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Nitrogen and iron interactions in filamentous cyanobacteria

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AN ABSTRACT OF THE THESIS OF David Allen Hutchins for the
Master of Science in Biology presented October 23, 1989.

Title: Nitrogen and Iron Interactions in Filamentous Cyanobacteria.

APPROVED BY THE MEMBERS OF THE THESIS COMMITTEE:

John G. Rueter, Chair

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Richard P. Sandstrom

John H. Golbeck

Iron nutrition and nitrogen fixation metabolism are known to be highly interdependent in cyanobacteria. This thesis investigated several aspects of this relationship in field collections of the marine genus *Trichodesmium* and cultures of the freshwater genus *Anabaena*.

Cellular nitrogen to iron ratios were the first parameter measured; these ratios were found to be between 200 and 700 for *Trichodesmium* and

between 75 and 400 for *Anabaena*. These results indicated that *Trichodesmium* uses cellular iron supplies much more efficiently than *Anabaena*, a conclusion which correlates well with the iron supply of their respective habitats.

Also investigated was the relationship between siderophore-mediated iron uptake and diazotrophy in *Anabaena*. It was found that the strain of *Anabaena* used in these experiments (PCC strain 7120) is unable to make siderophores and fix nitrogen simultaneously, suggesting that previous work linking these two capabilities to competitive dominance may be in error or misinterpreted.

The final aspect of this problem which was examined was the possibility of gratuitous repression of *Anabaena* siderophore production by manganese in the manner previously described by other investigators in *E. coli*. No evidence for this type of molecular regulation was found, although manganese did appear to bind to siderophore in the water.

NITROGEN AND IRON INTERACTIONS IN FILAMENTOUS CYANOBACTERIA

by

DAVID ALLEN HUTCHINS

A thesis submitted in partial fulfillment of the
requirements for the degree of

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in
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1989

TO THE OFFICE OF GRADUATE STUDIES:

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CHAPTER I

INTRODUCTION

Nitrogen is an essential macronutrient for all living organisms. As one of the most important components of phytoplankton protoplasm, nitrogen is required in stoichiometric amounts relative to other elements such as oxygen, hydrogen, carbon, phosphorous and sulfur. The atomic ratio of carbon to nitrogen in marine phytoplankton varies somewhat but is about 41:7 (Russell-Hunter 1970), and similar ratios are observed in freshwater algae. As photoautotrophs, carbon is readily available to these organisms through fixation of atmospheric CO₂, except under light-limiting conditions. However, nitrogen is often depleted or unavailable, which may act to limit primary productivity. Many of the world's oceans are believed to be primarily limited by nitrogen availability, especially the tropical oceans (Ryther and Dunstan 1971, Sharp 1983). Freshwater ecosystems too may experience nitrogen limitation (Mulvey 1986).

The pools of nitrogen available to support phytoplankton growth include "fixed" sources that include ammonium, nitrate and nitrite, and dissolved organic nitrogenous compounds such as amino acids and nucleotides. Among phytoplankton, only certain cyanobacteria have access to the vast atmospheric reservoir of nitrogen gas (N₂). These diazotrophs, although restricted as to numbers of species, may become important contributors

to primary productivity when fixed nitrogen sources are limiting. Released from the growth-limiting effects of nitrogen deficiency, freshwater nitrogen fixers may come to dominate the ecosystem and can contribute significantly to new nitrogen input. Blooms of nitrogen fixing cyanobacteria, including the genus *Anabaena*, can pose significant difficulties with regard to water quality management. Other than the obvious aesthetic problems of unsightly and odorous water, the massive die-offs which often follow blooms can cause depletion of dissolved oxygen levels by heterotrophic bacteria as the large algal biomass decomposes. The resulting anoxic conditions frequently lead to losses of desirable species of invertebrates and gamefish. Thus, the mechanisms of cyanobacterial dominance have received a good deal of scrutiny, although the phenomenon is far from being well understood (Vincent et al 1987).

Equally intriguing is the conspicuous lack of diazotrophs in nitrogen-limited marine ecosystems. With the exception of the enigmatic genus *Trichodesmium* (*Oscillatoria*), few nitrogen fixers have been able to become widely established in pelagic ecosystems, despite the fact that nitrogen is often the limiting nutrient. Oceanographers have devised various theories to account for the absence of diazotrophs in the open ocean. Possibilities include phosphate limitation (Doremus 1982), copper toxicity (Rueter et al 1979), limitation by availability of organic nutrients and particulate surfaces (Paerl et al 1987), interference due to excessive water turbulence (Carpenter and Price 1976) and competitive limitation of molybdenum uptake by sulfate because of a low molybdate to sulfate ratio (Howarth and Cole 1985). Another leading hypothesis postulates that iron, required in large amounts by diazotrophs, may be the key to limitation of

nitrogen fixation in the ocean (Rueter and Petersen 1987, Rueter 1988a).

Iron is well known to be intimately involved in phytoplankton nitrogen metabolism (Rueter and Ades 1987). Both nitrate and nitrite reductase contain iron prosthetic groups (Falkowski 1983, Mendez and Vega 1981). In particular, diazotrophs have a disproportionately large requirement for iron (Carnahan and Castle 1958). The highly conserved oxygen-labile enzyme nitrogenase, virtually identical throughout most of the diverse group of diazotrophic prokaryotes, contains iron in both major subunits. Averill and Orme-Johnson (1978) have estimated that each nitrogenase molecule contains as many as 36 iron atoms, although this may vary with the source of the nitrogenase (Postgate 1987), and many investigators prefer a more conservative estimate of about 32 irons per molecule. Exacerbating this unusually high enzymatic iron requirement is the relative inefficiency of the nitrogenase enzyme. With a substrate turnover rate of only $1.25 \text{ N}_2 \cdot \text{sec}^{-1}$ (Postgate 1987), large amounts of the enzyme must be available to support rapid growth rates on gaseous nitrogen. The net effect of these two interacting factors is that nitrogen fixing laboratory cultures of cyanobacteria may require iron at levels of up to an order of magnitude higher than the same species grown on fixed nitrogen sources (Lammers 1982).

Evidence has accumulated in recent years that iron may be a limiting nutrient in many areas of the marine environment (Entsch et al 1983, Subba Rao and Yeats 1984, Martin and Gordon 1988, Rueter 1988a). Iron is extremely insoluble in aerobic aqueous solutions, especially at the relatively high pH of seawater (normally about pH 8.1), and any new soluble input is quickly oxidized and lost to precipitation (Gordon et al

1982). Despite the fact that iron is one of the most common elements in the Earth's crust, iron concentrations in the euphotic zone of the ocean (usually defined as the depth to which 1% of incident light penetrates) are extremely depleted and often on the verge of detectability by present day analytical techniques. Bruland (1983) has collected reports of Fe concentrations in North Pacific surface waters of only 0.15-0.40 nmol/kg. At these concentrations, it is entirely feasible that cyanobacteria are unable to obtain enough nutritional iron to support the elevated demands of diazotrophy.

Iron has also been proposed as limiting nitrogen fixation in lakes. Diazotrophy in freshwater has often been found to be stimulated by iron enrichment (Schelske 1962, Elder and Horne 1977, Wurtsbaugh and Horne 1983, Wurtsbaugh et al 1985, Rueter and Petersen 1987, Wurtsbaugh 1988). Iron availability has also been suggested as contributing to nuisance blooms (Morton and Lee 1974) and cyanobacterial dominance (Gerloff and Skoog 1957, Parr and Smith 1976). Although terrestrial input usually insures that iron is more available in freshwater ecosystems than in the ocean, the evolutionary persistence of specific iron acquisition and conservation systems in many freshwater algae is a powerful argument for selective pressure through iron limitation in lakes.

The physiological consequences of iron deficiency in cyanobacteria have been studied by many investigators. In general, iron limitation has been found to result in a suite of deleterious effects. Among these are depressed levels of photosynthetic electron transport (Sandmann 1985) and growth rates (Sandmann and Malkin 1983), decreased cyanophycin content and degenerative morphological characteristics (Douglas et al

1986), and reductions in chlorophyll and phycobilin synthesis (Rueter 1988a). Adaptations have also been found which serve to decrease cellular iron demand, including switching from the iron-containing electron carrier ferredoxin to flavodoxin (Fillat et al 1988, Laudenbach et al 1988). Although flavodoxin is a less efficient electron transport protein, the fact that it requires no iron is an advantage under iron-limiting conditions.

Uptake of iron by freshwater and marine phytoplankton has been investigated by many researchers. Although some species have been found to have apparent access only to iron adsorbed on cell surfaces (Anderson and Morel 1982), many phytoplankton have been demonstrated to synthesize high-affinity iron selective chelators. Known as siderophores or siderochromes, these chelators are released into the environment where they bind to Fe^{3+} . When the iron-siderophore complex diffuses back to the cell surface, membrane-bound transport proteins take up the bound iron, probably with a simultaneous reduction of the metal to Fe^{2+} (Allnutt and Bonner 1984). Siderophores, by definition, are inducible by iron deprivation and repressible by iron availability. This excludes such noninducible extracellular compounds as citrate, which weakly binds iron but is usually not regulated on the basis of iron availability. Much of the pioneering work on siderophores in both heterotrophic and autotrophic prokaryotes has come out of the laboratory of J.B. Neilands (Neilands 1981). Although there is some evidence of siderophore production by eukaryotic algae (Trick et al 1983a, Trick et al 1983b, Allnutt and Bonner 1984), these iron chelators are especially widespread among the cyanobacteria (Lange 1974, Simpson and Neilands 1976, Armstrong and Van Baalen 1979, Goldman et al 1983). The type of siderophore produced by

both eukaryotic and prokaryotic algae contains the hydroxamate iron-chelating functional group, typified by schizokinen from *Anabaena* (see Figure 1). Although many other types of siderophores have been discovered in heterotrophic eubacteria (Neilands 1981, Lemos et al 1988, Moody 1986, Riuox et al 1986), only hydroxamate siderophores have been found in cyanobacteria.

The fact that siderophore production seems to be common among cyanobacteria suggests some interesting evolutionary speculations. Since these prokaryotes are believed to have been the first oxygenic photosynthesizers (Bold and Wynne 1985), it is possible that the subsequent advent of an oxidizing atmosphere in the Precambrian (about 2 billion years ago) necessitated the evolution of some new means of obtaining iron from the environment. Soluble iron may be presumed to have been readily available under the reducing conditions of the early Earth, but photosynthetically produced oxygen would have resulted in oxidation and precipitation of this Fe source. Thus, early cyanobacteria were in effect making iron unavailable to themselves. It is possible that high affinity iron uptake mechanisms such as siderophores had to coevolve with oxygenic photosynthesis. Siderophore production may therefore be an extremely ancient physiological function and modern cyanobacterial siderophores may represent the persistence of a metabolic pathway as old as photosynthesis itself.

The production of siderophores has frequently been suggested as an adaptation for competitive dominance. Often cited is the work of Lange (1974) and Murphy et al (1976), who hypothesized that siderophores function both to obtain sufficient iron to support nitrogen fixation and to

Figure 1. Structure of the hydroxamate siderophore schizokinen. Fe^{3+} is chelated by the two hydroxamate functional groups (-CO-NOH-) and the α -hydroxycarboxylate functional group of the citrate backbone (Figure from Simpson and Neilands 1976).

deny iron to other algae. Both of these mechanisms could act to inhibit the growth of competing species while favoring bloom-forming cyanobacteria. This ecological argument is complicated by the fact that many microorganisms, including many bacteria (Moody 1986) and some cyanobacteria (Goldman et al 1983), have been found to have uptake systems for iron chelated to siderophores which they cannot produce themselves. This ability to use exogenous siderophores could mean that dominance is not just a simple function of chelator production but involves other variables such as individual specific metabolic capabilities and species composition of the entire ecosystem.

One of the central problems in this study will be to define the relationship between siderophore production and nitrogen fixation. Both of these processes are extremely expensive in terms of energetics and cell metabolism, but can allow growth to occur when other algae are limited by iron or fixed nitrogen. The nature of this relationship has implications for both cyanobacterial dominance in freshwater and iron limitation of nitrogen fixation in the ocean.

Two cyanobacteria were chosen for these investigations; these include the freshwater filamentous heterocystous *Anabaena* (Pasteur Culture Collection strain 7120) and the marine genus *Trichodesmium* (*Oscillatoria*), a filamentous nonheterocystous colony former. These two diazotrophs are ideal to investigate the two aspects of nitrogen fixation and iron nutrition of interest: *Anabaena* is a notorious nuisance bloom former and *Trichodesmium* is one of the few species which has been highly successful at overcoming the prevailing limitation on nitrogen fixation in the open ocean.

Planktonic *Trichodesmium* colonies sampled in this investigation probably consist of mixed collections of three sympatric species: *T. erythraeum* Ehrenberg, *T. hildenbrandtii* Gomont and *T. thiebautii* Gomont (Borstad 1982). Despite extensive research effort on this genus, the ecology and physiology of *Trichodesmium* are in many respects still a mystery. Contradictions seem to abound whenever this genus is studied. Although abundant and widespread in nature, all attempts at culturing *Trichodesmium* have met with failure with the exception of the laboratory of Ohki and Fujita in Japan (Ohki et al 1986). Apparently slow-growing, with a reported doubling time of 12 to 115 days (Carpenter 1973, Carpenter 1975), this cyanobacterium is nevertheless the only nitrogen fixer capable of forming vast blooms in the open ocean. A bloom of about 90,000 km² was recently reported after being spotted by the Nimbus 7 satellite off the coast of Australia (Dupoy et al 1988), and efforts are underway to monitor blooms on a regular basis using satellite imagery. Recently, an endogenous rhythm in the nitrogen fixing activity of *Trichodesmium* has been reported (Capone and O'Neil 1988), with significant levels occurring only during daylight hours regardless of illumination. This seems like a strange strategy for an alga with no known mechanism for protecting its nitrogenase from oxygen (Carpenter and Price 1976), as O₂ levels are undoubtedly higher during active photosynthesis than at night. Finally, the high iron content and nitrogen fixation rates in this genus seem to be at odds with its habitat, where dissolved iron is virtually non-existent (Symes and Kester 1985). There is some evidence that *Trichodesmium* is adapted to take advantage of sporadic aeolian deposits of continental dust (Rueter 1988a).

Anabaena 7120 is an isolate of R. Haselkorn. First described as *Nostoc muscorum*, this strain is now classified as an *Anabaena* species, possibly *A. variabilis*. This clone appears to be conspecific with PCC strains 6411, 7118 and 7119. Characteristics include lack of motility and akinetes, susceptibility to cyanophage N-1, and obligate photoautotrophy. The exact source for this strain is unknown (Rippka et al 1979). This genus includes the first cyanobacterium to be reported to produce the siderophore schizokinen, which it makes in copious amounts under iron limited conditions (Simpson and Neilands 1976).

The investigations described in this paper are an attempt to further define and quantify the interrelationship of nitrogen fixation and iron nutritional physiology in these two species. Chapter II will present and compare data on nutritional ratios of field collected *Trichodesmium* colonies and laboratory *Anabaena* cultures, with the intent of examining possible correlations between observed iron levels and protein nitrogen and chlorophyll concentrations, as well as nitrogen fixation rates. Chapter III is an examination of nitrogen fixation and siderophore production in *Anabaena* with emphasis on the possible implications of hypothesized synergistic effects of these two physiological capabilities on cyanobacterial dominance and bloom formation. Chapter IV will deal with the possibility of gratuitous manganese repression of *Anabaena* siderophore production in the manner described by Hantke (1987) for regulation of siderophore production in *E. coli*. The Conclusions chapter (Chapter V) will present a discussion of the results of these experiments in the context of current problems in cyanobacterial physiology, ecology and evolution. It is hoped that a significant contribution can be made to

our understanding of the related problems of cyanobacterial dominance in freshwater ecosystems and the scarcity of cyanobacterial nitrogen fixation in marine ecosystems.

CHAPTER II

IRON RATIOS IN *ANABAENA* AND *TRICHODESMIUM* (CYANOPHYTA)

ABSTRACT

Laboratory cultures of the freshwater cyanobacterium *Anabaena* 7120 and field-collected samples of the marine cyanobacterium *Trichodesmium* were compared for iron use efficiencies and dependence of nitrogen fixation rates and chlorophyll content on cellular leachable iron levels. *Anabaena* was found to have a protein iron to nitrogen ratio, described here as an envelope from a maximum ratio of about 400 to a minimum of around 75. Protein N to Fe ratios in *Trichodesmium* ranged from about 700 to 200, indicating that *Trichodesmium* is much more iron efficient than *Anabaena*. A similar envelope was found for chlorophyll to iron ratios in *Anabaena* but not in *Trichodesmium*, which suggests that *Anabaena* chlorophyll levels are more sensitive to iron levels. Nitrogen fixation could not be described as a strict function of cellular labile iron concentrations in either genus; however, elevated N₂ fixation rates could not be demonstrated in low-iron samples. These results are discussed with respect to the distribution and ecology of both freshwater and marine cyanobacteria.

INTRODUCTION

The major elements which make up living phytoplankton are often found in an approximate ratio of 106 C: 263 H: 110 O: 16 N: 1P: 0.7 S, although there is considerable variability between species and populations (Redfield 1963). Balanced growth by phytoplankton requires uptake of all nutrients in the same ratios found in living cells; an element which constrains growth because it is unavailable in the required amounts becomes a limiting nutrient. Redfield observed that the typical 15:1 ratio of nitrogen to phosphorus often found in freshly upwelled seawater is very close to that observed in phytoplankton. Today it is believed that this reflects the interaction of planktonic algae and their environment. Phytoplankton both influence and are influenced by nutrient concentrations, leading to a situation of dynamic equilibrium between biotic and abiotic components of the marine environment. Long term geochemical cycles of major nutrient elements are intimately tied to biological processes in the photic zone of the sea.

In recent years the concept of cell elemental quotas and limiting nutrients has been extended to include micronutrients as well (Morel and Hudson 1984). Small amounts of metals such as iron, manganese and zinc are required for a variety of metabolic processes, especially those requiring oxidation/reduction reactions. Other trace elements also required as enzymatic cofactors include cobalt, molybdenum, copper and vanadium (Levinton 1982). Trace metal composition of phytoplankton has been investigated with the intent of assessing the effects of these organisms on cycling of trace metals in the ocean (Collier and Edmond 1984). Many trace

metals do in fact exhibit a "nutrient" type vertical profile in their distributions in the sea, with significant depletion in surface water (Bruland 1983). This suggests that, like macronutrients, these trace metals may be in a delicate state of balance determined by input, uptake by phytoplankton, particulate sinking rates and recycling through upwelling in the water column.

While the concept of Redfield ratios (Redfield 1963) can easily be extended to include iron, there are some basic differences in the way iron is incorporated into this system. These are primarily the result of the particular chemistry of iron in sea water. Unlike most nutrients, iron is highly insoluble in oxygenated seawater. New input, whether from rivers, atmospheric dust or seafloor vent activity, quickly oxidizes and precipitates out as ferric hydroxides. Iron tends to sink out and may accumulate in a reduced form in suboxic deep water or sediments (Bruland 1983), with little of the recycling apparent for most soluble nutrients in the photic zone. Thus, iron can be thought of as making a one-way trip through the marine biosphere; phytoplankton must obtain what they can from the input sources available before the inevitable losses to particulate sinking.

Much evidence has been gathered recently that iron may be a limiting nutrient in large areas of the open sea (Subba Rao and Yeats 1984, Martin and Fitzwater 1988, Martin and Gordon 1988, Rueter 1988a). To further explore this concept, a thorough examination of iron quotas of phytoplankton is necessary. How much iron do algae need in respect to other nutrients such as nitrogen? How do cellular iron ratios affect processes such as photosynthesis, nutrient uptake and growth? A special case is growth

through fixation of atmospheric nitrogen (diazotrophy), which requires far more iron than growth using fixed nitrogen sources (Raven 1988). Does iron availability limit the amount of diazotrophy which can occur in the sea, as has been proposed (Rueter 1982, Rueter and Petersen 1987, Rueter 1988a)?

This paper will present data on iron ratios of one of the few organisms which has been able to successfully adapt to fix nitrogen in the open sea, the cyanobacterium *Trichodesmium*. This data will be compared to similar data sets from cultures of the freshwater diazotroph *Anabaena*. Freshwater ecosystems are probably far less likely to experience iron limitation than marine systems due to the constant input from terrestrial sources. However, iron-limited freshwater systems have been described (Schelske 1962, Wurtsbaugh et al 1985, Wurtsbaugh 1988). The existence of a high-affinity siderophore iron-uptake system in *Anabaena* (Simpson and Neilands 1976) and other freshwater cyanobacteria is good evidence that iron limitation is a selective force here as well.

A comparison of protein nitrogen to iron and chlorophyll to iron ratios and nitrogen fixation rates in these two organisms is a step towards defining the similarities and differences in their strategies with respect to iron and diazotrophy. This paper describes an "envelope" of ratios encompassing the observed ratios in nitrogen fixing cells for both species with special attention to maximum nitrogen to iron ratios which allow appreciable nitrogen fixation rates. Nitrogen fixation rates were examined with respect to iron content and chlorophyll to iron ratios are presented as a possible indicator of the effect of iron on pigment levels.

This approach is useful in that it allows an inference to be made between the measured physiological parameters of these species and the

environmental conditions under which they exist in nature. Differences in iron strategies and selected ratios reflect different adaptations to particular environmental conditions. These results were examined for clues as to how *Trichodesmium* and *Anabaena* manage to thrive by fixing nitrogen in two environments characterised by two very different iron regimes.

MATERIALS AND METHODS

Trichodesmium colonies were collected and analyzed during a research cruise to the western Caribbean in November of 1988. Cyanobacterial colonies were obtained using plankton net tows and were manually separated from zooplankton and debris using a plastic inoculating loop. Only surface tows were used to avoid performing analyses on possibly light-limited colonies.

It is important to note the limitations of comparing field-collected samples to laboratory cultures. While cultures are maintained under carefully controlled nutrient and light regimes, the history and growth conditions of field samples are not known. In addition, the limited duration and relatively difficult working conditions of research cruises often means that fewer data points can be obtained than is possible with laboratory culture work.

Cultures of *Anabaena* 7120 obtained from the Pasteur Culture Collection were grown in a modified version of FRAQUIL medium (Petersen 1982) under continual cool white fluorescent light at a photon flux density of 50-100 $\mu\text{Einstein}/\text{m}^2/\text{sec}$. The medium was cleansed of contaminating trace metals by passing it through a Chelex 100 column (Morel et al 1979), and

was subsequently equilibrated to pH 7.8-8.0 and filter sterilized. All cultures were grown without a fixed nitrogen source and iron was added as FeEDTA. Cultures were grown under positive air pressure using a laminar flow hood to minimize contamination problems.

Data from two types of cultures are presented. In the first, cultures were grown either with 1 μM iron or without iron; these cultures were sampled on 1/19, 2/2, 2/10, 5/10, 5/14, and 5/18 (1989). In the second cultures were grown at a range of iron concentrations; the range used was 10 μM , 1 μM , 0.5 μM , 0.1 μM and 0.01 μM . These experiments were performed on 4/9, 4/12, 4/15 and 4/18 (1989). Other than iron concentrations, the basal medium for each experiment was identical. Culturing and field collection techniques are presented in more detail in Appendix A.

All culture and assay equipment was acid washed and rinsed six times in reagent grade distilled deionized water (Nanopure system). The data presented in this study represent the mean (plus or minus standard error) of two replicate measurements.

Iron measurements were made using the colorimetric ferrozine technique modified from the methods of Stookey (1970) and Gibbs (1979). Samples were extracted by overnight leaching in 1 N HCl at 60°C. For *Trichodesmium*, 200 colonies were placed directly into the acid for each replicate. For *Anabaena*, 50 to 100 mL of culture was filtered onto 0.2 μm Nucleopore filters which were then extracted in the acid. Filter blanks were also run and subtracted from the culture values obtained. After leaching, the extracts were desk-top centrifuged at high speed for five minutes and colorimetric ferrozine analysis was performed on the

supernatant. Standard curves for iron were run using the same procedure outlined above with $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$.

Total protein measurements were made using the BCA Protein Assay (bicinchoninic acid) available from Pierce Chemical. *Trichodesmium* colonies were individually pipetted onto Whatman GF/A filters, frozen and ground in 1% SDS using a tissue homogenizer. This extract was then centrifuged at high speed on a desktop centrifuge for five minutes and the BCA Enhanced Protocol (30 minutes at 60°C) was performed on the supernatant. *Anabaena* cultures were analyzed by the same procedure except that 25 mL samples were suction-filtered onto the glass fiber filters. Protein standard curves were prepared using defatted bovine serum albumen.

Protein nitrogen was calculated using information from Dayhoff et al (1965) which indicates that the average nitrogen content of cell protein is about 14% (weight to weight). Calculations based on this assumption undoubtedly underestimate the total nitrogen content of cells, as nucleic acids may account for 11-17% of total cell nitrogen in phytoplankton (Wheeler 1983). Another smaller fraction is accounted for by nitrogen in chlorophyll. Protein is the major cell sink for nitrogen, however, making up 70-90% of the total according to Wheeler. Estimates of nitrogen content based on protein measurements will be referred to in this paper as protein nitrogen.

Chlorophyll a measurements on *Trichodesmium* colonies were performed using near-boiling methanol extracts according to the methods of Jensen (1978). *Anabaena* culture chlorophyll a concentrations were assayed flourometrically according to the methods of Parsons et al (1984). Briefly,

90% acetone extracts were performed on samples filtered onto GF/A filters with the addition during filtering of saturated MgCO_3 . A Turner Designs fluorometer was used and fluorescence was measured before and after addition of 5% HCl. Culture chlorophyll *a* concentrations were calculated according to the methods of Parsons et al (1984).

Nitrogen fixation rates were estimated by measuring the reduction of acetylene to ethylene using a Shimadzu GC Mini2 Chromatograph with a Porapak N 80-100 mesh column. The carrier gas used was ultra-high purity nitrogen and ethylene standards were run to calculate sample reduction rates. Ethylene peak size was quantified using a Shimadzu C-R3A Chromatopac integrator. *Trichodesmium* colonies were run in sealed serum vials on freshly collected colonies under cool white fluorescent illumination. All *Anabaena* analyses used polypropylene Oak Ridge tubes illuminated during incubation using cool white fluorescent light; values obtained from these analyses were corrected for the small amount of leakage which is unavoidable with these tubes.

Based on electron transfer a molar ratio of 3:1 should apply for acetylene reduced to nitrogen atoms assimilated during N_2 fixation (Postgate 1987). In practice however a higher ratio is usually observed, probably because hydrogen gas is evolved during N_2 fixation but not during acetylene reduction. For the purposes of converting measured acetylene reduction rates to nitrogen fixation rates a 1 to 4 conversion of mols ethylene produced to mols nitrogen atoms fixed (not N_2) was used (Taylor 1983).

For complete descriptions of analytical techniques, see Appendix B.

RESULTS

Trichodesmium in nature grows in discrete bundles of filaments known as colonies, while *Anabaena* 7120 in culture grows dispersed throughout the medium as individual filaments. The results of *Trichodesmium* analyses are reported on a per colony basis while *Anabaena* results are quantified per mL of culture. Because of the dissimilar growth habits of these two organisms, it is difficult to make meaningful physiological comparisons of absolute values for the various analyses performed. For this reason, all comparisons will be between elemental and physiological ratios of the two genera examined and not between absolute measured values.

Anabaena 7120 Results

Data for *Anabaena* 7120 laboratory cultures are presented in Table I. Since these cultures were grown under defined medium conditions, iron levels added to the culture medium are reported. It should be noted that this table comprises data collected from three sets of experiments; each data set is made up of consecutive measurements on one of the experiments. No attempt will be made to relate measured physiological values to temporal changes in each experiment, although this exercise could be instructive concerning relationships between physiological ratios and growth. For a temporal series on *Anabaena*, see Chapter III.

The range in leachable iron per milliliter for *Anabaena* cultures is between 0.032 and 2.90 nmol/mL and closely follows the iron levels added to the medium, with cultures grown in high iron showing the highest leachable cellular iron concentrations and cultures grown with no added iron

TABLE I
IRON, PROTEIN, AND CHLOROPHYLL *a* CONTENT, NITROGEN
FIXATION RATES, AND PHYSIOLOGICAL
RATIOS OF *ANABAENA* 7120

Date and iron added to medium	Fe/mL culture (nmol)	Protein N/mL culture (nmol)	Chl <i>a</i> /mL culture (nmol)	Nitrogen fixa- tion rate (nmol N fixed/mL culture/hour)	N:Fe (mol:mol)	Chl <i>a</i> :Fe (mol:mol)	mol N fixed/ mol Fe/hour
1/19/89:							
10 ⁻⁶ M	0.142	42	0.224	0.1295	296	1.58	0.91
0M	0.034	19	0.103	0.0360	544	3.03	1.06
2/2/89:							
10 ⁻⁶ M	0.488	152	0.729	0.1010	311	1.49	0.21
0M	0.054	19	0.090	0.0285	352	1.67	0.53
2/10/89:							
10 ⁻⁶ M	0.555	172	1.030	0.0463	309	1.85	0.08
0M	0.032	32	0.124	0.0198	583	3.85	0.62
4/9/89:							
10 ⁻⁸ M	0.174	46	0.154	0.0100	264	0.89	0.06
10 ⁻⁷ M	0.188	55	0.158	0.0090	293	0.84	0.05
5x10 ⁻⁷ M	0.213	49	0.140	0.0030	230	0.65	0.01
10 ⁻⁶ M	0.232	51	0.260	0	220	1.12	0
10 ⁻⁵ M	0.361	35	0.166	0.0045	96	0.46	0.01
4/12/89:							
10 ⁻⁸ M	0.197	78	0.221	0.0705	396	1.12	0.36
10 ⁻⁷ M	0.259	92	0.275	0.1310	355	1.06	0.51
5x10 ⁻⁷ M	0.285	93	0.178	0.0600	326	0.63	0.21
10 ⁻⁶ M	0.299	68	0.155	0.0380	227	0.52	0.13
10 ⁻⁵ M	0.678	94	0.243	0.0970	139	0.36	0.14

TABLE I

IRON, PROTEIN, AND CHLOROPHYLL *a* CONTENT, NITROGEN
FIXATION RATES, AND PHYSIOLOGICAL
RATIOS OF *ANABAENA* 7120
(continued)

Date and iron added to medium	Fe/mL culture (nmol)	Protein N/mL culture (nmol)	Chl <i>a</i> /mL culture (nmol)	Nitrogen fixa- tion rate (nmol N fixed/mL culture/hour)	N:Fe (mol:mol)	Chl <i>a</i> :Fe (mol:mol)	mol N fixed/ mol Fe/hour
4/15/89:							
10 ⁻⁶ M	0.228	92	0.297	0.0640	404	1.30	0.28
10 ⁻⁷ M	0.367	105	0.401	0.0970	286	1.09	0.26
5x10 ⁻⁷ M	0.474	87	0.335	0.0980	184	0.71	0.21
10 ⁻⁶ M	0.836	73	0.330	0.1115	87	0.40	0.13
10 ⁻⁵ M	1.230	96	0.409	0.0890	78	0.33	0.07
4/18/89:							
10 ⁻⁶ M	0.300	96	0.364	0.0515	320	1.21	0.17
10 ⁻⁷ M	0.389	109	0.493	0.0665	280	1.27	0.17
5x10 ⁻⁷ M	0.443	83	0.366	0.0215	188	0.83	0.05
10 ⁻⁶ M	0.758	145	0.447	0.0810	191	0.59	0.11
10 ⁻⁵ M	2.90	163	0.413	0.0690	56	0.14	0.02
5/10/89:							
10 ⁻⁶ M	0.441	34	No Data	0.7650	77	No Data	1.73
OM	0.108	48	No Data	0.0630	444	No Data	0.58
5/14/89:							
10 ⁻⁶ M	0.623	68	No Data	0.0635	109	No Data	0.10
OM	0.126	41	No Data	0.0305	325	No Data	0.24
5/18/89:							
10 ⁻⁶ M	0.677	60	No Data	0.0065	89	No Data	0.01
OM	0.124	53	No Data	0.0290	427	No Data	0.23

TABLE I

IRON, PROTEIN, AND CHLOROPHYLL *a* CONTENT, NITROGEN
FIXATION RATES, AND PHYSIOLOGICAL
RATIOS OF *ANABAENA* 7120
(continued)

Date and iron added to medium	Fe/mL culture (nmol)	Protein N/mL culture (nmol)	Chl <i>a</i> /mL culture (nmol)	Nitrogen fixa- tion rate (nmol N fixed/mL culture/hour)	N:Fe (mol:mol)	Chl <i>a</i> :Fe (mol:mol)	mol N fixed/ mol Fe/hour
Mean	0.444	77	0.312	0.0778	265	1.12	0.29
Standard Deviation	±0.521	±40	±0.207	±0.1310	±136	±0.82	±0.37
Standard Error	±0.092	±70.7	±0.041	±0.023	±24	±0.161	±0.065
n	32	32	26	32	32	26	32

showing the lowest values. Cultures grown at a range of iron concentrations (4/9, 4/12, 4/15 and 4/18) also exhibited good correlation between measured cellular iron concentrations and iron added to the growth medium. *Anabaena* protein N concentrations were between 19 and 172 nmol/mL. In some cases (for instance, 1/19, 2/2 and 2/10) protein nitrogen concentrations paralleled iron added to the medium but in other cases they did not. Chlorophyll *a* per milliliter varied between 0.090 and 1.03 nmol. Rates of nitrogen fixation ranged from 0 to 0.765 nmol N/mL/ hour. Nitrogen fixation rates could not be described as a direct function of culture iron concentrations. Some samples were fixing very little or no nitrogen, indicating the effects of serious iron deficiency, transfer shock or other problems. For cultures which were fixing substantial levels of nitrogen (arbitrarily defined as greater than 0.050 nmol N fixed/mL culture/hour), the minimum iron content was 0.108 nmol/mL and the minimum protein N concentration for this subset was 34 nmol/mL.

A minimum N:Fe molar ratio of 56 for iron-sufficient cultures and a maximum of 583 for severely iron-limited cultures was observed, with an average ratio of 265. For cultures fixing nitrogen (more than 0.050 nmol N/mL/hour), the largest N:Fe ratio recorded was 444 and the lowest was 56. Chlorophyll *a* to iron ratios were between 0.14 and 3.85 and were substantially lower with higher iron concentrations in the medium.

Nitrogen to iron ratios for *Anabaena* cultures are shown in Figure 2. The envelope of ratios is quite well defined, with a maximum ratio of approximately 400 for iron-limited cultures and a minimum of about 75 for cultures grown with high iron concentrations. Since these cultures were grown under controlled culture conditions and encompass a wide range of

Figure 2. Protein nitrogen to iron ratios in *Anabaena* 7120. Molar ratios of cellular protein nitrogen to acid-leachable iron can be described by an envelope with an upper limit of 400 and a lower limit of 75.

added iron concentrations (0 to 10 μM FeEDTA), we are confident that the envelope described by this data represents real limits to N:Fe ratios during diazotrophy. Cultures which fall along the line of maximum ratios were all grown with very low added iron levels (0 to 0.01 μM), supporting the idea that this line closely coincides with the line of actual iron limitation. Two cultures which exceeded the maximum ratio were not fixing substantial levels of nitrogen and were severely iron-limited. This N:Fe ratio of 400 approximately describes the limits of iron efficiency for diazotrophic growth while the lower limit is probably represents a structural minimum for cell protein and/or an iron storage maximum.

Anabaena chlorophyll *a* to iron ratios are represented in Figure 3. Unlike field-collected *Trichodesmium* samples (see below), these cultures show a pattern very similar to that observed with nitrogen to iron ratios. Both the upper and lower limits of this envelope show some hint of an exponential relationship. Although chlorophyll *a* content seems to be dependent on iron concentrations at low iron concentrations, above about 0.5 nmol cellular Fe/mL chlorophyll *a* levels no longer show this dependency. The lower limits of this envelope are defined by a chlorophyll *a* to Fe ratio of about 0.35. This could represent either the point where iron storage capacity is saturated or where chlorophyll *a* levels are at the minimum necessary to support photoautotrophic growth.

Trichodesmium Results

Results of the *Trichodesmium* analyses are summarized in Table II. Latitudes and longitudes of sampling stations where collections were made are shown for comparative purposes. Acid-leachable iron levels varied from

Figure 3. Chlorophyll a to iron ratios in *Anabaena* 7120. Molar Chl a to acid leachable iron ratios exhibit an envelope similar to that seen for protein nitrogen to iron ratios, except that there is some indication of an exponential relationship in the upper and lower boundaries

TABLE II

IRON, PROTEIN, AND CHLOROPHYLL *a* CONTENT, NITROGEN
FIXATION RATES, AND PHYSIOLOGICAL
RATIOS OF *TRICHODESMIUM*

Sampling station, date, time	Latitude and Longitude	Fe/colony (nmol)	Protein N/ colony (nmol)	Chl <i>a</i> colony (nmol)	Nitrogen fixa- tion rate (nmol N fixed/ colony/hour)	N:Fe (mol:mol)	Chl <i>a</i> :Fe (mol:mol)	mol N fixed/ mol Fe/hour
5 11/6/88 8:30 a.m.	21° 37.258 N 74° 27.390 W	0.140	99	0.069	0.0162	707	0.493	0.12
6 11/6/88 2:08 p.m.	21° 09.410 N 73° 55.750 W	0.333	204	0.090	0.0124	613	0.270	0.04
9 11/7/88 12:43 p.m.	18° 52.826 N 76° 34.761 W	0.135	75	0.096	0.1384	556	0.709	1.03
16 11/9/88 12:45 p.m.	16° 50 N 83° 40 W	0.470	78	0.060	0.0914	166	0.128	0.19
25 11/14/88 8:30 a.m.	13° 48.080 N 81° 02.279 W	0.400	99	0.098	0.1248	248	0.245	0.31
26 11/14/88 12:30 p.m.	13° 27.953 N 81° 13.396 W	0.170	108	0.098	0.1064	635	0.578	0.63
28 11/15/88 8:30 a.m.	10° 22.072 N 82° 43.168 W	0.220	57	0.090	0.6616	259	0.410	3.01
29 11/17/88 10:00 a.m.	10° 36.267 N 82° 26.225 W	0.210	121	0.078	0.0790	576	0.372	0.38
30 11/17/88 1:00 p.m.	10° 54.576 N 82° 10.330 W	0.175	63	No Data	0.0054	360	No Data	0.03

TABLE II

IRON, PROTEIN, AND CHLOROPHYLL *a* CONTENT, NITROGEN
FIXATION RATES, AND PHYSIOLOGICAL
RATIOS OF *TRICHODESMIUM*
(continued)

Sampling station, date, time	Latitude and Longitude	Fe/colony (nmol)	Protein N/ colony (nmol)	Chl <i>a</i> / colony (nmol)	Nitrogen fixa- tion rate (nmol N fixed/ colony/hour)	N:Fe (mol:mol)	Chl <i>a</i> :Fe (mol:mol)	mol N fixed/ mol Fe/hour
32 11/18/88 9:30 a.m.	12° 26.203 N 80° 32.711 W	0.220	78	No Data	0.1426	355	No Data	0.65
33 11/18/89 7:00 p.m.	13° 32.017 N 79° 41.021 W	0.220	117	0.078	No Data	532	0.355	No Data
37 11/20/88 7:00 p.m.	18° 36.713 N 74° 48.040 W	0.175	117	No Data	No Data	669	No Data	No Data
39 11/21/88 8:30 a.m.	20° 21 N 73° 25.3 W	0.140	51	0.044	0.1064	364	0.314	0.76
Mean		0.231	97	0.080	0.136	465	0.387	0.65
Standard Deviation		±0.105	± 40	±0.018	±0.181	± 180	±0.170	±0.85
Standard Error		±0.029	± 11	±0.006	±0.055	± 50	±0.054	±0.26
n		13	13	10	11	13	10	11

0.135 to 0.470 nmol/colony while the range in protein nitrogen content is between 51 and 204 nmol/colony. Colony chlorophyll *a* content was between 0.044 and 0.098 nmol/colony while nitrogen fixation rates ranged between 0.0054 and 0.6616 nmol N fixed/colony/hour. These measured values show considerable variation due to the variable size of colonies collected at each site as well as because of real differences in physiological parameters. This again emphasizes the importance of ratio comparisons over measured values in order to fully appreciate the magnitude of physiological variability within and between natural populations.

Trichodesmium protein N to Fe ratios also show considerable variability, from a minimum of 166 (mol:mol) to a high of 707, with a mean value of 465. Molar chlorophyll *a* to Fe ratios are between 0.128 and 0.709. Although all samples, except for those collected at night (Stations 33 and 37) were fixing significant levels of nitrogen, no direct relationship between measured iron content and nitrogen fixation rates could be ascertained.

Protein nitrogen versus cellular iron content is plotted in Figure 4 on a mole to mole basis. All the data points can be fit into an envelope of ratios between 700 and 200. The upper boundary (N:Fe=700) describes cells with very low iron levels (on a per protein N basis), perhaps close to the minimum necessary for this organism to support nitrogen fixation metabolism. The minimum boundary observed was an N:Fe ratio of around 200. This probably does not represent a physiological limitation but rather a structural one. Colonies must have a certain minimum protein content regardless of iron levels since a minimum amount of protein is necessary to make up crucial structural and enzyme systems.

Figure 4. Protein nitrogen to iron ratios in *Trichodesmium*. The apparent limits of protein nitrogen to cellular acid-leachable iron ratios in these samples were about 700 and 200.

Since this data represents field collections, samples were not grown under known conditions and it cannot be assumed that the maximum and minimum ratios described here represent absolute limits. In addition, fewer analyses could be performed on *Trichodesmium* than was possible with laboratory cultures of *Anabaena*. It is possible that *Trichodesmium* populations exist which fall outside the ratio limits observed in these collections.

Chlorophyll *a* to iron molar ratios for *Trichodesmium* collections are presented in the same manner in Figure 5. It is obvious that there is considerable scatter in this data and that no firm envelope of ratios can be described. Although there is a wide range of chlorophyll *a* levels over all colony iron levels, except for two outliers most of the points do cluster in the upper left hand region of the graph. This indicates that *Trichodesmium* can achieve a high chlorophyll *a* content on relatively low levels of iron. This data should probably not be interpreted to indicate the existence of an inverse relationship between iron and chlorophyll *a* in *Trichodesmium*. A more likely interpretation is that in this organism chlorophyll *a* synthesis is not dependent on a high cellular iron content.

DISCUSSION

The upper limit for N:Fe molar ratios represents the minimum iron per nitrogen that is necessary to support diazotrophic cell growth. The ratios for *Anabaena* cultures presented here represent real physiological limits while those for *Trichodesmium* are less certain due to the random nature of field sampling. Nevertheless, this data is an important first step in

Figure 5. Chlorophyll a to iron ratios in *Trichodesmium*. Molar chlorophyll a to acid-leachable iron ratios in *Trichodesmium* could not be described as an envelope as was the case for *Anabaena* ratios.

describing the range of possible N:Fe ratios in *Trichodesmium*. The *Trichodesmium* ratios measured in this investigation can be enclosed in an envelope very similar in shape to that observed in *Anabaena* cultures grown under conditions where iron concentrations could be experimentally manipulated (see Figures 2 and 4). This suggests that the upper and lower limits to N:Fe ratios presented here for *Trichodesmium* may in fact be close to actual physiologically constraining limits.

The maximum N:Fe ratio for *Trichodesmium* in these experiments was about 700, somewhat lower than the limit of 1000 reported by Rueter (1988b). The fact that *Trichodesmium* has an apparent upper N:Fe limit which is at least 75% higher than *Anabaena* means that *Trichodesmium* is able to survive on proportionately far less iron than *Anabaena*. This is reasonable since *Trichodesmium* has evolved in an environment where iron is far less available than is usually the case for *Anabaena*.

In addition to requiring less iron, *Trichodesmium* appears to use iron more efficiently to fix nitrogen than does *Anabaena*. The average nitrogen fixation rate per mole iron for *Trichodesmium* in this experiment was 0.65 nmol N/hour/mol Fe but only 0.29 mol N/hour/mol Fe for *Anabaena*. This suggests that *Trichodesmium* is able not only to support diazotrophic growth with proportionately less iron but may also use this iron to fix nitrogen more efficiently.

It is interesting to compare these iron efficiencies to the calculations of Raven (1988) on theoretical iron use efficiencies during diazotrophy. His estimate, based on the iron content and turnover time of the nitrogenase enzyme and the additional iron costs of electron carriers during nitrogen fixation, is equivalent to 6.43 mol N fixed/mol Fe/ hour for balanced

photoautotrophic growth. The observed efficiencies for *Trichodesmium* in this experiment are only about 10.1% of Raven's ideal values; for *Anabaena*, the figure is about 4.5% of Raven's calculations. Why are these values so much less than theoretically possible? The answer may lie in the fact that much of the iron in photoautotrophs is unaccountable (Hutchins et al 1988). Hewitt (1983) has estimated that only about 28% of the total iron in plant leaves can be accounted for by known catalytic functions. It has been pointed out (Raven 1988) that a similar situation exists for phytoplankton. Paradoxically, even in demonstrably iron-limited cells an iron inventory can account for only a small fraction of the total cellular iron present (Hutchins et al 1988). Therefore it appears that photoautotrophs require far more iron than calculations based on known functions would suggest.

One possibility is that the excess iron is stored in a macromolecular form similar to ferritin, a large molecular weight iron protein which has been reported from higher plants (Van der Mark et al 1982) and eukaryotic algae (Pueschel and Cole 1980). Although ferritin has not been identified in cyanobacteria, Sandmann and Malkin (1983) reported the existence of membrane-bound iron storage in *Aphanocapsa*. In addition, a 90 kD iron protein with a possible storage function has been described in *Trichodesmium* (Hutchins et al 1988). However, if the "missing" iron is present as a storage protein then it is still difficult to explain why this iron seemingly cannot be remobilized to support growth under iron-limiting conditions.

Whatever the form of iron storage, there is little doubt that cyanobacteria are able to accumulate many times the minimum cell quota of iron. In this experiment, *Anabaena* cultures at the lower N:Fe border

(N:Fe=75) have about 5.3 times the minimum iron quota measured for the upper N:Fe border (N:Fe=400). *Trichodesmium* samples at the low end of the N:Fe scale have proportionately about 3.5 times the iron found in samples on the apparent iron limitation border (N:Fe=700). This ability to store iron may allow cells to take advantage of sporadic iron inputs and allow cell division to continue for generations before iron limitation is again evident. A hypothetical iron-sufficient population at the lower N:Fe limit would move progressively upwards towards the upper limit as cell iron is "diluted" by new growth. At this point additional input of iron is necessary, moving cellular N:Fe ratios downward, before growth can resume.

Iron storage may also help explain why, despite the known dependency of diazotrophy on adequate iron nutrition, there is little apparent correlation between iron content and nitrogen fixation rates. Samples with the highest cellular iron levels did not always have the highest fixation rates. Even populations with excess stored iron might still not be healthy enough to fix nitrogen at maximum rates due to other unknown environmental or physiological factors. Although high iron content did not necessarily mean high fixation rates, both *Trichodesmium* and *Anabaena* samples in the lowest iron ranges never exhibited the highest rates (see Tables I and II). It appears that adequate cellular iron is a necessary but not sufficient condition for rapid diazotrophic growth.

In *Trichodesmium*, dependence of chlorophyll *a* content on iron content seems to be much less than that observed in *Anabaena*. In addition, the fact that measured Chl *a*:Fe ratios tend to be higher in *Trichodesmium* suggests that chlorophyll *a* synthesis in *Anabaena* may be less efficient on a per iron basis. However, we have observed that photosynthetic pigments are much

more subject to the influence of environmental variables such as light regimes than is cell protein content (Hutchins unpublished data). This suggests that less certainty can be attached to inferences about the influence of iron, especially in field samples with an unknown history.

In summary, the data suggests that *Trichodesmium* may well be better adapted to iron-efficient growth than is *Anabaena*. This conclusion fits well with the iron supply of their respective habitats but does little to explain why few other diazotrophic cyanobacterial species have been able to develop similar iron-efficient strategies for growth in the ocean.

One possibly important factor may be the existence of the "unaccountable" iron fraction in photoautotrophs. Perhaps *Trichodesmium* has been able to develop a way to minimize this fraction, which seems to be necessary for growth but serves no known function. Such a strategy would allow more of the total cellular iron fraction to be used for nitrogen fixation and lead to improved iron efficiency. Future investigations may shed light on the role that this "excess" iron plays and whether this could be a factor in explaining the unique success of nitrogen-fixing *Trichodesmium* populations in exploiting iron-depleted marine environments.

Another missing piece to this puzzle may concern iron uptake mechanisms. *Anabaena* probably obtains its iron from the comparatively high levels of dissolved iron often available in freshwater ecosystems. If iron does become limiting, siderophores can be used to scavenge trace iron from the environment. Although the iron uptake mechanisms of *Trichodesmium* are not known with certainty, it seems doubtful whether the extremely low levels of dissolved iron in the tropical Atlantic (Symes and Kester 1985) are sufficient to support growth. *Trichodesmium* may be

able to obtain iron through physical capture of wind-deposited continental dust particles as they sink rapidly through the photic zone (Rueter 1988a). If true, this phenomenon might allow growth in areas where species lacking this ability would be limited by an insufficient dissolved iron supply.

This investigation has established apparent limits on N:Fe ratios found in nitrogen-fixing populations of these two cyanobacteria. Especially important are the maximum N:Fe ratios observed, 400 for *Anabaena* and 700 for *Trichodesmium*, since maximum N:Fe ratios mark the point where iron becomes a limiting nutrient. Maximum nitrogen to iron ratios place absolute constraints on the amount of iron which must be obtained to support nitrogen fixation. Whatever uptake mechanism is used must supply iron efficiently enough to maintain this ratio despite increases in cellular nitrogen content through diazotrophic growth. If iron acquisition systems fail to keep up with cellular nitrogen increases, iron limitation of further growth is the inevitable result. It is clear that maximum nitrogen to iron ratios are one of the most important governing factors in determining the possible mechanisms and regulation of iron uptake systems in diazotrophic cyanobacteria.

CHAPTER III

SIDEROPHORE PRODUCTION AND DIAZOTROPHY IN *ANABAENA* 7120

ABSTRACT

Cultures of the freshwater cyanobacterium *Anabaena* 7120 grown in chemically defined medium were used to investigate the relationship between siderophore production and nitrogen fixation. Cultures were grown with 300 μM nitrate or with no added nitrogen source and with 1 μM FeEDTA or with no added iron; all four possible combinations of these nitrogen and iron levels were examined. Parameters measured were siderophore production, protein and chlorophyll concentrations, acid-leachable cellular iron, and nitrogen fixation rates. Results indicated that only cultures grown with nitrate and without iron were able to produce siderophores, even under saturating light conditions. No substantial increases in growth rate were observed with the onset of siderophore production. Only cultures without nitrate but with iron were able to fix nitrogen effectively. Cultures grown without nitrogen or iron were unable to make siderophores or fix nitrogen and generally failed to thrive. The implications of the mutually exclusive relationship found between siderophores and nitrogen fixation are examined in the context of current theories concerning cyanobacterial dominance and bloom formation in freshwater and limitation of cyanobacterial nitrogen fixation in the marine environment.

INTRODUCTION

Cyanobacterial nitrogen fixation and iron nutrition are interdependent processes. Despite this, a paradoxical situation arises when cells must simultaneously fix nitrogen and take up iron using extracellular siderophore acquisition systems. Siderophore production represents a loss of cellular nitrogen in order to take up iron, while iron is required to obtain nitrogen through diazotrophy. The interrelationship of these two nutrient uptake systems increases the potential for environmental co-limitation by iron and nitrogen. The governing factor is the economics of cell nutrient utilization: how much iron is necessary to support maximum rates of nitrogen fixation and how much nitrogen must be invested to take up iron through siderophore production?

Adequate iron nutrition has long been known to be a crucial factor in nitrogen fixation metabolism in cyanobacteria (Carnahan and Castle 1958). Under nitrogen-fixing conditions, iron is required at levels far exceeding those needed for growth on fixed nitrogen sources (Lammers 1982, Raven 1988). Large amounts of iron are required both for synthesis of the nitrogenase enzyme (Averill and Orme-Johnson 1978) and to support the increased levels of photosynthetic electron transport necessary to provide reductant and ATP during diazotrophic growth (Raven 1988). Many of the same species that fix nitrogen are also known to have the ability to excrete iron-specific chelators known as siderophores under iron deficient conditions (Simpson and Neilands 1976). Uptake of siderophore-scavenged iron may allow growth to occur even under conditions of severe environmental iron depletion.

To many investigators, these two physiological capabilities have suggested a link between cyanobacterial dominance and siderophore production. It has been suggested that bloom-formers such as *Anabaena* may use siderophores to acquire enough iron to support nitrogen fixation (Simpson and Neilands 1976, Lean et al 1978, McKnight and Morel 1980), leading to exclusion of competing species (Lange 1974, Murphy et al 1976). Thus, siderophores could allow a species to overcome simultaneous iron and nitrogen limitation and favor the establishment of unialgal blooms.

Recent investigations using laboratory cultures of *Anabaena variabilis* have supported this hypothesis. Kerry et al (1988) have reported that this species produces significant levels of iron-binding compounds only when grown without a combined nitrogen source, and that growth rates dramatically increase with the onset of siderophore production. This seems to suggest that the physiological capability to produce siderophores may have evolved largely in response to selection for a means to scavenge sufficient iron to support diazotrophy. Increased growth rates are suggested to reflect a release from nutrient limitation as adequate iron is taken up through siderophore scavenging (Kerry et al 1988).

Studies in this laboratory with cultures of *Anabaena* 7120 suggest that this hypothesis is not valid. Observations showed that this strain was incapable of producing siderophores except when provided with high concentrations (greater than 50 μ M) of fixed nitrogen. Little or no siderophore production was evident in nitrogen-fixing cultures; iron limitation of these cultures led only to reduced levels of nitrogen fixation and growth, ultimately resulting in chlorosis and death. In addition, nitrate-grown iron-limited cultures exhibited no increases in growth with

the advent of siderophore production.

This study presents the results of a series of experiments in which we demonstrated that *Anabaena* 7120 is not able to produce siderophores while simultaneously fixing nitrogen. No increases in growth rates were observed when siderophores were produced in nitrate-grown cultures. We propose that the energetic cost of carrying out these two metabolically expensive processes (siderophore synthesis and nitrogen fixation) is simply too high to be practical, even under light-saturated growth conditions. Our results will be discussed in the context of a reexamination of the relationship between siderophores, nitrogen fixation, and cyanobacterial dominance.

MATERIALS AND METHODS

Unialgal cultures of *Anabaena* 7120 (obtained from the Pasteur Culture Collection) were maintained at 27°C under cool white fluorescent light. Photon flux density was 50 $\mu\text{Einsteins}\cdot\text{m}^2\cdot\text{sec}^{-1}$ in the low-light cultures and 400 $\mu\text{Einsteins}\cdot\text{m}^2\cdot\text{sec}^{-1}$ in the high-light cultures. The high-light photon flux density matched the photosynthetic saturation intensity for this strain as reported by Rueter (1988a).

Cultures were grown on FRAQUIL medium modified from the recipe described by Petersen (1982). The medium was equilibrated to pH 7.8-8.0, filter sterilized with 0.2 μm Nucleopore filters, and passed through a Chelex 100 column to remove contaminating metals (Morel et al 1979). To reduce the possibility of contamination, all culturing and sampling was carried out in plastic enclosure in which positive air pressure was maintained with a HEPA-filtered laminar flow hood. All polycarbonate

culture flasks and assay equipment were acid-soaked in 1% HCl and rinsed at least six times with Nanopure (Barnstead) distilled deionized water.

Four growth conditions were examined with respect to iron and nitrogen, and results for each condition are reported as the mean of two replicate cultures. The four conditions were: 300 μM NO_3^- , 1 μM Fe (+N,+Fe); 300 μM NO_3^- , no added Fe (+N,-Fe); 0 μM NO_3^- , 1 μM Fe (-N,+Fe); and 0 μM NO_3^- , no added Fe (-N,-Fe). Nitrate was added as NaNO_3 and iron was added as FeEDTA. Adequate concentrations of phosphate, molybdenum and other potential growth and nitrogen-fixation limiting nutrients were included to insure that only iron and/or nitrogen would be limiting (Petersen 1982). Inoculum was grown for one transfer on the same iron and nitrogen concentrations as the experimental cultures to minimize nutrient carry-over. For further details of culture conditions and medium, see Appendix A.

Measurements were made every other day on four of the five physiological parameters chosen to be indicative of the nitrogen and iron status and growth rates of the cells. These were siderophore production, nitrogen fixation, total cellular protein and chlorophyll *a*. Total acid-leachable iron was measured less frequently due to the large amount of culture consumed by this assay.

Siderophore production was monitored using a colorimetric assay (CAS/HDTMA) developed by Schwyn and Neilands (1987). The CAS/HDTMA shuttle solution, containing 4 mM 5-sulfosalicylic acid as an iron shuttle, was used to facilitate equilibration of the samples. Iron binding standard curves were generated using deferoxamine mesylate USP, marketed as

Desferal by CIBA GEIGY Ltd. Cell-free supernatants were prepared by manual filtration with Whatman GF/A filters through a syringe-injected swin-lock Nucleopore polycarbonate filter rig. Although this assay is a highly sensitive indicator of iron-binding ability, it does not identify the type of siderophore being produced. This was considered unnecessary as the siderophore produced by *Anabaena* (schizokinen) has already been well-characterized (Simpson and Neilands 1976, Goldman et al 1983). The measured iron binding capacity of 1 mole of Desferal was assumed to be equivalent to that of 1 mole of schizokinen, since both form iron complexes with stability constants many orders of magnitude higher than that of the CAS/HDTMA assay solution. Siderophores must be both inducible by iron deprivation and repressible by iron availability. To verify that iron binding capacities measured were in fact due to siderophore production, iron repression was verified by addition of FeEDTA to siderophore-producing cultures at the end of each experiment.

Nitrogen fixation rates were followed by acetylene reduction gas chromatography (Mague 1978) using a Shimadzu Mini 3 Gas Chromatograph with a Poropak N column. Acetylene and ethylene peak sizes were integrated with a Shimadzu C-R3A Chromatopac integrator, and ethylene (100 ppm ethylene in He) standards were used to quantify ethylene production. Ten mL of culture were used for each assay with a 20 mL headspace and a distilled water control was run simultaneously. All samples were manually agitated periodically during incubation to allow equilibration of gases with the liquid phase. Corrections were made for the minor leakage (about 5%) which occurred from the polycarbonate Oak Ridge tubes used in the incubation experiments. Estimates of ethylene production are probably low due to

absorption of ethylene by the teflon septa of the tubes and syringe used in the assays.

Total cell protein was monitored both as an indicator of cell N content and for growth rate calculations. The Pierce BCA Assay (Pierce Chemical Company) was chosen due to its compatibility with low concentrations of detergents (Smith et al 1985), allowing virtually complete solubilization of both hydrophilic and hydrophobic cell proteins. Cultures were suction filtered onto Whatman GF/A filters which were then frozen, ground with a Teflon pestle tissue grinder in 1% sodium dodecyl sulfate, and centrifuged to prepare a clear supernatant. Depending on the protein concentrations in the supernatant, the assay protocol was either the Room Temperature Protocol (100-1200 μg protein/ml) or the Enhanced Protocol (10-250 μg protein/ml), as described in the BCA assay reagent booklet. Protein standard curves were prepared using defatted bovine serum albumen.

Chlorophyll a concentrations were estimated on a Turner Designs fluorometer using 90% acetone extracts as described by Yentsch and Menzel (1963). Suction filtration onto Whatman GF/A glass fiber filters was followed by freezing and grinding in the acetone solution. Three to five minutes centrifugation at low speed with a desktop centrifuge was necessary to eliminate turbidity from cell debris and filter material before reading fluorescence of the extracts.

Cellular leachable iron levels in the cultures were determined using a modification of the ferrozine method described by Stookey (1970) and Gibbs (1979). Extreme care was taken throughout this part of the experiment to avoid iron contamination, including the use of 1 N HCl to acid wash all assay equipment. The ferrozine iron reagent was obtained from Hach Chemical

Company. Culture samples were filtered onto 1 μ M Nucleopore filters and acid-leachable iron was extracted by an overnight leaching in 1 N HCl at 60°C. The acid supernatant from these samples was then used in the colorimetric ferrozine analysis after partial neutralization. Iron standard curves were prepared using dilutions of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in the same acid that samples were run in. Acid and filter blanks were also run, although contamination from these sources was found to be negligible.

Analytical techniques are described more fully in Appendix B.

RESULTS

Culture chlorophyll *a* concentrations are illustrated in Figure 6A and 6B. Both the +N,+Fe and -N,+Fe cultures achieved substantial increases in chlorophyll *a* concentrations, although final yields were about one-third higher in the nitrate-grown cultures. Chlorophyll *a* increases in the -N,-Fe cultures were insignificant, with final yields just slightly higher than those measured on Day 0. The siderophore-producing +N,-Fe cultures were able to achieve somewhat greater chlorophyll *a* concentrations, although again here the effects of iron limitation act to constrain growth.

Protein concentrations mostly paralleled chlorophyll *a* levels, as shown in Figure 7A and 7B. Again, the +N,+Fe cultures attained the most rapid growth rates and the highest final concentrations, followed by the -N,+Fe cultures. The +N,-Fe cultures did show a substantially greater increase in protein than in chlorophyll *a*, while the -N,-Fe cultures were barely able to increase their protein concentrations over the course of the experiment.

Growth rates for the siderophore-producing cultures (+N,-Fe) did not

Figure 6. Chlorophyll *a* concentrations of low light *Anabaena* 7120 cultures. A. 300 μM nitrate cultures (+N,+Fe and +N,-Fe). B. 0 μM nitrate cultures (-N,+Fe and -N,-Fe). Note that the +N,+Fe cultures achieved higher chlorophyll *a* concentrations than the -N,+Fe cultures, and that the +N,-Fe cultures achieved higher concentrations than the -N,-Fe cultures.

Figure 7. Total protein concentrations of low light *Anabaena* 7120 cultures. A. 300 μM nitrate cultures (+N,+Fe and +N,-Fe). B. 0 μM nitrate cultures (-N,+Fe and -N,-Fe). Note that the +N,+Fe cultures achieved higher protein concentrations than the -N,+Fe cultures and that +N,-Fe cultures achieved higher concentrations than the -N,-Fe cultures.

show any substantial increase with the advent of siderophore production as reported by Kerry et al (1988). This held true whether rates were computed using chlorophyll *a* or protein increases. The specific growth rate (μ) for these cultures as calculated from chlorophyll *a* increases in the period before the onset of significant siderophore production (Day 0 to 10 in Figure 6 A) was $0.029 \cdot \text{day}^{-1}$, and increased just slightly to $0.033 \cdot \text{day}^{-1}$ during rapid siderophore production (Day 12 to 22). Specific growth rate calculated using culture protein concentrations (Figure 7 A) actually decreased by more than 50% with the onset of siderophore production, from $0.078 \cdot \text{day}^{-1}$ to $0.032 \cdot \text{day}^{-1}$.

Only cultures supplied with nitrate and lacking iron (+N,-Fe) exhibited siderophore production (Figure 8). Filtrates from cultures limited simultaneously by nitrogen and iron (-N,-Fe) failed to increase in iron-chelating ability over the course of the experiment. Cultures supplied with iron (+N,+Fe and -N,+Fe) did not produce siderophores (data not shown). Only +N,-Fe cultures were able to initiate and sustain siderophore synthesis. Iron additions at the end of the experiment suppressed further siderophore production, verifying that the iron-binding capacity measured was in fact due to siderophore production (data not shown).

Of the sets of cultures grown without nitrate, only those supplied with iron had measurable N_2 fixation rates, as illustrated by the nitrogen fixation graph (Figure 9). -N,-Fe cultures were able to reduce only trace amounts of acetylene, indicating only basal levels of nitrogenase activity. No reduction of acetylene was observed in cultures supplied with nitrate.

Iron uptake from the medium is illustrated in Figure 10A and 10B. Although few data points were taken due to the large amounts of culture

Figure 8. Siderophore production by low light *Anabaena* 7120 cultures. Iron-binding capacities of culture filtrates are expressed as equivalents of the commercially available siderophore Desferal and are assumed to be equivalent to equimolar amounts of schizokinen. +N,-Fe cultures produced siderophores while -N,-Fe cultures did not. Cultures with added iron (+N,+Fe and -N,+Fe) exhibited no production and are not shown.

Figure 9. Nitrogen fixation rates of low light *Anabaena* 7120 cultures. -N,+Fe cultures exhibited substantial nitrogen fixation rates while -N,-Fe cultures showed only basal levels. This illustrates that adequate iron nutrition is necessary to support diazotrophy. Cultures supplied with nitrate (+N,+Fe and +N,-Fe) did not fix nitrogen and are not shown.

Figure 10. Acid-leachable cellular iron levels of low light *Anabaena* 7120 cultures. A. 300 μM nitrate cultures (+N,+Fe and +N,-Fe). B. 0 μM nitrate cultures (-N,+Fe and -N,-Fe). Note that the -N,+Fe cultures achieved slightly higher leachable iron concentrations than the +N,+Fe cultures. Note also that siderophore-producing +N,-Fe cultures approximately doubled their leachable iron level over the course of the experiment while -N,-Fe cultures failed to obtain any iron from the medium.

needed for ferrozine analysis, it is evident that both cultures supplied with iron were able to assimilate large amounts of iron from the medium. In both the +N,+Fe and -N,+Fe cultures, a period of rapid initial uptake (Day 0 to 14) is followed by relatively stationary iron levels after the Day 14 measurements. This may indicate saturation of cellular iron storage mechanisms and/or depletion of iron from the medium. It is interesting to note that, while both these cultures achieved similar leachable cellular iron concentrations, the fact that the +N,+Fe cultures achieved greater final densities than those reached in the -N,+Fe cultures (see Figures 6 and 7) means that chlorophyll to iron and protein to iron ratios were higher in the nitrate-grown cultures. Final molar chlorophyll to iron ratios were 1.7 for the -N,+Fe cultures and 2.9 for the +N,+Fe cultures. Final protein to iron ratios, calculated using weight concentrations, were 588 for the -N,+Fe cultures and 952 for the +N,+Fe cultures.

The -N,-Fe cultures were unable to obtain iron from the medium, as is shown by the lack of any increase in acid-leachable cellular iron concentrations (Figure 10B). By contrast, the siderophore-producing +N,-Fe cultures approximately doubled cellular iron concentrations, although the total amount of uptake was modest (Figure 10A).

Results obtained under saturating light intensities did not significantly vary from the pattern observed at lower light intensities. Only +N,-Fe and -N,-Fe conditions were tested at these elevated illuminations, but the results again clearly demonstrate that only cultures supplied with nitrate produced siderophores (Figure 11). Substantial growth, as monitored by culture protein concentrations, occurred only in the nitrate-grown cultures (Figure 12). Although chlorophyll *a* concentrations were not monitored in

Figure 11. Siderophore production by high light *Anabaena* 7120 cultures. Cultures grown at saturating light intensities ($400 \mu\text{Einsteins}\cdot\text{m}^2\cdot\text{sec}^{-1}$) exhibited the same pattern of production seen in low light ($50 \mu\text{Einsteins}\cdot\text{m}^2\cdot\text{sec}^{-1}$) cultures. Only +N,-Fe cultures made siderophores.

Figure 12. Total protein concentrations of high light *Anabaena* 7120 cultures. Siderophore-producing +N,-Fe cultures showed substantial increases in total cellular protein levels while -N,-Fe cultures failed to show an increase over the course of the experiment.

this part of the experiment, -N,-Fe cultures quickly developed visible chlorosis and generally failed to thrive.

DISCUSSION

The results of these experiments conclusively demonstrate that this species is unable to produce siderophores and fix nitrogen simultaneously in culture. Extrapolation of these results to natural populations casts serious doubt on the ability of *Anabaena* to simultaneously overcome nitrogen limitation (through diazotrophy) and iron limitation (through siderophore production). This raises difficult questions for those who would link cyanobacterial competitive dominance with the use of siderophores to scavenge iron to support nitrogen fixation.

This data also serves to support previous work emphasizing the essential role of iron in nitrogen fixation (Carnahan and Castle 1958, Lammers 1982). Cultures grown without added iron were simply unable to support growth through diazotrophy. Equally important, -N,+Fe culture showed substantially lower chlorophyll *a* to iron and protein to iron ratios than the same species grown on nitrate and iron. This is presumably due to the considerably higher cell quota for iron under nitrogen-fixing conditions (Raven 1988).

While cultures grown without iron or nitrate were not able to assimilate any iron from the medium, siderophore-producing nitrate grown cultures were able to double their cellular iron in this experiment (see Figure 10 A and B). This underscores the value of siderophores as iron scavengers under iron-limiting conditions, allowing the cells to obtain sufficient iron for

limited growth even from the trace iron contamination in chelexed medium.

Chlorophyll *a* concentrations varied more in response to iron deprivation than did protein concentrations. The depressed levels of chlorophyll *a* synthesis observed in both sets of iron-limited cultures may be due to the fact that iron is required for several of the biosynthetic steps in the pathway leading to chlorophyll *a* synthesis (Spiller et al 1982, Chereskin and Castelfranco 1982), and in fact chlorosis is a common symptom of iron deprivation (Rueter 1988a). Protein, by contrast, is much more highly conserved under conditions of iron stress. We have found with a variety of phytoplankton that protein may be a better indicator of total algal biomass than photosynthetic pigments (Hutchins unpublished data), which vary not only with different light regimes but with iron nutrition and probably with other variables as well.

While Kerry et al (1988) found evidence of increased growth rates associated with iron limitation at very low iron concentrations, we have found no evidence for significantly increased growth rates associated with siderophore production at these iron levels. The notion of growth rates increasing at low iron concentrations is contrary to all our experience with laboratory cultures and natural populations of phytoplankton. In fact, the rather low specific growth rates observed for the iron-limited cultures in these experiments are a typical symptom of iron deprivation.

Our working hypothesis was that elemental allocation limitation was responsible for the mutually exclusive nature of siderophores and nitrogen fixation. Since each schizokinen molecule contains four atoms of nitrogen (Simpson and Neilands 1976), we reasoned that siderophore production represented an insupportable loss of nitrogen under limiting conditions.

However, rough calculations based on the cellular nitrogen content of the cultures (see Appendix B) and extracellular siderophore concentrations led us to estimate that only about 4% of cell nitrogen was lost as siderophore over the three week time frame of this experiment. It seems unlikely that siderophore production represents a substantial drain on the nitrogen reserves of this species. We now believe that the reason siderophore production is not feasible during nitrogen fixation is energetics limitation rather than elemental limitation.

Nitrogen fixation is undoubtedly the most energetically expensive route known for nitrogen acquisition. Each nitrogen molecule incorporated into ammonium via nitrogen fixation requires hydrolysis of about 16 ATPs (Postgate 1987), leading to a substantial loss of photosynthetically produced energy available to the cell for carbon fixation and growth. This expense is reflected in the lower growth rates of N_2 -fixing cultures when compared to those grown on nitrate. Similarly, siderophore production has been called the most energetically expensive route known for uptake of any required nutrient (Raven 1984). Siderophore production involves the synthesis and export of a large quantity of extracellular chelator, simply on the chance that some of the siderophore molecules will encounter iron and diffuse back to the cell surface for uptake. Each of the four nitrogen atoms of a schizokinen molecule hypothetically produced under nitrogen-fixing conditions would cost 16 ATPs; in addition, the citrate backbone of the siderophore represents the loss of another potential 20 ATPs (if the citrate had been used for oxidative phosphorylation). Thus, even if we assume a one-to-one ratio of siderophore produced to iron acquired, the cost of synthesizing the schizokinen necessary to take up enough iron to make up

the 36 iron atoms in each nitrogenase molecule would be at least 3,024 ATP. This cost represents a minimum break-even point for balanced growth. Of course, the true cost of nitrogen fixation and siderophore production is much higher, since this simplistic model neglects all the other energetics costs associated with these two processes. These include the energetic cost of making membrane receptor and uptake proteins, costs associated with coupling to drive siderophore synthesis and transport systems, costs of iron reduction and intracellular transport, and costs associated with synthesis of the protein portions of the nitrogenase molecule.

We attempted to find out whether this hypothesized energetics limitation could be overcome by maximizing photophosphorylation rates through growth at saturating light intensities. The fact that cultures were unable to simultaneously produce siderophores and fix nitrogen even at saturating light intensities may mean that this energetics limitation is absolute and cannot be overcome even at maximum photosynthetic rates.

A key assumption in this energetics limitation model is the ratio of iron taken up to siderophore exported. A one-to-one ratio seems highly unlikely to occur in the dilute conditions found in most natural waters. Most of the siderophore produced under natural conditions probably simply diffuses away, with only a very small proportion ever binding to iron in the environment and returning to the cell to support growth. Iron-limited cultures of *Anabaena* have been found to produce siderophores at molar concentrations from 10 to 1000 times greater than the molar concentrations of iron in the medium (Clarke et al 1987). If this is an accurate reflection of siderophore production levels by natural populations, the ATP costs calculated above are underestimated by one to three orders of

magnitude. Obviously, progressively greater dilutions of siderophore produced would soon make the cost of taking up iron by this route prohibitively expensive, especially if we consider the simultaneous cost of supporting growth through diazotrophy.

The very high affinity of the ferric schizokinen transport system for its substrate means that uptake is far more likely to be limited by the rate at which the iron-siderophore complex arrives at the cell surface than the rate at which iron is transported across the cell membrane. Kinetics of siderophore-mediated iron uptake in *Anabaena* have been investigated by Lammers (1982), who found the apparent K_M for schizokinen uptake to be very low, about 36 nM. This, coupled with the fact that the formation constant of the ferric schizokinen complex is very high ($K_f=10^{23}$), means that uptake is probably not the rate-limiting step for iron acquisition by siderophores. Uptake rates are essentially linear at any environmental iron concentration low enough to induce siderophore production. Since the rate at which the cell encounters siderophore-chelated iron is dependent on simple diffusion, substantial dilution of the siderophore would tend to severely limit the amount of iron that can be obtained. It appears that dilution could easily be a rate-limiting factor in the acquisition of iron through siderophores.

Energetics and the dilution effect may have implications for the production of siderophores by natural algal populations. There may be a threshold value for cell density which determines whether growth can be supported through siderophore-mediated iron uptake. Siderophore production may not be energetically feasible except under very dense bloom conditions where the dilution factor is not critical. At some point,

however, the effects of light limitation through self-shading would begin to counteract the advantages obtained from high siderophore concentrations. A balance point where cell densities are high enough to allow siderophore production to be energetically possible without being high enough to severely limit energy capture through reduced photon flux rates would seem to be the ideal situation. Although some cyanobacterial blooms may reach cell densities approaching those produced under laboratory culture conditions, it seems likely that dilution effects must discourage siderophore production when cell densities are low, as they are in most natural environments.

Constraints imposed by energetics and the dilution factor may be the key reasons why very few marine planktonic cyanobacteria have been found to produce siderophores. Investigations in our laboratory have failed to find any evidence of siderophore production by several strains of the ubiquitous unicellular marine cyanobacterium *Synechococcus* (data not shown), despite the fact that these strains come from oceanic environments where iron may well be limiting. Dilution of any siderophore by the huge volume of water in the photic zone of the sea may mean that the return of iron is simply insufficient to allow siderophore production to be advantageous. This may also be one reason why cyanobacterial diazotrophy is rare even in the nitrogen-limited tropical oceans, since planktonic cyanobacteria may be unable to use siderophores to obtain iron for nitrogen fixation.

Our work suggests that siderophore production and nitrogen fixation are not compatible processes in photoautotrophs. This necessitates a rethinking of proposed theories concerning competitive dominance and bloom maintenance through diazotrophy and siderophore production in

freshwater ecosystems, as well as raising some interesting questions for future researchers concerned with cyanobacteria, siderophores and nitrogen fixation in marine ecosystems.

CHAPTER IV

MANGANESE TOXICITY AND SIDEROPHORE PRODUCTION IN *ANABAENA* 7120

ABSTRACT

Iron-limited cultures of the freshwater cyanobacterium *Anabaena* 7120 were examined for gratuitous manganese repression of siderophore production. No evidence for this type of molecular regulation was found, although manganese did appear to bind to schizokinen in the medium, producing some interference with iron-binding assays. These results are discussed in light of their implications for genetic regulation of cyanobacterial siderophore synthesis and manganese toxicity to blue-greens.

INTRODUCTION

One of the factors which has been suggested as contributing to dominance shifts between cyanobacteria and eukaryotic algae is dissolved manganese ion concentrations. It has been hypothesized that high manganese levels may discourage the establishment of cyanobacterial blooms due to toxic effects, leading to dominance by diatoms (Patrick et al 1969). Although the mechanism of this increased sensitivity to manganese

in blue-greens is unknown, some clues have emerged from work on iron nutrition metabolism. Both iron and manganese are transition metals with similar atomic radii, weights and redox characteristics. There is some evidence to support the possibility that manganese acts as a competitive inhibitor of iron uptake. Gerloff and Skoog (1957) found that low iron-to-manganese ratios severely inhibited growth of cultures of the blue-green *Microcystis*, even when iron levels were the same as those that supported good growth in the absence of high manganese levels. They also measured manganese concentrations in lake water which could easily be toxic to cyanobacteria in soft water (low Ca^{2+}) lakes .

In light of this evidence for manganese toxicity to cyanobacteria, new evidence from unrelated studies of the molecular regulation of bacterial iron-uptake genes was of interest. The first thorough characterization of the genetic regulation of any siderophore system was carried out by J.B. Neilands and associates. They found that *E. coli* regulates iron uptake genes through a system known as *fur* (for ferric uptake regulation). The *fur* gene codes for a 17 kilodalton polypeptide which, along with the corepressor Fe^{2+} , represses expression of bacterial siderophore synthesis enzymes and membrane receptor genes (Bagg and Neilands 1987, Hantke 1984). Of particular interest was their observation that Mn^{2+} acts as a gratuitous repressor of the *E. coli* siderophore genes. Thus, suppression of siderophore synthesis can occur even under conditions of iron deprivation if high levels of manganese ions are present. This gratuitous repression is so pronounced that it has been used as a selection method for bacterial *fur* mutants (Hantke 1987).

The above work suggests that a possible mechanism of cyanobacterial

manganese sensitivity might be interference with siderophore-mediated iron uptake in a manner similar to that observed with the *fur* system. If manganese acts as a gratuitous repressor of siderophore-related gene expression in cyanobacteria, this could explain the reported observations of manganese toxicity in blue-greens. Manganese toxic effects might be due largely to interference with iron uptake by siderophore-producing cyanobacteria. To test this hypothesis, a set of experiments was devised to investigate the effects of various manganese levels on siderophore production by *Anabaena* 7120. In these experiments, siderophore production plus (+) and minus (-) manganese was measured. In addition, chlorophyll *a* levels were monitored to assess growth and toxic responses as well as to insure that any depression in siderophore production that might be observed was not simply the consequence of death of the cultures.

MATERIALS AND METHODS

Cultures of *Anabaena* 7120 (PCC) were grown in modified Fraquil medium (Petersen 1982) at pH 8 under cool-white fluorescent bulbs at a photon flux density of 50-100 $\mu\text{mol photons}\cdot\text{m}^2\cdot\text{sec}^{-1}$. Iron was omitted from the culture medium to promote induction of siderophore synthesis, and the medium was run through a Chelex 100 column to remove contaminating trace metals (Morel et al 1979). The medium contained normal Fraquil concentrations of nitrate, phosphate and trace metals. In addition, MnCl_2 was added to some cultures at a concentration of 100 μM or 10 μM , while others were grown minus added manganese as controls. The same pre-conditioned inoculum was used for all conditions in each

experiment. Details of the culture techniques used are presented in Appendix A.

Siderophore production was measured using the colorimetric CAS/HDTMA shuttle solution iron-binding assay (Schwyn and Neilands 1987). Culture supernatants were prepared by manual filtration through Whatman GF/A glass fiber filters and were added to an equal volume of the shuttle solution before equilibration at room temperature for two hours. Absorbance was measured at 630nm and culture siderophore production was calculated using a standard curve prepared with Desferal obtained from CIBA GEIGY Ltd.

Chlorophyll a concentrations were followed using 90% acetone extracts to measure fluorescence as described by Yentsch and Menzel (1963). Suction filtration of cultures onto Whatman GF/A filters with Mg^{2+} was followed by freezing and grinding in acetone. The extract was centrifuged at low speed on a desktop centrifuge for 5 minutes to clear out ground filter material and cell debris. Fluorescence was measured on a Turner Designs fluorometer. Culture chlorophyll a concentrations were calculated according to the method of Parsons et al (1984). Analytical techniques are described in greater detail in Appendix B.

RESULTS

These experiments were repeated four times. Since cultures were not replicated in each individual experiment, results are presented for two of these trials. In each case the result was the same: manganese additions failed to repress siderophore synthesis.

Immediately upon addition of manganese to the inoculated medium, a change in iron-binding capacity as measured by the CAS/HDTMA shuttle assay became evident. This is reflected by the lower levels of Desferal iron-binding equivalents in supernatants of plus manganese cultures, as shown in Figure 13. The decrease in iron-binding was greater for the 100 μM Mn cultures than it was for the 10 μM Mn cultures. It is unlikely that this reflects any real change in siderophore concentrations, since this decrease happened instantly on addition of manganese. A more likely explanation is that manganese binds to schizokinen and thereby interferes competitively with iron-binding capacity (see Discussion).

To examine the possibility of manganese binding to schizokinen in the medium, an iron-binding standard curve of Desferal plus and minus 10 μM Mn was run. The results, presented in Figure 14, show no apparent deviation between the standard curves plus and minus Mn. Apparently the trihydroxamate Desferal does not have significant Mn-binding ability, although the dihydroxamate schizokinen in the culture supernatants appeared to. Unfortunately, a purified schizokinen preparation was not available to compare iron-binding plus and minus Mn.

In Trial 1, production of siderophores was roughly parallel in the minus Mn and 100 μM Mn cultures, with the initial differences in iron-binding capacities being maintained throughout the course of the experiments (Figure 15). A different pattern was observed in the 10 μM Mn culture, which lagged in production at first and then exhibited strong siderophore production at the end of the experiment. In Trial 2 (Figure 16), this difference did not occur and siderophore production rates were approximately equal for each condition examined. A lag in siderophore

Figure 13. Manganese interference with the CAS/HDTMA iron-binding assay. Iron-binding capacity of the same schizokinen-containing culture filtrate decreases proportionately to the amount of manganese added. This suggests that manganese is binding to the siderophore and interfering with the ability of the siderophore to remove iron from the CAS/HDTMA shuttle solution (see Appendix B for a description of the chemistry of the CAS/HDTMA assay).

Figure 14. Desferal iron-binding curve with and without $10\ \mu\text{M}$ manganese. Iron-binding curves with the trihydroxamate siderophore Desferal were virtually identical with and without the addition of manganese.

Figure 15. Schizokinen production by *Anabaena* 7120 cultures with and without added manganese (Trial 1). 0 μM and 100 μM Mn cultures showed parallel rates of production, with the initial difference in iron binding (see Figure 13) being maintained throughout the experiment. The 10 μM culture lagged at first in siderophore production but eventually began producing schizokinen as well.

Figure 16. Schizokinen production by *Anabaena* 7120 cultures with and without added manganese (Trial 2). Production curves in all three cultures were roughly parallel, indicating that manganese does not repress schizokinen synthesis.

production is not an uncommon event in culture work and may be due merely to trace iron contamination of the medium or iron carry-over in the inoculum.

Except for the 10 μM Mn culture mentioned above, all the cultures exhibited a stationary phase of siderophore production which corresponded to the beginning of stationary growth phase as indicated by chlorophyll *a* levels, around day 8 for Trial 1 (see Figures 15 and 17). All cultures, plus or minus manganese, showed a similar pattern of chlorophyll *a* production, with an initial rapid growth phase being followed by a period of stationary phase and finally chlorosis and death. The symptoms of culture decline are identical to those commonly observed for iron-starved cultures and it is probable that this was the cause for the onset of senescence. In no case did manganese additions prevent growth from occurring, although the higher chlorophyll *a* concentrations achieved in the minus Mn cultures probably indicate that there were some sublethal toxic effects from the manganese concentrations used in the experiments.

DISCUSSION

No evidence was found for manganese repression of siderophore synthesis in *Anabaena* 7120. This seems to make it unlikely that schizokinen production is regulated in an identical manner to the *fur* iron uptake regulatory system of *E. coli*. This does not mean that regulation of siderophore synthesis does not occur through a similar repressor/corepressor mechanism, however. These experiments show only that manganese does not act as a gratuitous repressor in *Anabaena* ; in fact,

Figure 17. Chlorophyll *a* concentrations of *Anabaena* 7120 cultures with and without added manganese (Trial 1). Stationary growth phase, as indicated by culture chlorophyll *a* concentrations, occurred at about the same time as stationary phase of siderophore production (see Figure 15). Lower levels of chlorophyll *a* in cultures with added manganese probably reflects sublethal toxicity from the manganese additions.

a system which is similar in design but not in detail to *fur*-mediated regulation is still quite possible. Characterization of such a system would be much more difficult if no homology exists to the *fur* system, since this excludes the use of molecular biology techniques to locate the homologous cyanobacterial genes and regulatory regions. True characterization of the regulatory strategy of *Anabaena* siderophore production may have to await a more detailed description of the organization of the cyanobacterial genome.

Attempts are underway in this laboratory to repeat these experiments using other species of cyanobacteria, including the bloom-former *Microcystis* which was used in the iron/manganese work done by Gerloff and Skoog (1957). The use of species known to be sensitive to manganese toxicity will be helpful in assessing whether this sensitivity is due to interference with siderophore-mediated iron uptake or to another mechanism.

An interesting but unexpected observation from the results of this experiment is the fact that the algal dihydroxamate siderophore schizokinen appears to bind manganese, unlike the trihydroxamate Desferal used for an iron-binding standard. This result is similar to the experiments of McKnight and Morel (1980) and Clarke et al (1987) in which it was demonstrated that cultures of *Anabaena* were protected from copper ion toxicity by formation of a cupric schizokinen complex. The cupric complex was apparently not taken up by the algal cells, and the resultant reduction of free copper ion activity allowed siderophore-producing cultures to grow at copper concentrations which were lethal to non-producing cultures. Although the presence of the siderophore mediated the effects of copper

toxicity, it was not formed in response to copper exposure but only under iron-deficient conditions.

Since schizokinen also appears to bind manganese, it is possible that a similar detoxification mechanism may come into play. If, like the cupric schizokinen complex, the manganese schizokinen complex is not recognized or taken up by algal membrane receptors, it is likely that siderophore production may actually protect cells from the effects of high manganese concentrations. Thus, iron limitation may act to ameliorate the effects of toxic levels of manganese in the water column. This may help explain why our siderophore-producing cultures were able to continue growth (as indicated by increases in chlorophyll *a* levels) despite manganese concentrations as high as 100 μM . To determine whether schizokinen production acts to protect *Anabaena* from manganese toxicity, it would be necessary to grow iron-replete cultures (not producing siderophores) at identical manganese concentrations to those used in these experiments.

CHAPTER V

CONCLUSIONS

It is clear that cyanobacterial nitrogen fixation is dependent to a large extent on the ability to obtain sufficient iron. A critical parameter in any attempt to model this system is the cellular nitrogen to iron ratio. Since protein is the primary cell reservoir for nitrogen and many catalytic proteins (enzymes) require iron cofactors, including the nitrogenase enzyme, this physiological relationship is especially intimate. The nitrogen to iron ratio places constraints on the relative amounts of the two elements that must be obtained to support balanced growth and ultimately plays an important role in determining whether either nutrient becomes limiting. An examination of the range in these ratios allows us to predict with some confidence the iron conditions that a particular species requires to survive diazotrophically. High nitrogen to iron ratios (such as this investigation found in *Trichodesmium*) indicate selection for iron efficiency while low ratios (such as those measured in *Anabaena*) mean that only relatively iron-rich environments can be exploited while fixing nitrogen.

Another closely related factor which affects this system is the method by which iron is obtained. These experiments suggest that in some cases, as when iron must be obtained through siderophores, iron uptake is possible only when growth is supported by an exogenous supply of fixed nitrogen. Although it is unwise to assume that results obtained from one species

apply to all cyanobacteria, a qualified extrapolation of these results to natural environments is possible. If this mutually exclusive relationship between nitrogen fixation and siderophore production applies as a general rule, nitrogen and iron colimitation of cyanobacterial primary production becomes a likely consequence in many aquatic ecosystems. The crucial question here concerns the costs of iron uptake relative to the benefits. Any uptake system must be capable of providing iron in at least the minimum amounts demanded by the nitrogen to iron ratio of that species. Thus, *Anabaena* must obtain one iron atom for every 400 nitrogen atoms fixed in order to maintain balanced growth while *Trichodesmium* needs only one iron for about every 700-1000 nitrogens. At the same time, the cost of this uptake must be low enough that growth does not become energy limited, especially when added to the already high energetics cost of diazotrophic growth. Siderophore uptake is energetically very expensive and these costs increase proportionately in relation to dilution rates of the extracellular chelator. Consequently, dilution of siderophores may very well cause the costs of siderophore production to be prohibitive, except possibly in very dense blooms.

Although siderophore production in *Anabaena* does not seem to be regulated by manganese in the same manner that it is in *E. coli*, the fact that schizokinen appears to bind to manganese in the water has interesting implications for cyanobacterial evolution and ecology. Siderophores have already been shown to detoxify copper ions through complexation (McKnight and Morel 1980, Clarke et al 1987), and the possibility that this might also occur with manganese could mean that this detoxification role is more important than has been previously suspected. Detoxification of toxic

metals in the water is an additional benefit which, when added to the known advantages of allowing growth in iron-poor environments, might help make siderophores advantageous despite the high physiological costs of production. It is a common evolutionary occurrence that adaptations in response to a particular selective pressure (in this case, limitation by iron) are used for another purpose in the face of new selective pressures (in this case, metal toxicity). This is the well-known "Panda's Thumb" principle as formulated by Stephen Jay Gould (1980). Although *Anabaena* is not known to produce siderophores solely in response to toxic metal concentrations, it is possible that other species do. There is good evidence that most of the copper and zinc ions in seawater are tightly bound to uncharacterized dissolved organic ligands (Coale and Bruland 1988, Bruland 1989). Could these organic ligands be similar to or evolutionarily derived from siderophores? It is conceivable that strong selective pressure in the form of high environmental metal concentrations might favor constitutive siderophore mutants in which siderophore production is not regulated by iron availability. Elevated heavy metal input to some systems due to recent human activity may increase the possibility of evolutionary selection for constitutive siderophore mutants.

This question of whether siderophore production can protect cyanobacteria from toxic manganese levels is probably the most easily answered of any posed by these investigations. All that would be required would be to grow siderophore-producing and iron-sufficient (non-producing) cultures of the same species with various levels of manganese. If there is a detoxification effect, the non-producing cultures should exhibit symptoms of toxicity while the siderophore-producing cultures should not.

The experiments presented here can offer some insight into the problem of cyanobacterial dominance in freshwater ecosystems. The relatively low nitrogen to iron ratio of *Anabaena* suggests an adaptation to high iron environments and this observation fits in well with previous work suggesting that cyanobacteria are favored by high iron concentrations (Gerloff and Skoog 1957, Morton and Lee 1974, Parr and Smith 1976). The implications of the exclusive relationship between nitrogen fixation and siderophore production found in this strain of *Anabaena* depend on whether this exclusivity is a general rule or is specific to this strain. If only certain species or strains operate under this constraint, it may mean that different cyanobacterial strains will dominate under conditions of high or low iron and the in presence or absence of combined nitrogen sources. *Anabaena* 7120 may simply be a strain that is adapted to grow in nitrogen deficient environments only when iron is plentiful. If on the other hand, as we suspect, this relationship between siderophores and diazotrophy turns out to be widespread, the conclusion must be that cyanobacteria cannot dominate through fixing nitrogen in iron-poor environments. Iron and nitrogen colimitation would then be an insurmountable obstacle to competitive dominance by cyanobacteria. Either iron or nitrogen would have to be available in substantial amounts before cyanobacteria could successfully exclude other species. A series of experiments with cultures grown with and without iron and nitrogen (such as is presented for *Anabaena* 7120 in Chapter III) using a wide variety of siderophore-producing, diazotrophic cyanobacterial species and strains would help to answer this question. This would be an important step towards defining the conditions under which blooms are created and maintained.

The lack of cyanobacterial diazotrophs in the ocean also may be related to this inability to use siderophores to obtain iron while fixing nitrogen. The problem of siderophore dilution could be expected to be especially severe in open ocean systems. The fact that large areas of the ocean are depauperate in both nitrogen and iron may mean that the phenomenon of nitrogen and iron colimitation is the rule rather than the exception here. *Trichodesmium* is well adapted to this low iron environment, as evidenced by the very high nitrogen to iron ratios found in field samples. The question of whether this iron efficiency is due to less iron in the unaccountable cellular iron fraction, or to more efficient catalytic use of iron in known functions, still remains to be answered. Although little is known for certain about iron uptake in this genus, the postulated ability to obtain iron from sinking dust particles would go far towards explaining the unusual ability of *Trichodesmium* to fix nitrogen in the sea. In fact, the large size and profile of the macroscopic colonies would seem to make them ideal for capture of dust particles. The motile trichomes of this genus could conceivably envelop and retain dust, and something very close to this appears to be happening when colonies to which dust has been added are examined using scanning electron microscopy (John Rueter and Randy Smith personal communication). Once the dust is attached, iron could be mobilized through release of siderophore-like chelators at the cell surface or even solubilized by organic acids, significantly raising the local concentration of dissolved iron in the immediate vicinity of the colony. Bacteria have been shown to have the ability to obtain iron from a wide variety of natural minerals using siderophores (Ito 1987). The use of chelators to break down dust particles on the colony surface would probably be difficult to detect by

ordinary analytical techniques since the amounts required would be very small in comparison to conventional siderophore concentrations. Although this scenario remains pure speculation at present, a unique ability to utilize this source of iron which is not available to most cyanobacteria could well be a decisive selective advantage. The chief obstacle to answering this question is the difficulty which most investigators have experienced with culturing *Trichodesmium*. Until stable, healthy cultures can be maintained over an extended period of time it will be difficult to solve the question of iron uptake mechanisms.

The interplay between iron and nitrogen fixation metabolism is an extremely complex one. Determining factors include iron efficiency, iron storage, iron uptake mechanisms, and cell energetics. These factors are in turn affected by a host of environmental variables. Much work remains to be done before we can unravel the often subtle relationships between cyanobacterial iron and nitrogen utilization and fully apply this knowledge to the problems of cyanobacterial dominance in freshwater and limitation of nitrogen fixation in the marine environment.

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APPENDIX A

ALGAL CULTURE AND FIELD COLLECTION TECHNIQUES

ANABAENA CULTURE TECHNIQUES

The strain of *Anabaena* used in these experiments was *Anabaena* 7120 obtained through the Pasteur Culture Collection. Purity of the unialgal cultures was periodically verified by microscopic examination. Although cultures were not claimed to be axenic, bacterial contamination was very low and sometimes undetectable by visual microscopic examination.

Anabaena cultures were maintained in modified Fraquil medium (Petersen 1982) which was equilibrated to pH 8 and passed through a Chelex 100 column to remove contaminating trace metals as described in Morel et al (1979). Inoculum for experimental cultures was grown for at least one transfer on medium of the same iron and nitrogen content as the experimental cultures in order to allow the cells to become acclimated to experimental conditions and to minimize carry-over of nutrients and metals. The recipe of the modified Fraquil medium used to grow *Anabaena* is given in Table III. Modifications of the basic Fraquil recipe include reduction of silicate concentrations from $1.25 \cdot 10^{-5}$ M to $1.25 \cdot 10^{-6}$ M, addition of $2.5 \cdot 10^{-4}$ M KCl, and doubling of phosphate concentrations to $2 \cdot 10^{-5}$ M to avoid phosphate limitation during rapid growth, all of which have been found to be optimal for growth of *Anabaena* 7120. Nitrate-grown cultures were

TABLE III

MODIFIED FRAQUIL CULTURE MEDIUM FOR *ANABAENA* 7120

<u>Chemical component</u>	<u>Concentration (M)</u>
Ca^{2+}	$2.5 \cdot 10^{-4}$
Mg^{2+}	$1.5 \cdot 10^{-4}$
K^{+}	$2.5 \cdot 10^{-4}$
Na^{+}	$2.6 \cdot 10^{-}$
Fe^{3+}	$1.0 \cdot 10^{-6}$ or 0
Mn^{2+}	$2.3 \cdot 10^{-8}$
Co^{2+}	$2.5 \cdot 10^{-9}$
NH_3	$1.3 \cdot 10^{-9}$
CO_3^{2-}	$1.5 \cdot 10^{-4}$
SO_4^{4-}	$1.5 \cdot 10^{-4}$
Cl^{-}	$5.2 \cdot 10^{-4}$
PO_4^{3-}	$2.0 \cdot 10^{-6}$
B(OH)_4^{-}	$1.0 \cdot 10^{-7}$
MoO_4^{2-}	$1.5 \cdot 10^{-9}$
NO_3^{-}	$3.0 \cdot 10^{-4}$ or 0
EDTA	$5.0 \cdot 10^{-6}$
SiO_3^{2-}	$1.25 \cdot 10^{-6}$

maintained at a nitrate concentration of $3 \cdot 10^{-4}$ M NO_3^- . Nitrogen fixing cultures were grown on medium without nitrate and iron was omitted from iron-limited cultures.

Cultures were grown in a plastic enclosure with positive air pressure maintained by a HEPA-filtered laminar flow hood, allowing contamination problems to be kept to a minimum. All sampling and culture handling and inoculation were carried out in this enclosure. This is especially important when working with iron-limited cultures, as ordinary room dust contains enough iron to cause significant contamination. Light provided was continual cool-white fluorescent at a photon flux density of 50-100 $\mu\text{Einstein} \cdot \text{m}^2 \cdot \text{sec}^{-1}$ (as measured on a Li-Cor, Inc. Model Li-185B Quantum/Radiometer/ Photometer), except as otherwise stated. The temperature was maintained at 27°C for optimum growth. Culture vessels were 2 liter polycarbonate flasks, although culture volume was kept to 1.5 liters or less to allow ample room for gas exchange. Manual agitation at least once a day insured that all cells recieved adequate opportunity for light exposure and nutrient uptake.

TRICHODESMIUM COLLECTION TECHNIQUES

Field collections of *Trichodesmium* were made aboard the research vessel Columbus Islen which is owned by the National Science Foundation and operated by the University of Miami. Chief scientists on this cruise were Doug Capone and Ed Carpenter. Collections were made between November 4 and November 23 of 1988 and sampling stations were spread over a large portion of the northern and central Caribbean (see Table I,

Chapter II for latitude and longitude of stations). Plankton nets were used to collect colonies, which were then separated manually with a plastic inoculation loop from zooplankton and debris which was commonly included in the net tow samples. Colonies of the "tuft" morphology (linearly aligned trichomes) were chosen as many of the "puff" type colonies (radial morphology) are known to contain more commensal organisms such as hydroids (Gary Borstad personal communication), which would tend to skew protein, iron and chlorophyll measurements of algal biomass. All tows were taken from surface water (10 meters or less) to avoid the effects of light limitation on the cells.

APPENDIX B

ANALYTICAL TECHNIQUES

TRACE METAL CONTAMINATION CONTROL TECHNIQUES

All culture and assay laboratory equipment used in these experiments was acid washed in 1% HCl overnight and then rinsed in Nanopure distilled water six times before drying in a plastic-enclosed drying area. An exception to this were glass tubes used for acetone extractions of chlorophyll a which were not acid washed since trace amounts of residual acid could result in conversion of chlorophyll a to phaeophytin (see section on chlorophyll a assays). Another exception were the polystyrene tubes used for ferrozine iron analyses. Since this assay is particularly sensitive to iron contamination, these tubes were acid washed in 1 N HCl and then rinsed and dried as described above (see section on ferrozine analysis).

NITROGEN FIXATION: ACETYLENE REDUCTION GAS CHROMATOGRAPHY

Acetylene reduction measurements using gas chromatography were used to assay nitrogen fixation for both *Anabaena* and *Trichodesmium*. This technique is based on the fact that acetylene (C_2H_2) is a substrate analog for the nitrogenase enzyme, with reduction resulting in production of

ethylene (C_2H_4). Ethylene production over time is then measured using a gas chromatograph. The method has distinct advantages over ^{15}N isotope assays for diazotrophy, including greatly increased sensitivity and the fact that equipment is simple and portable, compared to mass spectrometry techniques required for isotope measurements. However, a problem with the acetylene reduction technique is uncertainty as to the conversion factor between the substrate analog and the natural substrate. A theoretical ratio of 3 moles C_2H_2 reduced to 1 mole N_2 should apply based on electron transfer; however the fact that H_2 is usually evolved in N_2 reduction but not with C_2H_2 means that observed ratios are often closer to 4 or 5 to 1 (Taylor 1983). For the purposes of this investigation, a ratio of 4 to 1 was assumed.

The equipment used for both field and laboratory measurements of acetylene and ethylene was a Shimadzu GC Mini2 Gas Chromatograph equipped with a Porapak N 80-100 mesh column operated at a temperature of $80^{\circ}C$. The carrier gas was ultra-high purity grade nitrogen and the hydrogen used for the burner was also ultra high purity. Column retention time was about 70 seconds for ethylene and 105 seconds for acetylene. An ethylene standard was used for calibration purposes, although it was not available until after some of the culture measurements were completed. Integration of ethylene peaks in order to estimate acetylene reduction rates was accomplished with a Shimadzu C-R3A Chromatopac integrator. All field measurements on *Trichodesmium* were performed by Doug Capone or Judy O'Neil and all laboratory measurements on *Anabaena* were performed by the author.

For field measurements, samples were incubated in 30 mL sealed serum vials, using 10-15 *Trichodesmium* colonies in 10 mL seawater, as described by Carpenter et al (1987). Laboratory cultures were incubated in 30 mL Nalgene polyethylene centrifuge flasks containing 10 mL well-mixed culture with a 20 mL headspace. A Teflon backed silicone septum was used for these measurements; although Carpenter et al (1987) found no difference in incubations performed in serum vials or in polyethylene tubes, the latter may show some minor leakage over the time course of the incubation (approximately 5%). The teflon septa used absorb some ethylene and so measurements of ethylene production are probably underestimates. After acetylene was generated by placing calcium carbide in distilled water in a specially designed plastic trap with a rubber septum, 2 mL of acetylene was withdrawn and injected into the experimental tubes; measurements were made between the second and third hours after injection to avoid any lag effects (Mague 1978). After incubation, 100 μ L of gas was withdrawn from each sample and injected into the gas chromatograph using a gas-tight 1750 syringe from Hamilton Co. of Reno, Nevada. Absorption of ethylene by the teflon septum of the syringe may be another source of error in ethylene measurements. Cultures were incubated under the same light conditions as those the cultures were grown under (see Appendix A). For field measurements, only colonies collected during daylight hours were used as *Trichodesmium* has been found to shut down nitrogen fixation at night, even when incubated under artificial lights (Capone and O'Neil 1988). Simultaneous controls were run with distilled water and acetylene or seawater and acetylene. A small ethylene peak was sometimes observed in the negative control which probably represents trace contamination of the

acetylene standard; only values in excess of the control were considered to be indicative of diazotrophy.

CHLOROPHYLL a : ACETONE AND METHANOL EXTRACTION ASSAYS

Chlorophyll a concentrations were determined in field samples using a methanol extraction spectrophotometric technique and in laboratory samples using fluourometry on acetone extractions. Both of these methods are standard techniques which have long been used by physiologists and oceanographers (Parsons et al 1984, Jensen 1978). Cyanobacteria do not contain accessory chlorophylls such as b and c; the role of these eukaryotic antenna pigments is filled by phycobilins and the only chlorophyll present in prokaryotes is Chl a (Bold and Wynne 1985). This means that correction for overlap of chlorophyll absorbance spectra is unnecessary.

Acetone extractions for chlorophyll a measurements were first introduced by MacKinney (1947). The method was refined to include the use of a fluourometer, which increased sensitivity considerably over spectrophotometric methods (Yentsch and Menzel 1963). In these investigations, a Turner Designs fluourometer was used, which makes the calibration of each door setting unnecessary. The method involves suction filtration of culture samples (usually 5 or 10 mL samples) onto Whatman GF/A glass fiber filters, with the addition of two drops of saturated MgCO_3 during filtering to make loss of the Mg^{2+} moiety of the chlorophyll a molecule thermodynamically unfavorable. This is necessary because the phaeophytin produced when the magnesium ion is removed has different fluorescence and absorbance characteristics than native chlorophyll a,

which will result in erroneous measurements. Filters were then either assayed immediately or frozen for future analysis. The next step involved brief grinding in 10 mL 90% acetone (v/v), taking care that the extract did not heat up from excess grinding. After centrifuging at high speed with a desk-top centrifuge for 5 minutes, fluorescence was measured before and after addition of two drops of 5% HCl, which produces phaeophytin. Dilution of extracts from concentrated cultures with 90% acetone was sometimes necessary. Micrograms per milliliter of the acetone extract was then determined using the following equation: $(\text{Fluorescence Before HCl} - \text{Fluorescence After HCl}) / 0.62$. Multiplication of this value by the volume of the acetone extract (10 mL) and division by the volume of culture filtered gave values in micrograms Chl *a* per milliliter of culture. If dilutions were necessary, this value was then multiplied by the appropriate dilution factor (Parsons et al 1984).

Methanol extractions of *Trichodesmium* were usually performed on 15 colonies; many of these analyses were performed by Nancy Walters. The colonies were hand picked onto Whatman GF/A glass fiber filters from net tow samples and frozen. 3 mL of methanol was added to each in a glass test tube and the tubes were held in a 75°C water bath just until bubbles began to appear (about 15-20 seconds). Boiling was avoided to prevent volume changes in the extract. The extract was then spun briefly on an Eppendorf centrifuge at 14,000 rpm to remove filter material. Absorbance was then read at 665 nm and 750 nm (as a turbidity correction). Milligrams per milliliter of extract was calculated with the following equation: $(\text{Absorbance at 665 nm} - \text{Absorbance at 750 nm}) \times 74.5$. Division of this value by the number of colonies used for the analysis yielded the number of

milligrams chlorophyll a per colony (Jensen 1978).

ACID-LEACHABLE CELLULAR IRON: FERROZINE ANALYSIS

The colorimetric ferrozine assay was used to determine total leachable iron content for both field and laboratory samples. This technique offers an alternative to atomic absorption spectrophotometry measurements which is both accurate over the range of Fe concentrations typically found in algal samples and simple enough to perform under difficult field conditions. Our technique was modified from the methods described by Stookey (1970) and Gibbs (1979).

Ferrozine reagent was obtained through Hach Chemical Company of Ames, Iowa. The chromogen is disodium 3-(2-pyridyl)-5,6-bis (4-phenyl sulfonic acid)-1,2,4-triazine, which forms a purple complex with Fe^{2+} . This complex exhibits a strong absorption peak at 562 nm with a molar extinction coefficient of 27,900 (Stookey 1970). Because the reagent is specific for ferrous ions under the reaction conditions used (Attari and Jaselskis 1972), measurements of total iron content depend on prior reduction of any Fe^{3+} present using 10% (w/v) hydroxylamine hydrochloride. Since copper, cobalt and nickel ions produce some interference at pH values close to neutral (Kundra et al 1974), the reaction mixture was re-equilibrated through addition of an ammonium acetate-ammonium hydroxide buffer (pH 8.7). Iron standards were prepared through serial dilutions of 1 mg/mL $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in the same 1 N HCl that samples were digested in.

Solubilization of iron in the samples was accomplished by a 24 hour leaching of sample material at 60°C in 1 N HCl. Due to the constant

potential problem of iron contamination all assay equipment was acid-washed in 1 N HCl and rinsed six times in Nanopure distilled water (see section on trace metal clean techniques).

Field collections of *Trichodesmium* were hand-plucked into 2 mL 1 N HCl using a plastic inoculating loop. Typically, 200 colonies were used for each replicate. After leaching, ferrozine analysis was carried out on 1 mL of sample supernatant (prepared by 5 minutes centrifugation at 14,000 rpm in Eppendorf tubes) to separate out cell debris. Values obtained by this method were presumed to represent half of the iron present in the 2 mL leached sample.

Laboratory cultures of *Anabaena* were suction-filtered onto 0.2 μ m Nucleopore filters and frozen for later analysis. Sample size was usually 50 or 100 mL. Leaching was carried out as previously described except that filter blanks were also run and these values were subtracted from the sample values. Contamination from iron in the nucleopore filters was however not a significant problem, as each filter was found to contain less than 0.9 ng iron. Filters were handled with nylon forceps to avoid possible contamination by metal forceps.

Reagents used were:

Hydroxylamine hydrochloride 100 g/L

Ferrozine 5.14 g/L

Buffer- 400 g ammonium acetate and 350 mL 58% ammonium hydroxide diluted to 1 L.

Iron standards- $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 1 N HCl made up to the following concentrations (μM): 55.86, 27.93, 13.97, 6.98, 3.49, 1.75 and 0.

Procedures were performed as follows:

1 mL of the sample or standard in 1 N HCl was mixed with 200 μ l each of buffer, hydroxylamine hydrochloride, and ferrozine reagent in that order. Absorbance was measured after 10 minutes in a 1 cm cell at 562 nm versus a distilled water blank. Samples were then plotted on a standard curve (molarity of iron standard versus absorbance at 562 nm). A typical standard curve is shown in Figure 18.

Because we cannot be certain that all cell iron is extracted with this acid-leaching method, measurements made on acid-leached extracts are reported as acid-leachable iron rather than as total cellular iron.

TOTAL CELLULAR PROTEIN: BICINCHONIC ACID ASSAY

The protein assay used in these experiments was the BCA Protein Assay available through Pierce Chemical Company of Rockford, Illinois. This assay has definite advantages over most traditional protein measurement methods. It is less sensitive to variability between proteins than the Coomassie Blue method (Pierce and Suelter 1977, Van Kley and Hale 1977) and is much more tolerant of interfering substances than the Lowry method (Smith et al 1985). In particular, the BCA assay can be used in the presence of fairly high concentrations of detergents, allowing complete solubilization of membrane-bound hydrophobic proteins. In these investigations, 1% w/v SDS (sodium dodecyl sulfate) was used to insure complete protein solubilization. Depending on the protocol chosen, the assay gives good results at protein concentrations ranging from 10 to 1200 μ g/mL.

The assay is based on the biuret reaction in which Cu^{2+} is reduced to Cu^{+}

Figure 18. Ferrozine iron standard curve. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 1 N HCl was used for an iron standard. Molar concentration of iron is proportional to absorbance at 562 nm.

by the action of protein under basic conditions. The sodium salt of bicinchonic acid then coordinates specifically with the cuprous ions produced with a 2:1 stoichiometry. This complex is characterized by a purple color which can be quantified by measuring absorbance at 562 nm in a 1 cm cell. The protein concentration of unknown samples is determined graphically using a standard curve generated with fatty acid-free bovine serum albumen.

In order to measure protein levels in *Anabaena* cultures in the laboratory, well-mixed cultures were suction filtered onto Whatman GF/A glass fiber filters. Depending on the density of the culture being sampled, 25 or 50 mL samples were used. The filters were then frozen and ground using a tissue homogenizer in 2 mL 1% SDS; one more mL of SDS solution was used to rinse the glass mortar into a clean plastic centrifuge tube for a final volume of 3 mL. The sample was then allowed to sit overnight in the refrigerator to allow for complete solubilization of the protein. The next day, 5 minutes of centrifugation at high speed in a desktop centrifuge was used to remove filter material and form a clear supernatant. 100 μ L of this supernatant was then mixed with 2 mL BCA Working Reagent (50 mL BCA detection reagent in an alkaline medium mixed with 1 mL 4% CuSO_4). Selection of the appropriate protocol was then made according to the expected levels of protein. The Enhanced protocol, used for lower protein concentrations (10-250 $\mu\text{g/mL}$), involves incubation at 60°C for 30 minutes before cooling to room temperature and reading absorbance at 562 nm. Sensitivity of the Enhanced protocol is about 0.032 A.U./ μg protein. The Room Temperature protocol was used for higher protein concentrations (100-1200 $\mu\text{g/mL}$); this version requires incubation at room temperature

for two hours and has a sensitivity of about 0.01 A.U./ μ g protein. Color stability of both protocols is fairly good according to the manufacturer's instruction booklet, with Room Temperature absorbances continuing to increase at 2.3% every 10 minutes and the Enhanced protocol increasing at the rate of only 0.1% every 10 minutes. Variability due to color instability was minimized by careful timing of sample incubations. Sample standard curves for both protocols using defatted bovine serum albumen are presented in Figures 19 and 20.

Field samples of *Trichodesmium* were treated in a manner identical to laboratory *Anabaena* samples except that individual colonies were hand-plucked onto the glass fiber filters using a plastic inoculating loop. A sample consisting of 50 colonies was found to fall towards the middle of the Enhanced protocol standard curve. In addition, solubilized *Trichodesmium* samples were separated out using 5 minutes of 14,000 rpm centrifugation in Eppendorf tubes rather than using a desktop centrifuge.

Culture protein nitrogen content was estimated using an assumption based on the average nitrogen content of protein as reported by Dayhoff et al (1965). This estimate places the average nitrogen content of protein at 14% (weight to weight). Protein nitrogen makes up 70-90% of total cell nitrogen in phytoplankton (Wheeler 1983), with the remainder mainly as nucleic acids and chlorophyll *a*. For this reason, estimates of total cell nitrogen using the information of Dayhoff et al (1965) are undoubtedly low. Because of this underestimation, this data is reported as total protein nitrogen rather than as total cell nitrogen.

Figure 19. Enhanced BCA protein standard curve. The Enhanced protocol was used for protein concentrations between 10 and 250 µg/mL. This protocol calls for samples to be incubated at 60°C for 30 minutes before cooling to room temperature and reading absorbance at 562 nm. Defatted bovine serum albumen was used as a protein standard.

Figure 20. Room temperature BCA protocol protein standard curve. This version of the BCA assay was used for protein concentrations between 250 and 1200 µg/mL and involves incubation at room temperature for two hours before reading absorbance at 562 nm. The protein standard used was defatted bovine serum albumen.

SIDEROPHORE PRODUCTION: CAS/HDTMA IRON-BINDING ASSAY

Iron-binding capacity of culture filtrates was measured using a colorimetric assay recently developed by Schwyn and Neilands (1987). This assay, known as the CAS/HDTMA method, measures the ability of a chelator to compete for chromophore-bound iron. The chromophore, chrome azurol S/iron(III)/hexadecyltrimethylammonium bromide, has a sharp absorption peak at 630 nm with a molar extinction coefficient of about $100,000 \text{ M}^{-1}$. As the iron is stripped from this complex by the higher affinity of the siderophore chelator, absorption at this wavelength decreases proportionally to the iron-binding ability of the solution. Thus, the lower the absorption of the equilibrated sample, the greater the concentration of siderophore in the solution being tested. This transition is visually apparent as a change in color from the deep blue of the iron-bound assay solution to an orange color after the iron has been completely removed by the siderophore (Schwyn and Neilands 1987).

The choice of this assay was dictated by the convenience and simplicity of the procedure. Other assays for siderophore production are available. Some, such as the Csaky test for hydroxamates, are more specific in identifying the chemical nature of the chelator (Csaky 1948, Gillam et al 1981). The CAS/HDTMA assay, on the other hand, accurately measures iron binding ability but gives no information as to the type of siderophore involved (Schwyn and Neilands 1987). Since the siderophore produced by *Anabaena* has been conclusively shown to be the hydroxamate siderophore schizokinen (Simpson and Neilands 1976, McKnight and Morel 1980, Goldman et al 1983), chemical characterization of the chelator was considered

unnecessary. This allowed us to avoid the cumbersome acid hydrolysis procedure necessary with the Csaky test while still giving precise measurements of siderophore production. Another alternative assay system is a bioassay using a mutant of the bacterium *Arthrobacter flavescens* (strain JG-9) which is a siderophore auxotroph. Since this organism is unable to take up iron without an exogenous source of chelator, siderophore production by algal cultures can be verified simply by adding culture filtrate to bacterial plate cultures and observing whether or not growth occurs (Lankford 1973, Lammers 1982). This procedure, however, has no advantages over the CAS/HDTMA assay since it also does not identify the type of siderophore produced and requires lengthy bacterial culture incubations and sterile techniques. The availability of the new Schwyn and Neilands assay used in these investigations has greatly simplified the process of screening for and quantifying siderophore production, although more precise characterization is still necessary in cases where the nature of the siderophore is unknown.

Iron-binding standard curves were generated using a trihydroxamate chelator produced by an actinomycete fungus which is sold under the brand name Desferal mesylate (deferroxamine mesylate USP). Desferal was obtained from CIBA-GEIGY Ltd. of Basel, Switzerland. A typical standard curve is illustrated in Figure 21. Although the curve has pronounced shoulders at Desferal concentrations above 3 μM and below 0.05 μM , it was found to be completely linear over the range of iron-binding capacity observed in the samples, equivalent to that obtained between 0.1 and 2 μM Desferal (Figure 22).

Chemicals for the CAS/HDTMA assay were obtained through Fluka

Figure 21. Desferal iron-binding standard curve. Desferal is a commercially available trihydroxamate siderophore, deferoxamine mesylate USP. Note the shoulders on this curve at Desferal concentrations above 3 μM and below 0.05 μM. Absorbance at 630 nm is inversely proportional to iron-binding capacity with the CAS/HDTMA assay.

Figure 22. Partial Desferal iron-binding standard curve. The portion of the Desferal standard curve which bracketed iron-binding capacities equivalent to the range found in *Anabaena* culture filtrates (0.1 to 2 μM Desferal) was found to be completely linear.

Biochemika of Switzerland. The reagent used in these experiments was the CAS/HDTMA shuttle solution, which in addition to the chromophore/iron complex contains 5-sulfosalicylic acid to act as an iron shuttle. The use of the shuttle solution greatly speeds up the process of attaining equilibrium, especially in samples of relatively low iron binding capacity.

Preparation of the CAS/HDTMA reagent involves placing 6 mL of 10 mM hexadecyltrimethylammonium bromide in a 100 mL volumetric flask and diluting the solution slightly with water. A mixture of 1.5 mL of 1 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10 mM HCl and 7.5 mL of 2 mM chrome azurol S in distilled water is then added slowly while stirring. 4.307 g of anhydrous piperazine is then dissolved in distilled water and 12 M HCl is added to a pH of 5.6 (about 6.25 mL acid is required). This buffer solution is then rinsed into the volumetric flask and distilled water is added to bring the final volume to 100 mL. This completes the preparation of the CAS/HDTMA assay solution; the shuttle solution is derived from this by the addition of 4 mM 5-sulfosalicylic acid. The shuttle solution should be stored in a dark polyethylene bottle at slightly above room temperature, as temperatures below 25°C cause a reversible precipitation of the sulfosalicylic acid. Cultures should be grown without high concentrations of chelators or phosphate and citrate buffers to avoid interference with the assay (Schwyn and Neilands 1987). Low concentrations of the chelator EDTA (5 μM) added to the culture medium with the trace metal solutions (see Appendix A) were not found to produce detectable levels of interference with this assay.

The siderophore assay procedure requires the preparation of a cell-free filtrate to avoid measuring iron binding occurring on the cell surfaces. This was accomplished by manual filtration of cultures using a syringe-injected

swin-lock Nucleopore polycarbonate filter rig with Whatman GF/A glass fiber filters. Filters were changed and the filtration apparatus rinsed well in distilled water between individual samples to avoid cross-contamination of culture filtrates, as the filter and apparatus were found to retain considerable amounts of liquid from the previous sample. One mL of the shuttle solution was then added to one mL of the culture filtrate, vortex mixed and allowed to equilibrate for two hours. Absorbance was then measured at 630 nm against a distilled water blank in a 1 cm cell.