooth, colourless or yellowish; trichomes uniform in width mostly straight, slightly bent, rarely spirally coiled, very easly broken into small fragments at the heterocysts; sheath firm, non-lamellated, colourless or yellowish.

3. This alga did not show growth under any of the culture conditions except salinity enrichments (Section 5.4), dominat all cultures at 0.5 M NaCl.

4. Recently Nordin and Stein (1980) made a taxonomic revision of a large number of *Nodularia* spp. using field material, herbarium specimens and laboratory cultures. They concluded that only two species of *Nodularia* exist, these are: *Nodularia harveyana* with vegetative cells 3.5-7.5 µm wide and *Nodularia spumigena* with vegetative cells 7.5-16 µm wide.

Nostoc spp.

Three *Nostoc* species were identified in the soil sample (Tabl 4.3). Two of them were brought into colonal (D586) and clonal axenic culture (D584).

Nostoc linckia D586

1. The alga grow as small globular colonies on the base of the culture flask in mixed populations, these structures became less apparent in the clonal culture and a

membrane-like structure formed with colonies scattered as nodules within the membrane. On solid medium the released hormogonia form minute colonies around the inoculum (Fig. 4.9). The colour of the plant mass is green, greenish or dull green.

2. Vegetative cells spherical, oval, barrel, quadrate or cylindrical, 2.5-4 µm wide, 2.5-6 µm long; heterocysts terminal and intercalary, terminal heterocysts spherical, sub-spherical or oval, 3-5 µm wide, 3-7 µm long, intercalary heterocysts are spherical or barrel shaped 3-5 µm wide, 3-7 µm long; akinetes spherical (mostly) barrel shaped occasionally, rarely cylindrical, 5-6 µm wide, 6-7 µm long; epispore smooth and colourless; trichomes twisted and densly entangled; sheath firm and colourless.

3. This alga is one of the dominant species in -N medium in most of the laboratory cultures it showed high tolerance to the reducing conditions (Section 5.5), salinity (Section 5.4) and to the high temperature treatments (Section 5.2).

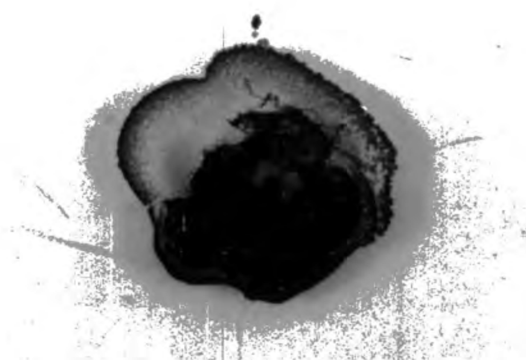
4. Kantz and Bold (1969) described a strain of this species under laboratory conditions on solid media as having a dull to shiny sturcture with a rough or broken margin. The vegetative cells, 3-4 µm wide, 3-5 µm long. They also mentioned that the plant mass in nature is initialy in the form of spherical colonies becoming irregularly expanded and gelatinous on aging.

Fig. 4.9 Growth forms and morphology of *Nostoc linckia* D586

1. Growth forms (a) on solid medium
   (b) in liquid medium

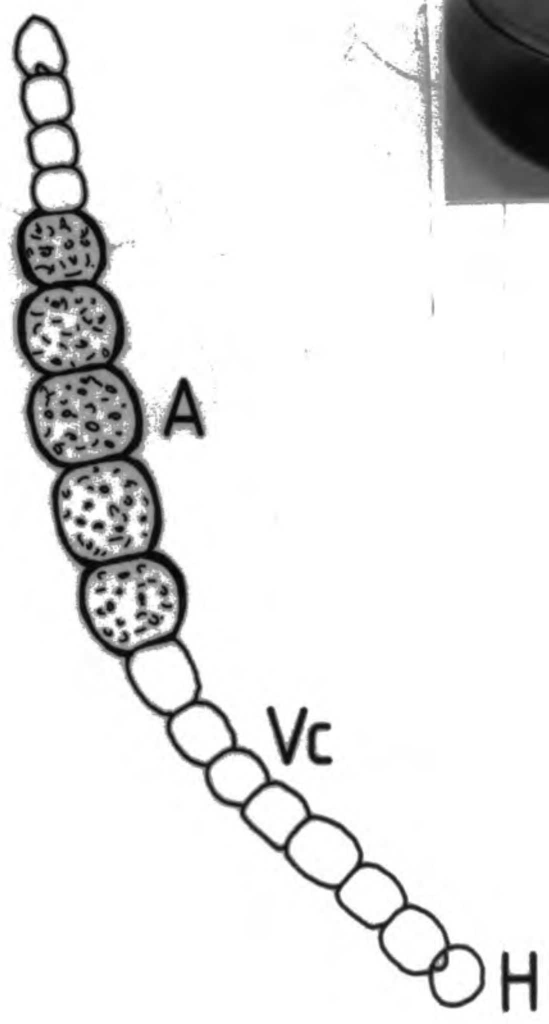
2. Morphology mature filament with akinetes

(H heterocyst, VC vegetative cell, A akinete)



(a)

(b)



Nostoc muscorum D584

1. In mixed populations, filaments entangled and formed small spherical colonies scattered on the base of the culture flask. Filaments may also form a mat on the surface of the liquid medium. After purification it forms a thick gelatinous mat on the surface of the liquid medium and is suspended in the medium. On solid medium it forms a mat slightly raized at the center with fimbriate margin. The colour of the plant mass is browm to brownish in liquid medium and dark brown on solid medium (Fig. 4.10).

2. Vegetative cells quadrate, isodiametric or cylindrical, 2-3 µm wide, 2-7 µm long; heterocysts terminal and intercalary, terminal heterocysts spherical, oval or oblong, 2-5 µm wide, 2-7 µm long, intercalary heterocysts cylindrical (mostly), barrel or spherical, 3-5 µm wide, 3-8 µm long; akinetes spherical, barrel or cylindrical in series, 4-6 µm wide, 5-15 µm long; epispore smooth and colourless to yellowish; trichomes straight, slightly bent or contorted, sheath firm and colourless to brownish.

3. This alga dominated almost all of the laboratory cultures (Table 4.4). It showed sensitivity toward both salinity and reducing conditions.

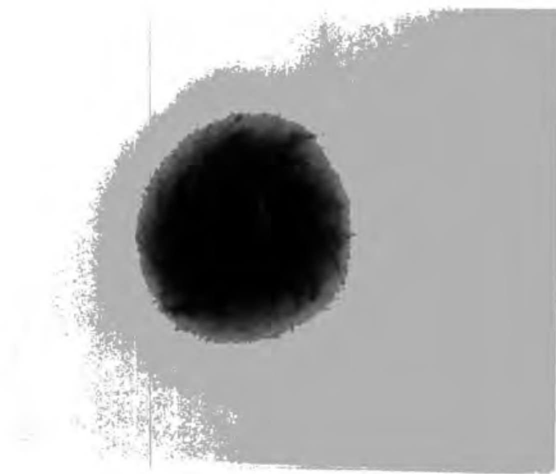
4. Kantz and Bold (1969) described a strain of this species

Fig. 4.10 Growth forms and morphology of *Nostoc muscorum* D584

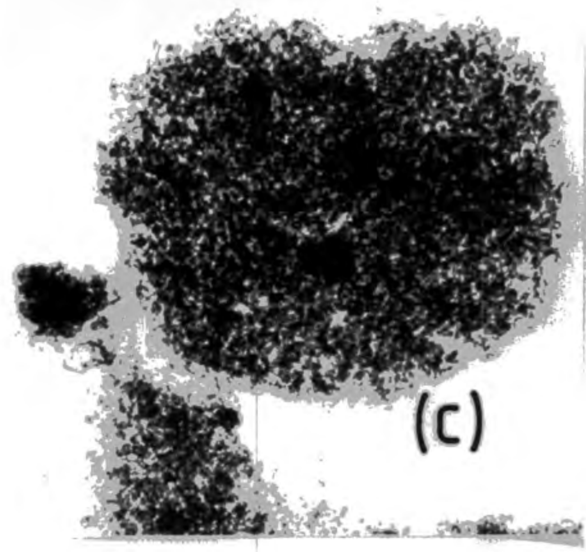
1. Growth forms (a) on solid medium
   (b) in liquid medium
   (c) colonial form in mixed populations

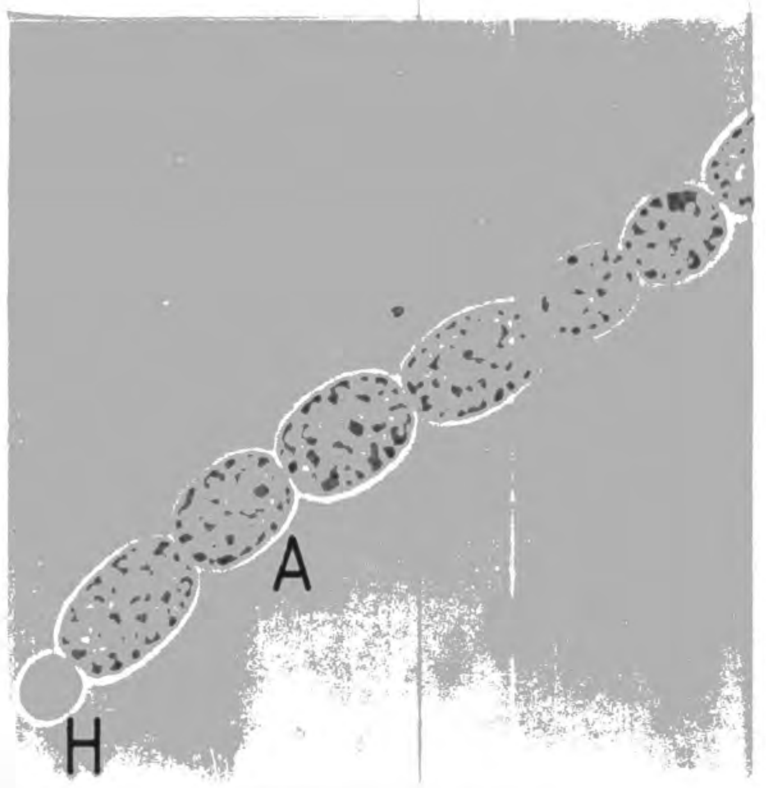
2. Morphology mature filament with akinetes

(H heterocyst, A akinete)


(a)

(b)


(c)



under laboratory conditions as having a rough or smooth margin on solid medium with vegetative cells 3-5 µm wide, 3-5 µm long.

B. Non-heterocystous forms

Lyngbya spp.

Five Lyngbya species were identified in the soil sample (Table 4.3). Two of them were brought into clonal cultures.

(i) Lyngbya aestuarii D638

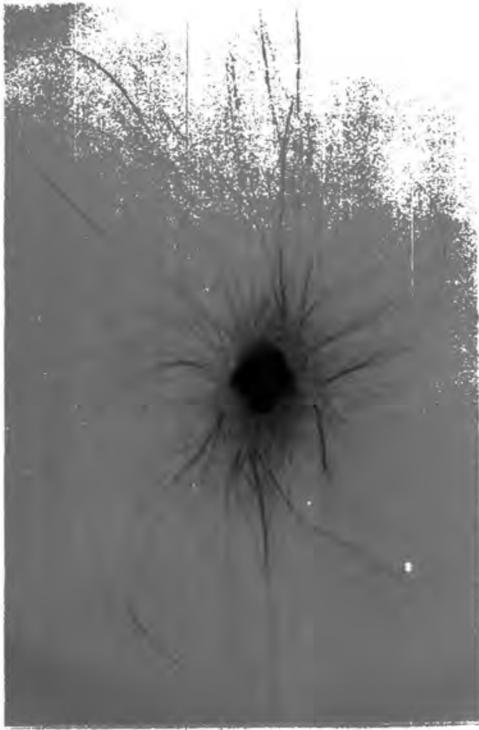
1. In mixed populations the alga grow as single or aggregated filaments which are sometimes entangled forming a mass on the surface of liquid medium or the base of the culture flask. The same growth form was found in the clonal culture (Fig. 4.11). On solid medium filaments grow in all directions from the inoculum material to form a shiny structure with fimbriate margin. The colour of the plant mass is green to dull green.

2. Vegetative cells shorter than broad, depressed and plate-like in shape, 16-18 µm wide, 2-3 µm long, end cell rounded; trichomes straight, contorted or coiled, sometimes having false branches, slightly or not constricted at the cross-wall; sheath thick, colourless at the beginning becoming yellowish to orange on aging, 3-5 µm in diameter; filaments upto 22 µm in diamter.

Fig. 4.11 Growth forms and morphology of *Lyngbya aestuarii* D638

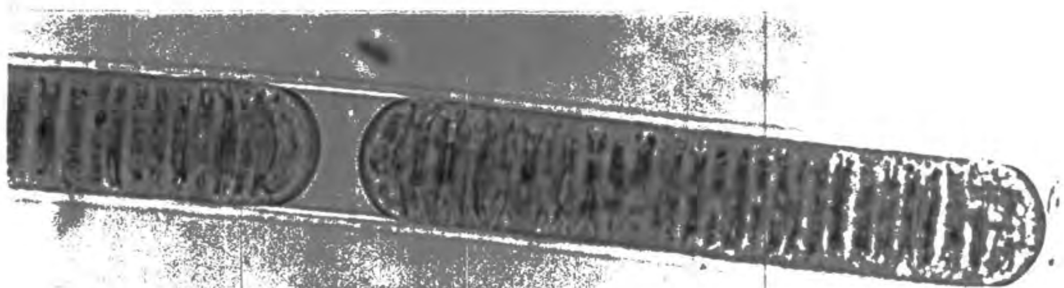
1. Growth forms (a) on solid medium
   (b) in liquid medium

2. Morphology (i) filament showing released hormogonium



(a)

(b)



(i)

3. This alga was dominant in the soil sample by direct inspection and in almost all of the laboratory cultures enriched with combined nitroge. It showed high abundance at low light conditions (15-20 µmol photon $m^{-2}s^{-1}$).

Lyngbya sp. D639

1. In mixed populations this alga forms a mass of densly entangled filaments on the base of the culture flask or on the surface of liquid medium. In clonal culture, mat like structures are formed on the surface of liquid medium and on the base of culture flasks. On solid medium shiny sturctures were formed (Fig. 4.12). The colour of the plant mass is green to greenish.

2. Vegetative cells are quadrate or cylindrical, 1-1.5 µm wide, 2-7 µm long, trichomes straight, twisted or spirally coiled and densly entangled, constricted at the cross-wall; sheath thick extended beyond the end of the trichomes, colourless to yellowish.

3. This alga dominated almost all of the laboratory cultures enriched with combined nitrogen together with L. aestuarii. It showed good tolerance to both salinity and reducing conditions.

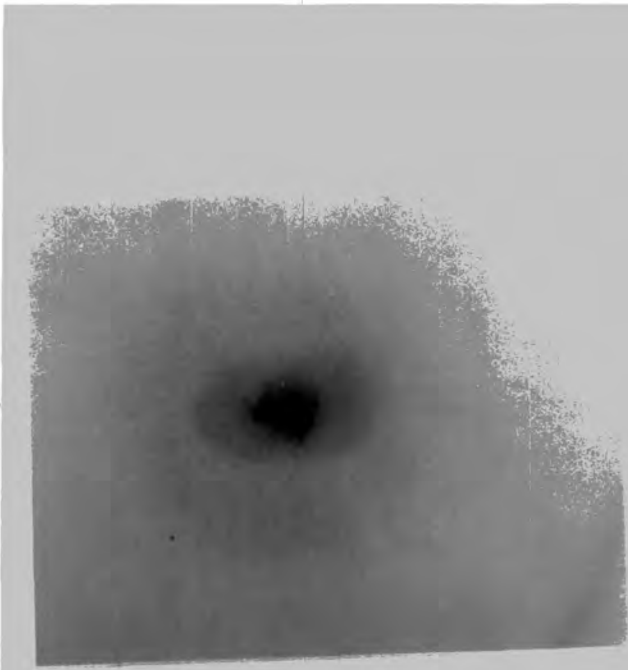
Microcoleus chthonoplastes D634

1. In several liquid cultures of mixed popualtions typical

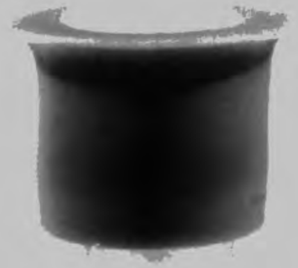
Fig. 4.12 Growth forms and morphology of *Lyngbya* sp. D639

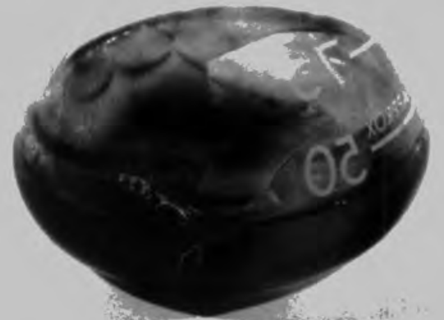
1. Growth forms (a) on solid medium
   (b) in liquid medium

2. Morphology (i) single filament showing long sheath



(a)





(b)

(i)

bundles of filaments with common firm, colourless or yellow to orange were seen (Fig. 4.13), single filaments with sheath extended beyond the end of the trichomes were also seen. When brought into clonal axenic culture, the bundle form was lost and the alga grew as single filaments densly entangled to form a mat-like structure on the surface of liquid medium. On solid medium filaments spreade in a shiny structure from the inoculum with diffuse or fimbriate margin. The colour of the plant mass was bright blue-green or green.

2. Vegetative cells, cylindrical (mostly) or quadrate, 4-5 µm wide, 4-13 µm long; trichomes straight, slightly bent or irregularly contorted, constricted at the cross-wall, apical cell conspicuously tapered or rounded; sheath thick and extende beyond the end of the trichomes.

3. It was the dominant alga in the field at the time of soil collection and in soil sample by direct inspection, it dominated some laboratory cultures (Table 4.4).

4.4 Influence of nitrogen sources on the morphology of the strains isolated

Several morphological changes were seen in mixed populations associated with media enriched with combined nitrogen, which made algal identification difficult in many cases. Therefore experiments were carried out to test the

Fig. 4.13 Growth forms and morphology of *Microcoleus chthonoplastes* D634

1. Growth forms (a) on solid medium
   (b) in liquid medium

2. Morphology (i) bundle of trichomes in mixed populations
   (ii) single filament showing long sheath in clonal axenic culture

(a)

(b)

(i)

(ii)

effect of combined nitrogen on the morphology of heterocystous forms of the strains isolated. Algae were cultured into AD-N, AD+NO_3-N and AD+NH_4-N (Section 2.45). All media were buffered with 5 mM HEPES at pH 7.6. Cultures were incubated in shaking tank at 32 °C and 90-120 µmol photon $m^{-2}s^{-1}$ with continuous shaking for 10-14 days. It was found that the growth of most algae was poor in media enriched with NH_4-N and the pH dropped to 4-5. In media enriched with NO_3-N the pH value wentup to 9-10. Morphological changes observations were therfore restricted to NO_3-N enriched media for all strains. The experiment was repeated with reduction of the nitrate concetration to 50 mg l^{-1} and the HEPES concentration increased to 10 mM. The pH values of media enriched with ammonium nitrogen dropped to 6.8-7.2 after 10-14 days and went up to 8-9 in media enriched with nitrate nitrogen. Morphological changes were almost the same for both nitrogen sources. The following morphological changes were recorded.

1. Growth form and colour of the plant mass

The growth form of axenic strains (Section 4.3) was almost the same in -N and +N media except in strain D636 in which growth was very poor in medium enriched with NH_4-N. However, clonal cultures of strains D586, D635 and D678 were unhealthy in media enriched with cobined nitrogen. Strains D635 and D678 tend to grow as a loose floc distributed

throughout the growth medium. The colours of the plant masses for strains D579, D580, D581 and D582 were green, dark-green to blue-green in +N medium. Strains D584, D585 and D636 have more or less the same colour in both media.

2. Heterocysts

Heterocysts formation was almost completely supressed in +N media for all strains tested under both nitrogen sources with ammonium nitrogen more effective. However, in all cases there were a few filaments which still had heterocysts. It has been found also that cultures showed few detached heterocysts.

3. Akinete

Akinete formation was markedly reduced in +N medium for all akinete-forming strains (D584, D586, D635, D636, D678). Strains D584 and D678 were less affected.

4. Hairs

D636 was the only hair-forming strain among the strains studied. In +N medium both hair length and the number of filaments with hairs was reduced. None of the Calothrix spp. showed hairs in +N medium.

5. Sheath thickness and colour

The sheath thickness was only slightly increased in all strians in +N medium.

6. Trichome shape

In general all strains form long gas-vacuolated hormogonia in +N medium. The length of the mature filaments was also greater in +N medium compared to -N medium. In strains D580, D583, D585, D636 the loss of heterocysts led to the formation of *Homoeothrix* or *Ammatoidea*-like trichomes. Strain D636 was less affected. In this strain most of the trichomes were short ending with short hairs; in addition there are many trichomes with *Homoeothrix* or *Ammatoidea* appearance.

7. Vegetative cells dimentions

No change in vegetative cells dimentions were found for strains D579, D582, D584, D586, D635, D678. Strains D580, D583, D636 the tapering charater become less apparent in +N medium.

8. Cyanophycin granules level

The level of cynanophycine granules increased markedly in all strains in +N media especially with NH_4-N.

4.5 Influence of phosphorus sources on the morphology strains isolated

Literature revied in Section 1.3 showed that tremendous morphological changes took place in blue-green algal species grown with different phosphorus concentrations. In addition phosphorus concentration in the growth meidum has been changed from 44.5 to 2.5 mg l^{-1} P in the experiments of Chapter 5 and subsequently to 1 mg l^{-1} for most of the experiments in Chapters 6 and 7. These changes were found to affect the morphology of some of the strains isolated. In order to have a clear idea about the influence of phosphorus concentration and form, experiments were conducted in which all strains isolated (Table 2.7)- except strain D693 which was isolated toward the end of the present study (Section 4.3)- were cultured in AD-N or AD+N medium with two initial phosphorus concetrations 1 and 44.5 mg l^{-1} P. Both media were buffered with 5 mM HEPES at pH 7.6. Cultures were then incubated in a growth room with 32 ^{o}C and 40-50 μmol photon $m^{-2}s^{-1}$. The morphological features of the strains were recorded after 14-17 days from the second subculturing. Axenic cultures of the isolated strains were also cultured in AD-N or AD+N (strain D634) in which phosphorus—phosphate was replaced by a number of organic and anhydrous phosphorus compouds (Table 2.5). Cultures were incubated at 32 ^{o}C, 90-120 μmol photon $m^{-2}s^{-1}$ and continuous shaking. The morphologies of the strains were recorded after

10-12 days. In growth curve experiments of strains D579, D584, D585 (Section 6.2), algae were cultured in AD-N medium with two initial phosphorus concentration 1 and 10 mg l^{-1} P the morphological changes of these strains were followed over 28 days.

The major morphological changes followed were the same for the influence of nitrogen sources (Section 4.4).

1. Growth form and colour of plant mass

The growth form of all strains were similar for both phosphorus concentrations; the colour of the plant mass was, however, largely different. It was greenish for strains D579, D580, D581, D582, D586, D634, D635, D638, D639, D678 under low phosphorus concentration (1 mg l^{-1} P). It was green to dark green or blue-green under high phosphorus concentrations (10 and 44.5 mg l^{-1} P). However strain D582 did not grow well under the high phosphorus concetration and the colour of the plant mass was the same. The colour of the plant mass of strains D583, D584, D585, D636 was brown to yellow-brown in low phosphorus concentrations and brown to dark-brown or red-brown in the higher phosphorus concentrations (10 and 44.5 mg l^{-1} P).

2. Heterocyst

Heterocyst formation was slightly reduced for strains D580, D581, D583, D585, D636 and markedly for strains D579, D582, D635, D584, D586, D678 under the low phosphorus

concentration (1 mg l^{-1} P). For example, the number of filaments with double heterocysts in strains D579 and D678 was about 50% of total number of filaments under high phosphorus concentration, while only 10% of the filaments showed double heterocysts under low phosphorus concentrations.

3. Akinete

D584, D586, D635, D636, D678 were the only akinete-forming strains. All lost their ability to form akinetes when cultured with a high phosphorus concentration (44.5 mg l^{-1} P). However, it is worth mentioning that strain D678 continued to form akinetes under this concentration, gradually reducing over several subculturings. The number of filaments with akinete was even reduced under low phosphorus concentrations with time. The was a delay and reduction, but not loss in akinete formation of strain D584 cultured with 10 mg l^{-1} P (Section 6.2).

4. Hairs

D636 was the only hair-forming strain. Both the number of trichomes with hairs and the length of the hairs was markedly reduced at high phosphorus concentrations (44.5 mg l^{-1} P). None of the _Calothrix_ species formed hairs at low phosphorus concentration.

Strains D585 and D636 showed a marked increase in the thickness of the sheath under low phosphorus concentrations, strains D580 and D583 only slightly increased their sheath thickness under low phosphorus conditions. The colour of the sheath was hyaline for strains D585 and D636 at high phosphorus concentrations becoming yellowish at low phosphorus concentrations.

6. Trichome shape

No change in trichome shape was seen in any of the strains studied. However, strain D580, D636 showed a slight reduction in the tapering pattern of the trichomes under at phosphorus concentrations.

7. Dimensions of vegetative cells

No major changes took place in the dimenSions of the vegetative cells of any strains studied.

8. Cyanophycin granules

The level of cyanophycin granules increased slightly or markedly in all strains studied at low phophorus concentrations.

The morphological changes took place under organic and anhydrous phosphorus enrichments were the same as those mentioned above for the low concentration of inorganic phosphorus (PO_4-P). However, no morphological observation was made for strain D582 under inositol hexaphosphate enrichment because of lack of growth under these conditions (Table 6.8).

CHAPTER 5. INFLUENCE OF ENVIRONMENTAL FACTORS ON ALGAE IN RICE-FIELD SOIL

5.1 Introduction

There are large daily and annual variations in the physico-chemical conditions of the rice-field in the southern marshes of Iraq (Section 3.2). On the other hand, the literature reviewed in Section 1.21 showed that the growth and abundance of many algae is limited by the physical or chemical factors or combinations of them in any area. Studies were therefore planned to investigate the influence of environmental factors on algae in rice-field soil. This is in order to get an idea about the distribution of the algae under different environments most likely to be important in the fields and to select for detailed studies those strains which show relatively high abundance in a wide range of environments.

5.2 Temperature

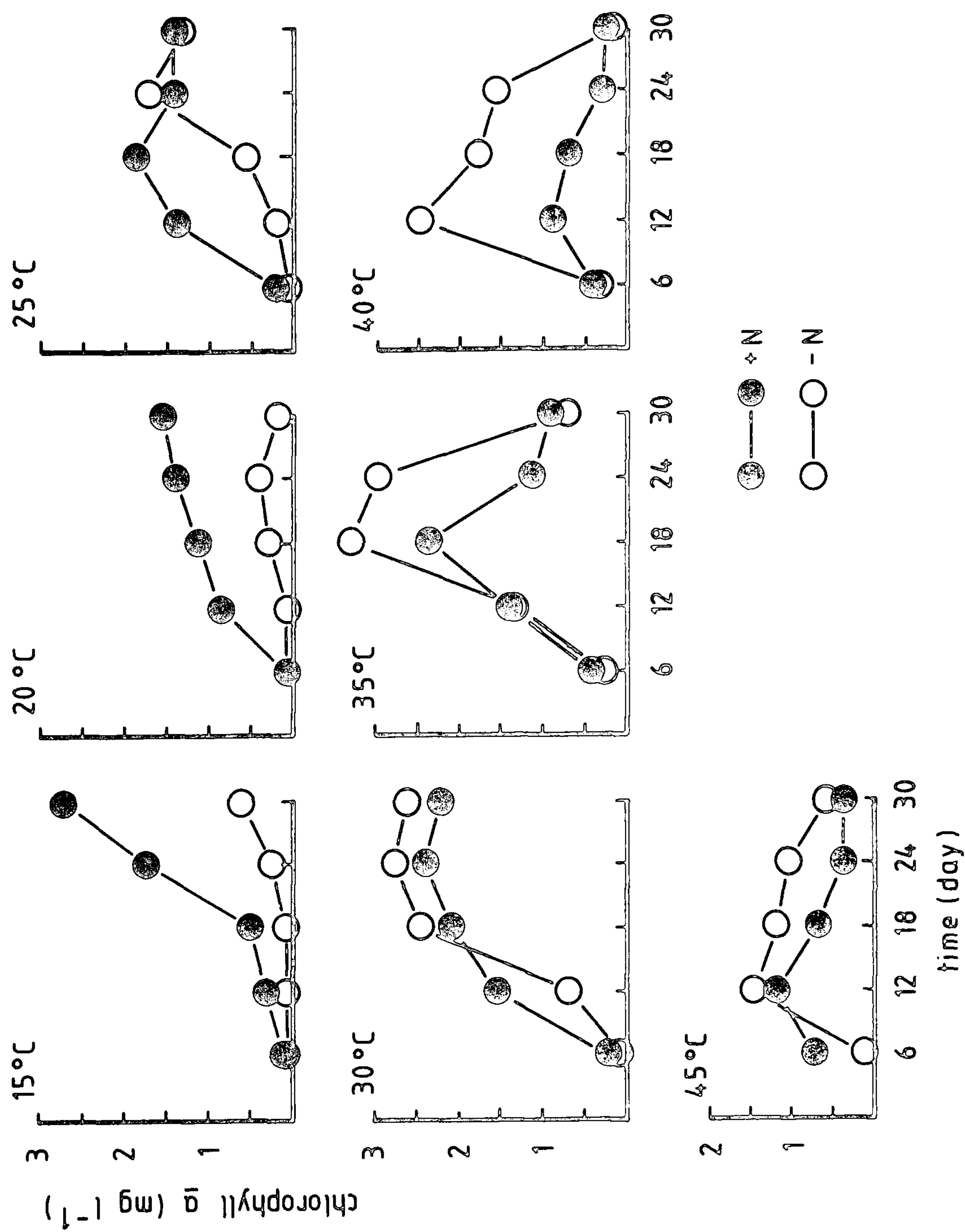
The effect of a wide range of temperature (5-50 °C) on the growth and composition of the algal community was studied. Experiments were carried out in the temperature-gradient apparatus and/or an illuminated incubator (Section 2.52). No algae grew at 50 °C. At 48 °C slight growth was evident in both media 3 days after

inoculation, but subsequently the cultures died. Substantial growth occurred at 45 °C and this was chosen as the upper temperature for the detailed experiments. Algal biomass (estimated as chlorophyll a) of cultures incubated at 5 °C was less than 0.05 mg l^{-1} chl a (detection limit of the method for this experiment) over 30 days. However, in addition to the taxa seen by direct viewing of the soil sample (Table 4.1) two more strains were seen in cultures incubated at 5 °C .These are Tribonema minus and Chlorococcum humicola. At 10 °C the algal biomass in AD-N was less than 0.05 mg l^{-1}. AD+N cultures collected on days 24 and 30 yielded 0.78 and 0.82 mg l^{-1} chl a, respectively. Most of the cultures incubated at 10 °C were dominated by T. minus or C. humicola. Some cultures, however, were dominated by Lyngbya spp., Aphanothece stagnina or Nostoc spp. Growth occurred in both -N and +N media over temperatures ranging between 15-45 °C (Fig. 5.1), but the relative effect of the two media differed according to the temperature. Due to the complexity of the ecosystems inside the culture flasks, too much attention cannot be placed on small differences, but two aspects seem to be established clearly:

(i) Initial growth was always faster in +N medium and the difference was greater the lower the temperature.

(ii) Growth continued to the end of the experimental period

Fig. 5.1 Infuence of a) temperature and b) presence or absence of combined nitrogen on growth yield of algal community developing on inoculation of soil sample (90-120 µmol photon $m^{-2}s^{-1}$)


15°C
20°C
25°C
30°C
35°C
40°C
45°C
chlorophyll a (mg l-1)
time (day)
6 12 18 24 30
+N
-N

of 30 days at 15 ^{o}C in both media, but at higher temperatures most of these mixed cultures showed a marked drop in crop (estimated as chl a) towards the end of the 30 days period. The total number of taxa was almost the same in both media and was higher at low temperatures (Fig. 5.2).

5.3 Light

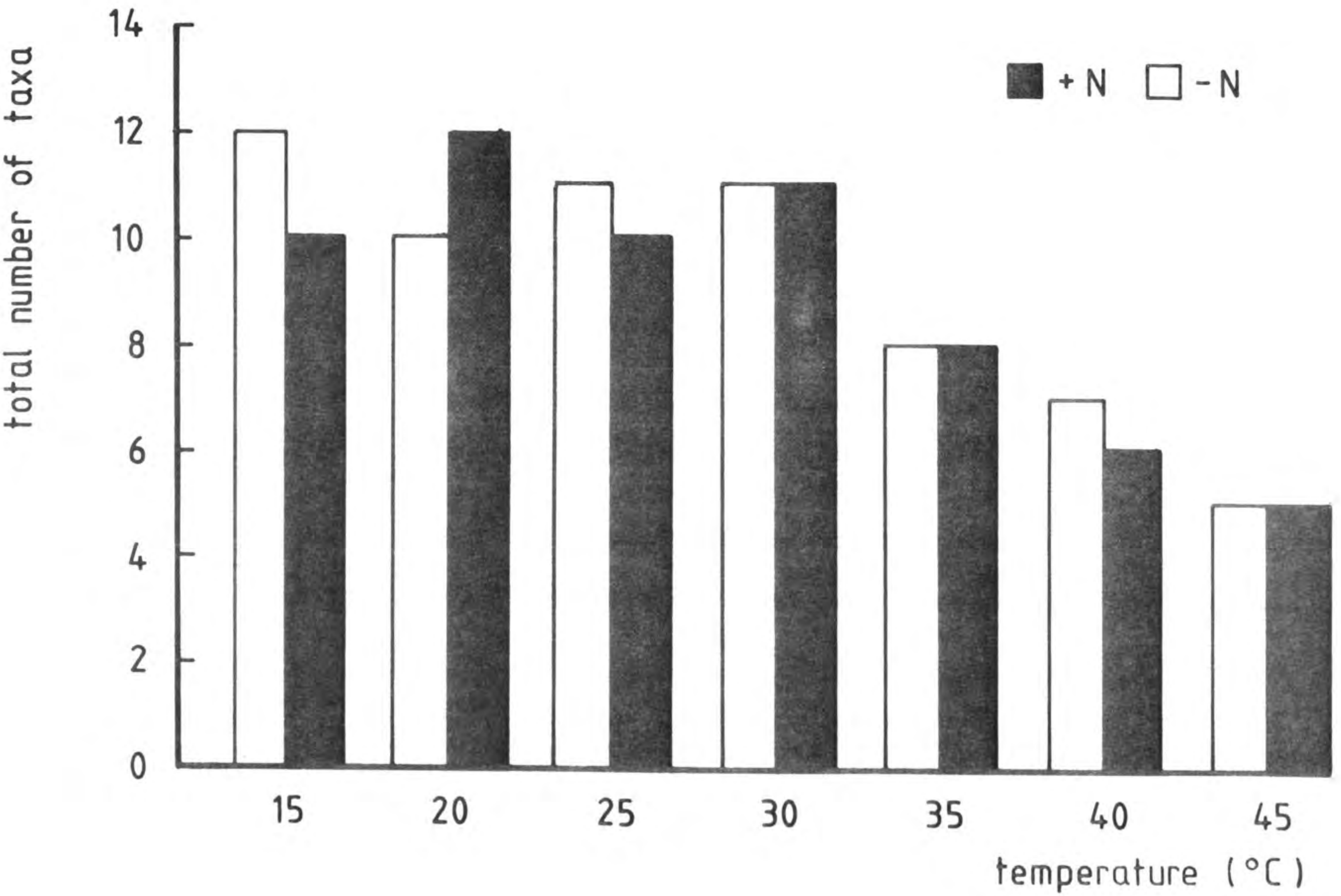
In the rice-fields algae grow under different light regimes (Section 3.2). To study the effect of different light conditions on the growth and distribution of algae in the soil of rice-fields, experiments were conducted in which media inoculated with soil were incubated under different light regimes.

5.31 Quantity

(i) Photon flux density

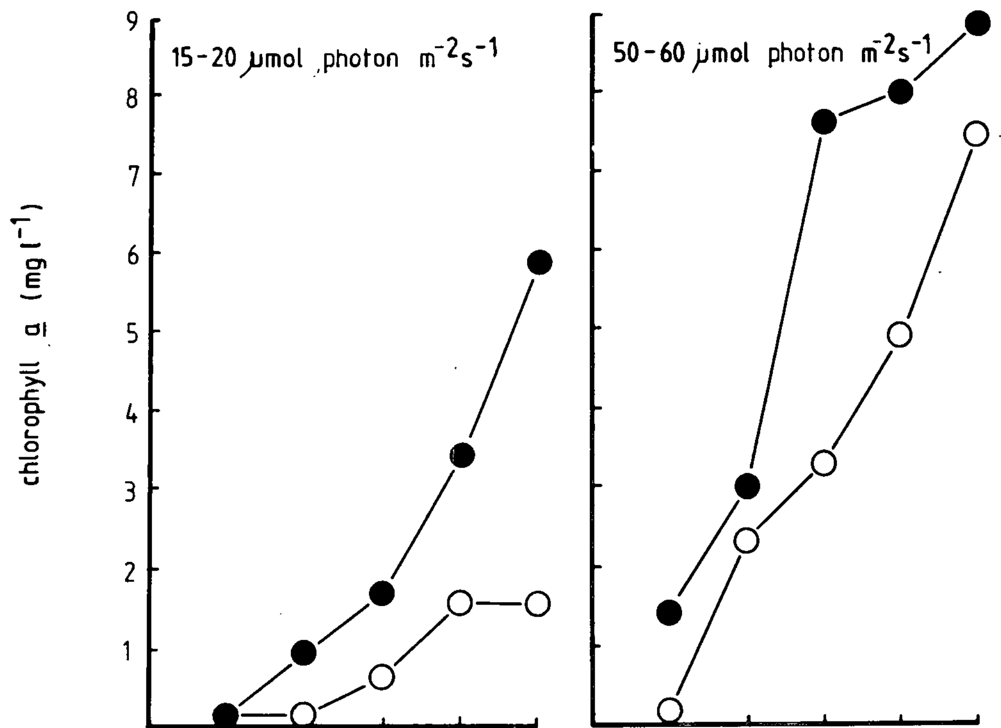
The influence of photon flux density on algal growth is shown in Fig.5.3. The highest yield was at light regime 50-60 μmol photon $m^{-2}s^{-1}$ and the lowest yield was at light regime 200-250 μmol photon $m^{-2}s^{-1}$. There were no large differences in the total number of taxa under different light regimes, but the dominant algae were different (Table 5.1). In general non-heterocystous filamentous blue-green algae dominated most of the cultures at the low photon flux

Fig. 5.2 Infuence of a) temperature and b) presence or absence of combined nitrogen on total number of taxa noted on all five days at which sample harvested (90-120 µmol photon $m^{-2}s^{-1}$)



+N
-N
total number of taxa
0
2
4
6
8
10
12
14
15
20
25
30
35
40
45
temperature (°C)

Fig. 5.3 infuence of photon flux density on growth yield of algal community developing on inoculation of soil sample (35 °C ± 1; in presence or absence of combined nitrogen)


15-20 µmol photon m-2s-1
50-60 µmol photon m-2s-1
chlorophyll a (mg l-1)
9
8
7
6
5
4
3
2
1

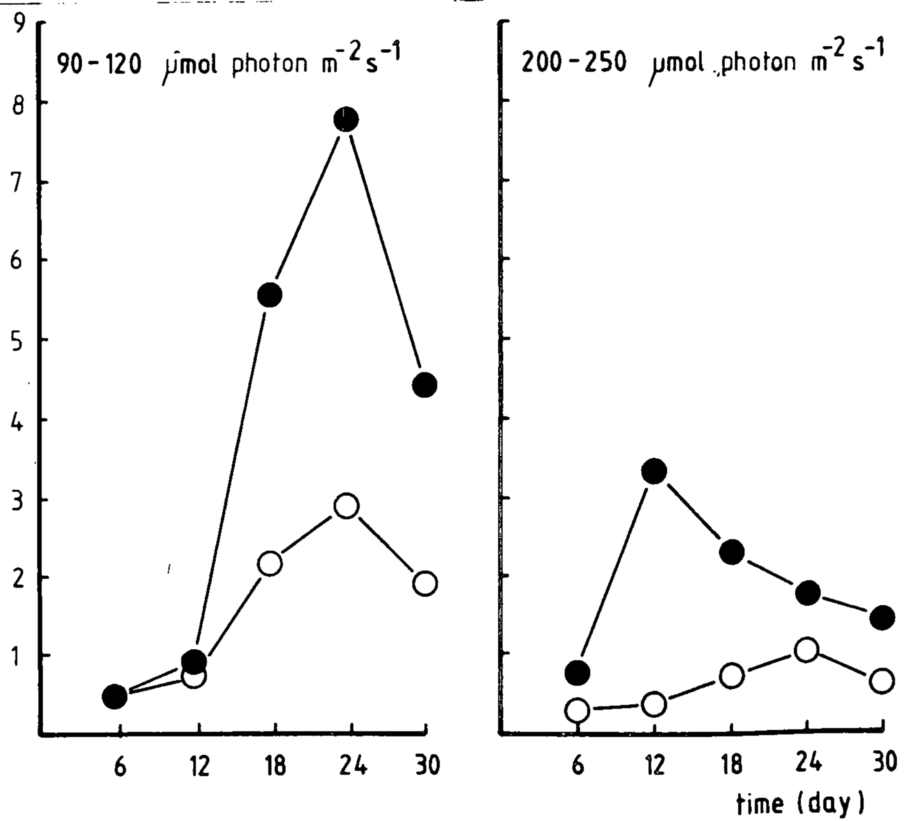

90-120 µmol photon m-2s-1
200-250 µmol photon m-2s-1
9
8
7
6
5
4
3
2
1
6
12
18
24
30
6
12
18
24
30
time (day)

Table 5.1 Influence of photon flux density on the dominance of algae in the mixed population (35 °C ± 1; 90-120 μmol photon $m^{-2}s^{-1}$; in presence or absence of combined nitrogen)

photon flux density μmol (photon $m^{-2}s^{-1}$)	-N dominant 1	-N dominant 2	+N dominant 1	+N dominant 2
15-20	*Nostoc* spp.	*Lyngbya* spp.	*Lyngbya*>1≤2um	*Lyngbya*>2≤4um
50-60	*Nostoc* spp.	*Anabaena cylindrica*	*Lyngbya* spp.	*Nostoc* spp.
90-120	*Cylindrospermum*	*Nostoc muscorum*	*Lyngbya* spp.	*Nostoc linckia*
200-250	*Nostoc muscorum*	*Anabaena cylindrica*	*Scenedesmus*	*Chlorococcum*

density. At high photon flux density heterocystous blue-green algae dominated the -N media and Chlorophyta dominated the +N media.

(ii) Dark/light cycle

In nature algae grow under an alternation of light and dark periods. Almost all of the experiments in this chapter, however, were carried out under continuous light. Some algae might grow only under dark/light conditions and their growth therefore might be inhibited by continuous illumination. To investigate this possibility an experiment was carried out in which cultures were incubated under photon flux density 90-120 μmol photon $m^{-2}s^{-1}$ for 12h followed by 12h incubation in the dark (Section 2.52). Algal yield under the dark/light treatment (Fig. 5.4) was higher than that of the continuous light at photon flux density of 200-250 μmol photon $m^{-2}s^{-1}$, but lower than that of the two low photon flux densities (15-20 and 50-60 μmol photon $m^{-2}s^{-1}$) (compare Fig. 5.4 and Fig. 5.3). Nostoc spp. dominated the -N media and Lyngbya spp. the +N media.

5.32 Quality

Algae in the rice-field might grow under conditions in which green light is dominant as a result of reflection from and/or filtration by rice plants, so an experiment was set

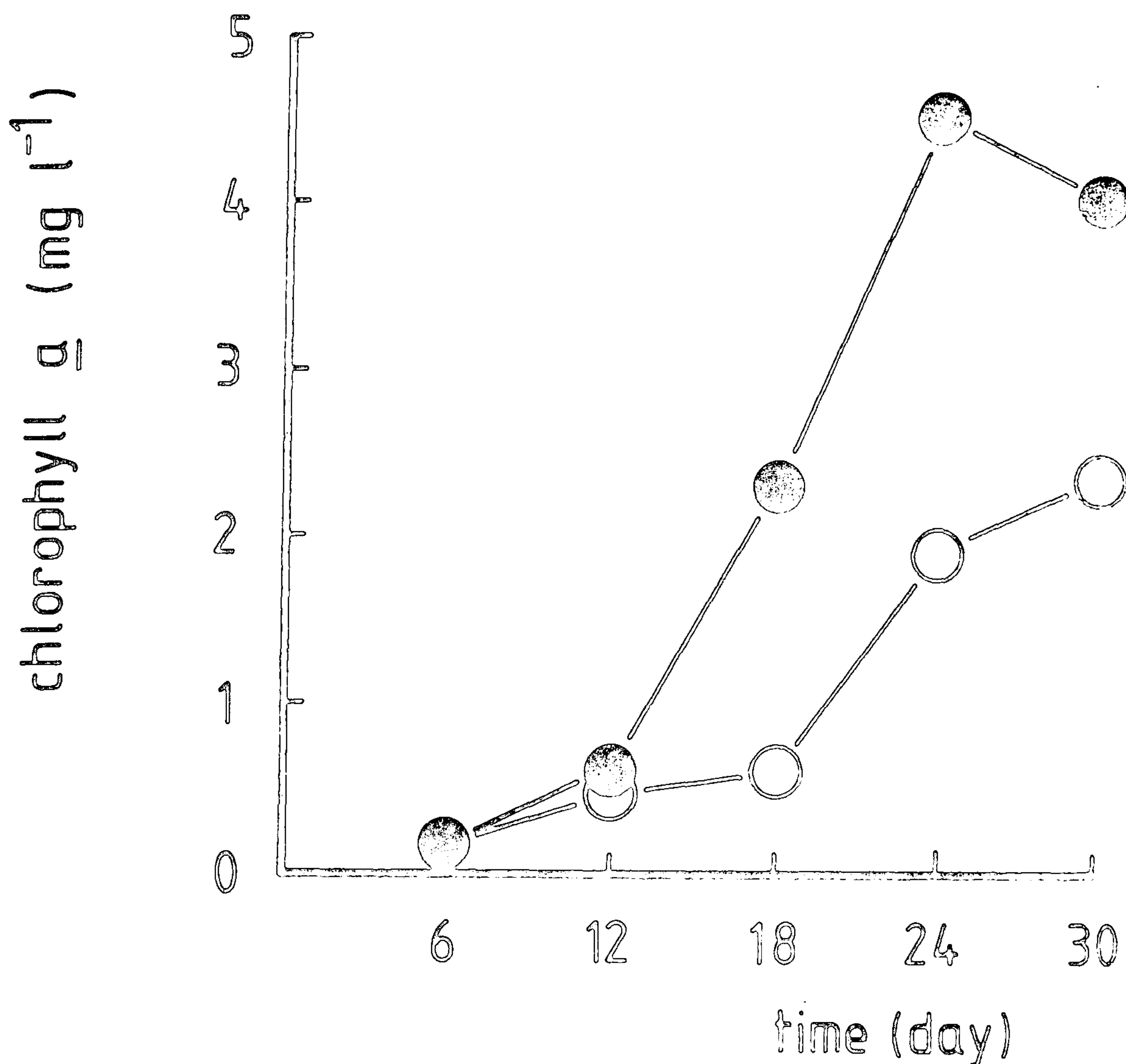


Fig. 5.4 Infuence of dark/light cycle (12h:12h) on growth yield of algal community developing on inoculation of soil sample (35 °C ± 1; photon flux density during light period was 90-120 μmol photon $m^{-2}s^{-1}$; in presence or absence of combined nitrogen)

up to study the influence of green light (465-570 nm) using a green filter (Section 2.52). The photon flux density was 15-20 µmol photon $m^{-2}s^{-1}$. Algal biomass under green light (Table 5.2) was very low compared to the biomass of the algae under full light of the same photon flux density (Fig. 5.3). There were no major differences in the relative abundance of algae under the two light qualities, but Gloeotrichia sp. D636 showed high abundance in old cultures under green light enrichment.

Table 5.2 Influence of green light on growth yield of algal community developing on inoculation with soil sample (at 35 $^{o}C \pm 1$; 15-20 μmol photon $m^{-2}s^{-1}$; in presence or absence of combined nitrogen)

time	mg chl a l^{-1}	
(day)	-N	+N
6	< 0.05	< 0.05
12	< 0.05	19.5
18	34.69	46.88
24	35.56	388.28
30	30.36	388.28

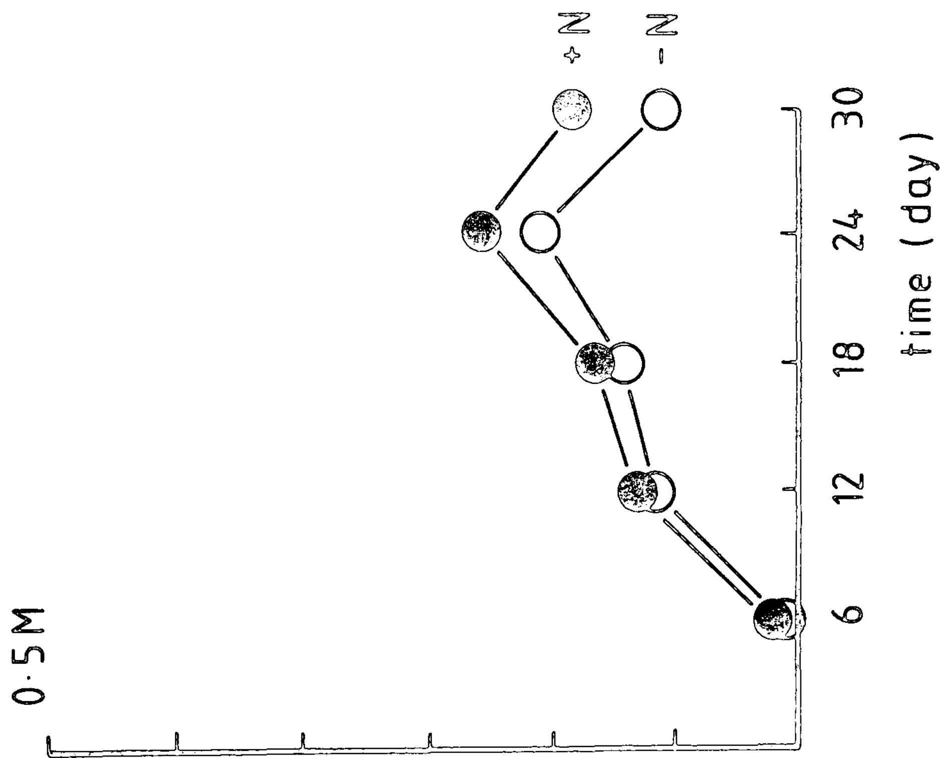
5.4 Salinity

Dissolved salts are probably at their lowest concentration during peak flooding of the Tigris and Euphrates where rice-fields are covered by water (Section 3.2). This water recedes in about 6-7 months and the rice-fields start to dry out. These changes are associated with tremendous variation in the salt content of both water and soil in the rice-fields. Salinity therefore may be an important factor influencing growth of some algae in the rice-field. An experiment was carried out to see the effect of salinity (using NaCl) on growth and abundance of the algae of the rice-fields soils. The growth was inhibited almost completely at 1.0 M NaCl. At 0.1 M growth was higher in -N medium; at 0.5 M addition of combined nitrogen improved the growth of algal community though slightly (Fig. 5.5). However, increasing the NaCl level brought about a marked reduction in the total number of taxa of both media (Table 5.3).

5.5 Reducing conditions

Experiments were carried out to study the effect of three reducing chemicals on the growth of the algal community. These were sulphide, sulphite and thiosulphate. All were added as sodium salt at 0, 0.1, 0.5, 1.0 and 5 mM. They were sterilized by filtration and added aseptically

Fig. 5.5 Infuence of NaCl on growth yield of algal community developing on inoculation of soil sample (35 $^{o}C \pm 1$; 90-120 μmol photon $m^{-2}s^{-1}$; in presence or absence of combined nitrogen)


0·5M
+N
−N
6
12
18
24
30
time (day)

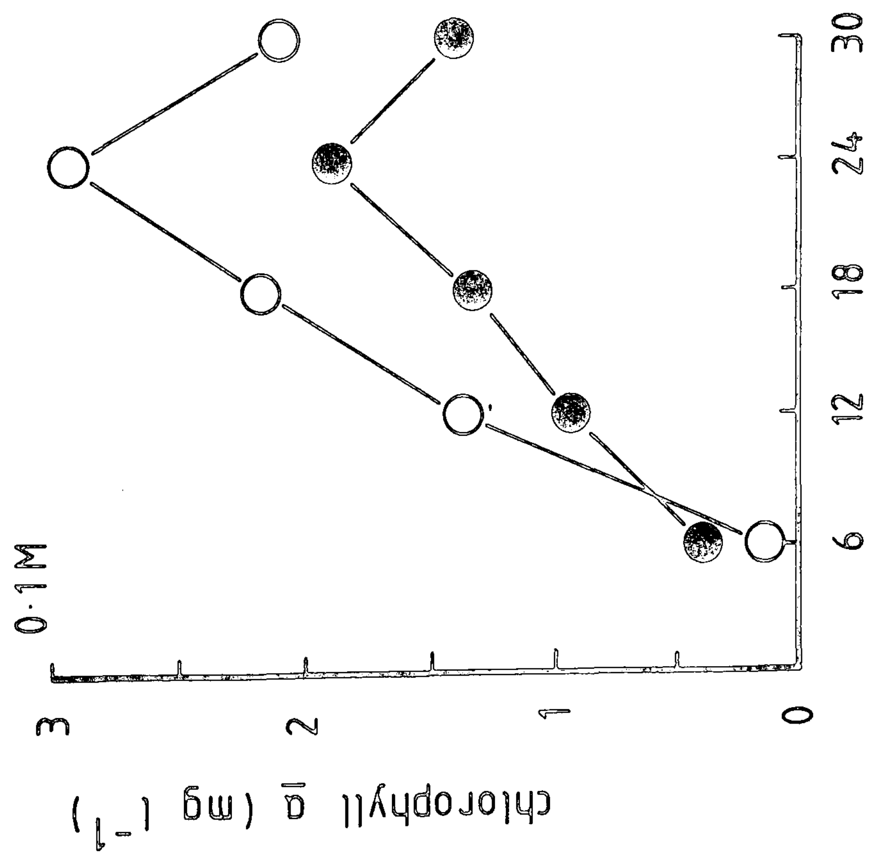

0·1M
0
1
2
3
chlorophyll a (mg l⁻¹)
6
12
18
24
30

Table 5.3 Taxa present on day 30 in media enriched with NaCl (35 + 1 C; 90-120 µmol photon $m^{-2}s^{-1}$; in presence or absence of combined nitrogen; relative abundace expressed on 1-5 scale)

taxa	NaCl concentration (M) 0.1 -N	0.1 +N	0.5 -N	0.5 +N	1.0 -N	1.0 +N
Anabaena cylindrica	5					
Cylindrospermum muscicola	4					
Chlorococcum humicola		3		*		
Lyngbya ≤ 1 µm	3	4	2	2	2	3
Lyngbya > 1 ≤ 2 µm	2	2	4	4	5	4
Lyngbya > 2 ≤ 4 µm	1	1				
Microcoleus chthonoplastes	1					
Nostoc linckia	2	5	3	2	4	5
N. muscorum	3	2	3	3		
Nodularia harveyana			5	5		

* *Chlorococcum* dead by day 30, but live cells abundent on day 24.

after inoculation of soil into the flask . Two flasks were used for each treatment. One was closed with silicon bungs (aerobic conditions) and the other with suba seal bungs (anaerobic conditions). There were no changes in the pH and appearance of the media after the addition of sulphite and thiosulphate. Addition of sulphide (1.0 and 5.0 mM) however increased the pH values to 8 and 10, respectively. The appearance of the media enriched with sulphide changed to milky, grey, dark-grey and dark-blue after the addition of 0.1, 0.5, 1.0 and 5.0 mM sulphide, respectively. The colour of the media under aerobic conditions, however, changed gradually to normal (colourless)at the low concentrations of sulphide (0.1 and 0.5 mM). There was very slow change toward the normal colour under anaerobic conditions. There were no obvious differences in the algal communities of either media enriched with any of the reducing chemicals under the aerobic conditions. However in media enriched with 0.1 and 0.5 mM sulphide and combined nitrogen there was slight increase in the abundance of the non-heterocystous filamentous blue-green algae under anaerobic conditions (Table 5.4). In -N media, *Cylindrospermum* was the most sensitive to the sulphide enrichment followed by *Anabaena*; *Nostoc linckia* was the most tolerant.

TABLE 5.4 Influence of sulphide on the abundance of taxa of algal community developing after 10 days from inoculation with soil sample ($35^{o}C \pm 1$; 90-120 μmol photon $m^{-2} s^{-1}$; in presence or absence of combined nitrogen; relative abundance expressed on 1-5 scale)

taxa	-sulphide (control)		+ sulphide (mM)							
			0.1		0.5		1.0		5.0	
	-N	+N	-N	+N	-N	+N	-N	+N	-N	+N
<u>Anabaena cylindrica</u>	3	2	3	1	3	2	2			
<u>Aphanothece stagnina</u>				2		2				
<u>Cylindrospermum muscicola</u>	4	3	2		2					
<u>Chlorococcum humicola</u>		5		1						
<u>Lyngbya</u> ≤ 1 μm	2	4	3	5	4	5	5	5	2	2
<u>Lyngbya</u> > 1 ≤ 2 μm	1	2	2	3	5	3	3	4	1	1
<u>Lyngbya</u> > 2 ≤ 4 μm	1	2		2		2	2	3		
<u>Microcoleus chthonoplastes</u>		2	1	4	2	4	2	2		
<u>Nostoc muscorum</u>	5	3	4	2	2	3	4	1		
<u>Nostoc linckia</u>	3	1	5	2	3	1	2	1		

5.6 Nitrogen sources

The influence of various environmental factors was investigated in -N or +N medium. At the beginning both nitrate-nitrogen and ammonium-nitrogen were used in unbuffered media (Section 2.45). This led to a dramatic changes in the pH of the media after short period from soil inoculation (6-12 days). The pH values of media enriched with NH_4-N dropped to 4-5 and went up to 9-10 in NO_3-N containing media. After the reduction of the phosphorus concentration to 2.5 mg l^{-1} P (Section 5.7) all media were buffered with 5 mM HEPES. In this case, there was only a slight change in the pH of media enriched with NH_4-N at the beginning only (pH dropped to 6-7 after 6 days) followed by gradual decrease to pH 4 after 12 days from inoculation; this was associated with chlorosis and death of the algal community. In media enriched with NO_3-N the pH was usually about 8 throughout the growth period. The initial algal community, howewver, was same with either nitrogen source. Therefore only NO_3-N was used for the work described in this chapter. Addition of combined nitrogen was included to study the effect of environmental factors on algal communities other than the heterocystous blue-green algae.

5.7 Phosphorus

In all of the experiments in this Chapter phosphorus was supplied as orthophosphate. In the earlier parts of

this Chapter (Sections 5.2 and 5.4) AD medium with 44.5 mg l^{-1} P was used, a concentration seldom likely to be found in the field. Therefore an experiment was carried out in which phosphorus concentration was reduced to 0.5, 2.5 and 10 mg l^{-1}. AD medium with 44.5 mg l^{-1} P was used as control. All of the media in this experiment were buffered with 5 mM HEPES; potassium was adjusted to that of the cotrol using KCl. The result (Fig. 5.6) showed that the highest yield of the algal community is that of the control up to day 18, but this drops sharply afterward. Growth of algal community at the other concentrations (i.e 2.5 and 10 mg l^{-1} P) continued until the end of the experimental period of 30 days and gave a final yield which was more or less similar to that of the control. At the lowest concentration (0.5 mg l^{-1} P), however, algal community yield dropped after a relatively short time (18 days). However, total number of taxa at the two lower concentrations (0.5 and 2.5 mg l^{-1} P) was higher than that of the other concentrations (i.e. 10 and 44.5 mg l^{-1} P) (Fig. 5.7). Therefore the phosphorus concentration in AD medium was reduced to 2.5 mg l^{-1} P and the medium buffered with 5 mM HEPES for the rest of the experiments in this Chapter.

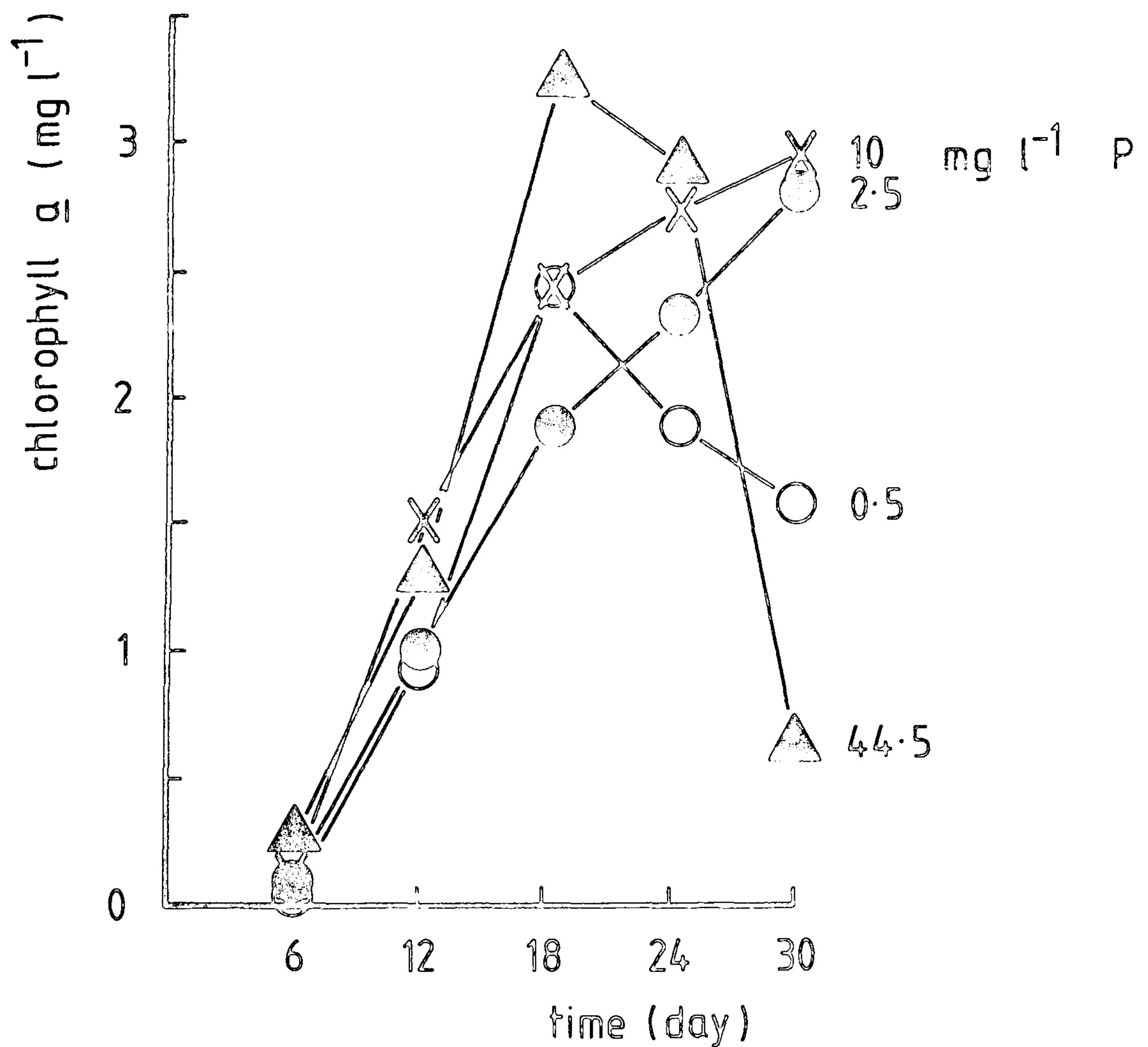
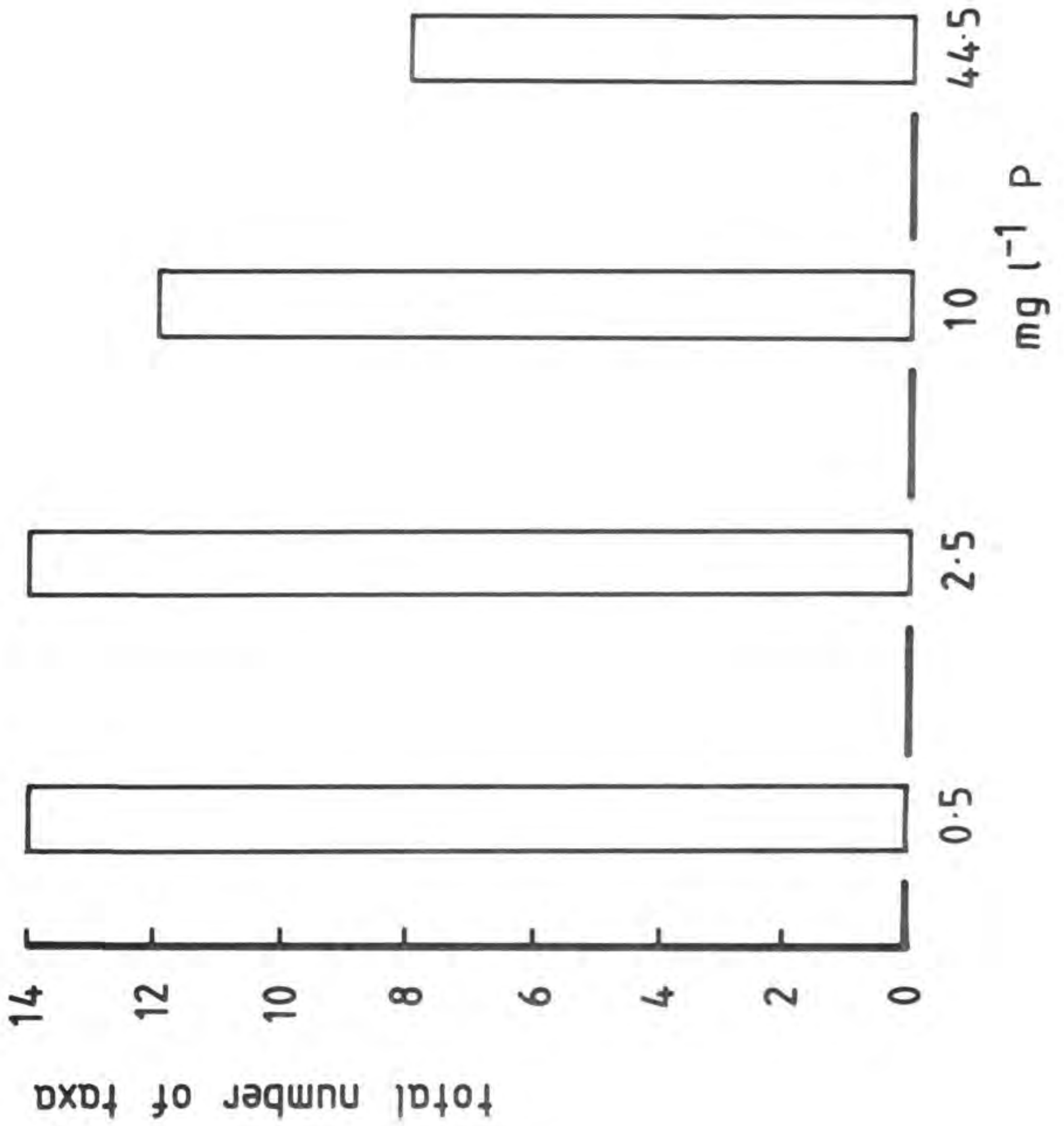


Fig. 5.6 Infuence of phosphorus concentration on growth yield of algal community developing on inoculation of soil sample (35 ^{o}C $\pm$ 1; 90-120 μmol photon m^{-2}s^{-1} in absence of combined nitrogen)

Fig. 5.7 Infuence of phosphorus concentration on total number of taxa noted on all five days at which samples harvested (35 $^{o}C \pm 1$; 90-120 μmol photon $m^{-2}s^{-1}$; in absence of combined nitrogen)



total number of taxa
0
2
4
6
8
10
12
14
0·5
2·5
10
44·5
mg l-1 P

CHPATER 6. INFLUENCE OF PHOSPHORUS ON STRAINS ISOLATED

6.1 Growth

In order to determine the optimum time for the measurement of the growth rates of all strains isolated, detailed experiments were carried out on three strains. These experiments were designed to examine the differences between the growth rates of the strains under the standard growth conditions; such results might be helpful in the interpretation of the successive appearance and dominance of algae in mixed populations (Chapter 5). The three selected strains were Calothrix sp. D585, Cylindrospermum muscicola D579 and Nostoc muscorum D584. The growth rates and mean doubling time were determined as described in Section 2.673. The highest growth rates for all strains were recorded on day two of the experiment (Table 6.1). On day four growth rates dropped to about 83% of that of the day two. After that growth rates of all strains decreased gradually. Growth rates of all strains (including Calothrix parietina D550 as cotnrol) were determined also after two days from inoculation. Large differences in growth rates were found between the strains (Table 6.2); the highest growth rate was that of strain D579 and the lowest was that of strain D580. The other strains could be divided into two groups; strains D583, D585 and D636 with growth rates ranging between 0.33

Table 6.1 Growth rate, $\grave{K}$ and mean doubling time (G) of *Calothrix* sp. D585, *Cylindrospermum muscicola* D579 and *Nostoc muscorum* D584; algae cultured in AD-N medium with initial phosphorus concentration of 1 mg l^{-1} P (32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)

time	*Calothrix* sp.		*Cylindrospermum*		*Nostoc*	
(day)	$\grave{K}$	G(h)	$\grave{K}$	G(h)	$\grave{K}$	G(h)
2	0.33	21.89	0.54	13.38	0.45	16.05
4	0.27	26.76	0.46	15.70	0.37	19.52
6	0.26	27.78	0.33	21.89	0.26	27.78
8	0.22	32.84	0.25	28.90	0.20	36.12
10	0.17	42.49	0.20	36.12	0.16	45.15
12	0.13	55.57	0.17	42.50	0.14	51.60

(strains)

Table 6.2 Growth rate, $\grave{K}$ and mean doubling time (G) of Iraqi isolate and Calothrix parietina D550 as control; algae cultured in AD-N medium with initial phosphorus concentration of 1 mg l^{-1} P (32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)

strains	Durham culture No.	$\grave{K}$	G(h)
<u>Anabaena</u> <u>cylindrica</u>	582	0.48	15.05
<u>Calothrix</u> <u>parietina</u>	☆550	0.44	16.42
<u>Calothrix</u> sp.	580	0.27	26.75
<u>Calothrix</u> sp.	583	0.33	21.89
<u>Calothrix</u> sp.	585	0.36	20.07
<u>Cylindrospermum</u> <u>muscicola</u>	579	0.54	19.52
<u>Gloeotrichia</u> sp.	636	0.37	19.52
<u>Hapalosiphon</u> <u>welwitschii</u>	581	0.40	18.06
<u>Nostoc</u> <u>muscorum</u>	584	0.45	16.02

☆ control

and 0.36 strains; D582, D550, D581 and D584 with growth rates ranging between 0.40 and 0.48.

6.2 Physiological changes

After the measurement of the growth rates of all strains (Section 6.1), detailed physiological changes were followed under two phosphorus concentrations over 28 days for the three selected strains. Experiments were carried out under the standard growth conditions (Section 2.52). Cultures were harvested every four days, taking four replicate flasks.

(i) Chl a and dry weight

Chlorophyll a and dry weight were determined as described in Sections 2.671 and 2.672, respectively. Figs 6.1, 6.2 and 6.3 showed the chl a and dry weight contents of strains D585, D579 and D584, respectively. Chl a as a percentage of dry weight for all strains was shown in Table 6.3

All figures were characterized by an initial increase in both chl a and dry weight. The length of this initial peak was a function of the initial phosphorus concentration in the growth medium. However, there was no immediate response to the high phosphorus concentration by any of the strains studied. Strains showed obvious differences under

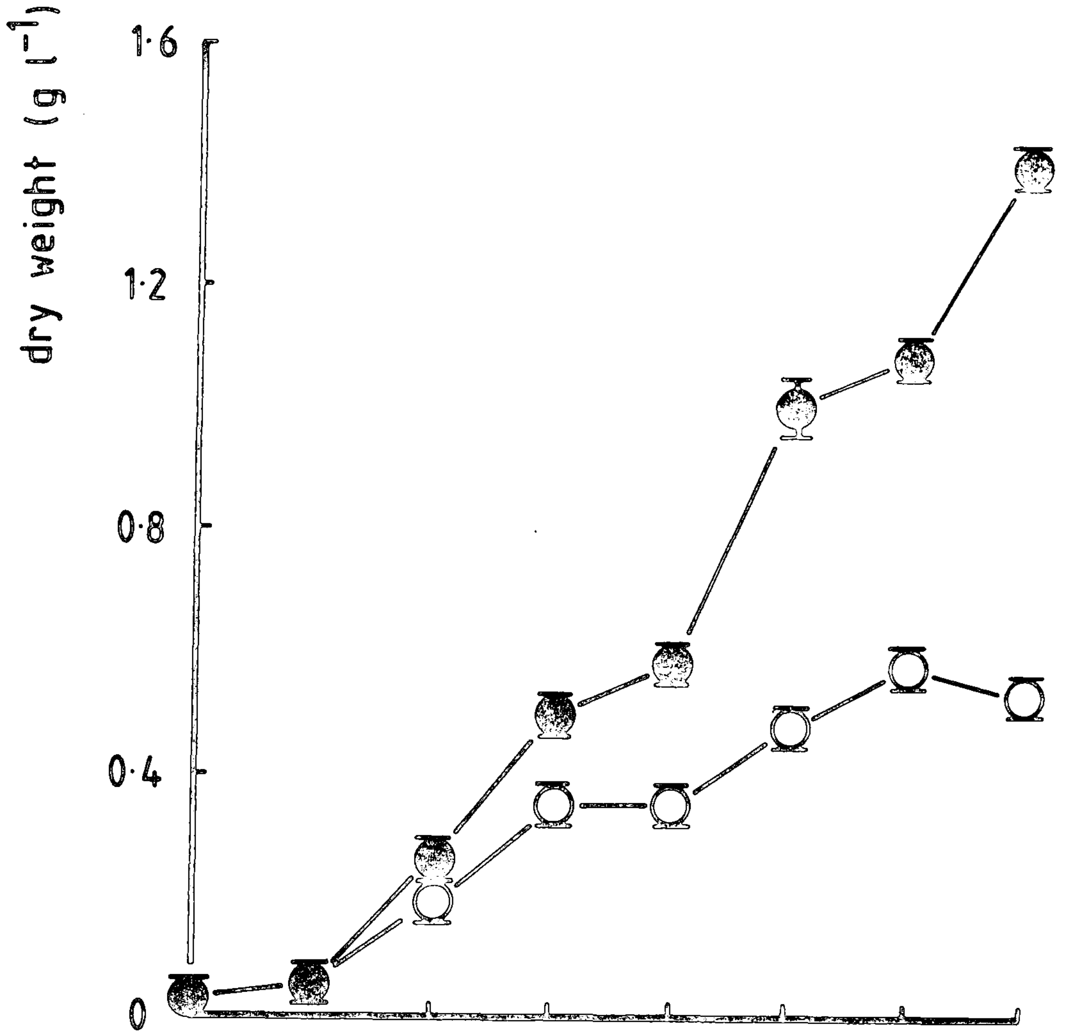
the two phosphorus concentrations at the late stage of growth. The maximum chl *a* contents, for example, of strains D585, D579 and D584, under a high phosphorus concentration were about 3, 6 and 2 times higher than their contents under low phosphorus concentration. The results showed that the strains D585 and D579 had doubled their maximum dry weight under high phosphorus concentration while there was only a slight difference between the dry weight of strain D584 under the two phosphorus concetrations.

The chl *a* content as a percentage of dry weight showed considerable differences between strains under the two phosphorus concentrations (Table 6.3), with low phosphorus, it remained around 1% until day 16 and did not drop to <0.5% throughout the study for the strain D585; it dropped to <0.5% on day 8 for strain D579 and it was <1% throughout the experiment for strain D584 and dropped to <0.5% at the end of the experiment. When phosphorus in excess, on the other hand, it was >1% for strain D585 and D584 until days 20 and 24, respectively; strain D579 had a chl *a* content <0.1% on day 12 and <0.5% on day 28.

(ii) Alkaline phosphatase activity

Alkaline phosphatase activity was determined as described in Section 2.81 except for cellular activity which was calculated as the difference between total and

Fig. 6.1 Comparison of growth yield of *Calothrix* sp. D585 grown with two initial phosphorus concentration (O) 1.0 and (O) 10.0 mg l^{-1}P (32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)


dry weight (g l⁻¹)
1·6
1·2
0·8
0·4
0

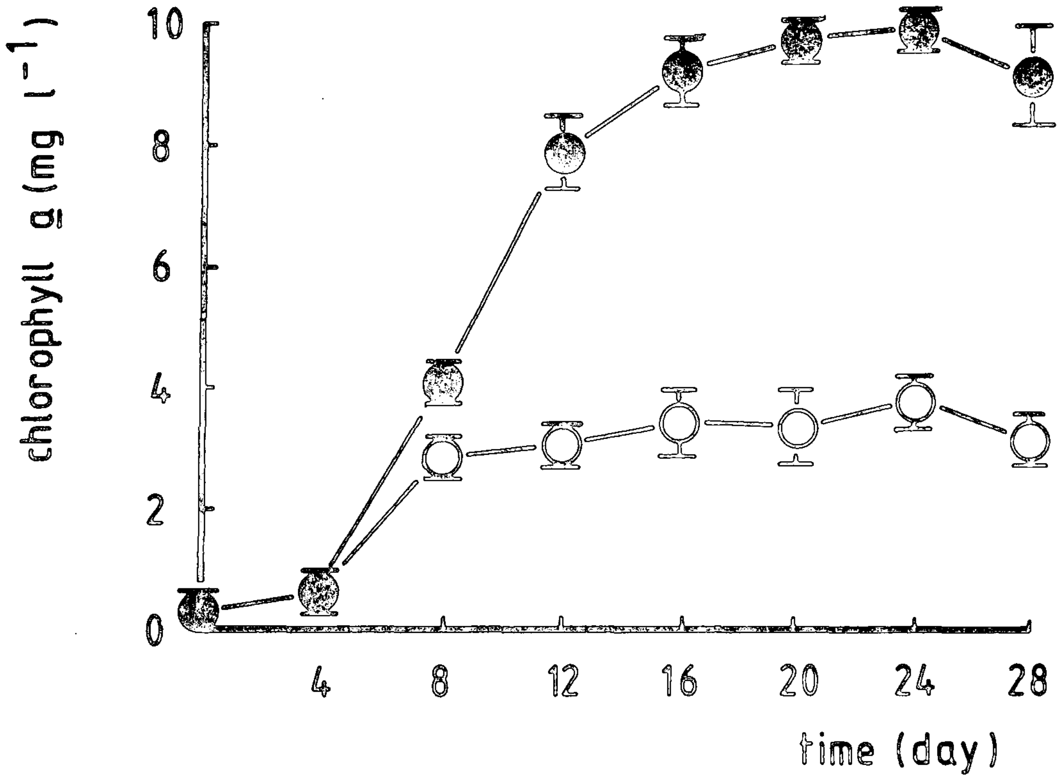
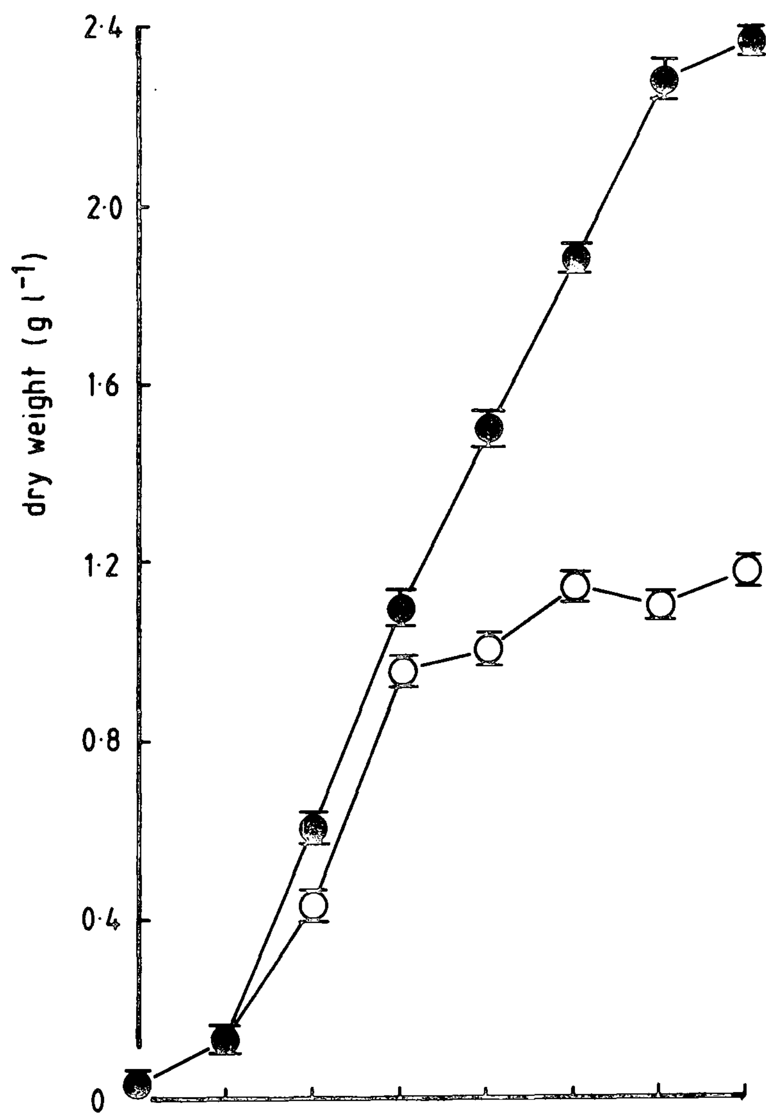

chlorophyll a (mg l⁻¹)
10
8
6
4
2
0
4
8
12
16
20
24
28
time (day)

Fig. 6.2 Comparison of growth yield of *Cylindrospermum muscicola* D579 grown with two initial phosphorus concentrations (O) 1.0 and (O) 10.0 mg l^{-1}P (32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)


2·4
2·0
1·6
1·2
0·8
0·4
0
dry weight (g l⁻¹)

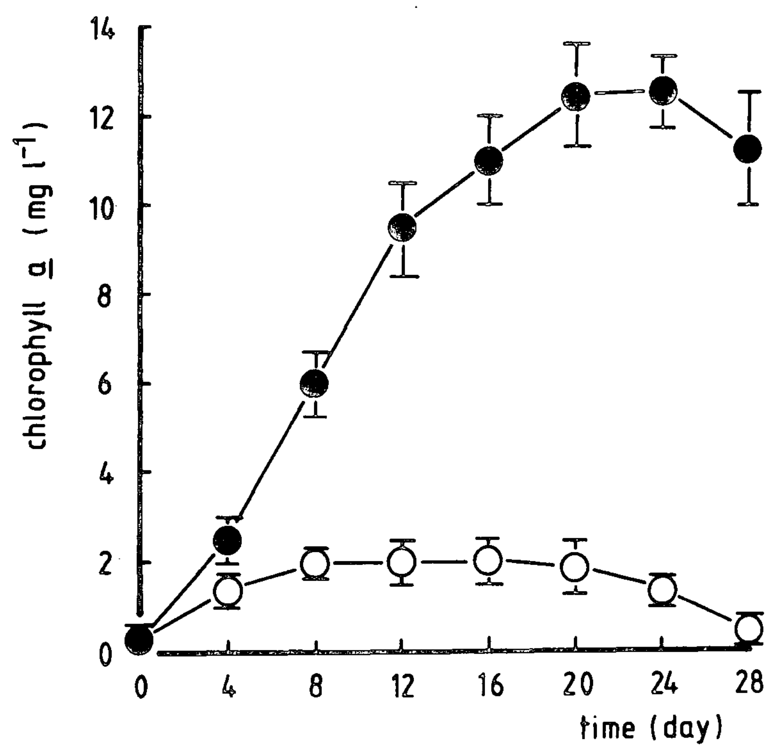
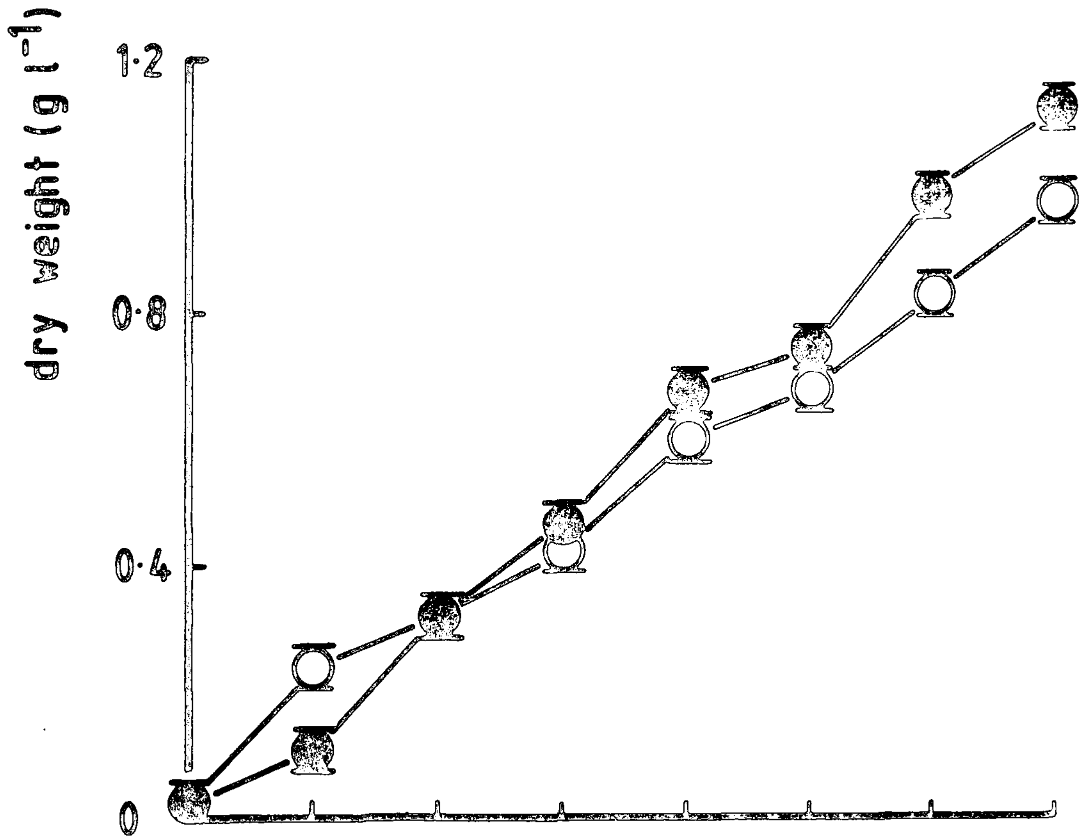

14
12
10
8
6
4
2
0
chlorophyll a (mg l⁻¹)
0
4
8
12
16
20
24
28
time (day)

Fig. 6.3 Comparison of growth yield of *Nostoc muscorum* D584 grown with two initial phosphorus concentrations (O) 1.0 and (O) 10.0 mg l^{-1} P (32 ^{o}C; 90-120 μmol photon m^{-2} s^{-1}; continuous shaking)


dry weight (g l⁻¹)
1·2
0·8
0·4
0

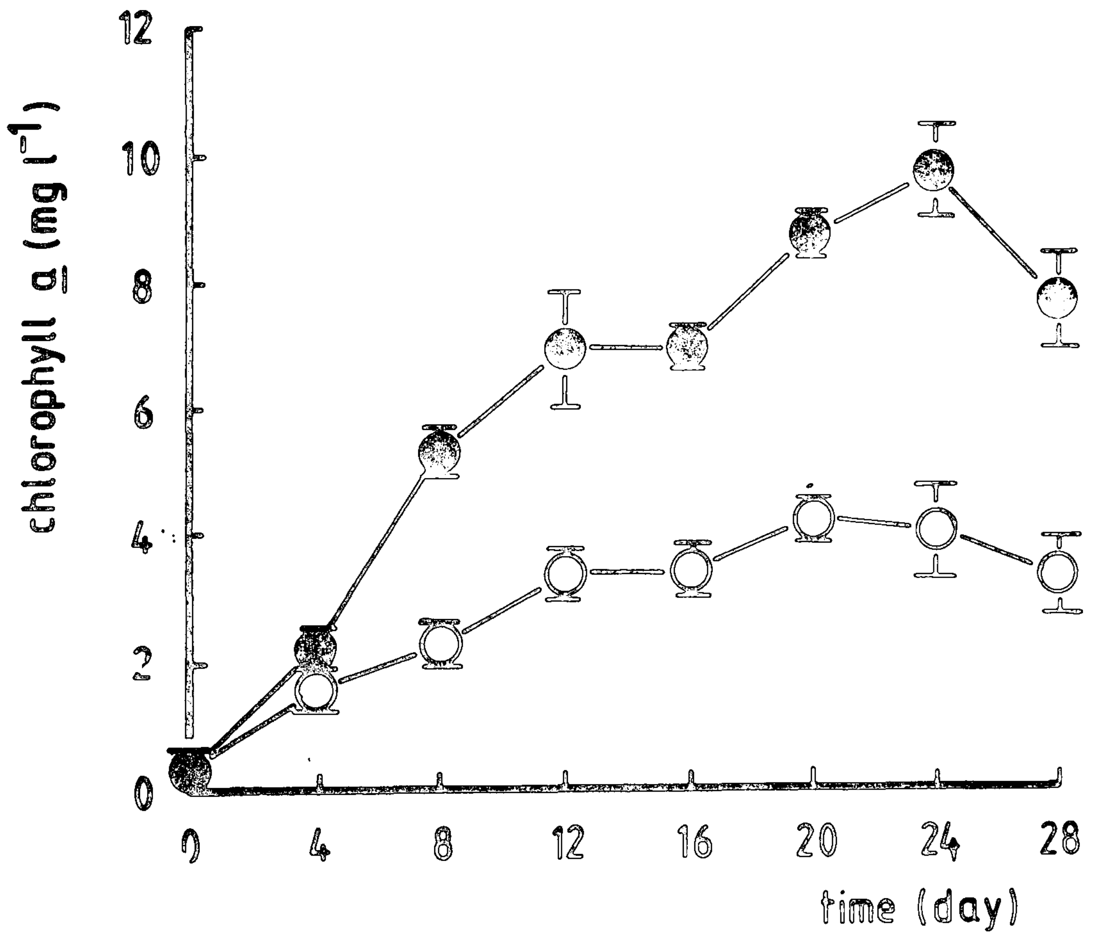

chlorophyll a (mg l⁻¹)
12
10
8
6
4
2
0
0
4
8
12
16
20
24
28
time (day)

Table 6.3 Chl *a* as % of dry weight of *Calothrix* sp. D585, *Cylindrospermum muscicola* D579 and *Nostoc muscorum* D584 grown with two initial phosphorus concentrations 1.0 and 10 mg l^{-1} P in AD-N (32 ^{o}C; 90-120 µmol photon $m^{-2}s^{-1}$; continuous shaking)

time (day)	phosphorus concentration (mg l^{-1}) 1.0 *Calothrix*	1.0 *Cylindrospermum*	1.0 *Nostoc*	10.0 *Calothrix*	10.0 *Cylindrospermum*	10.0 *Nostoc*
4	1.00	1.16	0.66	1.16	2.28	1.98
8	1.55	0.47	0.72	1.58	1.02	1.26
12	0.88	0.21	0.80	1.59	0.86	1.50
16	1.01	0.21	0.57	1.55	0.73	1.05
20	0.71	0.16	0.63	0.95	0.66	1.20
24	0.66	0.12	0.50	0.85	0.55	1.00
30	0.61	0.03	0.37	0.64	0.47	0.70

filterable activities (Section 5.6) (Figs 6.4, 6.5, 6.6). The strains showed marked differences in, for example, maximum activity, the extent to which the enzyme was extracellulary released, the degree of inhibition of enzyme by the high phosphorus concentration.

The highest activity was that of the strain D584 and the lowest was that of the strain D579. The activity of strain D584 was about 2 and 4 times higher than that of strain D579 and D585, respectivily.

Almost all of the enzyme activity of strain D585 is associated with the algal materials, strain D579 showed filtrable activity of 20% and 28% of the maximum activity under high and low phosphorus concetrations, respectivilely. Strain D584 has much higher filtrable enzyme activity than both strains under the two phosphorus concentrations.

The high phosphorus concentration inhibits enzyme activity at the early stage of growth for all of the strains. Strain D579 was the most affected, where the total maximum activity was reduced to 65% of that of the low phosphorus concentration. The total maximum acivity of strains D585 and D584 were only slightly reduced.

(iii) Phosphorus content of the algae

Five different methods (Section 2.72) were tested for the analysis of algal phosphorus. The mean and the

Fig. 6.4 Infleunce of phosphorus on alkaline phosphatase activity of *Calothrix* sp. D585 (A) 1.0 and (B) 10.0 mg l^{-1}P (alga cultured and assay carried out at 32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)

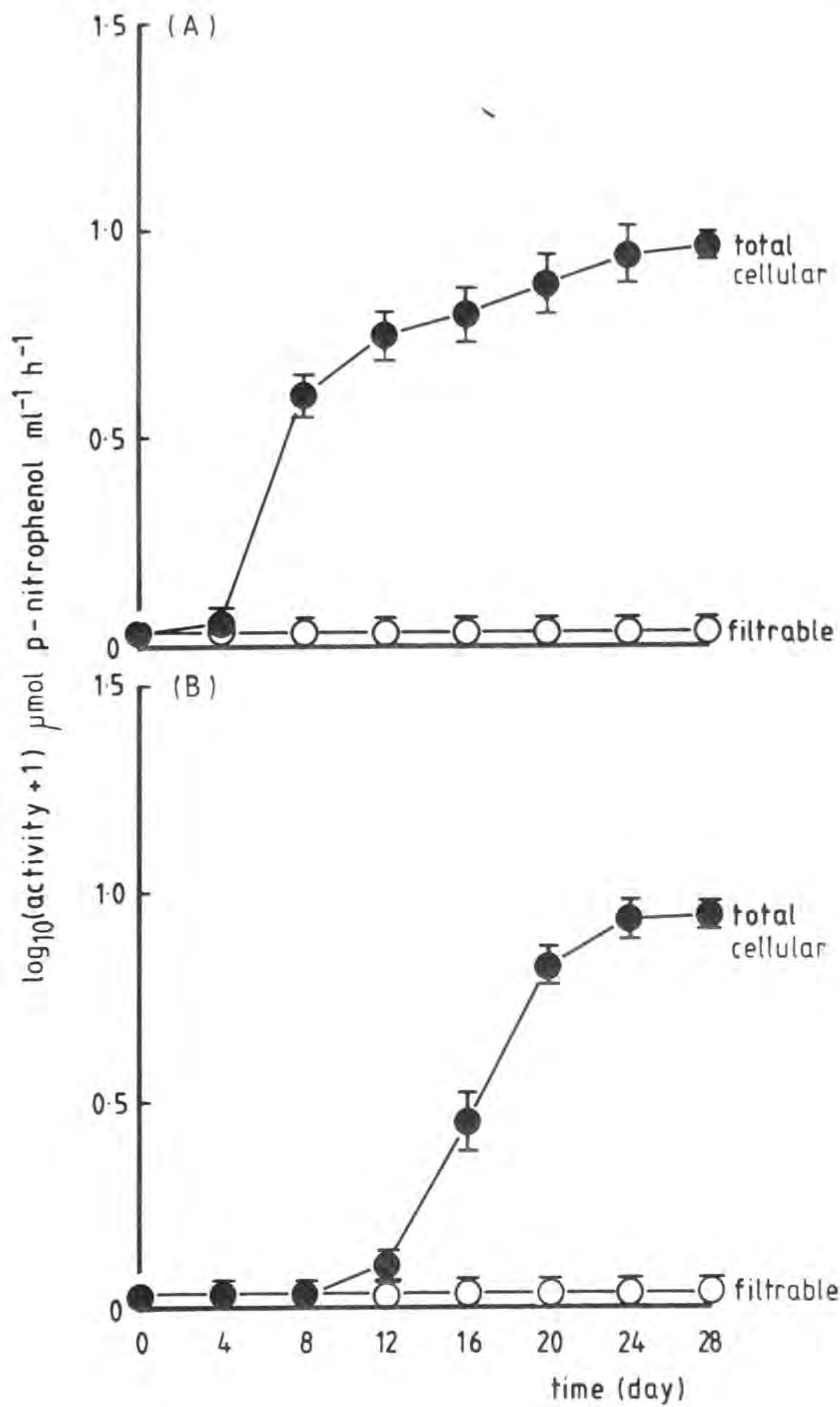
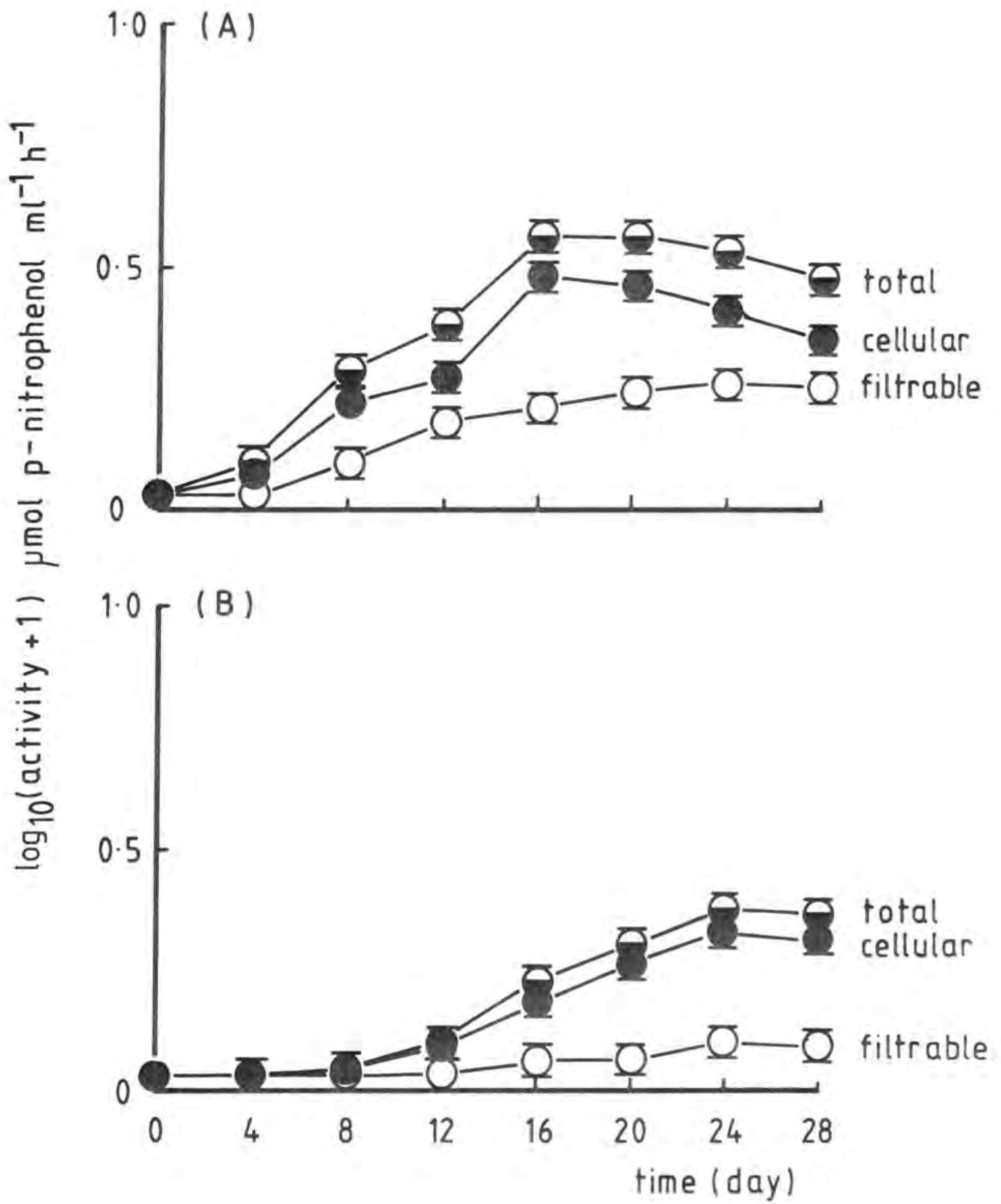
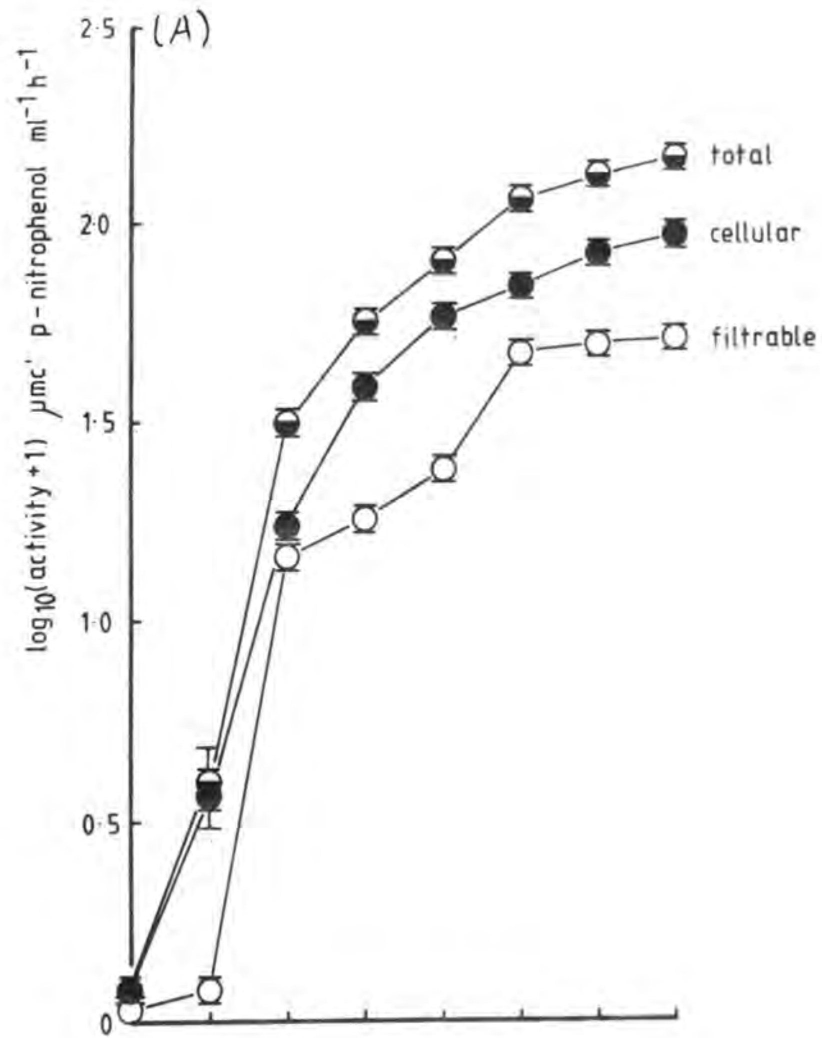

(A)
(B)
1·5
1·0
0·5
0
total cellular
filtrable
log10(activity + 1) μmol p-nitrophenol ml⁻¹ h⁻¹
0
4
8
12
16
20
24
28
time (day)

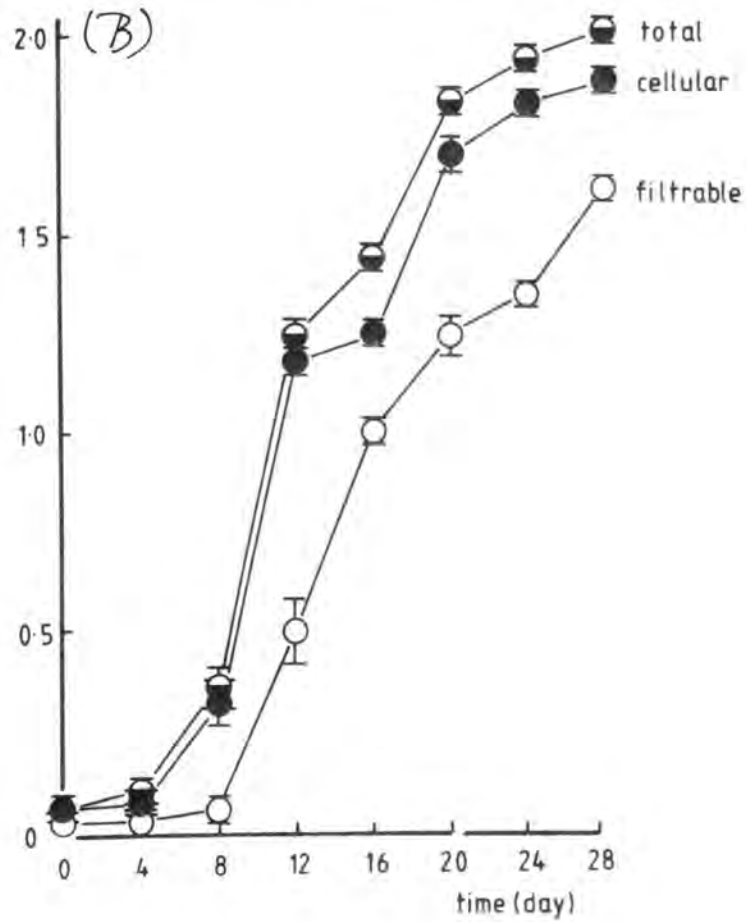
Fig. 6.5 Infleunce of phosphorus on alkaline phosphatase activity of *Cylindrospermum muscicola* D579 (A) 1.0 and (B) 10.0 mg l^{-1}P (alga cultured and assay carried out at 32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)



(A)
(B)
1·0
0·5
0
total
cellular
filtrable
log10(activity + 1) μmol p-nitrophenol ml-1 h-1
0
4
8
12
16
20
24
28
time (day)

Fig. 6.6 Influence of phosphorus on alkaline phosphatase activity of Nostoc muscorum D584 (A) 1.0 and (B) 10.0 mg l^{-1}P (alga cultured at 32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)


(A)
log10(activity +1) μmc' p-nitrophenol ml⁻¹ h⁻¹
2·5
2·0
1·5
1·0
0·5
0
total
cellular
filtrable


(B)
2·0
1·5
1·0
0·5
0
total
cellular
filtrable
0
4
8
12
16
20
24
28
time (day)

percentage recovery of the total phosphorus using the differents methods are shown in Table 6.4. Method 5(A) was chosen and used throughout the study because it is simple, quick and less dangerous chemicals are involved. The phosphorus content of the three strains showed a peak on day four of the experiment (Figs 6.7, 6.8, 6.9). The contents of all strains decreased after that, although the phosphorus content of the growth medium, under the high phosphorus concentration, is still high (Tables 6.5, 6.6, 6.7). The maximum concentrations of phosphorus in the three strains were 42, 24, 36 ug mg dry weight for strains D585, D579 and D584, respectively. The minima were about 2 μg mg^{-1} dry weight in each case.

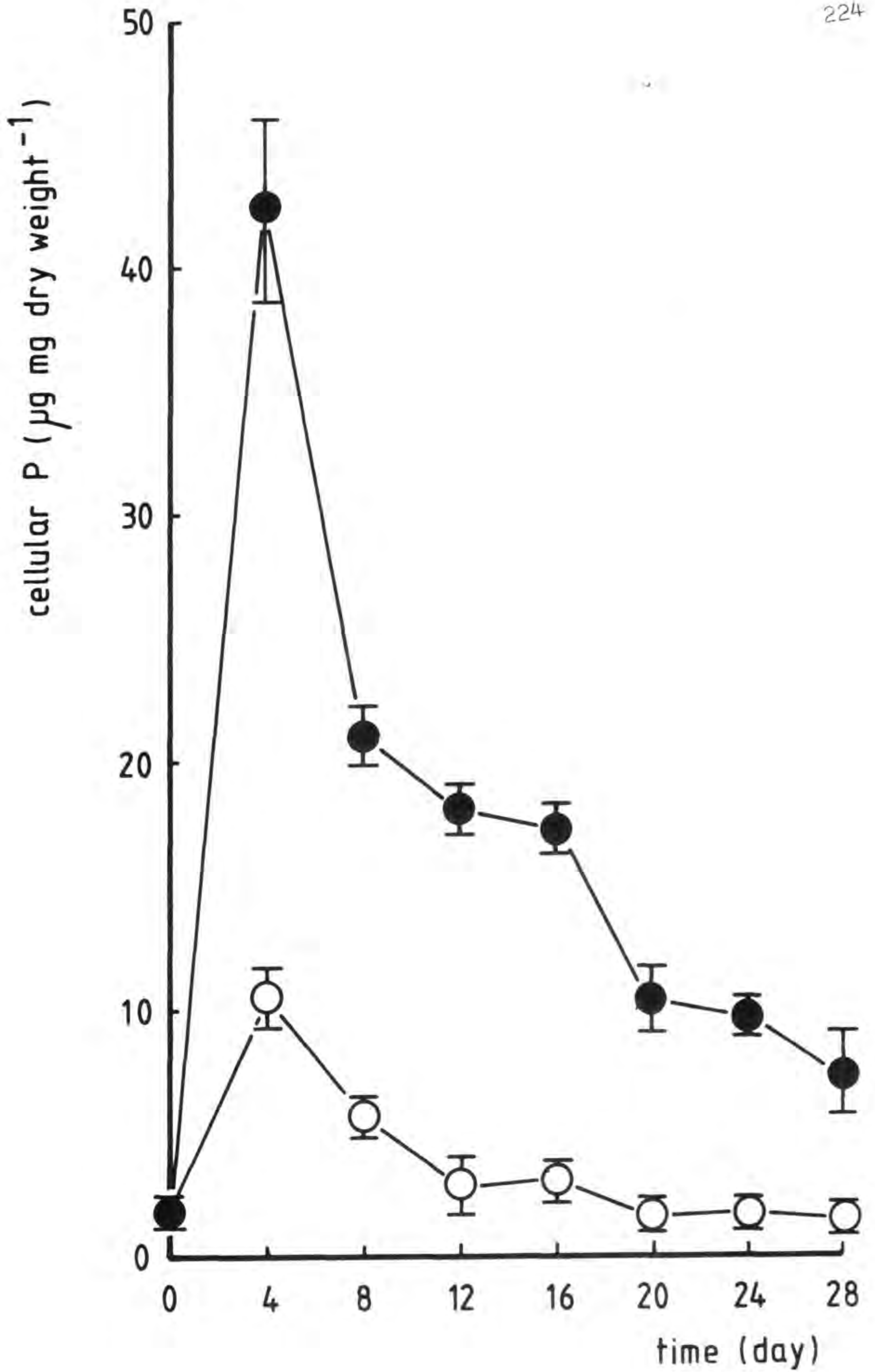
(iv) Phosphorus depletion of growth medium

The phosphorus content of the growth media was determined as described in Section 2.72. The phosphorus contents of the growth media of all strains dropped to below the detection limits of the method used (Section 2.72) on day four of the experiment under low phosphorus concentration (Tables 6.5, 6.6, 6.7) ; at the same time it dropped to 7.3, 6.91 and 5.4 mg l^{-1} for strains D585, D579 and D584, respectively, under the high phosphorus treatment. On day eight only strain D585 had measureable phosphorus in the growth medium. Only small amounts of organic phosphorus

Table 6.4 Mean and percentage recovery of total phosphorus of *Nostoc muscorum* D584 grown in AD-N medium with initial phosphorus concentration of 44.5 mg l^{-1} for 24 days; phosphorus analysed by five different methods mentioned in Section 2.72.

method		μg P mg^{-1} dry weight $\bar{x}$	(n)	% recovery
1		16.2	(3)	99%
2	A	16.32	(3)	100%
	B	14.16	(2)	87%
3	A	16.32	(3)	100%
	B	15.30	(2)	94%
4		16.08	(2)	99%
5	A	15.92	(2)	98%
	B	15.83	(2)	97%

Fig. 6.7 Comparison of cellular phosphorus content of *Calothrix* sp. D585 grown with two initial phosphorus concentrations (O) 1.0 and (O) 10.0 mg l^{-1}P (32 ^{o}C; 90-120 umol-photon $m^{-2}s^{-1}$; continuous shaking)


50
40
30
20
10
0
cellular P (μg mg dry weight ⁻¹)
0
4
8
12
16
20
24
28
time (day)

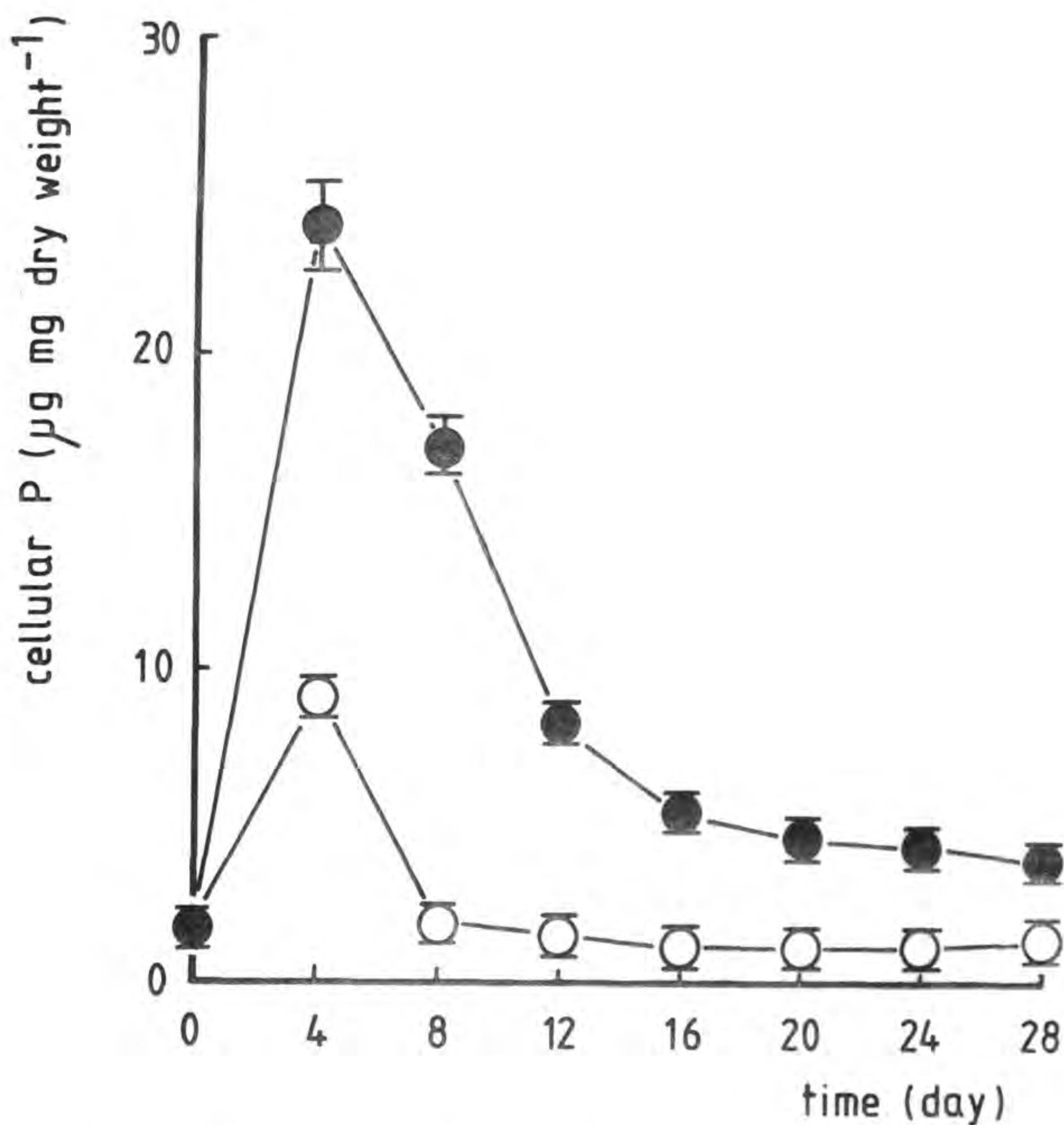


Fig. 6.8 Comparison of cellular phosphorus content of <u>Cylindrospermum</u> <u>muscicola</u> D579 grown with two initial phosphorus concentrations (O) 1.0 and (●) 10.0 mg l^{-1}P (32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)

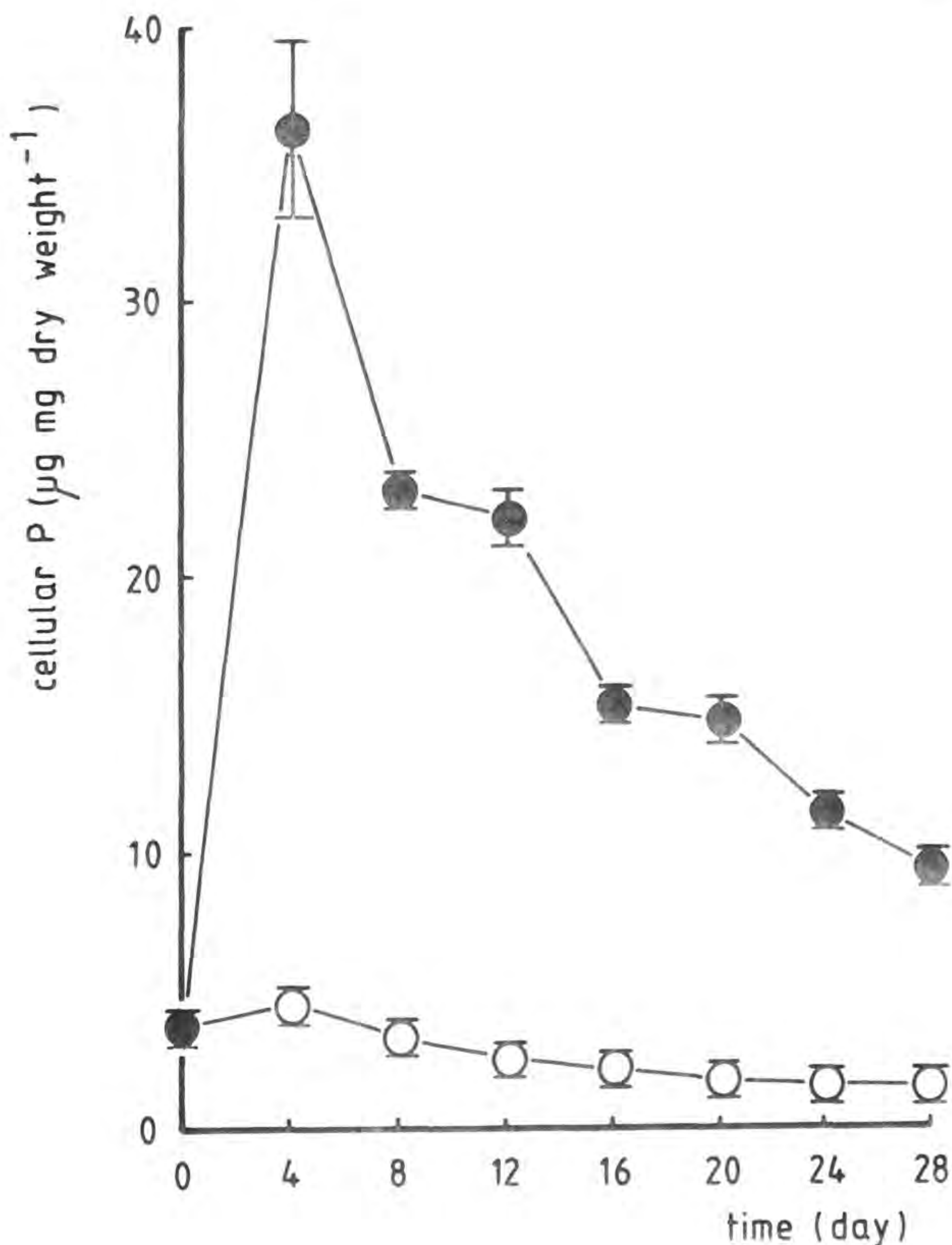


Fig. 6.9 Comparison of cellular phosphorus content of *Nostoc muscorum* D584 grown with two initial phosphorus concentrations (O) 1.0 and (●) 10.0 mg l^{-1}P (32 °C; 90-120 µmol-photon $m^{-2}s^{-1}$; continuous shaking)

Table 6.5 Filtrable reactive phosphorus (FRP) and total filtrable phosphorus (TFP) in the growth medium of *Calothrix* sp. D585 grown with two initial phosphorus concentrations 1.0 and 10.0 mg l^{-1} P (32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking in AD-N medium)

time (day)	1.0 mg l^{-1} FRP $\bar{x}$	± SD	TFP $\bar{x}$	± SD	10.0 mg l^{-1} FRP $\bar{x}$	± SD	TFP $\bar{x}$	± SD
4	<0.005	-	<0.005	-	7.27	0.19	7.27	0.19
8	<0.005	-	<0.005	-	4.35	0.37	4.59	0.40
12	<0.005	-	0.007	0.001	0.34	0.11	0.57	0.30
16	<0.005	-	0.007	0.001	<0.005	-	0.022	-
20	<0.005	-	0.023	0.005	<0.005	-	0.03	0.01
24	<0.005	-	0.020	0.005	<0.005	-	0.052	0.01
28	<0.005	-	0.030	0.005	<0.005	-	0.046	0.007

Table 6.6 Filtrable reactive phosphorus (FRP) and total filtrable phosphorus (TFP) in the growth medium of *Cylindrospermum muscicola* D579 grown with two initial phosphorus concetrations 1.0 and 10.0 mg l^{-1} P (32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking in AD-N medium)

time (day)	1.0 mg l^{-1} FRP $\bar{x}$	± SD	TRP $\bar{x}$	± SD	10.0 mg l^{-1} FRP $\bar{x}$	± SD	TFP $\bar{x}$	± SD
4	<0.005	-	0.025	0.018	6.91	1.05	6.91	1.15
8	0.01	-	0.054	0.008	0.02	0.001	0.21	0.008
12	0.017	-	0.09	0.01	0.04	0.006	0.13	0.01
16	0.01	-	0.17	0.01	0.041	0.006	0.20	0.02
20	0.01	-	0.21	0.03	0.053	0.008	0.23	0.03
24	0.055	0.03	0.17	0.006	0.058	0.02	0.29	0.05
28	0.085	0.01	0.08	0.003	0.12	0.01	0.11	0.01

Table 6.7 Filtrable reactive phosphorus (FRP) and total filtrable phosphorus (TFP) in the growth medium of <u>Nostoc muscorum</u> D584 grown with two initial phosphorus concentrations 1.0 and 10.0 mg l^{-1} P (32 ^{o}C; 90- 120 µmol photon $m^{-2}s^{-1}$; continuous shaking in AD-N medium)

time	1.0 mg l^{-1}				10.0 mg l^{-1}			
	FRP		TFP		FRP		TFP	
(day)	$\bar{x}$	$\pm$ SD	$\bar{x}$	$\pm$ SD	$\bar{x}$	$\pm$ SD	$\bar{x}$	$\pm$ SD
4	<0.005	-	<0.005	-	5.40	0.07	5.95	0.65
8	0.039	0.016	0.084	0.018	0.03	0.01	0.05	0.01
12	<0.005	-	0.015	-	<0.005	-	0.013	0.007
16	0.007	0.003	0.014	0.005	0.014	0.003	0.024	0.010
20	<0.005	-	0.01	0.005	<0.005	-	0.013	0.002
24	<0.005	-	0.018	0.005	<0.005	-	0.021	0.003
28	<0.005	-	0.014	0.003	<0.005	-	0.024	0.006

were detected throughout the experiments under either phosphorus concetration for all the strains studied.

Microscopical inspection was made at the different intervals of collection. The major morphological changes were the same as those mentioned in Section 4.5 .

6.3 Comparison of phosphorus sources

Phosphorus may be present in a wide variety of forms in nature (Section 1.32). Experiments were carried out to study the ability of axenic cultures Iraqi isolates (Table 2.7) and two commonly used research organisms Anabaena cylindrica D2 and Anacystis nidulans D33, to grow on different organic and anhydrous phosphorus compounds. The compounds, except p-nitrophenyl phosphate and bis-nitrophenyl phosphate (Section 2.81), were sterilized by filtration (Section 2.46) and added aseptically to AD-P medium or ACM-P medium. Cultures were incubated under the standard growth conditions (Section 2.52).

All of the Iraqi isolates and the two common research organisms were capable of growth on different phosphorus sources (Table 6.8). However, one of the Iraqi isolates (A.cylindrica D582) did not grow on the substrate inositol hexaphosphate; this was repeated twice. All of the tested compounds were fairly stable under the experimental conditions (Table 6.9). The stability of the compounds was

Table 6.8 Phosphorus compounds utilized by axenic cultures of Iraqi isolates and two common research organisms *Anabaena cylindrica* D2 and *Anacytis nidulans* D33 (32 °C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking; compounds sterilized by filtration and added aseptically into AD-P and ACM-P medium)

enzyme	substrate
phosphomonoesterases	p-nitrophenyl phosphate
	β-glycerophosphate
	inositol hexaphosphate *
	glucose^{-1}-phosphate
phosphodiesterases	bis-nitrophenyl phosphate
	DNA
	phosphatidyl choline
anhydrous hydrolases	pyrophosphate
	metaphosphate
	polyphosphate

* not utilized by *Anabaena cylindrica* D582

TABLE 6.9 Total phosphorus (TP), soluble reactive phosphorus (SRP) and SRP as % of TP of various phosphorus compounds at different collection times; compounds filter sterilized and added into AD-P medium or ACM-P medium; flasks incubated under the following conditions (32°C; 90-120 μmol photon m^{-2} s^{-1}; continuous shaking)

	time (day)																			
	0					5					10					15				
	TP		SRP		% $\frac{SRP}{TP}$	TP		SRP		% $\frac{SRP}{TP}$	TP		SRP		% $\frac{SRP}{TP}$	TP		SRP		% $\frac{SRP}{TP}$
Phosphorus compounds	mg l^{-1}					mg l^{-1}					mg l^{-1}					mg l^{-1}				
	$\bar{x}$	± SD	$\bar{x}$	± SD		$\bar{x}$	± SD	$\bar{x}$	± SD		$\bar{x}$	± SD	$\bar{x}$	± SD		$\bar{x}$	± SD	$\bar{x}$	± SD	
β-glycerophosphate	1.19	0.005	0.01	0.001	0.84	1.21	0.06	0.02	0.002	1.65	1.18	0.04	0.01	0.002	0.85	1.20	0.04	0.013	0.001	1.08
DNA	1.22	0.01	0.03	0.005	2.46	1.22	0.08	0.05	0.01	4.10	1.13	0.03	0.06	0.005	5.31	1.19	0.13	0.07	0.03	5.90
inositol hexaphosphate	1.15	0.03	0.013	0.005	1.13	1.04	0.02	0.01	0.005	1.96	1.07	0.08	1.01	0.002	0.93	1.15	0.01	0.03	0.01	2.61
lecithin	1.05	0.02	0.017	0.002	1.62	1.04	0.11	0.013	0.001	1.25	1.03	0.04	0.01	0.002	0.97	1.02	0.03	0.012	0.001	1.18
glycose-1-phosphate	1.13	0.06	0.04	0.005	3.54	1.00	0.07	0.022	0.01	2.20	1.13	0.07	0.05	0.001	4.42	1.01	0.03	0.014	0.003	1.40
pyrophosphate	1.30	0.05	0.08	0.001	6.15	1.19	0.005	0.10	0.01	8.40	1.20	0.001	0.12	0.001	10.0	1.19	0.02	0.12	0.002	10.0
metaphosphate	0.90	0.01	0.04	0.001	4.44	0.90	0.02	0.03	0.002	3.33	0.93	0.03	0.04	0.001	4.30	0.92	0.005	0.05	0.004	5.43
polyphosphate	1.07	0.001	0.04	0.001	3.74	1.07	0.03	0.061	0.001	5.70	1.03	0.03	0.11	0.01	10.68	1.01	0.01	0.16	0.01	16.00

judged from the amount of the soluble reactive phosphorus in solution. This amount did not change appreciably for any organic phosphorus compounds throughout the experiment. Among anhydrous compounds, pyrophosphate and polyphosphate were less stable than metaphosphate.

6.4 Phosphatase activity

The results summarized in Section 6.4 indicated that the strains might have a wide range of phosphatases. More detailed experiments were conducted therefore to study different aspects of alkaline phosphatase of the Iraqi isolates.

(i) Alkaline phosphatase activity of young and old cultures

In this experiment, alkaline phosphatase activity of young (5-8) and (20-23) day old cultures of Iraqi isolates and Anabaena cylindrica D2 was determined as described in Section 2.81.

All strains showed enzyme activity in both cultures, but activity was much higher in the latter (Table 6.10). The activity of cellular and filtrable enzyme of the various strains shows considerable differences. In particular the amount of the enzyme released into the growth medium at the two stages of growth by various strains varies widely. For example, strains D585 and D583 and the control organism

TABLE 6.10 Alkaline phosphatase activity of young (5-8 days old) and old (20-23 days old) cultures and chl*a* as % dry weight of the Iraqi isolates and *Anabaena cylindrica* D2 as a control; algae cultured in AD-N medium with initial phosphorus concentration of 1 mg l^{-1} P (32°C; 90-120 μmol photon m^{-2} s^{-1}; algal material collected on 0.45 μm Millipore filter; assay carried out under same culture conditions using tris-HCl buffer 0.1M, pH 8.5).

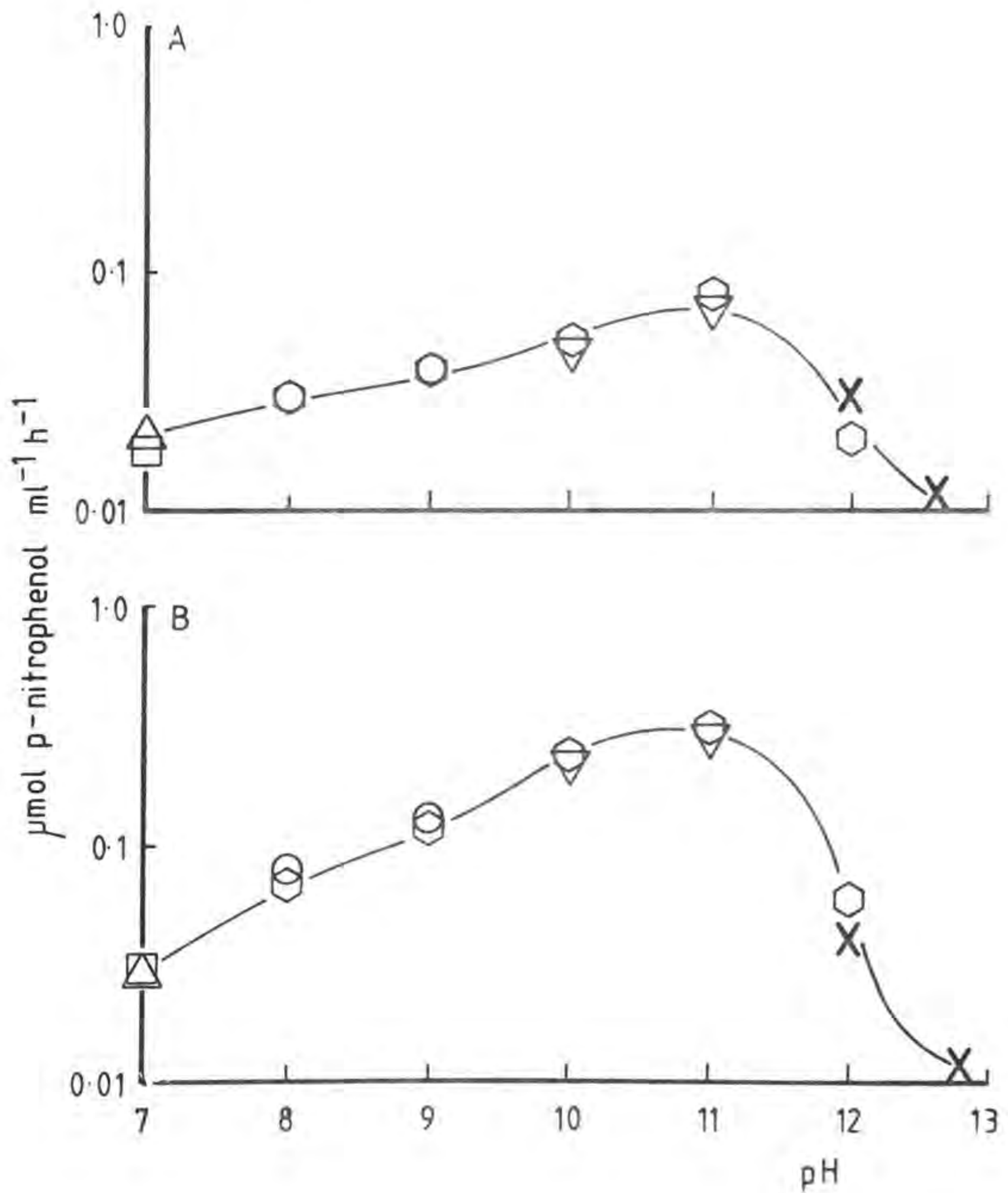
strains	Durham Culture No.	alkaline phosphatase activity (μmol p-nitrophenol ml^{-1} h^{-1})								$\frac{\text{chlorophyll } a}{\text{dry weight}} \times 100$	
		young culture				old culture					
		cellular		extracellular		cellular		extracellular		young culture	old culture
		$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD		
Anabaena cylindrica	2	0.03	0.003	0.007	0.001	0.38	0.01	0.02	0.006	0.63%	0.18%
Anabaena cylindrica	582	0.43	0.06	0.05	0.007	1.36	0.21	0.51	0.01	0.94%	0.20%
Calothrix sp.	580	0.20	0.03	<0.005		2.31	0.23	0.58	0.02	0.88%	0.10%
Calothrix sp.	583	0.05	0.01	<0.005		0.28	0.01	<0.005	-	0.77%	0.20%
Calothrix sp.	585	0.69	0.20	<0.005		1.53	0.18	0.02	0.003	0.69%	0.17%
Cylindrospermum muscicola	579	0.08	0.003	<0.005		0.50	0.07	0.35	0.01	1.32%	0.24%
Hapalosiphon welwitschii	581	0.10	0.02	<0.005		1.44	0.02	1.15	0.01	0.72%	0.31%
Nostoc musconum	584	0.72	0.05	0.02		3.44	0.2	2.3	0.03	0.73%	0.28%

(strain D2) did not show large increases in the amount of extracellular enzyme. The rest of the strains release large amounts of enzyme into the growth medium to about 50% or more of the cellular enzyme. Increased enzyme activities in old cultures is associated with sharp decreases in the chl a/dry weight ratio.

(ii) Influence of pH

The activity of cellular and filtrable enzymes of the Iraqi strains and Anabaena cylindrica D2 was assayed over a wide range of pH values using two different buffers at least for each pH (Table 2.4). Strains were cultured under the standard growth conditions (Section 2.52) and after 20-23 days, enzyme assay was carried out as described in Section 2.81, except that the reaction mixture voulume was 3 ml instead of 2 ml as $CaCl_2$ was added separately. The optimum pH of all strains and the control organism ranges between pH 9 and 11 (Figs 6.10-6.17). However, the pH optima of the cellular and filtrable enzymes of each strain were more or less same. The filtrable enzyme of strain D581 only showed two peaks of activity, one between pH 8 and 9 and the other between 11 and 12 (Fig.6.16)

Fig. 6.10 Infuence of pH on alkaline phosphatase activity of *Anabaena cylindrica* D2 as a control (A) filtrable (GF/C) and (B) cellular (alga cultured and assay carried out at 32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)


DMGA
HEPES - NaOH
borax - HCl or NaOH
glycine - NaOH
tris - HCl
KCl - NaOH
A
B
1·0
0·1
0·01
μmol p-nitrophenol ml^{-1} h^{-1}
7
8
9
10
11
12
13
pH

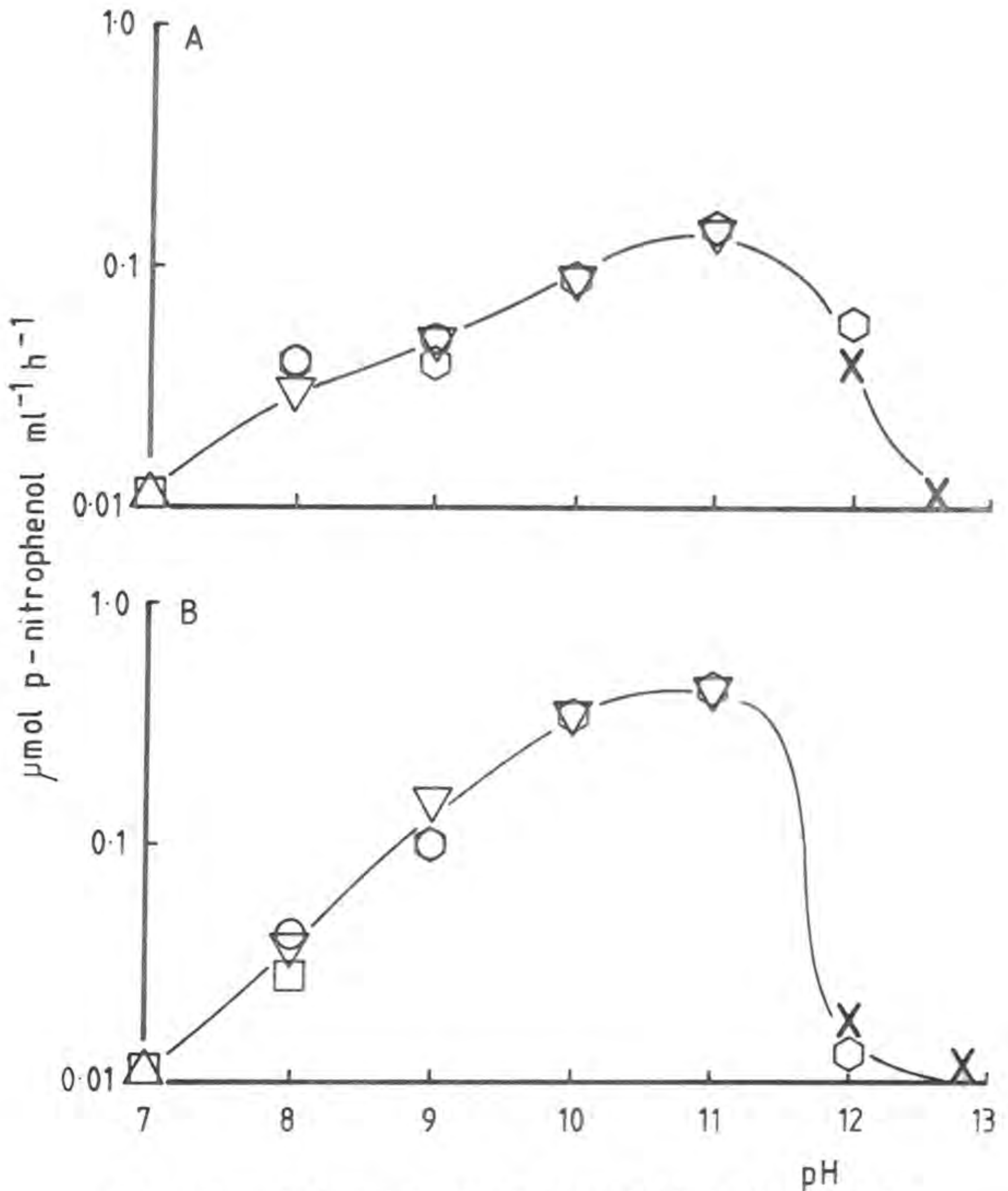


Fig. 6.11 Influence of pH on alkaline phosphatase activity of <u>Anabaena</u> <u>cylindrica</u> D582 (A) filtrable (GF/C) and (B) cellular (alga cultured and assay carried out at 32 °C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)

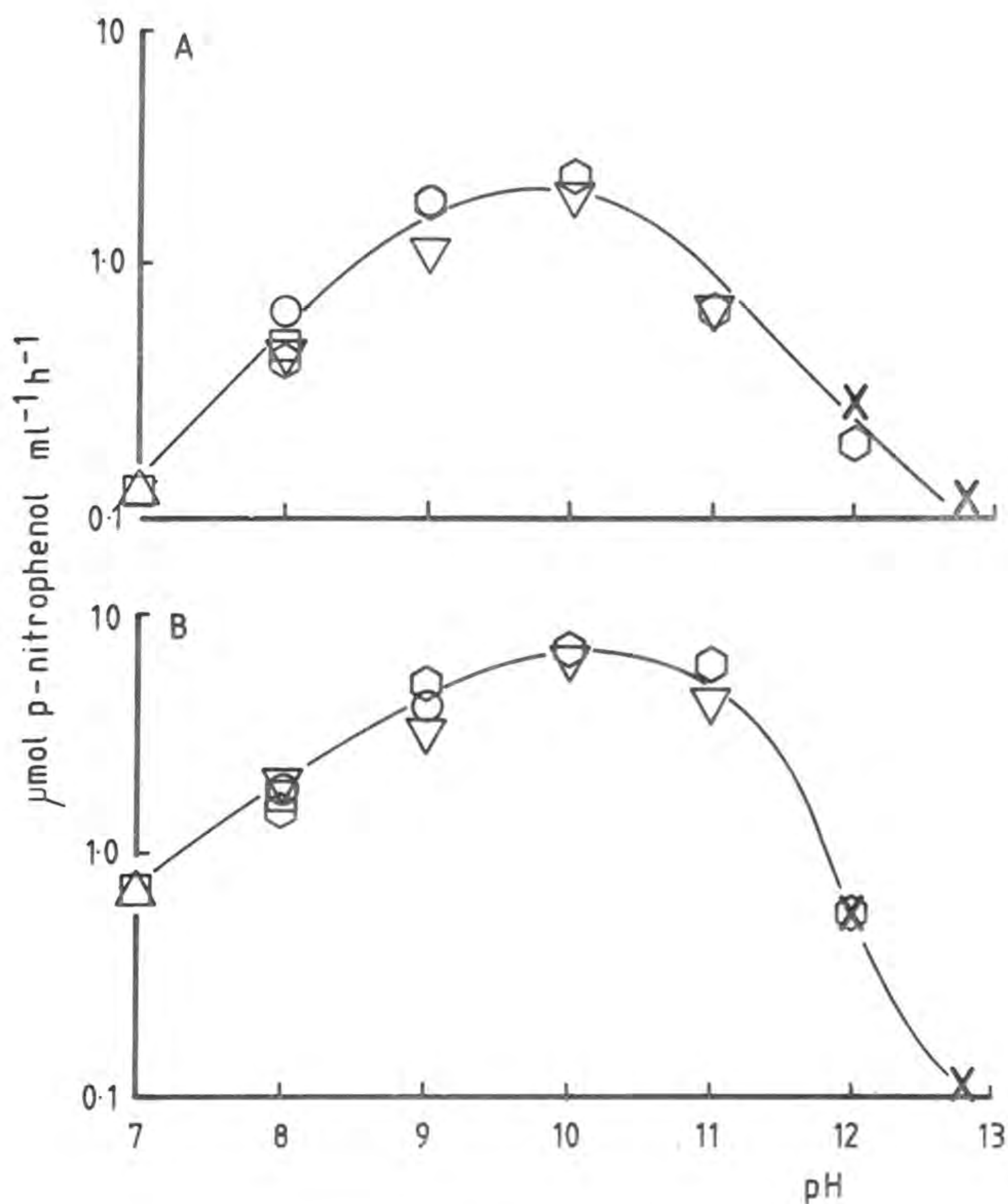


Fig. 6.12 Infuence of pH on alkaline phosphatase activity of Calothrix sp. D580 (A) filtrable (GF/C) and (B) cellular (alga cultured and assay carried out at 32 °C; 90-120 µmol photon $m^{-2}s^{-1}$; continuous shaking)

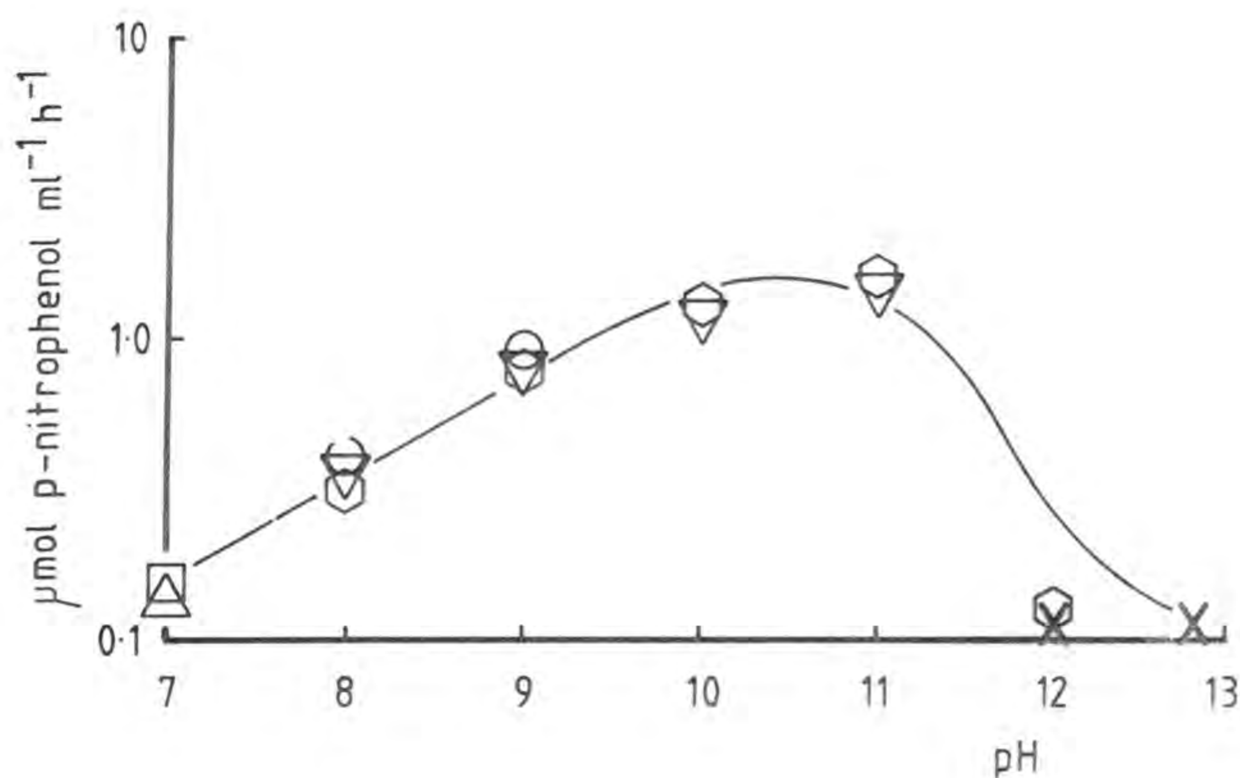


Fig. 6.13 Influence of pH on alkaline phosphatase activity (cellular) of Calothrix sp. D583 (alga cultured and assay carried out at 32 °C; 90-120 µmol-photon $m^{-2}s^{-1}$; continuous shaking)

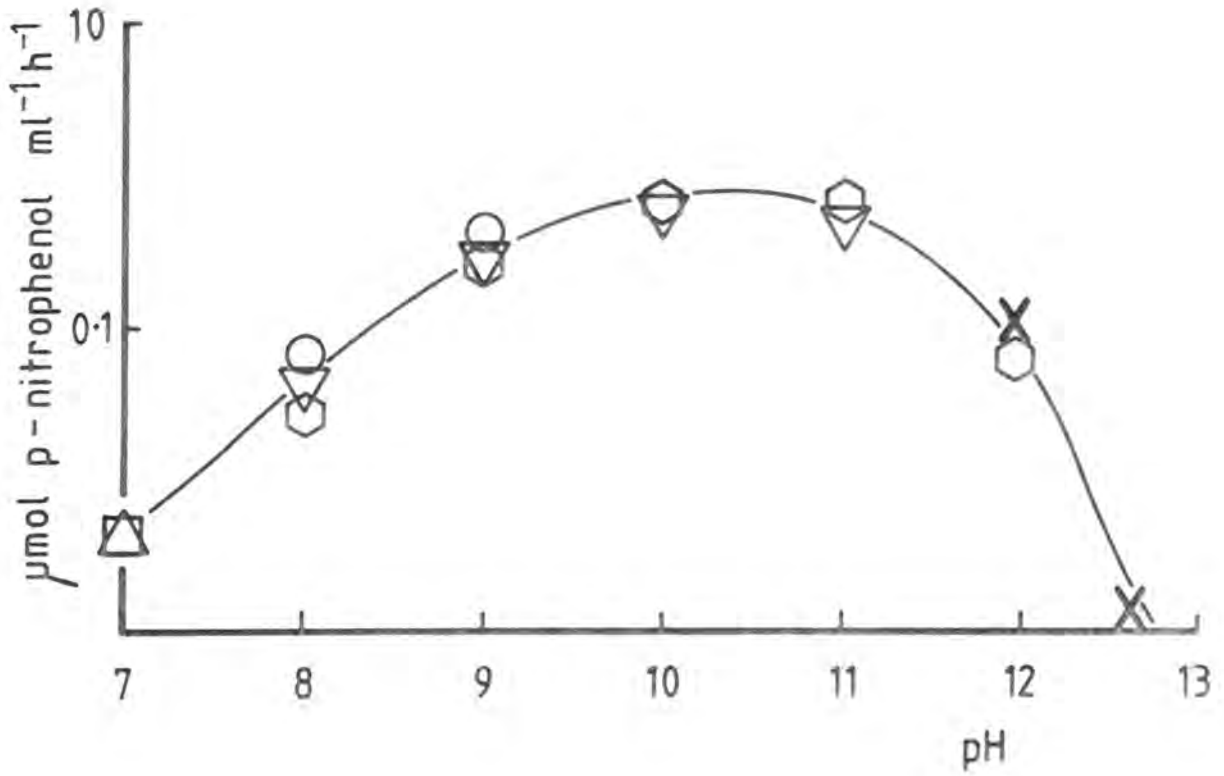
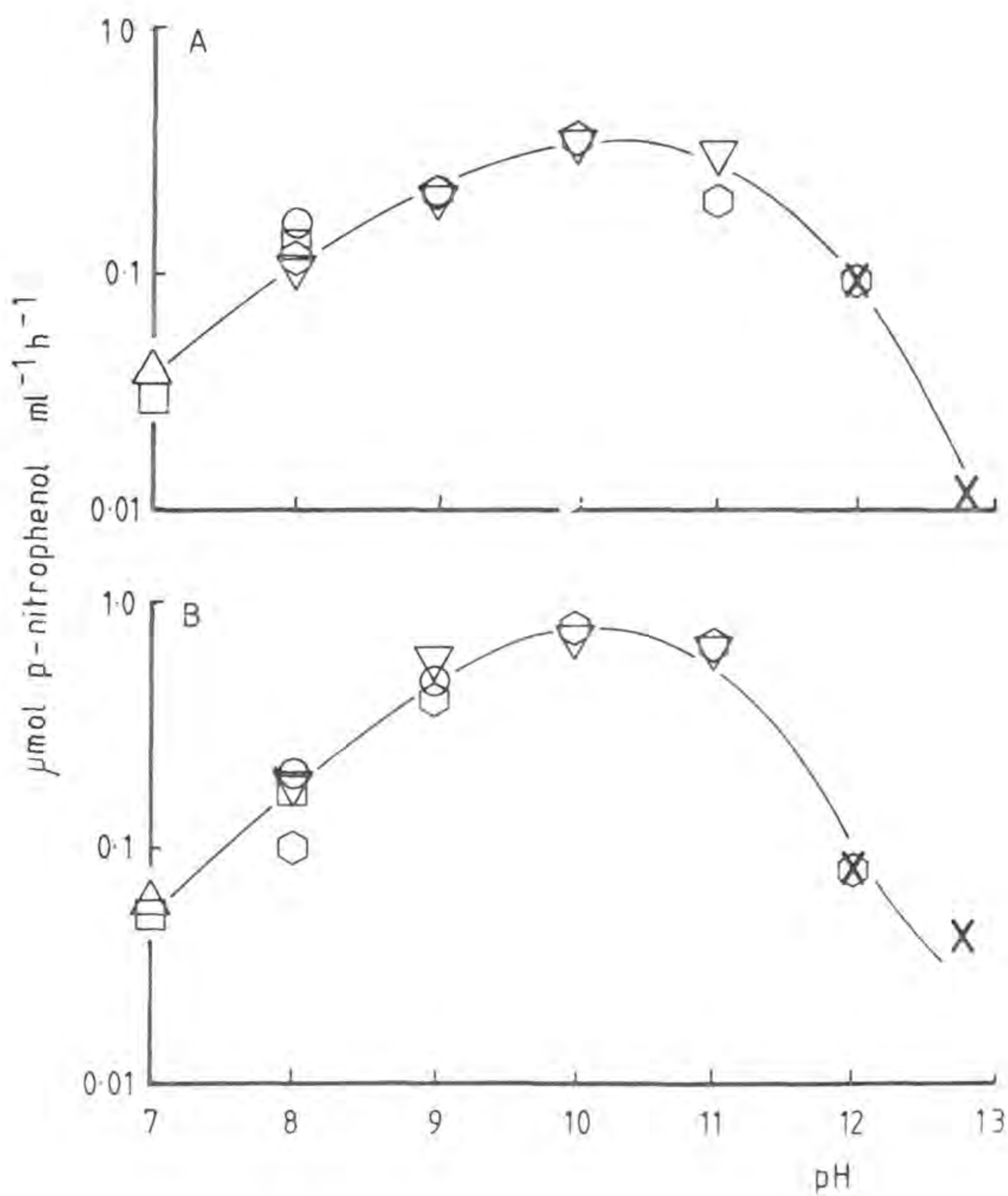


Fig. 6.14 Influence of pH on alkaline phosphatase activity (cellular) of Calothrix sp. D585 (alga cultured and assay carried out at 32 °C; 90-120 µmol-photon $m^{-2}s^{-1}$; continuous shaking)

Fig. 6.15 Influence of pH on alkaline phosphatase activity of *Cylindrospermum muscicola* D579 (A) filtrable (GF/C) and (B) cellular (alga cultured and assay carried out at 32 ^{o}C; 90-120 µmol photon $m^{-2}s^{-1}$; continuous shaking)



1·0
A
0·1
0·01
1·0
B
0·1
0·01
μmol p-nitrophenol ml^{-1} h^{-1}
7
8
9
10
11
12
13
pH

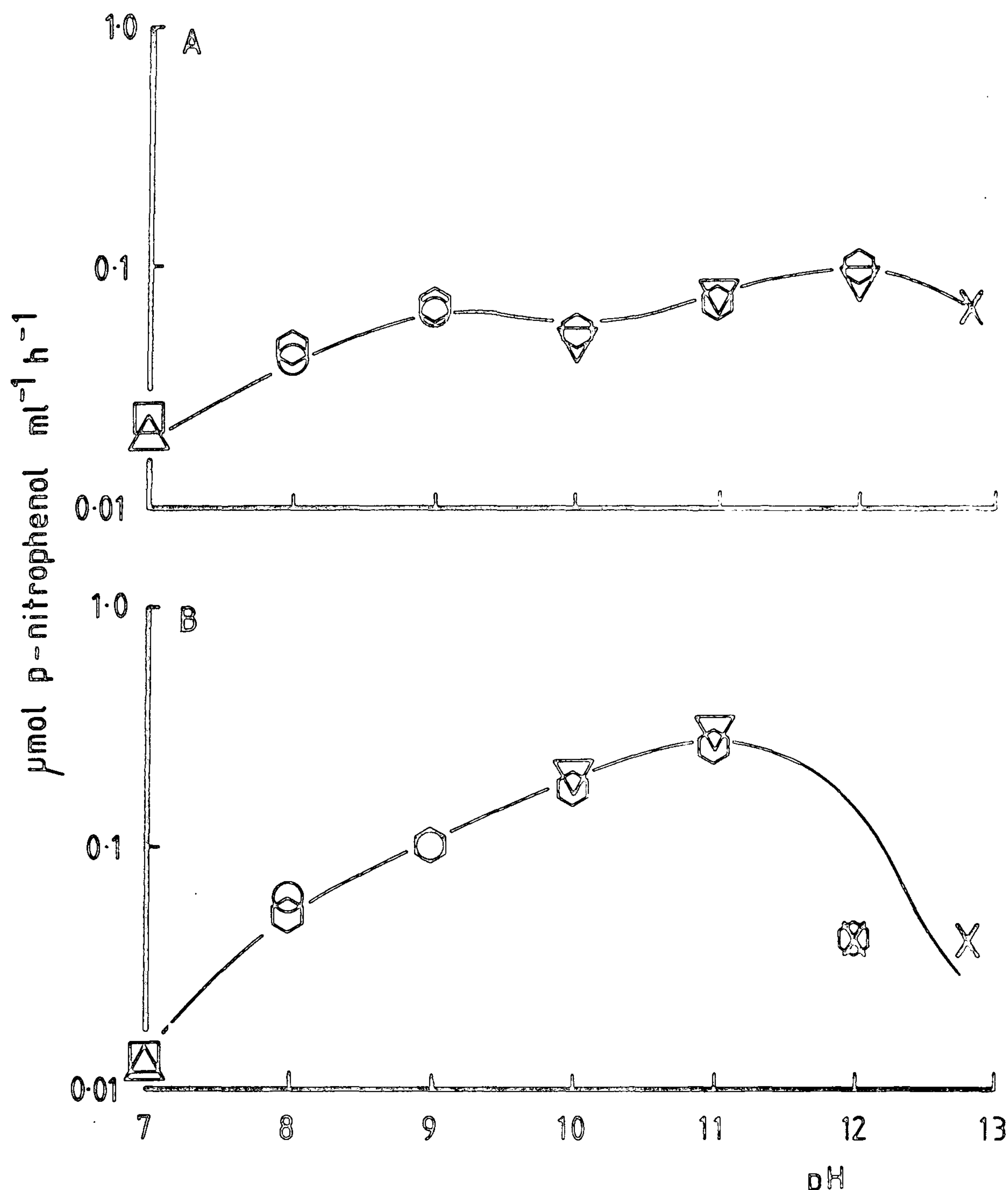


Fig. 6.16 Influence of pH on alkaline phosphatase activity of Hapalosiphon welwitschii D581 (A) filtrable (GF/C) and (B) cellular (alga cultured and assay carried out at 32 °C; 90-120 µmol photon m^{-2} s^{-1}; continuous shaking)

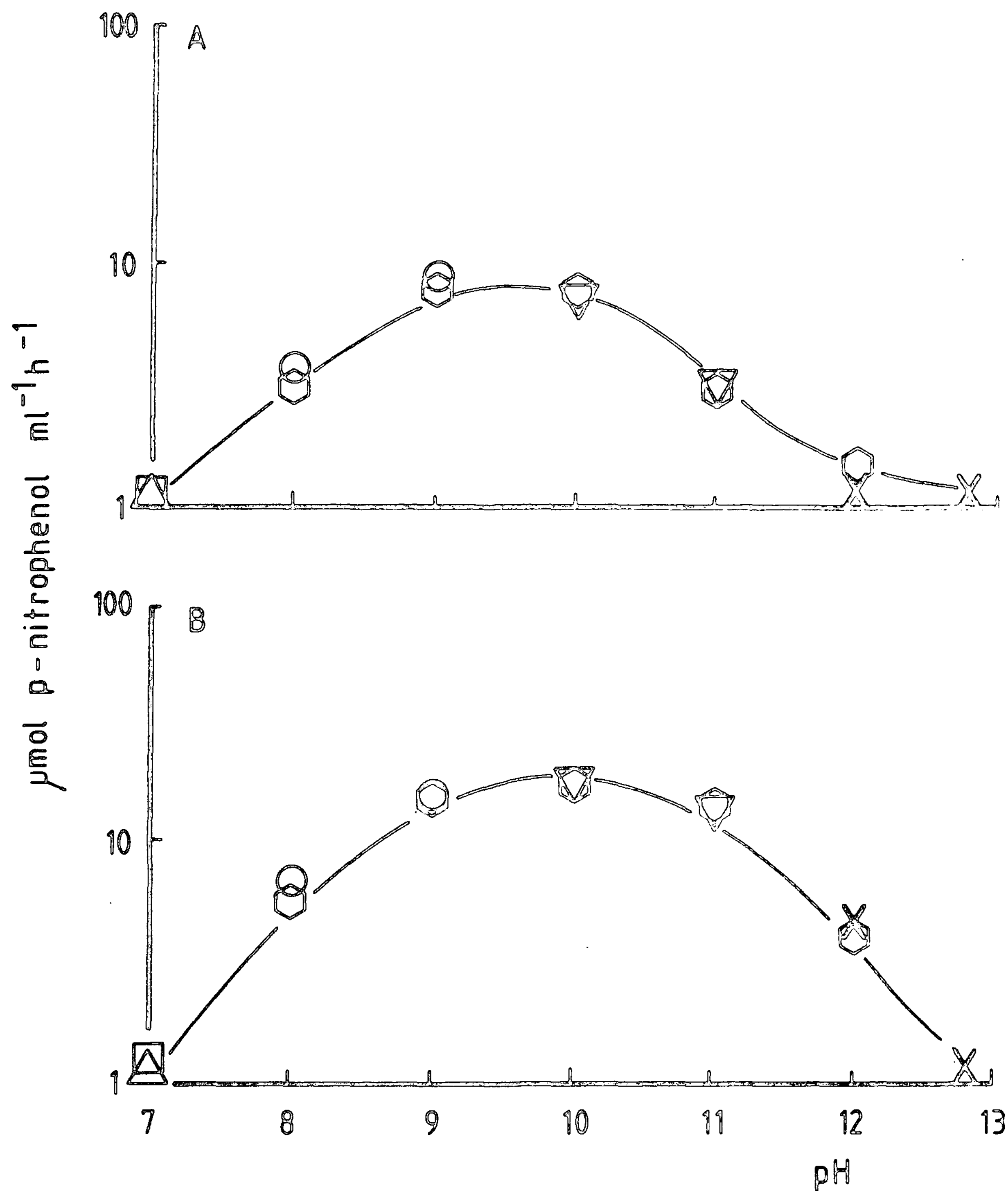


Fig. 6.17 Influence of pH on alkaline phosphatase activity of *Nostoc muscorum* D584 (A) filtrable (GF/C) and (B) cellular (alga cultured and assay carried out at 32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking)

(iii) Effect of calcium and magnesium

Calcium apparently has a stimulatory effect on the activity of all microorganisms, while magnesium has either a slight stimulatory effect or none (Section 1.4). It was thus of interest to study the effect of both elements on enzyme activity of the strains isolated (magnesium is added to the glycine buffer; see SIGMA Technical Bulletin no 104, 1974). Both elements were added as chloride salts to give a final concentration of 2 mM Ca or Mg. The enzyme assay (Section 2.81) was carried out on 13-16 day old cultures, incubated under standard conditions (Section 2.52). However, because of the complexity of the filtrate of the growth medium the assay was confined to the cellular enzyme. The addition of Ca stimulated the enzyme activity of all strains, whereas Mg had an inhibitory effect (Table 6.11). The degree of stimulation and inhibition varied from one strain to another.

To see whether the effect of Ca was specific to glycine-NaOH buffer (pH 10.5) another experiment was run. In all cases Ca stimulated the enzyme activity, especially at higher pH values (Table 6.12).

(iv) Effect of filter paper

In order to choose a filter to give an effective separation of cellular and filtrable enzyme, five different filters were tested. The results (Table 6.13) showed that

TABLE 6.11 Effect of calcium and magnesium (2mM added as chloride salt) on the activity of cellular alkaline phosphatase of the Iraqi isolates; algae cultured in AD-N medium with initial phosphorus concentration of 1 mg l^{-1} P for 13-16 days at 32°C; 90-120 μmol photon m^{-2} s^{-1}; continuous shaking; algal material collected on GF/C filter; assay carried out under same culture conditions using glycine-NaOH buffer 0.05M, pH 10.5 containing 0.1 mM $MgCl_2$)

STRAINS	Durham Culture No.	Alkaline phosphatase activity (μmol p-nitrophenol $m^{-1}h^{-1}$)							
		control		+ calcium			+ magnesium		
		$\bar{x}$	SD	$\bar{x}$	SD	% increase	$\bar{x}$	SD	% decrease
Anabaena cylindrica	582	2.86	0.17	4.29	0.28	50%	2.10	0.20	27%
Calothrix sp.	580	0.58	0.03	1.53	0.11	164%	0.34	0.04	41%
Calothrix sp.	583	1.50	0.12	2.16	0.10	44%	1.33	0.10	11%
Calothrix sp.	585	1.42	0.06	1.79	0.12	26%	1.19	0.04	16%
Cylindrospermum muscicola	579	0.55	0.02	0.90	0.01	64%	0.42	0.02	24%
Hapalosiphon welwitschii	581	0.78	0.05	1.00	0.02	28%	0.58	0.03	26%
Microcoleus chthonoplastes	634	0.14	0.01	0.17	0.002	21%	0.10	0.01	29%
Nostoc muscorum	584	3.25	0.37	4.25	0.34	31%	2.47	0.12	24%

Table 6.12 Effect of calcium (2 mM) on cellular alkaline phosphatase activity of *Nostoc muscorum* D584 at different pH values and buffers.

pH	buffer	molarity (M)	absorbance (410 nm) +Ca	-Ca	% increase
8.0	glycine-NaOH	0.05	0.27	0.31	15%
	tris-HCl	0.05	0.18	0.38	111%
	HEPES-NaOH	0.01	0.36	0.40	11%
9.0	glycine-NaOH	0.05	0.31	1.11	258%
	tris-HCl	0.05	0.35	1.16	231%
10.0	glycine-NaOH	0.05	0.37	1.20	224%

Table 6.13 Cellular and filtrable alkaline phosphatase activity of _Nostoc muscorum_ using different filters (glycine-NaOH 0.1M pH 10.5 was used; alga grown in AD-N for 15 days at 32 °C; 90-120 µmol photon $m^{-2}s^{-1}$; continuous shaking)

filtre	cellular	filtrable	cellular+filtrable	chl _a_ (mg l^{-1})
	µmol p-nitrophenol $ml^{-1}h^{-1}$			
Millipore 0.22 µm	3.04	0.28	3.32	0.43
" 0.45 "	6.2	1.51	7.71	0.43
" 0.8 "	6.91	7.93	14.84	ND*
" 1.2 "	4.17	3.32	7.49	0.43
Glass fibre GF/C	7.75	8.87	16.62	0.49
Whatman No.1	10.34	13.07	23.40	0.52

* ND not determined

the acivity of both enzymes decreased with the decrease in pore size of the filter. The percentage recovery of cellular + filtrable enzyme versus total of all strains using 0.2 μm Nuclepore filter ranged between 20% and 50% (Table 6.14).

(v) Effect of centrifugation

The growth media of the relatively young cultures (16 days old with no obvious cell lysis) were collected and centrifuged at different speeds (Table 6.15). Centrifugation of the growth media at speeds up to 40000xg for 30 min did not affect the activity of the enzyme. However, centrifugation at 110000xg for 60 minutes reduced the activity of the enzyme of all strains, though to different degrees.

From the results of the Sections (iv) and (v) it was decided to determine the activity of total and filtrable (GF/C) enzyme only and calculate the cellular activity as the difference (Section 6.2).

Table 6.14 percetage recovery of alkaline phosphatase activity of strains isolated (grown for 13-16 days in AD-N at 32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking; glycine-NaOH 0.1 M, pH 10.5 containing 0.1 mM $MgCl_2$ was used)

species	Durham code No.	total	μmol p-nitrophenol $ml^{-1}h^{-1}$ cellular	filtrable	cell.+fil.	%recovery
Anabaena cylindrica	582	1.38	0.14	0.26	0.4	29%
Calothrix sp.	580	4.47	1.06	0.23	1.29	29%
Calothrix sp.	583	0.34	0.07	<0.005	0.07	21%
Calothrix sp.	585	1.16	0.29	0.01	0.30	26%
Cylindrospermum muscicola	579	0.72	0.08	0.29	0.37	51%
Hapalosiphon welwitschii	581	0.17	0.01	0.05	0.06	35%
Nostoc muscorum	584	2.45	0.85	0.36	1.21	49%

TABLE 6.15 Effect of centrifugation speed on alkaline phosphatase activity in the supernatant of Iraqi isolates cultures; algae cultured in AD-N medium with initial phosphorus concentration of 1 mg l^{-1} P for 16 days (32°C; 90-120 μmol photon m^{-2} s^{-1}; continuous shaking; assay carried out under same culture conditions using glycine-NaOH buffer 0.1 M pH 10.5 containing 0.1 mM $MgCl_2$; the centrifugation time was 30 min. in all cases except for 110000xg which ran for 60 min., centrifugation carried out at 4°C)

Strains	Durham culture No.	alkaline phosphatase activity (μmol p-nitrophenol $ml^{-1}h^{-1}$) speed x 10^3g						
		5	10	15	20	40	110	$\frac{110}{5}$ x 100
Anabaena cylindrica	582	1.317	1.422	1.383	1.310	1.325	1.038	79%
Calothrix sp.	580	0.294	0.278	0.265	0.289	0.270	0.118	40%
Calothrix sp.	585	0.099	0.096	0.093	0.084	0.085	0.053	54%
Cylindrospermum muscicola	579	0.314	0.331	0.338	0.341	0.330	0.249	79%
Hapalosiphon welwitschii	581	0.462	0.518	0.456	0.448	0.481	0.336	73%
Microcoleus chthonoplastes	634	0.034	0.034	0.037	0.030	0.026	0.021	62%
Nostoc muscorum	584	1.037	1.092	1.052	0.954	1.007	0.635	61%

CHAPTER 7. NITROGENASE ACTIVITY OF IRAQI ISOLATES

7.1 Introduction

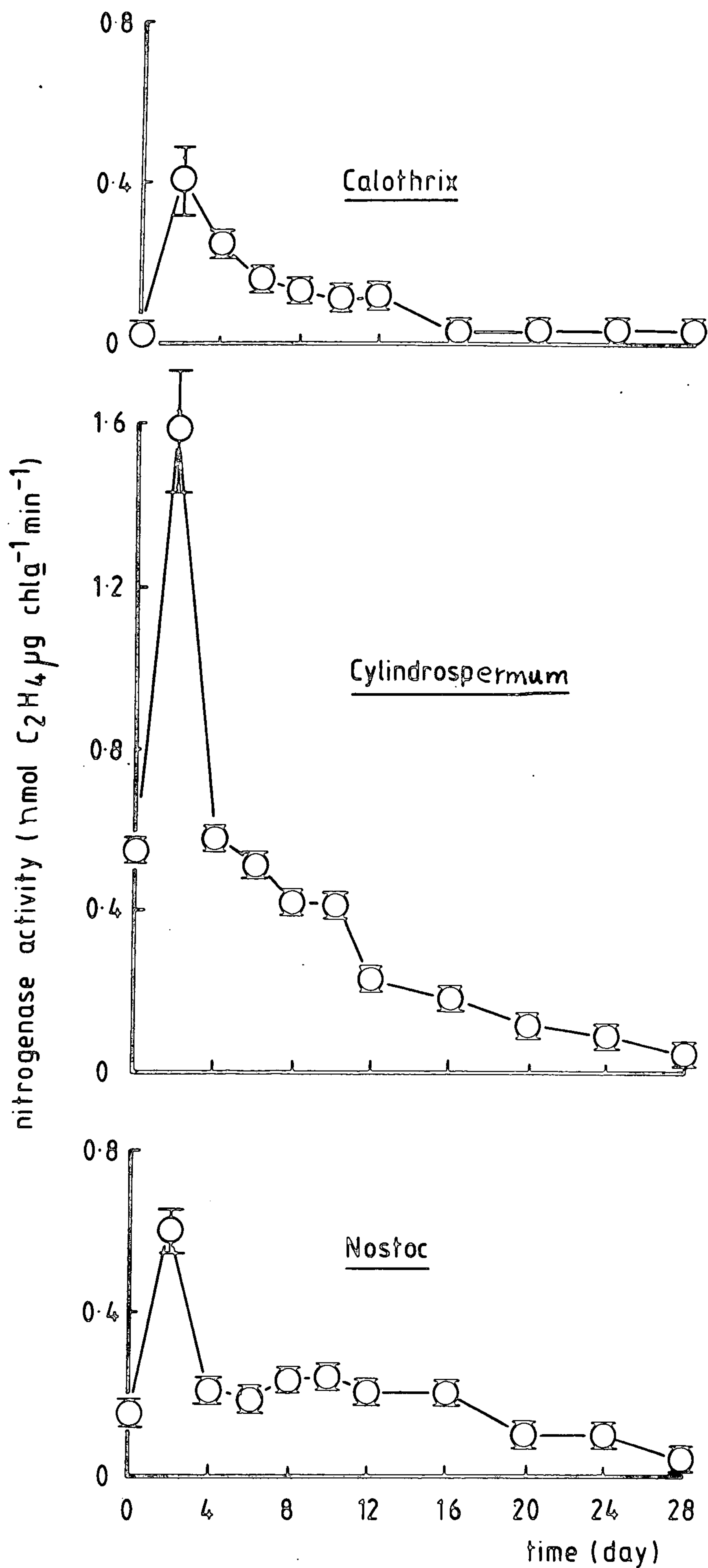
Studies reported in the previous chapters provided background about the effect of environmental factors on growth of algal communities and on physiology of the isolates. Experiments were therefore planned to investigate nitrogenase activity of the isolates. The majority of the experiments were carried out using axenic cultures. However, preliminary experiments were carried out on _Microcoleus chthonoplastes_ and two bacterised cultures of _Lyngbya_ spp.

7.2 Nitrogenase activity of heterocystous forms

In addition to the eight bacteria-free cultures of Iraqi isolates (Table 2.7), _Calothrix parietina_ D550 was included as a control in some experiments. Before making comparisons between the strains, nitrogenase activity was measured at intervals during the growth of the cultures of three strains. This was done to determine the stage of growth at which activity was at a maximum. Nitrogenase activity of all the three strains showed a peak on day two of the experiment (Fig. 7.1).

Nitrogenase activity of the other strains was determined two days after inoculation. There were marked

Fig. 7.1 Time course of the nitrogenase activity (acetylene reduction) of <u>Calothrix</u> sp. D585, <u>Cylindrospermum</u> <u>muscicola</u> D579 and <u>Nostoc</u> <u>muscorum</u> D584; algae cultured in AD-N medium with initial phosphorus concentration of 1 mg l^{-1}P (32 ^{o}C; 90-120 μmol-photon $m^{-2}s^{-1}$; continuous shaking; assay carried out for 60 or 120 min under same culture conditions except when 110 $\pm$ 10 μmol photon $m^{-2}s^{-1}$; n=4)



0·8
0·4
0
Calothrix
1·6
1·2
0·8
0·4
0
Cylindrospermum
nitrogenase activity (nmol C_2H_4 µg chl$\underline{a}^{-1}$ min^{-1})
0·8
0·4
0
Nostoc
0
4
8
12
16
20
24
28
time (day)

differeces between the strains (Table 7.1). For instance, the highest activity (strain D579) was about five times greater than the lowest activity (strain D580). Strains can be arranged as below according to their nitrogenase activity from high to low activity.

D579>D581>D550>D584>D636>D582>D583>D585>D580

Literature reviewed in Section 1.51 showed that there were large differences between strains in their response to environmental factors. Because of enormous daily and annual variation in the physico-chemical conditions of the study area (Section 3.2), it was decided to study the effect of environmental factors on nitrogenase activity of the isolates.

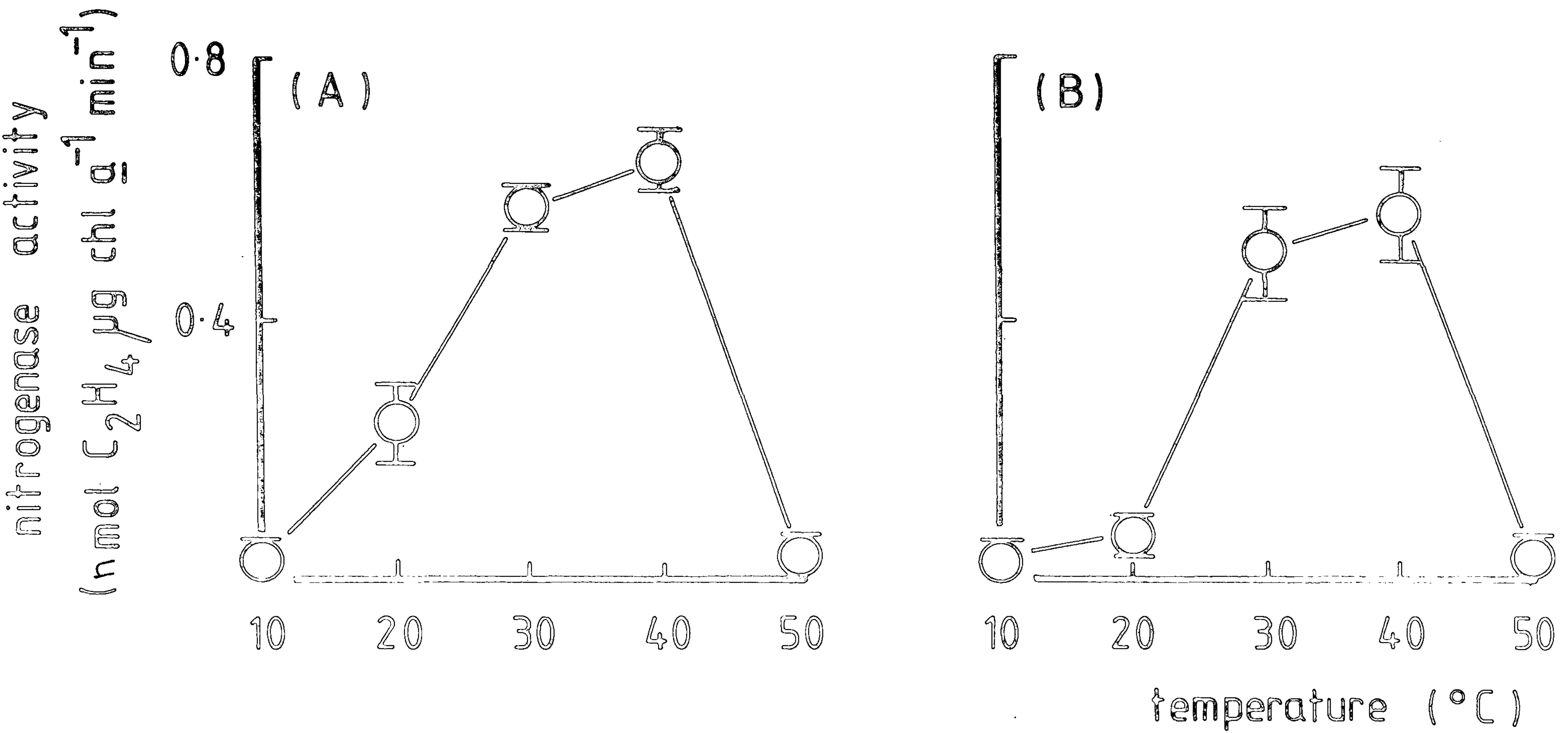
(i) Temperature

Experiments were conducted to study the effect of a wide range of temperatures (Section 2.52) on the nitrogenase activity (acetylene reduction) of two strains only. The activity of both strains was very low at 10 ^{o}C (Fig. 7.2) with a sharp increase in activity as the temperature increased to 20 and 30 ^{o}C, respectively. Increasing the temperature to 40 ^{o}C however led to a slight increase in the activity over 30 ^{o}C. At 50 ^{o}C the activity of both strains was inhibited almost completely.

Table 7.1 Nitrogenase activity (acetylene reduction) of Iraqi isolates and *Calothrix parietina* D550 as a control; algae cultured in AD-N medium for two days (32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking; assay caried out for 60 min under same culture conditios except 110 ± 10 μmol photon $m^{-2}s^{-1}$)

strains	Durham culture No.	nitrogenase activity (nmol C_2H_4 μg^{-1} chl *a* min^{-1}) $\bar{x}$	± SD
Anabaena cylindrica	582	0.47	0.05
Calothrix parietina	550	0.60	0.08
Calothrix sp.	580	0.31	0.02
Calothrix sp.	583	0.43	0.02
Calothrix sp.	585	0.41	0.10
Cylindrospermum muscicola	579	1.59	0.20
Gloeotrichia sp.	636	0.53	0.17
Hapalosiphon welwitschii	581	0.73	0.10
Nostoc muscorum	D584	0.55	0.13

Fig. 7.2 Infuence of temperature on nitrogenase activity (acetylene reduction) of Calothrix sp. D585 (A) and Nostoc muscorum D584 (B); algae cultured in AD-N medium with initial phosphorus concentration of 1 mg l^{-1}P for 2 days (32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking; pre-incubation and assay periods were 60 min under same culture condition except 110 ± 10 μmol photon $m^{-2}s^{-1}$; n=4)


nitrogenase activity
(nmol C2H4 /μg chl a-1 min-1)
0·8
0·4
(A)
(B)
10
20
30
40
50
temperature (°C)

(ii) Light quantity and quality

The influence of photon flux density and green light on the nitrogenase activity (acetylene reduction) of the two strains is shown in Table 7.2. The activity of both strains was nearly saturated at the photon flux density of 100 μmol photon $m^{-2}s^{-1}$. Reduction of the highest photon flux density by three fold (using a neutral density filter) caused 31% and 41% reduction in the highest activity of strains D585 and D584, respectively. Green light caused only a slight further reduction in the activity over the same light condition using a neutral density filter. Dark treatment on the other hand, reduced the highest activity of both strains by about 80-70%.

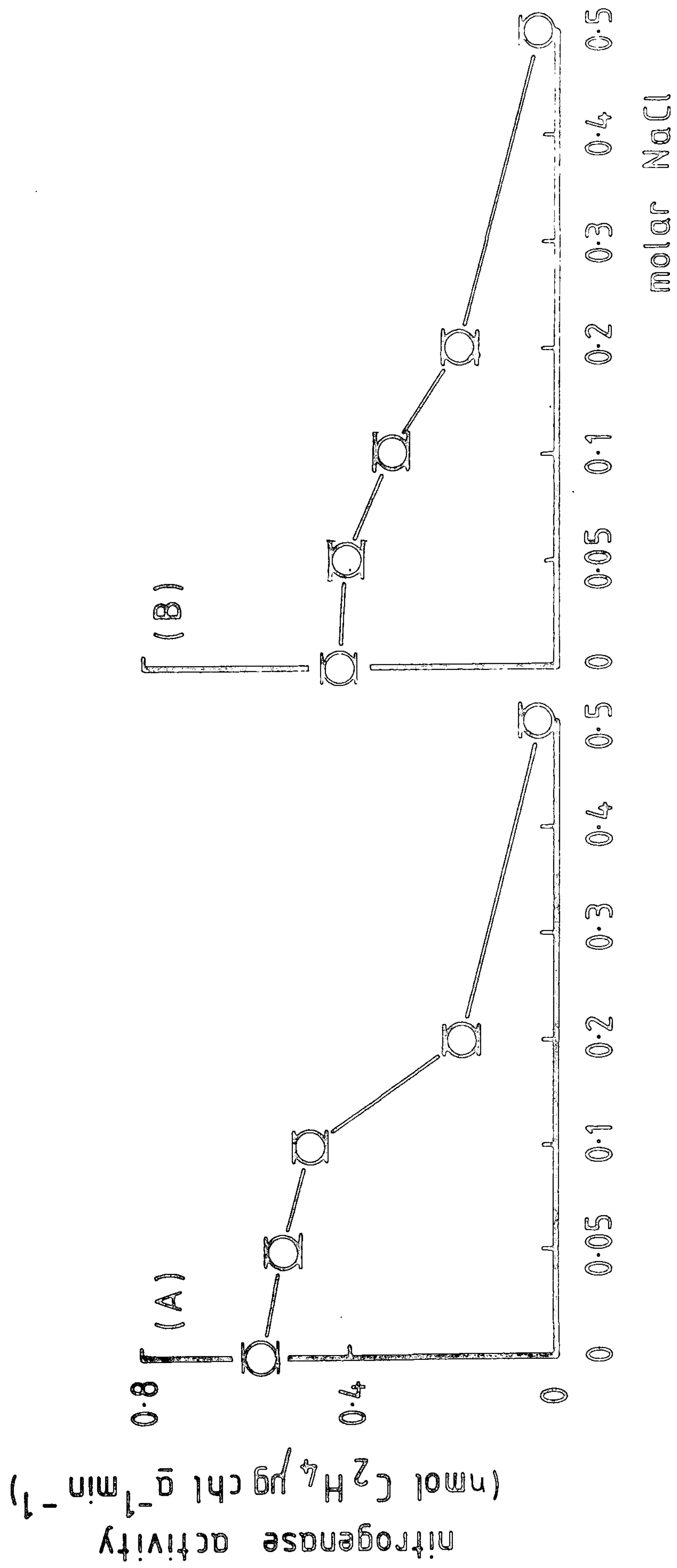
(iii) Salinity

The effect of five levels of salinity (Fig. 7.3) on the nitrogenase activity of two strains were tested. These were acheived by adding sodium chloride directly and aseptically to the cultures; cultures without added salts served as a control. There was only a slight reduction in the activity of both strains at the NaCl concentration of 0.05 M. However, the activity of strains D584 and D585 was reduced by 26% and 16%, respectively, at 0.1 M NaCl. At 0.5 M the activity of both strains dropped below the detection limits of the method used in this experiment (0.003 nmol C_2H_4 ug^{-1} chl a min-1).

Table 7.2 Effect of photon flux density and green light on nitrogenase activity (acetylene reduction) of Calothrix sp. D585 and Nostoc muscorum D584; algae cultured in AD-N medium for 2 days (23 °C; 90-120 µmol photon $m^{-2}s^{-1}$; continuous shaking; the pre-incubation and assay period were 60 min; neutral density and green filter, Section 2.52, were used for light reduction; n=4 in all cases)

photon flux density (µmol photon $m^{-2}s^{-1}$)	nitrogenase activity (nmol C_2H_4 µg^{-1} chl a min^{-1})			
	Calothrix sp		Nostoc	
	$\bar{x}$	± SD	$\bar{x}$	± SD
250	0.54	0.03	0.51	0.18
160	0.62	0.11	0.42	0.03
100	0.57	0.05	0.40	0.08
75	0.43	0.05	0.30	0.08
25	0.30	0.05	0.18	0.01
25 (green)	0.25	0.05	0.16	0.05
Dark	0.13	0.01	0.13	0.02

Fig. 7.3 Infuence of salinity on nitrogenase acivity (acetylene reduction) of _Calothrix_ sp. D585 (A) and _Nostoc muscorum_ D584 (B); algae cultured in AD-N with initial phosphorus concentration of 1 mg l^{-1}P for 2 days (32 ^{o}C; 90-120 µmol photon $m^{-2}s^{-1}$; continuous shaking; pre-incubation and assay periods were 60 min under same culture conditions except 110 $\pm$ 10 µmol photon $m^{-2}s^{-1}$; n=4 in all cases)



(A)
(B)
nitrogenase activity
(nmol C2H4 µg chl a-1 min-1)
0·8
0·4
0
0
0·05
0·1
0·2
0·3
0·4
0·5
molar NaCl

(iv) Nitrogen sources

The two nitrogen sources tested were ammonium-nitrogen (NH_4-N) and nitrate nitrogen (NO_3-N) (Section 2.44). The sodium concentration of all media was adjusted with NaCl to that of NO_3-N containing medium. Ammonium nitrogen inhibited almost completely the activity of 50% of the Iraqi isolates (Table 7.3). The inhibitory effect of nitrate-nitrogen, on the other hand, on the activity of the strains was different. For instance, there was 55% and 95% reduction in the activity of strains D584 and D579, respectively. It is of interest to mention that there was no appreciable change in the pH value of all media over the experimental period (2days).

(v) Phosphorus

An experiment was carried out to study the effect of phosphorus on the nitrogenase activity of Calothrix sp. D585. Twelve days-old cultures were used; this was chosen because of low nitrogenase activity shown by the alga at this stage of growth (Fig. 7.1), but before the alga reached the senescence stage of growth when any responses may be reduced or delayed. Phosphorus was added at different concentrations (Table 7.4); after 90 min algal material was

TABLE 7.3 Effect of nitrogen sources on nitrogenase activity (acetylene reduction) of axenic cultures of Iraqi isolates (heterocystons forms) and Calothrix parietina D550 as a control; algae subcultured from AD-N into AD-N + NaCl; AD + $NaNO_3$; Ad+NH_4Cl+NaCl all with initial phosphorus concentration of 1 mg l^{-1} P for 2 days (32°C; 90-120 μmol photon m^{-2} s^{-1}, continuous shaking; assays carried out for 60 mins. under same culture conditions except 110±10 μmol photon m^{-2} s^{-1}; n = 4).

strains	Durham culture No.	nitrogenase activity (nmol C_2H_4 μg chla^{-1} min^{-1})					
		AD-N + NaCl		AD + $NaNO_3$		AD + NH_4Cl + NaCl	
		$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD
Anabaena cylindrica	582	0.51	0.11	0.17	0.08	0.02	0.005
Calothrix parietina	550	0.66	0.10	0.30	0.03	0.007	0.0005
Calothrix sp.	580	0.30	0.05	0.04	0.004	<0.003	-
Calothrix sp.	583	0.60	0.10	0.07	0.01	<0.003	-
Calothrix sp.	585	0.51	0.02	0.01	0.005	<0.003	-
Cylindrospermum muscicola	579	1.51	0.34	0.07	0.005	0.02	0.001
Gloeotrichia sp.	636	0.65	0.12	0.20	0.02	0.03	0.007
Hapalosiphon welwitschii	581	1.03	0.15	0.25	0.03	0.007	0.0004
Nostoc muscorum	584	0.60	0.04	0.26	0.05	<0.003	-

separated by centrifugation and resuspended in phosphorus-free medium and acetylene reduction assays (Section 2.81) carried out. There was a slight response to the added phosphorus, but only at low concentrations (Table 7.4). The time course of the effect of the 5 mg $l^{-1}P$ on the nitrogenase activity of the same alga is shown in Table 7.5. Activity was reduced, although slightly, until 60 min when a slight increase occurred.

7.3 Nitrogenase activity of non-heterocystous forms

In view of the fact that substantial populations of non-heterocystous strains ocurred in the rice-field soil (Table 4.3) and because many non-heterocystous strains have been shown to possess nitrogenase activity under aerobic and/or microaerobic conditions (Section 1.51) it seems possible that the Iraqi strains might do so in the natural environments. Acetylene reduction assays were applied for the three non-heterocystous strains (Table 2.7). In order to check that the experimental conditions were favorable for nitrogenase activity Cylindrospermum muscicola D579 was included as a control. The non-heterocystous strains were cultured initially in AD+N medium (Section 2.45); after 10-12 days they were subcultured into AD-N medium. Acetylene reduction assays were carried out at two days intervals for 12 days. There was no indication of

Table 7.4 Effect of phosphorus concentration on nitrogenase acitivity (acetylen reduction) of Calothrix sp. D585; alga cultured in AD-N medium with initial phosphorus concentration of 1 mg l^{-1} P for 12 days (32 °C; 90-120 µmol photon m^{-2} s^{-1} ; continuous shaking; the pre-incubation period was 90 min after which the alga transfered to AD-P and assay carried out for 60 min under same culture condition except 110 ± 10 µmol photon m^{-2} s^{-1})

P (mg l^{-1})	nitrogenase activity (nmol C_2H_4 μg^{-1} chl a min^{-1}) $\bar{x}$	± SD
control	0.1	0.005
0.005	0.12	0.01
0.01	0.11	0.01
0.02	0.11	0.02
0.05	0.09	0.02
0.10	0.08	0.01
0.50	0.13	0.008

Table 7.5 Effect of pre-incubation period after the addition of 5 mg l^{-1} PO_4-P on nitrogenase acitivity (acetylene reduction) of *Calothrix* sp. D585 ;alga cultured in AD-N medium with initial phosphorus concentration of 1 mg l^{-1} P for 12 days (32 ^{o}C; 90-120 μmol photon $m^{-2}s^{-1}$; continuous shaking; after each incubation period the alga was re-suspended in AD-N-P and assay carried out for 60 min under same culture condition except 110 ± 10 μmol photon $m^{-2}s^{-1}$)

per-incubation period (min)	nitrogenase activity (nmol C_2H_4 ug^{-1} chl *a* min^{-1}) $\bar{x}$	± SD
0	0.16	0.01
10	0.11	0.02
20	0.10	0.02
40	0.09	0.03
60	0.10	0.01
120	0.12	0.001

nitrogenase activity by the non-heterocystous strains (Table 7.6). Under the same conditions nitrogenase activity was evident with C. muscicola.

The absence of demonstrable nitrogenase activity of the non-heterocystous strains studied in the experiment described above led to the selection for purification of Microcoleus chthonoplastes D634. This decision was taken for the following reasons. The alga was one of the dominant algae in the rice-field at the time of collection of the soil sample and under some laboratory conditions (Table 4.4); there was also evidence that some strains of the alga do fix nitrogen (Section 1.51). After being purified the alga was used in series of experiments designed to study the potential, if any, for the synthesis of nitrogenase.

(i) The results summarized in Section 5.5 showed that the addition of sulphide to the mixed population led to an increase, although slight, in the growth of the non-heterocystous strains. An experiment was therefore carried out to see if the alga could grow in -N media when enriched with sulphide. It was also decided to see the effects of dark/light cycle and flushing the media with nitrogen gas (oxygen-free) as well as deaerating the media with the same gas (Section 2.53). Two flasks were used for each treament, one kept standing at

TABLE 7.6 Time course of nitrogenase activity (acetylene reduction) and chlorophyll *a* content of *Cylindrospermum muscicola* D579; *Microcoleus chthonoplastes* D634; *Lyngbya aestuarii* D638 and *Lyngbya* sp. D639; algae except *Cylindrospermum* cultured in AD+N medium with initial phosphorus concentration of 1 mg l^{-1} PO_4 for 10-12 days (32°C; 90-120 μmol photon m^{-2} s^{-1}; continuous shaking; they were re-subcultured into AD-N with same phosphorus concentration and incubated under same conditions; assays carried out for 60 min. under same culture conditions except 110±10 μmol photon m^{-2} s^{-1}; n = 3)

time (day)	strains															
	Cylindrospermum				*Microcoleus*				*L. aestuarii*				*Lyngbya* sp.			
	chl *a* (mgl^{-1})		nmol C_2H_4 μg chl a^{-1} min^{-1}		chl *a* (mgl^{-1})		nmol C_2H_4 μg chl a^{-1} min^{-1}		chl *a* (mgl^{-1})		nmol C_2H_4 μg chl a min^{-1}		chl *a* (mgl^{-1})		nmol C_2H_4 μg chl a^{-1} min^{-1}	
	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD	$\bar{x}$	± SD
0	0.02	0.002	0.44	0.03	NR*		NR		NR		NR		NR		NR	
2	0.17	0.05	1.51	0.20	0.89	0.35	0.003	-	0.41	0.05	0.003	-	1.05	0.03	0.003	-
4	1.45	0.13	0.57	0.03	1.03	0.11	"	-	0.50	0.22	"	-	1.02	0.06	"	-
6	2.03	0.50	0.51	0.03	0.93	0.09	"	-	0.39	0.15	"	-	1.02	0.05	"	-
8	2.03	0.25	0.42	0.01	0.79	0.09	"	-	0.41	0.07	"	-	1.02	0.01	"	-
10	2.03	0.25	0.40	0.04	0.91	0.04	"	-	0.38	0.02	"	-	1.00	0.06	"	-
12	2.03	0.50	0.23	0.05	0.86	0.04	"	-	0.37	0.01	"	-	1.00	0.04	"	-

*NR not run

50-60 μmol photon $m^{-2}s^{-1}$, the other placed in a mechanical shaker at 10-20 μmol photon $m^{-2}s^{-1}$; both were incubated in a growth room of 32 °C. In all cases there was an initial growth, which stopped after 2-3 days (Table 7.7). However, growth was slightly better in cultures gassed with N_2. The colour of the material inoculated changed gradually from normal bright blue-green to green, greenish and finally yellow or brown.

(ii) The above results indicated that the alga might fix nitrogen at the beginning but eventually became limited by N_2 supply. There was also a possible limitation of CO_2 supply. Accumulation of O_2 as a result of photosynthesis might lead to inactivation or destruction of the nitrogenase. An experiment was therefore, carried out in which the study alga was subcultured into AD-N medium and enriched with one of the following carbon sources, sucrose, carbonate and bicarbonate. Sucrose was used because it was found that the alga grew better when cultured in AD+N medium enriched with either glucose or sucrose (Table 7.8). Sulphide (0.01 mM) was added, as $Na_2S.9H_2O$, at the beginning only in one set of flasks or the other set daily. Alkaline pyrogyllol was included in a separate container in the culture flasks. In all treatments there was a slight growth but only for the first 2-3 days of the experiment (Table 7.9).

Table 7.7 Effect of different enrichments on the growth of _Microcoleus chthonoplastes_ D634 in AD-N medium (32 ^{o}C; 50-60 or 10-20 µmol photon $m^{-2}s^{-1}$; shaking or standing; o = no growth bur alive, + = slight growth, ++ = good growth)

treatments	time (day) 7	15	30
AD-N medium (control)	+	o	o
+ sulphide (0.01 mM)	+	o	o
+ flushing with nitrogen (oxygen free) at beginning only	+	o	o
+ deaerated with nitrogen (oxygen free) for 60 min once only	++	o	o
+ dark/light cycle (12h:12h)	+	o	o

Table 7.8 Effect of sucrose, glucose and DCMU [3-(3-4 dichlorophenyl) -1, -1 dimethyl Urea] enrichments in AD+N medium on growth of Microcoleus chthonoplastes D634 after 10 days in the light and 21 days in the dark (32 °C; 50-60 μmol photon $m^{-2}s^{-1}$; without shaking)

treatments	chlorophyll a (mg l^{-1})			
	light		dark	
	$\bar{x}$	± SD	$\bar{x}$	± SD
AD+N (control)	0.80	0.04	<0.05	-
+ sucrose (0.01 M)	1.46	0.11	0.07	0.001
+ glucose (0.01 M)	1.19	0.02	0.05	0.001
+ DCMU (10^{-5}M)	0.69	0.07	<0.05	-
+ sucrose + DCMU	0.72	0.06	<0.05	-
+ glucose + DCMU	0.76	-	<0.05	-

Table 7.9 Comparison of growth of *Microcoleus chthonoplastes* D634 after 7 days in AD-N medium under different enrichments; AD+N medium was used as control (32 ^{o}C; 10-15 or 50-60 umol photon $m^{-2}s^{-1}$; without shaking; o= no growth but alive, + = slight growth, ++ = good growth; BBG = bright blue-green, G = green, GH = greenish; YH = yellowish)

treatments	photon flux density (µmol photon $m^{-2}s^{-1}$)	
	10-15	50-60
AD-N (untreated, control)	+ (GH)	+ (GH)
AD+N (untreated, control)	++ (BBG)	++ (BBG)
AD-N deaerated with N_2/ CO_2 at beginning only and pH adjusted with NaOH		
A. + alkaline pyrogallol	+ (G)	+ (G)
B. + sulphide (0.01 mM) added once only	+ (GH)	+ (G)
C. + sulphide (0.01 mM) added daily	+ (YH)	+ (YH)
AD-N deaerated with N_2 (oxygen free) at the beginning only + sucrose (0.01 M)		
A. + alkaline pyrogallol	+ (G)	+ (G)
B. + sulphide (0.01 mM) added once only	+ (G)	+ (GH)
C. + sulphide (0.01 mM) added daily	+ (GH)	+ (YH)
AD-N deaerated with N_2 (oxygen free) at the begininng only + carbonate (56 mM)		
A. alkaline pyrogallol	+ (G)	+ (G)
B. + sulphide (0.01 mM) added once only	+ (GH)	+ (GH)
C. + sulphide (0.01 mM) added daily	+ (YH)	+ (YH)
AD-N deaerated with N_2 (oxygen free) at the begininng only + bicarbonate		
A. + alkaline pyrogallol	+ (G)	+ (GH)
B. + sulphide (0.01 mM) added once only	+ (GH)	+ (GH)
C. + sulphide (0.01 mM) added daily	+ (YH)	+ (YH)

(iii) It was assumed that if the alga was not deficient in carbon, nitrogen or phosphate before being subcultured into medium free from combined nitrogen and cultured at low photon flux density. Under these conditions nitrogenase activity might have been expected. Therefore the alga was first subcutured into AD+N medium with 0.01 M sucrose, 0.01 M $NaNO_3$-N, 0.3 mM PO_4-P and incubated at 32 ^oC and 50-60 μmol photon $m^{-2}s^{-1}$ for 10 days. after this it was transfered into AD-N medium without sucrose but phosphorus was added at 0.03 mM. Flasks were incubated at 10-15 μmol photon $m^{-2}s^{-1}$ and acetylene reduction assays (Section 2.81) were carried out after 0, 15, 30, 60, 120 and 180 min and after 24, 48, 72 and 96 hours. In no case was there any indication of nitrogenase activity.

(iv) It seemed possible that calcium might be required at a high concentration for the nitrogenase activity of the alga. When calcium concentration was increased from 18 mg l^{-1} (in the normal medium Table 2.3) to 36 mg l^{-1} no response was found. Sodium was also suspected to be required at high concentration. An experiment was therefore set up in which the sodium concentration in AD-N medium was increased to 0.1, 0.5 and 1.0 M Na using NaCl. There was no growth in any of the concentrations tested. It was also suggested that DCMU [(3-3-4dichlorophenyl) -1,1- dimethyl

urea)] might be necessary for the inhibition of photosystem II and thus activation of nitrogenase of the alga grown in -N medium. When this was added at a concentration of 10^{-5} M, no growth occurred in -N medium, but growth was normal in +N medium and DCMU.

It is not possible from the results of the previous experiments to rule out conclusively the ability of the alga to fix nitrogen. It can be said, however, that the alga did not show nitrogenase activity under the experimental conditions tested. In addition, in a preliminary experiment, the same alga showed positive nitrogenase activity (acetylene reduction) (Table 7.10). The $^{15}N_2$ enrichment experiment, however, showed that none of the cultures tested were significantly enriched (Table 7.11). The alga in these experiments were pre-incubated under a gas phase consisting of 0.7 atm Ar, 0.2 atm N_2 and 0.1 atm CO_2 for 17 h at 32 ^{o}C and 100 $\pm$ 10 μmol photon $m^{-2}s^{-1}$ with continuous shaking. The assays were started by injection of acetylene (0.1 atm) into one set of the flasks and $^{15}N_2$ enrichment (30 atm% excess) into another set. Five flasks were used for each treatment. Flasks were re-incubated under the assay conditions for 20 h. Nitrogenase activity (acetylene reduction) was very low in comparison with other nitrogen-fixing algae (Table 7.1) and the rates were highly

Table 7.10 Nitrogenase activity (acetylene reduction) of <u>Microcoleus chthonoplastes</u> D634 (32 °C; 110 ± 10 µmol photon $m^{-2}s^{-1}$; continuous shaking; 20h incubation)

replicates	nitrogenase activity (nmol C_2H_4 μg^{-1} chl a min^{-1})
1	0.00045
2	0.000046*
3	0.000027*
4	0.000207
5	0.000240
$\bar{x}$	= 0.000194
SD	= 0.000172

* rates <0.0005 did not show an increase in the amount of C2H4 compared with the blank

Table 7.11 Nitrogenase activity ($^{15}N_2$ assays) of Microcoleus chthonoplastes D634 (32 ^{o}C; 110 ± 10 µmol photon $m^{-2}s^{-1}$; continuous shaking; 20h incubation; 30% atom excess enrichment)

replicates	$^{15}N_2$ enrichment	
	acetylene reduction	$^{15}N_2$ assay
1	0.3614	0.3602
2	0.3604	0.3600
3	0.3608	0.3636
4	0.3581	0.3635
5	0.3632	0.3634
x	0.3608	0.3621
SD	0.001842	0.001865
enrichment	0.0013	

variable. On the other hand, if the difference in $^{15}N_2$ enrichment is taken as 0.0013 atm% excess (Table 7.11), the amount of N per flask as 953 µg (mean of 5 replicates), the amount of chlorophyll a as 111 µg (mean of 5 replicates) and C_2H_4: N_2 conversion factor of 3 (Stewart et al., 1968) then the amount of N_2 fixed is equivalent to 0.00003 nmol C_2H_4 μg^{-1} chl a min^{-1}. This is comparable with the minimum increase of C_2H_4 taken to be due to acetylene reduction (Table 7.10).

CHAPTER 8. DISCUSSION

8.1 Influence of environmental factors on algae in rice-fields

It is clear from the experimental results given here (Chapters 4 & 5) that changes in the environment can bring about marked differences in species composition of a single sample from the rice-field in the marshes.

The algal flora revealed in laboratory cultures was much richer than that found by direct inspection of the sample or that developed in the wet sample after two weeks incubation at 32 °C and 30-40 µmol photon $m^{-2}s^{-1}$ (Tables 4.1, 4.2, 4.3). A similar effect has been found by other workers, who have reported that direct inspection of natural samples (soil or water) usually reveals very low numbers of species compared with enrichment cultures (Allen and Stanier, 1968; Fogg et al. 1973; Round, 1975).

In the present study almost every factor showed a selective effect on the growth of different taxa (Table 4.4). At low temperature, for example, algae other than the blue-greens were abundant, especially in cultures enriched with combined nitrogen. As the temperature increased to 25 °C or more heterocystous blue-green algae dominated -N cultures during the early stages of growth. However after a period of akinete formation by some of theses species,

non-heterocystous filamentous forms came to dominate old cultures. Patrick(1969), who studied the algal commmunities of the Sabine River in the United States of America, mentioned that green algae tend to grow best at temperatures lower than that of the blue-green algae but higher than that of the diatoms. Kratz and Myers (1955) suggested that blue-green algae may have a temperature range significantly higher than that of most other algae. Fogg (1956) mentioned that the temperatures which blue-green algae are able to tolerate are much higher than those of other groups obtained from similar environments. The growth of eukaryotic algae has been found by Allen and Stanier (1968) and Antarikanonda and Lorenzen (1982) to be completely inhibited at 35 ^{O}C in cultures free from combined nitrogen. The highest temperature at which growth of blue-green algae occurred was 40 ^{O}C and no blue-green algae developed at 45 ^{O}C (Allen and Stanier, 1968). In the present study the highest temperature at which growth occurred, although only for a very short period, was 48 ^{O}C. However, substantial growth occurred at 45 ^{O}C (Fig. 5.1). On the other hand, the lowest temperature at which algae grew was 10 ^{O}C, yet several forms were found alive at 5 ^{O}C. According to Fogg _et al._ (1973) blue-green algae show a wide distribution along the temperature gradient from polar regions to hot springs of up to 73 ^{O}C. In the rice-fields of the study area, enormous

diel and annual temperature variations are common, with temperatures dropping to nearly freezing in the winter and rising to 50 °C on the soil surface in summer (Section 3.2). During the peak flood of the Tigris and Euphrates, March-May, rice is planted in the fields which are covered by water. The recorded water temperature during most of the cultivation period in the rice-fields and the surrounding marshes ranged between 24-29 °C (Al-Kaisi, 1976; Maulood et al., 1981; Al-Saadi et al., 1981). This range favours the growth of the blue-green algae, as they tended to dominate cultures grown at or above 25 °C and more in the present study. At the end of the period of rice cultivation the soil dries out and after harvest is exposed to direct sunlight and temperatures between 40 and 50 °C may occur. According to the result of the present study nitrogen-fixing blue-green algae grew well up to 45 °C (Fig. 5.1).

The ability of two of the major nitrogen-fixing blue-green algae found in the present study to form akinetes at 45 °C (Section 4.3) may enable them to resist the extreme temperatures that occur in the rice-fields and act as inoculum for the next cycle of rice-cultivation. It was found, for example, that the spores of *Cylindrospermum muscicola* can be kept alive for three years in completely dry conditions at 32 °C. The green algae most commonly found in the present study, *Chlorococcum* and *Ulothrix*, are

known to be members of the soil algae (Bold and Wynne, 1978). The former usually grows luxuriantly in liquid medium (Archibald and Bold, 1970).

The algal flora of the sample under investigation showed marked response to changes in the light conditions (Table 5.1). At the lowest photon flux density (15-20 µmol photon $m^{-2}s^{-1}$), non-heterocystous blue-green algae were dominant in +N medium, increasing the photon flux density to 50-120 µmol photon $m^{-2}s^{-1}$ led to a marked increase in the growth of the heterocystous blue-green algae. At the highest photon flux density used (200-250 µmol photon $m^{-2}s^{-1}$) blue-green algae dominated the cultures at the beginning but they quickly underwent chlorosis in +N medium and were replaced by other forms of the green algae (mainly Scenedesmus and Chlorococcum). Although certain blue-green algae are tolerant of high light conditions (Traore et al., 1978; Nordin and Stein, 1980) the majority are generally light sensitive and usually regarded as low light forms (Brown and Richardson, 1968). The effect of different light conditions on the growth of green and blue-green algae was demonstrated by the work of Mur et al., (1978) who grew the blue-green alga Oscillatoria agardhii with the green alga Scenedesmus protuberans in continuous culture under different photon flux densities. They found that the former grew better and dominated the culture under

low light conditions (1 W m^{-2} s or 4 μmol photon $m^{-2}s^{-1}$) while the latter grew better and dominated the culture at the highest light condition tested (39 W m^{-2} s or 156 μmol photon $m^{-2}s^{-1}$). Several workers have found that the light level at which half-maximum growth rate is reached for a number of blue-green algae is low compared with green algae (van Liere and Mur, 1980). However, different species of blue-green algae have different light requirements (van Liere and Walsby, 1982). Thus Foy and Gibson (1982) found that Oscillatoria species were more efficient in harvesting low light irradiance than species of Anabaena and Anabaenopsis. Zevenboom and Mur (1980) suggest that even in the presence of nitrate nitrogen-fixing blue-green algae have a higher light requirement than that of non-nitrogen-fixing forms.

Because of the pigment composition of blue-green algae, certain forms can undergo a complementary chromatic adaptation which enables them to utilize different quantities and qualities of light (Brown and Richardson, 1968). For example, the development of c-phycoerythrin under green light can be used to scavenge the green light unused by most of other algal groups (van Liere and Walsby, 1982). The use of green light enrichment led to a marked increase in the growth of Gloeotrichia sp. D636, yet no critical experiments were carried out to test the effect of green light on the growth of this alga and other isolated

strains. In natural conditions algae normally experience alternating light and dark periods. Testing this factor did not show any major changes in the species composition, although a slight increase in the chlorophyll a content was found (Fig 5.4). Applying the results of the effect of light on the species composition to the natural condition of the rice-fields may give an impression that green algae are expected to be dominant in the rice-fields at the early stage of rice planting, because of the high photon flux density and little shading by rice plants. However, as the rice plants grow the light condition may be gradually reduced, favouring the growth of the blue-green algae as was found in many rice-fields elswhere (Section 1.22). This may also explain the dominance of blue-green algae in the rice-field at the time of soil collection (Al-Mousawi and Whitton, 1983) where the rice plants form a dense vegetation in the field (Fig. 3.5).

Increasing salinity led to a decreasing number of algal species (Table 5.3), but even at salinities well above sea water (1 M NaCl, 58.44‰), several blue-green algal species survived. At 0.5 M NaCl (29.22‰) Nodularia harveyana dominated almost all of the cultures. However, it was completely absent from cultures enriched with 0.1 M NaCl (0.58‰). The contrast between the abundance of N.harveyana at 0.5 M and its absence at 0.1 M was striking. Presumably

it was unable to compete effectively with Anabaena, Cylindrospermum and Nostoc at the lower salinity. Nordin and Stein (1980) mentioned that moderate salinity is essential for the growth of Nodularia spp. Toward the end of the present study it was found that Anabaena (probably A. oscillarioides) grew well at 1 M NaCl enrichment and dominated the algal flora which included in addition to the A. oscillarioides two non-heterocystous filamentous blue-green algae (Lyngbya > 1 ≤2 µm and Arthrospora sp.). Several workers have found that Anabaena spp. are one of the genera of blue-green algae commonly found in saline soil and several other species show an absolute requirement for salinity for their growth (Section 1.22). The effect of salinity on the algal flora (mainly blue-green algae) showed that algae can be divided into two groups. These are a low salinity group which includes most of the strains identified in the present study (Table 5.3) and a high salinity group which includes three heterocystous forms and two non-heterocystous forms. The first group is most likely to be found in the rice-fields at the period of flood, where low salinity water (Maulood et al., 1979) covers the rice-field. The second group is most likely to grow better in the soil which was characterized by moderate to high salinity (Buringh, 1960; Al-Kaisi, 1976).

The algal flora of the soil sample showed only slight changes after enrichment with the reducing agent (sulphide), which led to a slight increase in the growth of non-heterocystous forms (mainly *Microcoleus chthonoplastes*). Heterocystous forms responded differently, with *Cylindrospermum* appearing very sensitive and *Nostoc linckia* more resistant. Howsley and Pearson (1981) found that heterocystous blue-green algae were the most sensitive to sulphide inhibition, unicellular types were the least sensitive, non-heterocystous filamentous forms being intermediate. Kashyap *et al.* (1983) found large differences between a group of heterocystous blue-green algae in their tolerance to the sulphide enrichments with *Nostoc calcicola* being the most tolerant species. Al-Kaisi (1976) mentioned that during July and August hydrogen sulphide can be smelled frequently in the rice-fields of the south eastern marshes of Iraq. This may indicate that reducing conditions are widespread in the rice-fields of these marshes and that non-heterocystous blue-green algae are likely to consist of a significant part of the algal flora and contribute to soil fertility since large numbers of them are known to fix nitrogen under microaerobic or anaerobic conditions (Section 1.5). This may explain the dominancy of *Microcoleus chthonoplastes* beside *Nostoc muscorum* in the field (Al-Mousawi and Whitton, 1983)

Enrichment of culture media with combined nitrogen led to the dominance of algae other than the blue-greens at low temperatures (Table 4.4). At high temperatures non-heterocystous filamentous blue-green algae became dominant. Similar observations were reported under both laboratory and field conditios (Section 1.22). In the present study combined nitrogen was added at a high concentration (140 mg l^{-1} NO_3-N), a concentration presumably seldom found in the field. However, it was added on the assumption that yield under laboratory conditions is not likely to exceed 2 g l^{-1} dry weight and about 7% of the algal dry weight is nitrogen (Section 2.45). Under these conditioins algae other than nitrogen-fixing blue-green algae are not likely to be limited by nitrogen supply.

An increased phosphorus concentration caused a decrease in the total number of taxa (Fig. 5.7). Among the blue-green algae inhibited by high phosphorus concentration were Calothrix spp., while other algae, e.g., Cylindrospermum, Anabaena and Nostoc grew better and dominated cultures with high phosphorus. The inhibitory effect of high phosphorus concentrations on the growth of several blue-green algae was mentioned in Section 1.3. In the present study (Section 6.2) one of the Calothrix spp. (Calothrix sp. D585) accummulated more phosphorus than Cylindrosperemum and Nostoc (Figs. 6.7, 6.8 & 6.9). This

may indicate that Calothrix species spend much of their energy on the uptake and storage of phosphorus and hence grow slowly while other forms, like Cylindrospermum spend much of their energy in cell division and reproduction using less phosphorus and dominating the cultures where factors other than phosphorus will limit the growth of Calothrix species. In the earlier parts of the present study it was noticed that the heterocystous blue-green algae dominated almost all of the cultures at high temperatures (30-45 ^{o}C). This was unexpected as the presence of a nitrogen source is known to enhance the growth of algae other than the blue-greens at low temperatures and non-heterocystous blue-green algae at high temperatures (Section 1.22). However, in the older cultures non-heterocystous forms dominated the cultures enriched with combined niterogen. Knowing that in the earlier stage of growth phosphorus was added as 44.5 mg l^{-1} P and nitrogen as 140 mg l^{-1} NO_3-N give N/P loading ratio by weight of about 3. When the phosphorus concentration was reduced to 2.5 mg l^{-1} P (Section 5.7) and the nitrogen concentration remained the same the ratio wentup to 56. Several workers have found that an N/P loading ratio below 5 favoured the growth of heterocystous forms (Schindler and Fee, 1974; Schindler, 1975,1977) while a loading ratio greater than 5 supports the growth of non-nitrogen-fixing forms (Brunskill, 1973).

The species composition of the soil sample showed several features of particular interest. For example, apart from low temperatures and high light condition enrichments, blue-green algae dominated almost all the cultures. The total number of species (Table 4.5) also showed that blue-green algae were overwhelmingly predominant. Al-Kaisi (1976) found that blue-green algae comprise 86% of the total number of algal taxa identified during July and August in the rice-fields of the south east marshes of Iraq. These results are to be expected since most of the common ecological requirments of blue-green algae (Fogg _et al._, 1973; Fay, 1983) are available in the rice-field studied (Sections 1.2,3.2). In addition the sample was collected in September from the margin of the rice-field (Section 3.3) where the dense growth of rice plants (Fig. 3.5) may have reduced the photon flux density and favoured the growth of blue-green algae (Section 1.22). This was evident from the dense growth of presumably nitrogen-fixing blue-green algae seen in the field (Al-Mousawi and Whitton, 1983). However, the total number of blue-green algal species identified is relatively small compared to the total number found in the general survey of Al-Kaisi (1976).

The complete absence of diatoms in the sample studied was unexpected. Diatoms are widely distributed in the rice-fields (Al-Kaisi, 1976) as well as the surrounding

marshes (Pankow et al. 1979; Hinton and Maulood, 1980; Maulood et al., 1981). The possibility that their growth was limited by the absence of silicate in the basic growth medium used (Tables 2.2, 2.3) was eliminated by the use of a silicate-containing medium (Chu 10D, Tables 2.2, 2.3). However, most of the diatoms are very thinly coated with mucilage (Round, 1975) and are probably in general less tolerant of desiccation (Lund, 1962; Trainor, 1970).

The number of green algal taxa found in the present study was also low compared with field studies of the rice-fields and the marshes of Al-Kaisi (1976) and Maulood et al. (1981). No doubt one of the factors responsible was the fact that the sample was collected during part of the year when temperatures were very high (Section 3.2), a factor known to inhibit the growth of green algae (Section 1.22).

(ii) Growth of algal community

The growth rate and growth yield (chlorophyll a content) of the mixed population responded differently to the effect of the environmental factors studied. However, although the ecosystem inside the culture flasks was very complex and too much emphasis can not be placed on small differences, several aspects seem to be established clearly. For example, initial growth was always faster in +N medium,

particularly at low temperatures (Fig. 5.1) and low light conditions (Fig. 5.3); initial growth was also faster the higher the temperature (Fig. 5.1). Of particular interest is the apparent contradiction between results of Fig. 5.1 and Fig. 5.3. Thus, the yield of mixed populations at 35 °C (Fig. 5.1) showed a higher value in -N medium than in +N medium. This experiment was carried out under a photon flux density of 90-120 µmol photon $m^{-2}s^{-1}$. Under the same conditions (Fig. 5.3) the yield is higher in +N medium than in -N medium. As mentioned above in the earlier experiments (Fig 5.1) the N/P loading ratio was about 3, which favoured the growth of the heterocystous blue-green algae, while in the later experiments (Fig. 5.3) this ratio was 56, which favoured the growth of non-heterocystous blue-green algae.

The main heterocystous blue-green algae (*Cylindrospermum*, *Anabaena* and *Nostoc*) in the earlier experiments show akinete formation in +N medium earlier than in -N medium. Rapid growth and fast spore formation is often associated with the addition of an exogenous nitrogen source (see Nicholas and Adams, 1982 for references). The characteristic features of the growth yield of mixed populations under most of the environmental factors studied is a progressive increase to a peak value followed by a gradual or sharp drop. The size of this peak and the duration of the subsequent drop were affected differently by

the variuos factors. For instance, growth yield continued to increase to the end of the experimental period of 30 days in several experiments, 15 °C (Fig. 5.1), low photon flux density (Fig. 5.3) and low phosphorus concentration (Fig. 5.6). Microscopical inspection showed that several biotic factors (mainly protozoa) were apparently responsible for the decrease in the size of the growth yield in old cultures. Their sparse occurrence at 15 °C probably explains why the maximum yield reached in +N medium was at this temperature. Antarikanonda and Lorenzen (1982) found that the main contaminants of the algal cultures from the rice-field soil in Thailand are bacteria, fungi and amoeba. Effects of biotic factors on the blue-green algae in the rice-fields were mentioned in Section 1.22.

In addition to biotic factors, both physical and chemical factors played important roles in determininig the yield of the mixed population. Increasing the temperature to 40 and 45 °C, for example, led to a reduction in the yield (Fig. 5.1). Microscopical inspection showed that the higher the temperature the earlier the spore formation. Wolk (1965) and Fernandes and Thomas (1982) have reported on the enhancement of spore formation in *Anabaena cylindrica* and *A. torulosa* with increasing temperature. In the present study in +N medium yellowing of the Oscillatoriaceae species took place rapidly at high temperature.

In general the growth of mixed population increased as the photon flux density increased (Fig. 5.3). However, the highest yield was found at a photon flux density of 50-60 μmol photon $m^{-2}s^{-1}$. Increases in the chlorophyll content of photosynthetic algae under low light were found by several workers (Brown and Richardson, 1968; Foy and Gibson, 1982). At the highest photon flux density (200-250 μmol photon $m^{-2}s^{-1}$) reduction of the yield was evident. This may be due to photodestruction of the chlorophyll content of the algae, a phenomenon commonly observed both in field and laboratory studies under high light conditions (Abeliovich and Shilo, 1972; Eloff et al . 1976). Belay and Fogg (1978) found that photooxidation of photosynthetic pigments increased as the temperature increased.

The yield of the mixed population was hardly affected at the low salinity (0.1 M NaCl). However, increasing salinity to 0.5 M led to a large reduction in the yield (Fig. 5.5). The yield, however, is slightly higher in +N medium which may indicate sensitivity of nitrogen fixation processes to the salinity more than other aspects of algal metabolism (Whitton and Sinclair, 1975).

Increasing the phosphorus concentration from 0.5 to 44.5 mg l^{-1} P led to an increase in the yield of the mixed population (Fig. 5.6). However, the yield of the

mixed population at the highest phosphorus concentration dropped rapidly. This may be due to the rapid growth of the mixed population at this high concentration which may have led to the development of mutual shading (Antarikanonda and Lorenzen, 1982) or a nutritional factor other than phosphorus becoming limiting to the growth of the algae. On the other hand, the rapid drop of the yield at the lowest phosphorus concentration is most probably due to phosphorus deficiency, a factor known to enhance spore formation in many of the blue-green algae (Section 1.33).

Comparison of the results of the effect of the environmental fators on the yield of the mixed population to what would be expected in the field is of particular interest. Thus temperature is not likely to play an important role in determining the algal yield during most of the cultivation period when rice-fields are covered with water. This was based on the temperature data of the water which show little variation during most of the growing season of the rice plants (Al-Saadi et al.,1981). It may have an important role as the soil dries out and large variations occur (Section 3.2). Light on the other hand, may be the most important factor determining the size of the yield during the cultivation period. At the beginning, high photon flux density was expected which may have a deleterious effect, but as the rice plants grow and form

dense vegetation (Fig 3.5) a reduction in the light quantity occurs which could increase the algal yield. As in the case of temperature, salinity may have either little or no effect during the flood period where low salinity water covered the rice-fields (Section 3.2). However, as the soil dries out, and especially after harvest, high salinity in the soil surface accompanied by high temperature and high photon flux density may severly reduce the algal yield. The lack of sufficient data on the concentration of different phosphorus fractions makes it difficult to comment on the role of phosphorus in determining the size of algal yield (Section 8.2).

8.2 Influence of phosphorus

Little is known about the phosphorus status in the marshes around the rice-fields and there are no data on the phosphorus content of rice-field soils. Therefore, study of the physiological behaviour of a relatively large number of strains isolated from the rice-fields is of a significant importance (Whitton and Carr, 1982). In the present study a special effort was given to bring into culture the main strains of blue-green algae.

The growth rate of the strains isolated showed marked differences (Table 6.2) with some strains growing quite fast (mean doubling time 13-19h) and others very slowly.

Generally, Rivulariaceae strains had the lower growth rates. The different culture conditions in which the individual experiments were carried out make it very difficult to compare the results with those reported in the literature.however,the mean doubling time for Anabaena cylindrica D582 was 15 h (Table 6.2); is similar to that for the same species (14.1 h) found by Weare and Benemann (1973). Peterson and Burris (1976) found that the mean doubling time of Anabaena 7120 under nitrogen-fixing conditions is 12 h. Recently much faster growing strains of Anabaena have been isolated from rice-fields elsewhere (Antarikanonda and Lorenzen, 1982; Chen, 1983). However, it is worth mentioning that the growth rate of the strains reported in the literature are those under the optimum growth conditions. The growth rates of the strains isolated may be much higher if studied under their optimum conditions.

There are several approaches to the identification of growth-limiting nutrients. None of them is ideal and applicable for all conditions (Healey, 1973, 1978). Two main approches were applied in the present study. The major morphological changes associated with phosphorus deficiency are the stimulation of akinete formation and reduction of heterocystous frequency in spore forming heterocystous strains (Section 4.5). Strains (D585, D636) also showed a

marked increase in the sheath material. The only hair forming strain D636 showed an increase in hair formation under phosphorus-deficient conditions. The level of cyanophycin granules was found to increase slightly or markedly in all of the strains studied. These morphological changes are similar to those reported in the literature (Section 1.33).

Several physiological changes took place under phosphorus-deficient conditions. Three strains were used for detailed study. They responded to phosphorus deficiency by a reduction in growth rate (Table 6.1), change of chlorophyll a and dry weight content (Table 6.3, Figs 6.1, 6.2, 6.3), development of phosphatase activity (Figs 6.4, 6.5 , 6.6) and changes of cellular phosphorus content (Figs 6.7, 6.8 , 6.9). In all of these physiological changes differences between strains were evident. These differences were more obvious with respect to phosphatase activity and the cellular phosphorus content of the strains. The cellular phosphorus content at which detectable cellular alkaline phosphatase activity developed for strains D585, D579 and D584 was 18, 8 and 36 μg P mg^{-1} dry weight, respectively. According to Healey (1982) the approximate limits for P-deficiency and P-sufficiency in the blue-green algae are <4 and >7 μg P mg^{-1} dry weight, respectively. Livingstone *et al.* (1983) found that *Calothrix parietina*

D550 developed alkaline phosphatase at a cellular phosphorus content of about 10 $\mu g\ mg^{-1}$ dry weight. the extremely high cellular phosphorus concentration at which strain D584 developed phosphatase activity (36 $\mu g\ mg^{-1}$ dry weight) may be an indicator of the constitutive rather than inducible enzyme in this strain. None of the three strains studied release appreciable amounts of organic or inorganic phosphorus to the growth medium throughout the time course of the experiments (Tables 6.6, 6.7, 6.8). Similar results were found for some prokaryotic and eukaryotic microorganisms (Section 1.31)

Alkaline phosphatase assays of other strains showed that they all have cellular and extracellular enzyme (Table 6.11). The extracellular enzyme was found to be, at least partly, a true soluble enzyme (Table 6.16). The pH optima of both cellular and extracellular enzyme of the isolated strains lie between 9-11 (Figs 6.10-6.17). The activity of the cellular enzyme is stimulated by Ca^{++} and inhibited by Mg^{++} (Tables 6.12, 6.13). The characteristic features of the alkaline phosphatase of the strain isolated are similar to those reported for other algae (Kuenzler and Perras, 1965; Healey, 1973; Doonan and Jensen, 1980). However the inhibitory effect of Mg^{++} was unexpected . Magnesium was found to have either no or a slight stimulatory effect on the alkaline phosphatase of several blue-green algae

(Section 1.4).

Apart from strain D582 which could not grow when phosphorus was supplied in the form of inositol hexaphosphate (phytic acid) all other strains grew well and utilized a wide range of phosphorus sources (Table 6.9). The phosphorus compounds tested were fairly stable under the experimental conditions (Table 6.10). However, the soluble reactive phosphorus detected in the solutions of all of the phosphorus compounds might be a result of the effect of the acidic reaction environment during analysis of phosphorus (Stainton et al., 1977). Similar observations were made by Chu (1946) and Potman and Lijklema (1983). Fogg (1973) mentioned that due to the possession by algae of surface phosphatases they can utilize phosphorus from sources other than orthophosphate.

The ability of the strains isolated to utilize a wide range of phosphorus compounds is of special ecological importance. This is because the concentration of phosphorus (PO_4-P fraction) in the marshes and presumably in the rice-fields during peak flood, is rather low (Maulood et al, 1979, 1981). The organic phosphorus content may be high, especially in the rice-fields, where large amounts of organic matter were noticed by Al-Kaisi (1976). The present results of organic and anhydrous phosphorus utilization and phosphatase activities indicated that one beneficial effect

of blue-green algae on rice plants may be the mineralization of organic and anhydrous phosphorus sources.

8.3 Nitrogen fixation in the rice-fields

Various reports indicated that blue-green algae may be important agents in maintenance of the nitrogen balance of soil in rice-fields (Section 1.5). The time course of the nitrogenase activity of three strains showed a peak after 2 days from culturing followed by a gradual decrease in the acivity (Fig. 7.1). As mentioned above, the cellular phosphorus content of the same strains showed a similar sequence (Figs 6.7, 6.8, 6.9).

The nitrogenase activity (acetylene reduction) of the strains isolated was in the range of 0.31-1.59 nmol C_2H_4 μg^{-1} chl _a_ min^{-1} (Table 7.1).

Some comparison can be made with other literature assuming a typical blue-green algal cell during a logarithmic growth phase consist of 50% of dry weight as protein (Collyer and Fogg, 1955) and 1% as chlorophyll _a_ (Fay, 1969). _In vivo_ nitrogenase activity (acetylene reduction) in the laboratory is in a typical range of 1-10 nmol C_2H_4 mg^{-1} protien min^{-1} (Stewart, 1973b) (i.e. 0.05-0.5 nmol C_2H_4 μg^{-1} chl _a_ min^{-1}). Accordingly the results obtained in the present study are within and some even higher than those mentioned above. Similar results

were found by Antarikanonda and Lorenzen (1982) for several strains of blue-green algae isolated from rice-fields in Thailand (8-20 nmol C_2H_4 mg^{-1} protein min^{-1}) (0.4-1.0 nmol-μg^{-1} chl a min^{-1}) assays carried out at 37 °C. Chen(1983) found that nitrogenase activity of three Anabaena strains isolated from rice-fields in Taiwan ranged between 0.7-2.3 nmol C_2H_4 μg^{-1} chl a min^{-1} when assayed at 32 °C.

The results of the effect of environmental factors on the nitrogenase activity of some isolates (Figs 7.2, 7.3; Table 7.2) showed that only extrem conditions have an inhibitory effect. These conditions are not likely to be found in the field during most of the cultivation period of rice plants(Section 3.2). However, the effect of combined nitrogen (Table 7.3) showed that some strains, e.g. D584 has relatively high activity when combined nitrogen was supplied as nitrate, although a very high concentration was used. These results are important since the need for blue-green algae strains capable of fixing nitrogen under conditions riche in combined nitrogen is ecologically important (Stewart, 1973a).

The results of nitrogenase activity of Microcoleus chthonoplastes under microaerobic conditions (Table 7.10) are also important since this alga is dominant in the field where microaerobic and/or anaerobic conditions likely to be prevalent due to the presence of sulphide (al-Kaisi, 1976).

However, the high variation in the results may be due to the relatively short incubation period (37h). Several workers have found that long incubation period (72-120h) is required for active nitrogenase activity of non-heterocystous strains of blue-green algae to develope measurable nitrogenase activity (Stewart and Lex, 1970; Kenyon et al., 1972).

The abundance of Nostoc muscorum in the field sample, and the presence of at least 17 other heterocystous blue-green algae in the small sample (1 g) used for the present study, together suggest that blue-green algal nitrogen fixation is likely to play an important role at this site.

8.4 Concluding remarks

1. The present study showed that the soil sample studied is relatively rich in algae compared to the field reports on the same area. Thus, it is likely that the algae identified constitute an important proportion of the field flora.

2. Blue-green algae overwhelmingly dominated the algal flora of the sample and consisted of 71.5% of the total taxa identified. This is in agreement with the known environmental features of the study area which favour the growth of the blue-green algae.

3. Heterocystous, and presumably nitrogen-fixing blue-green algae constituted 50% of the total blue-green algal taxa found in the sample. These algae dominated cultures laking

combined nitrogen under most of the environmental factors tested.

4. Of the environmental factors studied; light is likely to play an important role in the early stage of rice cultivation when the rice-fields were covered by water. Temperature and salinity would be critical at the later stages and after harvest harvest, when the soil dries out and high temperature and salinity occurs.

5. The ability of Nodularia harveyana and Anabaena oscillarioides to grow at high levels of salinity is of particular importance in an area where salinity is the major agricultural problem. These organisms require further study.

6. It seems likely that alkaline phosphatase activity and organic and unhydrous phosphorus utilization are important phenomena in the field. One of the beneficial effects of algae in the rice-fields may be the mineralization of phosphorus compounds.

7. Active nitrogenase activity of heterocystous forms, together with the ability of some of these strains to produce akinetes suggested that these strains are capable to withstand the extreme environmental conditions could and act as an inoculum for the next cultivation cycle.

8. There is an urgent need to study the role of algae in these fields over a whole year. Thus, it could be

established whether changes in cultivation practice could be used to increase the phosphorus and nitrogen status of the soil for both rice plants and winter crops without the necessity to introduce chemical fertilizers of these essential elements.

SUMMARY

1. An intensive laboratory study was made of algae in one soil sample taken from a small area in a field of relatively mature rice in the southern marshes of Iraq.

2. From this sample 49 species were identified after various enrichment culture techniques as opposed to 11 by direct microscopic inspection of a wetted sample.

3. 35 species were Cyanophyta, 12 Chlorophyta, one Euglenophyta and one Xanthophyta. 18 of the Cyanophyta were heterocystous.

4. Detailed descriptions were made of the major blue-green algal species both in mixed populations and when brought into clonal and/or axenic culture. These descriptions were based both on growth under standard growth conditions and under a range of phosphorus and nitrogen enrichments. Many of the strains isolated showed marked morphological variability.

5. The effects of a wide range of environmental factors likely to be important in the field were tested on mixed populations (commencing with 5 mg of thoroughly mixed soil).

6. Increase in algal biomass was negligable at 5 ^{o}C. At 48 ^{o}C live filaments of <u>Lyngbya</u> ssp. were at first visible, but subsequently these all died. Rapid growth of the mixed

populations occurred at temperatures between 15°C and 45 ^{o}C.

7. Blue-green algae dominated most of the cultures at temperaturs between 25-45 ^{o}C, with heterocystous forms predominant in - N medium. Eukaryotic algae dominated cultures in + N medium at low temperatures.

8. Non-heterocystous forms dominated most of the cultures at low photon flux densities (15-20 umol photon $m^{-2}s^{-1}$) while green algae dominated cultures at high photon flux densities (200-250 umol photon $m^{-2}s^{-1}$). Heterocystous blue-green algae dominated -N cultures at photon flux densities between 50-120 umol photon $m^{-2}s^{-1}$.

9. Neither dark/light cycles nor the use of green light affected markedly the algal flora, but the yield of mixed populations was very low under green light.

10. Six taxa showed good growth in medium enriched with 0.5 M NaCl; three taxa grew in 1 M NaCl, but the growth was very slow.

11. Reducing conditions (sulphide) enhanced only slightly the growth of non-heterocystous filamentous blue-green algae in + N medium.

12. A high phosphorus concentration (44.5 mg l^{-1} P) led to a reduction of the total number of taxa detected at the end of growth in batch culture as compared with growth under low concentrations (0.5 and 2.5 mg l^{-1}P).

13. All strains isolated in axenic culture showed alkaline phosphatase activity associated with algal material and all, except strains D583 and D585, released appreciable amounts of the enzyme to the growth medium under phosphorus-deficient conditions.

14. Very high phosphorus concentrations (44.5 mg l^{-1}P) inhibited the formation of akinetes and hairs and the synthesis of alkaline phosphatase.

15. The strains isolated were capable of utilizing a wide range of phosphorus compounds.

16. The ability of the axenic cultures of heterocystous forms to fix nitrogen was demonstrated using acetylene reduction assays. The rates ranged between 0.31-1.59 nmol C_2H_4 ug^{-1}chl a min^{-1}.

17. Nitrogenase activity of all strains was almost completely inhibited when these strains were cultured in media with high concentrations of combined nitrogen (140 mg l^{-1} NO_3-N or 50 mg l^{-1} NH_4-N).

18. High concentrations of combined nitrogen led to a marked reduction in heterocyst differentiation and of the formation of trichomes resembling those of *Homoeothrix* or *Ammatoidea* in tapered strains.

19. None of the non-heterocystous strains showed nitrogenase activity under aerobic conditions. However, positive results

were found for _Microcoleus_ _chthonoplastes_ under microaerobic conditions.

20. The abundance of blue-green algae in the soil sample studied, their ability to produce alkaline phosphatase and to utilize a wide range of phosphorus compounds suggests that blue-green algae are likely to play an important role in soil fertility through nitrogen fixation and hydrolysis of organic phosphorus compounds. It is suggested that morphological features may provide a means of assessing nutrient status in the field.

REFERENCES

Aaronson S. and Patni N. J. (1976) The role of surface and extracellular phosphatases in the phosphorus requirement of Ochromonas. Limnol. Oceanogr. 21(6), 838-845.

Abeliovich A. and Shilo M. (1972) Photooxidative death in blue-green algae. J. Bact. 111, 682-689.

Ahlgren G. (1978) Growth of Oscillatoria agardhii in chemostat culture. 2. Dependence of growth constants on temperature. Mitt. int. Verein. theor. angew. Limnol. 21, 88-102.

Ali S. and Sandhu G. R. (1972) Blue-green algae of the saline soils of the Punjab. Oikos 23, 268-272.

Al-Kaisi K. A. (1976) Contribution to the algal flora of the rice-fields of the southeastern Iraq. Nova Hedwigia 27, 813-827.

Al-Kholy A. A. (1956) On the assimilation of phosphorus in Chlorella pyrenoidosa. Physiol. Plant. 9, 137-143.

Allen M. B. (1963) Nitrogen-fixing organisms in the sea. Oppenheimer C. H. (Ed.) Symposium on Marine Microbiology. pp. 85-92. Springfield, Illinois.

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

Allen M. M. (1968) Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. 4, 1-4.

Allen M. M. and Stanier R. Y. (1968) Selective isolation of blue-green algae from water and soil. J. gen. Microbiol. 51, 203-209.

Al-Mousawi A. H. A. and Whitton B. A. (1983) Influence of environmental factors on algae in rice-field soil from the Iraqi marshes. Arab Gulf J. Scientific Res. 1(1), 237-253.

Al-Saadi H. A., Antoine S. E. and Nurul Islam A. K. M. (1981) Limnological investigation in Al-Hammara marsh area in southern Iraq. Nova Hedwigia 35, 157-166.

American Public Health Association (1981) Standards Methods for the Examination of Water and Wastwater, 15th Edn. 1143 pp. American Public Health Association, Inc., New York.

Anagnostidis K., Economou-Amilli A. and Tsangridis A. (1981) Taxonomic and floristic studies on algae from rice-fields of Kalochorion-Thessaloniki, Greece. Nova Hedwigia 34, 1-66.

Anantani Y. S. and Vaidya B. S. (1983) Growth characteristic and pigment levels in a halotolerant cyanophyte. Phykos 22, 101-104.

Antarikanonda P. (1982) Effect of salinity on growth, nitrogen fixation and sodium uptake of rapidly growing nitrogen-fixing blue-green alga Anabaena sp. TA1. Microbios 34, 177-184.

Antarikanonda P. and Lorenzen H. (1982) Nitrogen-fixing blue-green algae (Cyanobacteria) of high efficiency from paddy soils of Bangkok, Thailand: characterization of

species and nitrogen-fixing capacity in the laboratory. Arch. Hydrobiol. suppl. 63(1), 53-70.

Apte S. K. and Thomas J. (1980) Sodium is required for nitrogenase activity in Cyanobacteria. Curr. Microbiol. 3, 291-293.

Archibald P. A. and Bold H. C. (1970) Pycological studies Xl. The genus Chlorococcum meneghini. The University of Texas Publication 7015. 115pp.

Armstrong F. A. J. (1965) Phosphorus. In: Riley J. P. and Skirrow G. (Eds) Chemical Oceanography vol. 1. pp. 323-364. Academic Press, New York.

Arora S. K. (1969) Blue-green algae: a source of nitrogen and organic matter in paddy fields. Riso 18, 63-66.

Balandreau J. P., Rinaudo G., Oumarov M. M. and Dommergues Y. R. (1976) Asymbiotic nitrogen fixation in paddy soils. In: Newton W. E. and Nyman C. J. (Eds). Proceeding of the First International Symposium on Nitrogen Fixation. pp. 611-618. Washington State University Press, Pullman.

Barraquio W. L., de Guzman M. R., Barrion M. and Watanabe I. (1982) Population of aerobic heterotrophic nitrogen-fixing bacteria associated with wetland and dryland rice. appl. environ. Microbiol. 43(1), 124-128.

Batterton J. C. and Van Baalen C. (1968) Phosphorus deficiency and phosphate uptake in blue-green alga Anacystis nidulans. Can. J. Microbiol. 14, 341-348.

Belay A. and Fogg G. E. (1978) Photoinhibition of photosynthesis in Asterionella formosa (Bacillariophyceae). J. Phycol. 14, 341-347.

Bernstein L. and Hayward H. E. (1958) Physiology of salt tolerance. A. Rev. Plant Physiol. 9, 25-46.

Bold H. C. and Wynne M. J. (1978) Introduction to the Algae. 706pp. Prentice-Hall, Inc., Englewood, New Jersey.

Bone D. H. (1971a) Relationship between phosphates and alkaline phosphatase of Anabaena flos-aquae in continuous culture. Arch. Microbiol. 80, 147-153.

Bone D. H. (1971b) Nitrogenase activity and nitrogen assimilation in Anabaena flos-aquae growing in continuous culture. Arch. Microbiol. 80, 234-241.

Bose P., Nagpal U. S., Venkataraman G. S. and Goyal S. K. (1971) Solubilization of tricalcium phosphate by blue-green algae. Curr. Sci. 5(7), 165-166.

Bothe H. (1982) Nitrogen Fixation. In: Carr N.G. and Whitton B.A. (Eds.) The Biology of Cyanobacteria. pp. 87-104. Blackwell, Oxford.

Bothe H. and Eisbrenner G. (1977) Effect of 7-azatryptophan on nitrogen fixation and heterocyst formation in the blue-green alga Anabaena cylindrica. Biochem. Physiol. Pflanz. 171, 323-332.

Bottomley P. J., Grillo J. F., Van Baalen C. and Tabita F. (1979) Synthesis of nitrogenase and heterocysts by Anabaena sp. (CA) in presence of high level of ammonia J. Bact. 140, 938-943.

Bottomley P. J. and Stewart W. D. P. (1977) ATP and nitrogenase activity in nitrogen-fixing blue-green algae. New Phytol. 79, 625-638.

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.

Bradley S. and Carr N. G. (1976) Heterocyst and nitogenase development in Anabaena cylindrica. J. gen. Microbiol. 96, 175-184.

Brill W. J. (1975) Regulation and genetics of nitrogen fixation. A. Rev. Microbiol. 29, 109-129.

Brown R. M., Jr and Bischaffe H. W. (1962) A new and useful method for obtaining axenic cultures of algae. Phycol. News Bull. 15(4), 43-44.

Brown T. E. and Richardson R. L. (1968) The effect of growth environment on the physiology of algae, light intensity. J. Phycol. 4, 38-54.

Brunskill G. J. (1973) Rates of supply of nitrogen and phosphorous to Lake Winnipeg, Manitoba, Canada. Verh. int. Verein. theor. angew. Limnol. 18, 1755-1759.

Bunt J. S. (1961) Nitrogen-fixing blue-green algae in Australian rice soils . Nature, lond. 192, 479-480.

Buringh P. (1960) Soils and Soil Conditions in Iraq. Longman Green and Co. Ltd., London.

Carpenter E. J. and Price C. C. (1976) Marine Oscillatoria (Trichodesmium): explanation for aerobic nitrogen fixation without heterocysts. Science, N.Y. 191, 1278-1280.

Castenholz R. W. (1973) Ecology of blue-green algae in hot springs. In: Carr N. G. and Whitton B. A. (Eds). The Biolgoy of Blue-green Algae. pp. 379-414. Blackwell, Oxford.

Catling H. D., Martinez M. R. and Islam Z. (1981) Survey of algae associated with deepwater rice in Bangladesh. Cryptogamie, Algologie 11(2), 109-121.

Chakravarty H. L. (1976) Plant Wealth of Iraq, Vol. 1. pp. 398-432. Botany Directorate, Baghdad, Iraq, pp.398-432.

Chang T. P. (1979) Zur morphologie von Gloeotrichia echinulata (Smith) Richter in axenischer Kulture. Nova Hedwegia 31, 265-284.

Chapman V. J. and Chapman D. J. (1973) Soil algae and symbiosis. In: Chapman V. J. and Chapman D. J. (Eds). The Algae. pp. 381-387. MacMillan, London.

Chen M. (1974) Kinetics of phosphorous absorption by Corybacterium bovis, Microb. Ecol. 7, 164-175.

Chen P. C. (1983) Effect of nitrogen sources on nitrogen fixation of Anabaena species isolated from paddy soils Proc. Nat. Sci. Counc. B. Roc. 7(3), 317-321.

Cheshire M. V. and Anderson G. (1975) Soil polysaccharides and carbohydrates phosphates. Soil Sci. 119(5), 356-362.

Chu S. P. (1946) The utilization of organic phosphorous by phytoplankton. J. mar. Biol. Ass. U.K. 26, 285-295.

Clesceri N. L. and Lee G. F. (1965) Hydrolysis of condensed phosphates. II. Sterile environment. Int. J. Air Wat. Pollut. 9, 743-751.

Collyer D. M. and Fogg G. E. (1955) Studies on fat accumulation by algae. J. exp. Bot. 6, 256-275.

Cosgrove D. J. (1967) Metabolism of organic phosphates in soil. pp. 216-228 In: McLaren A. D. and Peterson G. H. (Eds) Soil Biochemistry. Edward Arnold, London.

Cox R. M. and Fay P. (1969) Special aspects of nitrogen fixation by blue-green algae. Proc. R. Soc. Lond. B. 142, 357-366.

Coxson D. S. and Kershaw K. A. (1983) Nitrogenase activity during Chinook snowmelt sequences by Nostoc commune in Stipa-Bouteloa grassland. Can. J. Microbiol. 29(8), 938-944.

Davey A. (1983) Effects of abiotic factors on nitrogen fixation by blue-green algae in Antarctica. Polar Biol. 2, 95-100.

Davis E. M. and Wilcomb M. J. (1967) Enzymatic degradation and assimilation of condensed phosphates by green algae. Wat. Res. 1, 335-350.

Davis E. M. and Wilcomb M. J. (1968) Condensed phosphate degradation and assimilation by selected blue-green algae. Wat. Res. 2, 311-324.

Dawson R. M. C. and Elliott W. H. (1959) III. Buffers and physiological media. In: Dawson R. C. M., Elliott D. C., Elliott W. H. and Jones K. M. (Eds). Data For Biochemical Research. pp. 139-209. Oxford University Press.

De P. K. and Mandal L. N. (1958) Fixation of nitrogen by algae in rice soil. Soil Sci. 81(6), 453-458.

Desikachary T. V. (1959) Cyanophyta. 686pp. Indian Council of Agricultural Research.

Doonan B. B. and Jensen T. E. (1979) Effects of ions on the activity of the enzyme alkaline phosphatase from Plectonema boryanum. Microbios 25, 177-186.

Doonan B. B. and Jensen T. E. (1980) Pysiological aspects of alkaline phosphatase in selected Cyanobacteria. Microbios 29, 185-207.

Dugdale R. C., Menzel D. W. and Ryther J. H. (1961) Nitrogen fixation in the Sargasso Sea. Deep-Sea Res. 7, 298-300.

Ehrlich H. L. (1981) Geomicrobial transformations of phosphorus compounds. Advan. Microbiol. Ecol. 5, 63-89.

Eisenreich S. J., Bennerman R. T. and Armstrong D. E. (1975) A simplified phosphorus analysis technique. Environ. Lett. 9(1), 43-53.

El-Nawawy A. S., Ibrahim A. N. and Aboul-Fadl M. (1968) Nitrogen fixation by Calothrix sp. as influenced by certain sodium salts and nitrogenous compounds. Acta Agron. Acad. Sci. Hung. 17(3-4), 323-327.

Eloff J. N., Steinitz Y. and Shilo M. (1976) Photooxidation of Cyanobacteria in natural conditions. appl. environ. Microbiol. 31, 119-126.

Fay P. (1969) Cell differentiation and pigment composition in Anabaena cylindrica. Arch. Mikrobiol. 67, 62-70.

Fay P. (1976) Factors influencing dark nitrogen fixation in a blue-green alga. appl. environ. Microbiol. 31, 376-379.

Fay P. (1983) The Blue-Greens. Studies in Biology no. 160, 88pp. Edward Arnold, London.

Fay P., Stewart W. D. P., Walsby A. E. and Fogg G. E. (1968) Is the heterocyst the site of nitrogen fixation in blue-green algae? Nature, Lond. 220, 810-812.

Feder J. (1973) The phosphatases. In: Griffith E. J., Beeton A., Spencer J. M. and Mitchell D. T. (Eds). Environmental Phosphorus Handbook. pp. 475-508. John Wiley and Sons, New York.

Fernandes T. A. and Thomas J. (1982) Control of sporulation in the filamentous cyanobacterium Anabaena torulsa J. Biosci. 4 (1), 85-97.

Fernandez M. P., Gascon S. and Schwencke J. (1981) Some enzymatic properties of vascular alkaline phosphatase from yeast. Curr. Microbiol. 6, 121-126.

Fisher R. W. and Wolk C. P. (1976) Substance stimulating the differentiation of spores of the blue-green alga Cylindrospermum licheniforme. Nature, Lond. 259, 394-395.

Fitzgerald G. P. (1970) Evaluations of the availability of sources of nitrogen and phosphorus for algae. J. Phycol. 6, 239-247.

Fitzgerald G. P. and Nelson T. C. (1966) Extractive and enzymatic analyses for limiting or surplus phosphorus in algae. J. Phycol. 2, 32-37.

Fogg G. E. (1956) The compararive physiology and biochemistry of the blue-green algae. Bact. Rev. 20, 148-165.

Fogg G. E. (1969) The physiology of an algal nuisance. Proc. R. Soc. London B. 173, 175-189.

Fogg G. E. (1973) Phosphorus in primary aquatic plants. Wat. Res. 7, 7-91.

Fogg G. E. (1974) Nitrogen fixation. In : Stewart W. D. P. (Ed.) Algal Physiology and Biochemistry. pp. 560-582. Blackwell, Oxford.

Fogg G. E. (1975) Algal cultures and Phytoplankton Ecology. 175pp. 2nd Edn. University of Wisconsin Press, Wisconsin, U.S.A.

Fogg G. E. and Stewart W. D. P. (1968) In situ determination of biological nitrogen fixation in Antarctica. Br. Antarctic Surv. Bull. 15, 39-46.

Fogg G. E., Stewart W. D. P., Fay P. and Walsby A. E. (1973) The Blue-Green Algae. 459pp. Academic Press, London.

Foy R. H. and Gibson C. E. (1982) Photosynthetic characteristics of planktonic blue-green algae: The response of twenty strains grown under high and low light. Br. phycol. J. 17(2), 169-182.

Frémy P. (1933) Cyanophycees. 235pp. des cotes d Europe. Mem. Soc. Nat. et math. Cherbourg. 41.

Gallon J. R., Kurz W. G. W. and Larue T. A. (1973) Isocitrate supported nitrogenase activity in Gloeocapsa sp. LB794. Can. J. Microbiol. 19, 461-465.

Geitler L. (1932) Cyanophyceae. 1196pp. L. Rabenhorst s Kryptogamen-Flora vol. 14. Akadememische Verlagsgellschaft, Leipzig.

General Establishment for Studies and Design (1979) Shatt Al-Arab Project, Vol. 3 Part A Text, Basrah, Iraq.

Gentile J. H. and Maloney T. E. (1969) Toxicity and environmental requirements of a strain of _Aphanizmenon flos-aquae_ (L.) Ralfs. Can. J. Microbiol. 15, 165-173.

Glew R. H. and Heath E. C. (1971) Studies on the extracellular alkaline phosphatase of _Micrococcus sodenensis_. J. Biol. Chem. 246(6), 1556-1565.

Gerloff G. C., Fitzgerald G. P. and Skoog F. (1950) The isolation, purification and culture of blue green algae. Amer. J. Bot. 37, 216-218.

Golachowska J. B. (1979) Phosphorus forms and their seasonal changes in water and sediments of lake PluBsee. Arch. Hydrobiol. 86, 217-241.

Good N. E., Winget G. D., Winter W., Connoly T. N., Izawa S. and Singh R. M. M. (1966) Hydrogen ion buffers for biological research. Biochemistry, N.Y. 5(2), 467-477.

Gotto J. W., Tabita F. R. and Van Baalen C. (1979) Isolation and characterization of rapidly-growing marine nitrogen-fixing strains of blue-green algae. Arch. Microbiol. 121, 155-159.

Grillo J. F. and Gibson J. (1979) Regulation of phosphate accumulation in the unicellular cyanobacterium <u>Synechococcus</u>. J. Bact. 140, 508-517.

Guerts van Kessel A. H. M., Mullahy J. H. and Linjkens H. F. (1977) The relation of heterocysts and hormogonia to nitrogen fixation and reproduction in blue-green algae. Plant Syst. Evol. 128, 171-176.

Guest E. (1966) <u>Flora of Iraq</u>. vol 1. 213pp. Ministry of Agriculture, Baghdad, Iraq.

Gupta A. B. (1966) Algal flora and its importance in the economy of rice-fields. Hydrobiologia. 28,213-222.

Habte M. and Alexander M. (1980) Nitrogen fixation by photosynthetic bacteria in lowland rice culture. appl environ. Microbiol. 39(2), 342-347.

Hamdi Y. A., Yousef A. N., Al- Azawi S.,Al-Tai A. and Al-Baquari M. S. (1978) Distribution of certain non-symbiotic nitrogen-fixing organisms in Iraqi soils. In: Environmental role of nitrgen-fixing blue-green algae and asymbiotic bacteria. Ecol. Bull., Stockholm 26, 110-115.

Happey-Wood C. M. (1982) "The slime-problem"-algal ecology of rice-fields in southeastern Australia (Abstract). Br. phycol. J. 17(2), 233.

Hardy R. W. 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.

Harold F. M. and Spitz E. (1975) Accumulation of arsenate, phosphate and aspartate by Streptococcus faecalis. J. Bact. 122, 266-277.

Harwood J. E., van Steenderen R. A. and Kuhn A. L. (1969) A comparison of some methods for total phosphorus analyses. Wat. Res. 3, 425-432.

Healey F. P. (1973) Characteristics of phosphorus deficiency in Anabaena. J. Phycol. 9, 383-394.

Healey F. P. (1975) Physiological indicators of nutrient deficiency in algae. Fish. Mar. Serv. Res. Tech. Rep. 585, 30pp.

Healey F. P. (1978) Physiological indicators of nutrients in algae. Mitt. Int. verein. Limnol. 21, 34-41.

Healey F. P. (1982) Phosphate. In: Carr N. G. and Whitton B. A. (Eds) The Biology of Cynanobacteria. pp. 105-124. Blackwell, Oxford.

Healey F. P. and Hendzel L. L. (1975) Effect of phosphorus deficiency on two algae growing in chemostats. J. Phycol. 11, 303-309.

Heath R. T. and Cooke G. D. (1975) The significance of alkaline phosphatase in a eutrophic lake. Verh. int. Verein. theor. angew. Limnol. 19, 959-965.

Heckman C. W. (1979) Rice field ecology in the North eastern Thailand. Monog. Biol. 34, 1-22.

Hinton G. C. F. and Maulood B. K. (1980) Some diatoms from brakish water habitats in southern Iraq. Nova Hedwigia 33, 487-497.

Holmes N. T. H. and Whitton B. A. (1981) Phytobenthos of the River Tees and its tributaries. Freshwater Biol. 11, 139-163.

Holst R. W. and Yopp J. H. (1979) Environmental regulation of nitrogenase and nitrate reductase as systems of nitrogen assimilation in the _Azolla-mexicana-Anabaena azollae_ symbiosis. Aquat. Bot. 7, 369-384

Horne A. J. (1972) The ecology of nitrogen fixation on Signy Island, South Orkney Island. Br. Antart. Surv. Bull. 27, 1-18.

Hoshaw R. W. and Rosowski J. R. (1973) Methods for microscopic algae. In: Stein J. R. (Ed.) _Handbook of Phycological Methods_. pp. 53-67. Cambridge University Press, Cambridge.

Howsley R. and Pearson H. W. (1981) Ecological and physiological considerations of sulphide toxicity in different groups of blue-green algae. (Abstract) Br. phycol. J. 16(2), 137.

Hutchinson G. E. (1957) _A Treatise on Limnology_. Vol. 1. John Wiley and Sons, Inc., New York.

Ichimura S. (1954) Ecological studies on the plankton in paddy fields. I. Seasonal fluctuations in the standing crop and productivity of plankton. Jap. J. Bot. 14, 269-279.

Ihlenfeldt M. J. A. and Gibson J. (1975) Phosphate utilization and alkaline phosphatase activity in _Anacystis nidulans_ (_Synechococcus_). Arch. Microbiol. 102, 23-28.

Jensen T. E. and Sicko L. M. (1974) Phosphate metabolism in blue-green algae. I. Fine structure of the "polyphosphate overplus" phenomenon in _Plectonema boryanum_. Can J. Microbiol. 9, 1235-1239.

Jensen T. E. and Sicko-Coad L. M. (1976) Phosphate metabolism in blue-green algae. II. Changes in phosphate distribution during starvation and the "polyphosphate overplus" phenomenon in _Plectonema boryanum_. Amer. J. Bot. 63, 183-188.

Johannes R. E. (1964) Uptake and release of dissolved organic phosphorus by representatives of a costal marine ecosystem. Limnol. Oceanogr. 9, 224-234.

Johannes R. E. (1965) Influence of marine protozoa on nutrient regenaration. Limnol. Oceanogr. 10, 434-435.

Jones K. (1977) The effects of light intensity on acetylene reduction by blue-green algal mats in sub-tropical grassland. New Phytol. 78, 427-431.

Jones K. and Stewart W. D. P . (1969) Nitrogen turnover in marine and brakish habitats. III. The production of extracellular nitrogen by Calothrix scopulorum. J. mar. Biol. Ass. U. K. 49, 475-488.

Kantz T. and Bold H. C. (1969) Morphological and taxonomical investigations of Nostoc and Anabaena in culture. Univ. Texas Publ. No. 6924, 1-67.

Kashyap A. K., Pandey K. D. and Rai R. (1983) Sulphide dependent growth and nitrogen fixation by heterocystous blue-green algae. Plant and Nature 1(1), 44-52.

Kenyon C. N., Rippka R. and Stanier R. Y. (1972) Fatty acid composition and physiological properties of some filamentous blue-green algae. Arch. Mikrobiol. 83, 216-236.

Ketchum B. H. (1954) Mineral nutrition of phytoplankton. A. Rev. Plant Physiol. 5, 55-74.

Khan M. and Mathur A. (1976) Algal flora of the rice-fields around Dehrandum, India. Rev. Algol. N. S. 11, 333-337.

Khoja T. M., Livingstone D. and Whitton B. A. (1984) Ecology of a marine Rivularia population. Hydrobiologia. 108, 65-73.

Kikuchi E., Furusaka C. and Kurihara Y. (1977) Effects of Tubificids (Branchiura sowerbyi and Limnodrilus socialis) on the nature of a submerged soil ecosystem. Jap. J. Ecol. 27, 163-170.

Kirkby S. M. (1975) Biological studies on the Rivulariaceae. 393 pp. Ph. D. Thesis, University of Durham, England.

Kobayashi M. and Hague M. Z. (1971) Contribution to nitrogen fixation and soil fertility by photosynthetic bacteria. In: Lie T. A. and Mulder E.G. (Eds) Biological Nitrogen Fixation in Natural and Agricultural Habitats. pp. 443-456. Nijhoff, The Hague.

Kratz W. A. and Myers J. (1955) Nutrition and growth of several blue-green algae. Amer. J. Bot. 42(3), 282-287.

Kuenzler E. J. (1965) Glucose-6-phosphate utilization by marine algae. J. Phycol. 1, 156-164.

Kuenzler E. J. (1970) Dissolved organic phosphorus excretion by marine phytoplankton. J. Phycol. 6, 7-13.

Kuenzler E. J. and Perras J. P. (1965) Phosphatases of marine algae. Biol. Bull. Mar. biol. Lab., Woods Hole 128, 271-284.

Kuhl A. (1962) Inorganic phosphorus uptake and metabolism. In: Lewin R. A. (Ed.) Physiology and Biochemistry of Algae. pp. 211-229. Academic Press, New York.

Kuhl A. (1974) Phosphorus. In: Stewart W. D. P. (Ed.) Algal Physiology and Biochemistry. pp. 636-654. Blackwell, Oxford.

Kulasooriya S. A., Roger P. A. and Watanabe I. (1980) Relationship between blue-green algae growth and the standing crop of wetland rice fields. Int. Rice Res. News Lett. 5(1), 18-19.

Lam C. W. Y. (1979) Phosphate requirement of Anabaena oscillarioides and its ecological implications. Hydrobiologia 67(1), 89-96.

Langowska I. (1982) Utilization of organic phosphorus by selected bacteria and algae. Wat. Res. 16, 161-167.

Lean D. R. S. (1973) Movements of phosphorus between its biologically important forms in lake water. J. Fish. Res. Bd Can. 30, 1525-1536.

Lean D. R. S. and Nalewajko C. (1976) Phosphate exchange and organic phosphorus excretion by fresh water algae. J. Fish. Res. Bd Can. 33, 1312-1323.

Livingstone D., Khoja T. M. and Whitton B. A. (1983) Influence of phosphorus on physiology of a hair-forming blue-green alga (Calothrix parietina) from an upland stream. Phycologia 22(4), 345-350.

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

Lund J. W. G. (1962) Soil algae. In Lewin R. A. (Ed.) Physiology and Biochemistry of algae. pp. 759-770. Academic Press, New York

Mackereth F. J. (1953) Phosphorus utilization by Asterionella formosa Hass. J. exp. Bot. 4(12), 296-313.

Mackereth F. J. H., Heron J. and Talling J. F. (1978) Water Analysis: Some Rivised Methods for Limnologists. Freshwater Biol. Assoc. U. K. Sc. Publ. No. 36.

Marker A. F. H., Nusch E. A., Rai H. and Riemann B. (1980) The measurement of photosynthetic pigments in freshwaters and standardization of methods: conclusions and recommendations of the workshop. Arch. Hydrobiol./Beih. Ergebn. Limnol. 14, 91-106.

Materasi R. and Balloni W. (1965) some observations on the presence of autotrophic nitrogen-fixing microorganisms in paddy soils (Abstract). Ann. Inst. Pasteur. 109, 218-223.

Maulood B. K., Hinton G. C. F., Kamees H. S., Saleh F. A. K., Shaban A. A. and Al-Shahwani S. M. H. (1979) An ecological survey of some aquatic ecosystems in southern Iraq. Tropical Ecology 20(1), 27-40.

Maulood B. K., Hinton G. C. F., Whitton B. A. and Al-Saadi H. A. (1981) On the algal ecology of the lowland Iraqi marshes. Hydrobiologia 80, 269-276.

Metting B. (1981) The systematics and ecology of soil algae. Bot. Rev. 47, 195-312.

Miller J. D. A. and Fogg G. E. (1957) Studies on the growth of Xanthophyceae in pure culture. I. The mineral nutrition of Monodus subterraneus Peterson. Arch. Mikrobiol. 28, 1-77.

Moore A. W. (1969) Azolla: biology and agronomic significance. Bot. Rev. 35, 17-34.

Mullineaux P. M., Gallon J. R. and Chaplin A. E. (1981) Nitrogen fixation in cultures of the Cyanobacteria Gloeocapsa (Gloeothece) sp. 1430/3 incubated in the dark. J. gen. Microbiol. 124, 141-146.

Mur L. R., Gons H. J. and van Liere L. (1978) Competition of the green alga Scenedesmus and the blue-green Oscillatoria. Mitt. int. Verein. theor. angew. Limnol. 21, 473-479.

Murphy J. and Riley J. P. (1962) A modified single solution method for the determination of phosphate in natural waters Anal. Chim. Acta 27, 31-36.

Murry M. A., Jensen D. B. and Benemann J. R. (1983) Role of ammonia in regulation of nitrogenase synthsis and activity in Anabaena cylindrica. Biochim. Biophys. Acta 756, 13-19.

Myers J. (1951) Physiology of the algae. A. Rev. Microbiol. 5, 157-180.

Nagatani H. H. and Haselkorn R. (1978) Molybdenum independence of nitrogenase component synthesis in the non-heterocystous Cyanobacteria Plectonema. J. Bact. 134,597-605.

Nicholas J. M. and Adams D. G. (1982) Akinetes In: Carr N. G. and Whitton B. A. (Eds) The Biology of Cyanobacteria. pp. 387-412. Blackwell, Oxford.

Nordin R. N. and Stein J. R. (1980) Taxonomic revision of *Nodularia* (Cyanophyceae/Cyanobacteria). Can. J. Bot. 58(11), 1211-1224.

Ogawa R. E. and Carr J. E. (1969) The influence of nitrogen on heterocyst production in blue-green algae. Linmol. Oceanogr. 14, 342-351.

Ohmori M. and Hattori A. (1972) Effect of nitrate on nitrogen fixation by the blue-green alga *Anabaena cylindrica*. Pl. Cell Physiol., Tokyo 13, 589-599.

Ohmori M. and Hattori A. (1974) Effect of ammonia on nitrogen fixation by the blue-green alga *Anabaena cylindrica*. Pl. Cell Physiol. Tokyo 15, 131-142.

Ohmori M. and Hattori A. (1978) Transient change in ATP pool of *Anabaena cylindrica* associated with ammonium assimilation. Arch. Microbiol. 117, 17-20.

O'Kelley J. C. (1973) Phosphorus nutrition of algae. In: Griffith E. J., Beeton A., Spencer J. M. and Mitchell D.

T. (Eds) Environmental Phosphorus Handbook. pp. 443-449. John Wiley and Sons, New York.

Okuda A. and Yamaguchi M. (1952) Algae and atmospheric nitrogen fixation in paddy soils. II. Relation between the growth of blue-green algae and physical and chemical properties of soil and effect of soil treatments and inoculation on the nitrogen fixation. Mem. Res. Inst. Food Sci. 4, 1-11.

Pandey D. C. (1965a) A study on the algae from paddy field soils of Ballia and Ghazipur districts of Uttar Pradesh, India. I. Culture and ecological conditions. Nova Hedwigia. 9, 299-334.

Pandey D. C. (1965b) A study on the algae from paddy field soils of Ballia and Ghazipur districts of Uttar pradesh, India. II(A). Taxonomic considerations-Cyanophyceae. Nova Hedwigia. 10, 177-209.

Pandey R. K. and Talpasayi E. R. S. (1980) Control of sporulation in a blue-green alga *Nodularia spumigena* Mertens. Ind. J. Bot. 3, 128-133.

Pankow H., Al-Saadi H. A., Huq M. F. and Hadi R. A. M. (1979) On the algal flora of the marshes near Qurna (southern Iraq). Willdenowia 8, 493-506.

Patni N. J. and Aaronson S. (1974) Partial characterization of the intra and extracellular acid phosphatase of an alga, Ochromonas danica. J. gen. Microbiol. 83, 9-20.

Patrick R. (1969) Some effect of temperature on fresh water algae. In: Krenkel P. A. and Parker F. L. (Eds) Biological Aspects of Thermal Pollution. pp. 161-185. Vanderbilt University Press, Nashville, Tenn.

Patterson G. (1983) Effects of heavy metals on freshwater Chlorophyta. 212pp. Ph. D. Thesis, University of Durham, England.

Pearson H. W., Malin G. and Howsley R. (1981) Physiological studies on in vivo nitrogenase activity by axenic culture of the blue-green alga Microcoleus chthonoplastes. (Abstract). Br. phycol. J. 16, 139.

Perry M. J. (1972) Alkaline phosphatase activity in subtropical central North Pacific waters using a

sensitive fluorometric method. Mar. Biol. 15, 113-119.

Peters G. A. (1978) Blue-green algae and algal association. Bio. Sci. 28,580-585.

Peterson R. B. and Burris R. H. (1976) Properties of heterocysts isolated with colloidal silica. Arch. Microbiol. 108, 35-40.

Peterson R. B., Friberg E. E. and Burris R. H. (1977) Diurnal variation in nitrogen fixation and photosynthesis by aquatic blue-green algae. Plant Physiol. 59, 74-80.

Petrovska L. and Stojanov P. (1972) Periodchni promeni vo alginite zaednitsi na orizishtata vo Kotsansko. (Periodische veranderung der algengemeinschaften auf den Riesfeldern in der umgebung von Kotschani). Skopje Univ. Prir. Mat. Fak. God. Zb. Biol. Annu. Biol. 24, 103-111. (cited by Tsangridis, 1982).

Postgate J. R. (1972) The acetylene reduction test for nitrogen fixation. In: Norris J. R. and Robisons D. W. (Eds) Methods In Microbiology. Vol. 6B. pp. 343-365. Academic Press, New York.

Potman W. and Lijklema L. (1983) Inositolphosphate, hydrolysis, stability and analysis. Wat. Res. 17(4), 411-414.

Potts M. (1977) Studies on blue-green algae and photosynthetic bacteria in the lagoon of Aldabra Atoll. 421pp. Ph. D. Thesis, University of Durham, England.

Potts M. and Friedmann E. I. (1981) Effect of water stress on cryptoendolithic Cyanobacteria from hot desert rocks. Arch. Microbiol. 130, 267-271.

Potts M. and Whitton B. A. (1977) Nitrogen fixation by blue-green algal communities in the intertidal zone of Aldabra Atoll. Oecologia, Berl. 27, 275-283.

Potts M. and Whitton B. A. (1980) Vegetation of the intertidal zone of the lagoon of Aldabra, with particular reference to the photosynthetic prokaryotic communities. Proc. Roy. Soc. Lond. B. 208, 13-55.

Provasoli L. (1958) Nutrition and ecology of protozoa and algae. A. Rev. Microbiol. 12, 279-308.

Rai A. K., Pandey K. D. and Kashyap A. K. (1978) Heterocyst differentiation and polarity in Calothrix. New Phytol. 81, 647-651.

Ramos J. L. and Guerrero M. G. (1983) Involvement of ammonium metabolism in the nitrate inhibition of nitrogen fixation in Anabaena sp. strain ATCC 33047. Arch. Mikrobiol. 136, 81-83.

Rao P. S. N. and Talpasayi E. R. S. (1982) Culture studies on Anabaena fertilissima from Sambhar salt work. Ind. J. exp. Biol. 20,588-589.

Rhee G. Y. (1973) A continuous culture study of phosphate uptake, growth rate and polyphosphate in Scenedesmus sp. J. Phycol. 9, 495-506.

Rinaudo G. (1974) Biological nitrogen fixation in three types of rice soil in Ivory Coast. (Summary). Rev. Ecol. Biol. Sol. 11, 149-168.

Rippka R., Deruelles J., Waterbury J. B., Herdman M. and

Stanier R. Y. (1979) The Cyanobacteria. J. gen. Microbiol. 111, 1-61.

Rippka R., Neilson A., Kunisawa R. and Cohen-Bazire G. (1971) Nitrogen fixation by unicellular blue-green algae. Arch. Mikrobiol. 76, 341-348.

Rippka R. and Stanier R. Y. (1978) The effects of anaerobiosis on nitrogenase synthesis and heterocyst development by Nostocacean Cyanobacteria. J. Gen. Microbiol. 105, 83-94.

Rippka R. and Waterbury J. B. (1977) The synthesis of nitrogenase by non-heterocystous Cyanobacteria. FEMS. Microbiol. Lett. 2, 83-86.

Rivkin R. B. and Swift E. (1980) Characterization of alakline phosphatase and organic phosphorus utilization in the oceanic dinoflagellate Pyrocystis noctiluca. Mar. Biol. 61, 1-8.

Robertson B. R. and Button D. K. (1979) Phosphate-limited continuous culture of Rhodotorula rubra: kinetics of transport, leakage and growth. J. Bact. 138, 884-895.

Rodhe W. (1948) Environmental requirements of freshwater planktonic algae. Symb. Bot. Ups. 10, 1-49.

Roger P. A. and Kulasooriya S. A. (1980) Blue-Green Algae and Rice. 112 pp. International Rice Research Institute, Los Baños, Philippines.

Roger P. and Reynaud P. A. (1976) Dynamics of the algal populations during a culture cycle in a Sahel rice-field. (Summary). Rev. Ecol. Biol. Sol. 13, 545-560.

Roger P. and Reynaud P. A. (1977) Algal biomass in rice-fields of Senegal: relative importance of Cyanophyceae that fix nitrogen. (Summary). Rev. Ecol. Biol. Sol. 14, 519-530.

Roger P. A. and Reynaud P. A. (1979) Ecology of blue-green algae in paddy fields. In: Nitrogen and rice pp. 257-310. International Rice Research Institute. . Los Baños, Philippines.

Round F. E. (1975) The Biology of Algae. 278pp. Edward Arnold, London.

Sankaram A., Mudholkar N. J. and Sahay M. N. (1966) Algae as aids in rice production. Ind. Farming. 16, 37-38.

Schindler D. W. (1975) Whole-lake eutrophication experiments with phosphorus, nitrogen and carbon. Verh. int. verein. theor. angew. Limnol. 19, 3221-3231.

Schindler D. W. (1977) Evaluation of phosphorus limitation in lakes. Science, N. Y. 195, 260-262.

Schindler D. W. and Fee E. J. (1974) Experimental lake area: whole lake experiments in eutrophication. J. Fish. Res. Bd Can. 31, 937-953.

Schmidt G. and Laskowski M. Sr. (1961) Phosphate ester cleavage. In: Boyer P. D., Lardy H. and Myrback K. (Eds). The Enzymes. vol. V. pp. 3-35. Academic Press, New York.

Selitrennikoff C. P. and Sonneborn D. R. (1977) Alkaline phosphatase of Blastocladiella emersonii: partial purification characterization. J. Bact. 130, 249-256.

Senft W. H. (1978) Dependance of light-saturated rates of

algal photosynthesis on intracellular concentration of phosphorus. Limnol. Oceanogr. 23, 709-718.

Sharma V. K. and Gaur Y. S. (1981) Nitrogen fixation by pesticide-adapted strains of paddy-field Cyanophyta. Int. J. Ecol. env. Sci. 7, 117-122.

Shehata F. H. A. and Whitton B. A. (1981) Field and laboratory studies on blue-green algae from aquatic sites with high levels of zinc. Verh. int. Verein. theor. angew. Limnol. 21, 1466-1471.

Shtina E. A. (1972) Some peculiarities of the distribution of nitrogen-fixing blue-green algae in soils. In: Desikachary T. V. (Ed.) Taxonomy and Biology of Blue-Green algae.pp. 294-295. Madras, India.

Simonis W., Bornefeld T., Lee-Kader J. and Majumdar K. (1974) Phosphate uptake and photophosphorylation in the blue-green alga Anacystis nidulans. In: Zimmerman U. and Dainty J. (Eds) Membrane Transport In Plants.pp. 220-225. Springer, Berlin.

Sinclair C. and Whitton B. A. (1977a) Influence of nutrient

deficiency on hair formation in the Rivulariaceae. Br. phycol. J. 12, 297-313.

Sinclair C. and Whitton B. A. (1977b) Influence of nitrogen sources on morphology of Rivulariaceae (Cyanophyta). J. Phycol. 13, 335-340.

Singh P. K. (1973) Nitrogen fixation by the unicellular blue-green alga Aphanothece. Arch. Microbiol. 92, 59-62.

Singh R. N. (1950) Reclamation of (usar) lands in India through blue-green algae. Nature, Lond. 165, 325-326.

Singh R. N. (1961) Role of Blue-Green Algae in Nitrogen Economy of Indian Agriculture. 175pp. Indian Council of Agricultural Research. New Delhi.

Singh R. N. and Viswanathan I. (1972) Heterocyst differentiation in Camptylonema lahorense ghosei. In: Desikachary T. V. (Ed.) Taxonomy and Biology of Blue-Green Algae. pp. 209-217. Madras, India.

Singh S. P. (1978) Growth, nitrogen fixation by blue-green algae under reducing conditions. Act. Bot. Ind. 6(supl.), 1-6.

Smart M. M., Rada R. G. and Donnermeyer G. N. (1983) Determination of total nitrogen in sediments and plants using persulphate digestion; an evaluation and comparision with the Kjeldahl procedure. Wat. Res. 17(9), 1207-1211.

Smith R. V. and Foy R. H. (1974) Improved hydrogen ion buffering of media for the culture of freshwater algae. Br. phycol. J. 9, 239-245.

Soeder C. J. and Stengel E. (1974) Physico-chemical factors affecting metabolism and growth rate. In: Stewart W. D. P. (Ed.) Algal Physiology and Biochemistry. pp. 714-740. Blackwell, Oxford.

Sorokin C. (1959) Tabular comparative data for the low and high temperature strain of Chlorella. Nature, Lond. 184, 613-614.

Sprent J. I. (1979) The Biology of Nitrogen-Fixing Organisms. 196pp. McGraw-Hill Book Company, U. K.

Srinivasan S. (1978) Distribution of types of blue-green algae under fertilizer practices. Aduthurai Rep. 2(11), 132-134.

Stacey G., Van Baalen C. and Tabita F. R. (1977) Isolation and characterization of a marine Anabaena sp. capable of rapid growth on molecular molecular nitrogen. Arch. Microbiol. 114, 197-201.

Stadtman T. (1961) Alkaline phosphatases. In: Boyer P. D., Lardy H. and Myrback K. (Eds) The Enzymes. Vol. V. pp. 55-71. Academic Press, New York.

Stainton M. P., Capel M. J. and Armstrong F. A. J. (1977) The Chemical Analysis of Freshwater. 2nd Edn. 180pp. Misc. Spec. Publs Fish. Mar. Serv. Can., No. 25 (2nd Edn.).

Stein J. R. (1973) Handbook of Phycological Methods. 445pp. Cambridge University Press, Cambridge.

Stevens S. E., Jr and Paone D. A. M. (1981) Accumulation of cyanophycin granules as a result of phosphate limitation in Agmenellum quadruplicatum. Plant Physiol. 67, 716-719.

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

Stewart W. D. P. (1970) Algal fixation of atmospheric nitrogen. Plant and Soil 32, 555-588.

Stewart W. D. P. (1973a) Nitrogen fixation by photosynthetic microorganisms. A. Rev. Microbiol. 27, 283-316.

Stewart W. D. P. (1973b) Nitrogen fixation. In: Carr N. G. and Whitton B. A. (Eds) The Biology of Blue-Green Algae. pp. 260-278. Blackwell, Oxford.

Stewart W. D. P. (1974) Blue-green algae. In: Quispel A. (Ed.) The Biology of Nitrogen fixation. pp.202-237. North-Holland Publ. Co., Amsterdam.

Stewart W. D. P. (1977) Present-day nitrogen-fixing plants. Ambio 6(2-3), 166-173.

Stewart W. D. P. (1980) Some aspects of structure and

function in nitrogen fixing Cyanobacteria. A. Rev. Microbiol. 34, 497-536.

Stewart W. D. P. (1982) Nitrogen fixation, its current relevance and future potential. Isr. J. Bot. 31, 5-44.

Stewart W. D. P. and Alexander G. (1971) Phosphorus availability and nitrogenase activity in aquatic blue-green algae. Freshwater Biol. 1, 389-404.

Stewart W. D. P., Haystead A. and Dharmawardene M. W. N. (1975) Nitrogen assimiation and metabolism in blue-green algae. In: Stewart W. D. P. (Ed.) _Nitrogen Fixation in Free-Living Microorganisms_. pp. 129-158.

Stewart W. D. P., Fitzgerald G. P. and Burris R. H. (1968) acetylene reduction by nitrogen-fixing blue-green algae. Arch. Mikrobiol. 62, 336-348.

Stewart W. D. P., Fitzgerald G. P., and Burris R. H. (1970) Acetylene reduction assay for determination of phosphorus availability in Wisconsin Lakes. Proc. natn. Acad. Sci. U. S. A. 66, 1104-1111.

Stewart W. D. P. and Lex M. (1970) Nitrogenase activity in the blue-green alga Plectonema boryanum strain 594. Arch. Mikrobiol. 73, 250-260.

Stewart W. D. P. and Pearson H. W. (1970) Effects of aerobic and anaerobic conditions on growth and metabolism of blue-green algae. Proc. Roy. Soc. Lond. B. 175, 293-311.

Stewart W. D. P., Pemble M. and Ul-Ugaily L. (1978) Nitrogen and phosphorus storage and utilization in blue-green algae. Mitt. Int. Verein. thoer. Limnol. angew. 21, 224-247.

Stewart W. D. P., Sampaio M. J., Isichei A. O. and Sylvester-Bradley R. (1977) Nitrogen fixation by soil algae of temperate and tropical soils. In: Dobereiner J., Burris R. H., Hollaender A., Franco A. A., Neyra C. A. and Scott D. B. (Eds) Limitations and Potentials for Biological Nitrogen Fixation in the Tropics. pp. 41-63. Procs. 15th Latin American Symposium. Plenum Press, New York.

Stulp B. K. and Stam W. T. (1982) General morphology and akinete germination of a number of Anabaena strains

(Cyanophyceae) in culture. Arch. Hydrobiol. Suppl. 63, 1/Algol. Studies 30, 35-52.

Subrahmanyan R., Relwani L. L. and Manna G. B. (1965) Fertility build-up of rice-field soils by blue-green algae. Proc. Ind. Acad. Sci. B 62, 252-277.

Sutherland J. M., Herdman M. and Stewart W. D. P. (1979) Akinetes of the Cyanobacterium Nostoc PCC 7524: macromolecular composition, structure and control of differentiation. J. gen. Microbiol. 115, 273-287.

Tchan Y. T. (1953) Relationship between redox potential, penetration of light and vertical distribution of algae in sandy soils. Aust. Conf. Soil Sci. 1, 371-373.

Tel-Or E. (1980) Response of nitrogen-fixing Cyanobacteria to salt. appl. environ. Microbiol. 40(4), 689-693.

Thesiger W. (1964) The Marsh Arabs. Longman, Green and Co., London.

Thirukkanasan A., Kulasooriya S. A. and Theivendirarjah K. (1977) A periodic survey of the blue-greens found

abundantly during the monsoon period (from September to January) in a paddy field near Jaffa Campus at Vaddukoddai and a general survey of blue-grees in the Jaffa peninsula during the same period. pp. 50-51. Proc. Ann. Sessions of the S. L. A. A. S. (cited by Roger and Kulasooriya, 1980).

Thomas J. and David K. A. V. (1971) Studies on the physiology of heterocyst production in the nitrogen-fixing blue-green alga Anabaena sp. L-31 in continuous culture. J. gen. Microbiol. 66, 127-131.

Tiwari G. L. (1972) A study of the blue-green from paddy field soils of India. Hydrobiologia. 39, 335-350.

Torriani A. (1960) Influence of inorganic phosphate in the formation of phosphatases by Escherichia coli. Biochim. Biophys. Acta 38, 460-469.

Trainor F. R. (1970) Survival of algae in desiccated soil. Phycologia 4, 111-113.

Traore T. M., Roger P. A., Reynaud P. A. and Sasson A. (1978) Nitrogen fixation by blue-green algae in a paddy

field in Mali. (Summary). Cah. ORSTOM Ser. Biol. 13(2), 181-185.

Tsangridis A. Th. (1982) On Cyanophyta and Chlorophyta from rice-field of Kalochorion-Thessaloniki. (Summary). 221pp. Doctoral Thesis. Institute of Systematic Botany, University of Athens.

Upchurch R. G. and Mortenson L. E. (1980) In vivo energetics and control of nitrogen fixation: changes in the adenylate energy change and adenosine 5-diphosphate/adenosine 5-triphosphate ratio of cells during growth on dinitrogen versus growth on ammonia. J. Bact. 143, 274-284.

Van Baalen C. and Edwards P. (1973) Light and temperature gradient plate. In: Stein J. R. (Ed.) Handbook of Phycological Methods. pp. 267-274. Cambridge University Press, Cambridge.

van Liere L. and Mur. L. R. (1980) Occurrence of Oscillatoria agardhii and some related species, a survey. In: Barica J. and Mur L. R. (Eds) Hypertrophic Ecosystem. pp. 67-77. Junk, Den Haag.

van Liere L. and Walsby A. E. (1982) Interaction of Cyanobacteria with light. In: Carr N. G. and Whitton B. A. (Eds) The Biology of Cyanobacteria. pp. 9-46. Blackwell, Oxford.

Venkataraman G. S. (1961) The role of blue-green algae in agriculture. Sci. Cult. 27, 9-13.

Venkataraman G. S. (1972) Algal Biofertilizers and Rice Cultivation. 75pp. Today and Tomorrow's Printers and Publishers, New Delhi.

Venkataraman G. S. (1975) The role of blue-green algae in tropical rice cultivation. In: Stewart W. D. P. (Ed.) Nitrogen Fixation by Free-living Microorganisms. pp. 207-218. Cambridge University Press, Cambridge.

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

Watanabe A. (1959) Distribution of nitrogen-fixing blue-green algae in variuos ares of south and east Asia. J. gen. appl. Microbiol. 5, 21-29.

Watanabe A., Ito R. and Sasa T. (1955) Micro-algae as a source of nutrients for daphnids. J. gen. appl. Microbiol. 1, 137-141.

Watanabe I., Lee K. K. and de Guzman M. R. (1978) Seasonal change of nitrogen-fixing rate in rice-field assayed by <u>in situ</u> acetylene reduction technique: II. Estimation of nitrogen fixation associated with rice plant. Soil Sci. Plant Nutr., Tokyo, 24(4), 465-471.

Watanabe A. and Yamamoto Y. (1971) Nitrogen fixation in the tropics. Plant and Soil (special volume), 403-413.

Weare N. M. and Benemann J. R. (1973) Nitrogen fixation by <u>Anabaena</u> <u>cylindrica</u>. I. Localization of nitrogen fixation in the heterocysts. Arch. Mikrobiol. 90, 323-332.

Weisshaar H. and Böger P. (1983) Nitrogenase activity of the non-heterocystous Cyanobacterium <u>Phormidium</u> <u>foveolarum</u>. Arch. Microbiol. 136, 270-274.

Whitford L. A. (1960) Ecological distribution of fresh water algae. In: Tryon C. A., Jr. and Hartman R. T. (Eds). <u>The</u>

Ecology of Algae. pp. 2-10. A symposium, University of Pittsburg.

Whitton B. A. (1967) Phosphate accumulation by colonies of Nostoc. Pl. Cell Physiol., Tokyo 8, 293-296.

Whitton B. A. (1969) The taxonomy of blue-green algae. Br. phycol. J. 4, 121-123.

Whitton B. A. and Carr N. G. (1982) Cyanobacteria: current perspectives. In: Carr N. G. and Whitton B. A. (Eds) The Biology of Cyanobacteria. pp. 1-8. Blackwell, Oxford.

Whitton B. A., Donaldson A. and Potts M. (1979b) Nitrogen fixation by Nostoc colonies in terresterial environments of Aldabra Atoll, Indian Ocean. Phycologia 18, 278-287.

Whitton B. A. and Sinclair C. (1975) Ecology of blue-green algae. Sci. Progr., Lond. 62, 429-446.

Whitton B. A., Diaz B. M. and Holmes N. T. H. (1979a) A computer orientated numerical coding system for algae. Br. phycol. J. 14, 353-360.

Wickstrom C. E. (1980) Distribution and physiological determinants of blue-green algal nitrogen fixation along a thermogradient. J. Phycol. 16, 436-443.

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

Wiedeman V. E., Walne P. L. and Trainor F. R. (1964) A new technique for obtaining axenic cultures of algae. Can. J. Bot. 42, 958-959.

Wolk C. P. (1965) Control of sporulation in blue-green algae. Devl Biol. 12, 15-35.

Wyatt J. T. and Silvey J. K. G. (1969) Nitrogen fixation by Gloeocapsa. Science, N. Y., 165, 908-909.

Yoshida T., Roncal R. A. and Bautista E. M. (1973) Atmospheric nitrogen fixation by photosynthetic microorganisms in a submerged Philippine soil. Soil Sci. Plant Nutr. 19, 117-123.

Zevenboom W. and Mur L. R. (1980) nitrogen-fixing Cyanobacteria: why they do not become dominant in Ducth hypertrophic lakes. In: Barica J. and Mur. L. R. (Eds). Hypertrophic Ecosystems. pp. 123-130. Junk, den Haag.