DEVELOPMENT OF A FIELD ASSAY OF IRON LIMITATION IN NUTRIENT RICH LAKES

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ABSTRACT

Severe blooms of cyanobacteria (blue-green algae) affect many nutrient-rich Oregon lakes. However, the availability of iron may limit bloom development in certain lakes. Iron uptake and assimilation by phytoplankton have been the subjects of a number of studies, particularly with regard to possible iron limitation of primary productivity. An understanding of iron assimilation by phytoplankton has been hindered by a lack of data on the biological availability of aqueous iron and the corresponding iron nutritional status of cells. The physiological response of phytoplankton to iron stress appears to be the best way of obtaining this information but analytical problems had previously limited this approach. In this report we demonstrate that cellular concentrations of the redox proteins ferredoxin and flavodoxin are practical indicators of iron stress, both in laboratory cultures and in natural populations of cyanobacteria.

Experiments with the N_2-fixing cyanobacterium Anabaena sp. 7120 reveal that ferredoxin levels decrease and flavodoxin levels increase in proportion to the overall iron stress of the cells. Iron stress is a function of both the iron provided in the medium (iron availability) as well as the iron requirements of the cells. Thus for a given level of available iron, N_2-fixing and nitrate-reducing cells, which have high iron requirements, exhibited greater iron stress than cells growing on ammonium. This indicates an important linkage between iron availability and nitrogen availability: N_2-fixation induces a greater need for iron, and low iron may limit the rates of N_2-fixation. Predictions of iron limitation in lakes must take into account the nitrogen resources of the lake. Conversely, studies of nitrogen assimilation in lakes should consider the effects of iron availability.

FOREWORD

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1. INTRODUCTION

WATER QUALITY PROBLEMS AND ALGAE

Many species of cyanobacteria (blue-green algae) can supply their needs for nitrogen by fixing nitrogen gas directly from the atmosphere (Bothe 1982). In phosphate-rich, nitrogen-limited lakes, N_2-fixing cyanobacteria may completely dominate the phytoplankton community (Schindler and Fee 1973; Smith 1983). The resulting dense cyanobacterial populations cause considerable practical problems in eutrophic lakes. Offensive tastes and odors may be imparted to drinking water supplies, the decomposition of dead cells kills fish by depleting dissolved oxygen (Rohlich 1969), and exuded toxins may poison fish and cattle (Carmichael 1981) and cause skin rashes among recreational users (Schwimmer and Schwimmer 1964). Understanding the factors that control the growth of phytoplankton such as cyanobacteria is essential for efficient, realistic control and regulation of water quality in lakes.

PURPOSE AND OBJECTIVES

As will be discussed in detail below, iron may be important in regulating the growth of cyanobacteria. Studies of iron and phytoplankton have been hindered by the difficulty of quantifying the amount of iron available to the algal cells. In our research we adopted a novel strategy of using biochemical indicators of iron stress in cyanobacteria. The objectives of this study were: 1) to verify the utility of certain cellular proteins as practical markers of iron stress, 2) to extend the method to field and laboratory studies of iron nutrition in nitrogen-fixing cyanobacteria, 3) to develop a rapid and convenient high-performance chromatography method of determining ferredoxin and flavodoxin concentrations in small cell volumes, and 4) to use these methods to explore the interactive relationship between nitrogen starvation and iron starvation in blue-green algae.

BACKGROUND AND RELATED RESEARCH

Phytoplankton productivity depends upon the availability, uptake, and assimilation of inorganic nutrients in the correct stoichiometric proportions (Redfield 1934; Thomas 1968). Nitrogen and phosphorus are well recognized as nutrients that limit carbon fixation but the role of the so-called micronutrients

in the regulation of productivity is still poorly understood. A number of growth-limiting micronutrients have been investigated (Goldman 1972), but iron is the most extensively studied and may have the greatest ecological significance among the trace elements.

Iron is essential and widely utilized in biological systems. Although relatively abundant, it occurs primarily as insoluble ferric oxyhydroxides under oxic, nonacidic conditions. The limited solubility is especially problematic for aquatic microorganisms and the possibility of iron limitation of primary productivity has been proposed for both lacustrine and marine ecosystems (Menzel and Ryther 1961; Schelske 1962; Glover 1978; Entsch et al. 1983; Wurtsbaugh and Horne 1983). However, iron limitation is difficult to confirm, as outlined below. Moreover, interest has shifted in recent years from the idea that iron uptake directly limits productivity to the more subtle notion that iron and other trace nutrients influence ecosystems by regulating the uptake and assimilation of major nutrients. For example, iron and molybdenum are key components of nitrogenase, the enzyme that fixes atmospheric nitrogen. It has been suggested that the low availability of one of these metals precludes the widespread fixation of nitrogen in marine systems and thereby indirectly regulates global carbon fixation (Howarth and Cole 1985; J. Rueter, pers. comm.). If iron is essential to the uptake of certain forms of nitrogen, then the ecological effect of iron stress cannot be known unless the nitrogen resources of the population are considered. Hence, knowledge of the ecological significance of iron depends more on understanding the assimilation processes that iron regulates than on simply demonstrating iron limitation of overall productivity.

In this report we describe the development of refined methods for determining iron stress in cyanobacteria. We applied these techniques to demonstrate that the effect of iron limitation on cyanobacterial physiology is strongly influenced by the induction of nitrogen fixation and by the form in which fixed nitrogen (as either nitrate or ammonium) is available. Conversely, iron stress may affect several key nitrogen-assimilation pathways.

INDICATORS OF IRON STRESS

Many methods for establishing the limitation of productivity by iron have been proposed but most are prone to artifacts. Concentrations of "soluble" (filtrable) iron comprise many colloidal and coordinated species and do not

reflect the biological availability. Photochemical cycling of Fe(III) and Fe(II) further complicates the question of availability (Miles and Brezonik 1980; Anderson and Morel 1982; Waite and Morel 1984). Iron enrichment of isolated phytoplankton cultures forms adsorbent colloids and alters the speciation of other nutritional or toxic metals. Therefore, the best determinants of iron limitation in aquatic ecosystems appear to be direct physiological indicators of iron nutritional stress in the phytoplankton population (Entsch et al. 1983).

Phytoplankton exhibit many responses to iron stress, several of which have been used as evidence of iron limitation. Iron stress causes a decline in cellular chlorophyll ("chlorosis") (Karali and Price 1963; Davies 1970) but this decrease is not uniquely associated with iron deficiency. Changes in iron-containing cell constituents are more specific indicators of iron stress (Entsch et al. 1983). Glover (1977) used the ratio of the heme-iron protein cytochrome f to chlorophyll a (cyt f/chl a) to identify potential iron limitation of marine phytoplankton. Because cytochrome f is membrane-bound and is not readily isolated or quantified from cell extracts, Glover (1977) made in vivo measurements of the protein by spectroscopic determination of the cytochrome absorption maximum at 410 nm, the so-called Soret absorption band. Unfortunately, the Soret band lies in a spectral region in which cell pigments, particularly the biliproteins of cyanobacteria, may also strongly absorb (J. G. Rueter, pers. comm.). Furthermore, although chl a is a convenient biomass normalization factor, its partial covariance with cyt f causes the cyt f/chl a ratio to vary less during iron stress than does the true cellular concentration of cyt f. This reduces the sensitivity of the ratio as a marker of iron stress.

Entsch et al. (1983) examined concentrations of pigments, cytochrome c, ferredoxin, and flavodoxin in coral reef algae and concluded that measurements of cellular ferredoxin and flavodoxin were the most reliable indicators of iron stress. Ferredoxin is an iron-containing redox protein in plants and bacteria. It is a key reductant in Photosystem I, in the nitrate assimilation pathway, and in N_2-fixation (Ramirez et al. 1966). Like other iron proteins, its concentration diminishes during iron stress (Hutber et al. 1977). However, ferredoxin is particularly useful as a marker of iron stress because it is soluble, can be quantitatively extracted and isolated, is relatively stable, and is readily identified and quantified by its distinctive visible absorption spectrum (Buchanan and Arnon 1971; Hutber et al. 1977; Ho et al. 1979).

Smillie (1965) discovered that the cyanobacterium Anacystis nidulans (now called Synechococcus strain 6301) substitutes a non-iron, flavin-containing redox protein for ferredoxin under iron limiting conditions. Smillie termed this protein phytoflavin but it was later found to be structurally and functionally similar to flavodoxin (first isolated from Clostridium pasteurianum by Knight and Hardy (1966)), and the latter name is now applied to all such proteins. Flavodoxin is structurally quite distinct from ferredoxin but their redox potentials are close (Eo = -0.4 mV) and in studies with chloroplasts isolated from numerous plant and algal species, flavodoxin catalyzed photo- synthetic reduction of $NADP^+$ at rates comparable to ferredoxin (Bothe et al. 1971). Studies of protein electrostatic-potential surfaces suggest that flavodoxin can substitute freely for structurally dissimilar ferredoxin because the intermolecular recognition between either of these proteins and their complementary electron acceptors is governed largely by electrostatic interactions at the active sites (P. Weber, pers. comm.). The substitution of flavodoxin for ferredoxin apparently enables cells to conserve iron. Unlike other marker compounds that decline during iron stress, flavodoxin is a positive cellular indicator of iron stress. Hutber et al. (1977) showed that diminishing the amount of iron added to cultures of Nostoc strain MAC resulted in decreased ferredoxin levels and elevated flavodoxin concentrations.

Although the utility of ferredoxin and flavodoxin measurements as markers of iron stress had been identified in limited laboratory and field studies, the method has not been applied previously to any detailed studies of phytoplankton growth and nutrition. In the section below we discuss refined analytical procedures using high-performance liquid chromatography that enable the quantifying of ferredoxin and flavodoxin from cell samples that are one to two orders of magnitude smaller than those of previously published methods. We applied these methods to detailed laboratory investigations of iron and nitrogen nutrition as well as to samples taken from natural algal populations in four Oregon lakes.

2. METHODS AND PROCEDURES

LABORATORY CULTURES

The cyanobacterium Anabaena strain 7120 was used for all laboratory culture experiments. This organism is capable of fixing N_2 and has a well characterized, siderophore-mediated iron-uptake system (Lammers and SandersLoehr 1982). Anabaena 7120 was maintained in axenic culture at 20°C on 1.5% agar slants of BG-11 medium (Stanier et al. 1971). Inocula from these slants were grown in liquid culture at 30°C on modified BG-11 medium in which citric acid, ferric ammonium citrate, and EDTA were omitted, and varying amounts of iron were added from a stock solution of 5 mM $FeCl_3$ in 25 mM $HClO_4$. For studies of nitrogen-fixing cells, sodium nitrate was omitted from the medium. For growth on ammonium, sodium nitrate was omitted and ammonium chloride substituted. Growth media (minus iron and trace metals) were sterilized by autoclaving and the metals added as sterile solutions passed through 0.22 μm filters (Millex-GS, Millipore Corp.). To reduce iron contamination, glassware was cleaned in 9M HCl and rinsed with high-purity deionized water (NanoPure System, Barnstead). Media were likewise prepared with high-purity water. The growth of cultures was monitored by measuring the optical density with a Klett-Summerson colorimeter fitted with a red no. 66 filter.

Cultures initially were transferred from slants to 10 mL culture tubes. When the inoculum cultures were well into log-growth phase (at about 60 Klett units) they were transferred to 125 mL sterile flasks containing 50 mL of medium. In cases where greater cell volumes were desired, cultures were transferred to larger sterile flasks with 250 mL or 500 mL of medium. For the first experiments in which large cell volumes were desired, 1-L cultures were prepared in 2-L flasks. Cells were harvested when the optical density reached 25-150 klett units, depending on the experiment (100 klett units = 0.15 mg acetone cell-powder (see below) per mL of culture). Cells were harvested by allowing the cells to settle out, siphoning off about 80% of the essentially cell-free supernatant, decanting the remainder into 25 mL or 250 mL polycarbonate centrifuge bottles, and centrifuging the cells into a soft pellet at 650 x g. The cells were resuspended in an equal volume of 50 mM Tris/HCl buffer, pH 7.8, and either frozen or processed immediately.

FIELD STUDY AREAS

Four Oregon lakes were sampled in the summers of 1985 and 1986. Cullaby Lake is a 76.1 ha lake located in Clatsop County at 45°05'38"N and 123°54'22"W. The lake is distinguished by a high concentration of dissolved organic carbon (DOC), primarily fulvic and humic acids. Consequently the water is slightly acidic and well buffered, with a year-round pH range of 6.5 to 7.0 (Johnson et al. 1985). Cyanobacterial blooms occur nearly every year but can be transient, with durations on the order of 2-3 weeks (R. Petersen, pers. comm.). Sampling was conducted from a dock at the northern end of the lake in August, 1985.

Upper Klamath Lake is a large (24,907 ha) lake located in Klamath County at 42°13'54"N and 121°48'19"W. It is a broad, shallow, hypereutrophic lake that receives high nutrient loading from natural sources (phosphate-rich ground water) in the summer and from anthropogenic sources (agricultural runoff) in the winter and spring (Johnson et al. 1985; Perdue et al. 1981). The lake is subject to massive cyanobacterial blooms from midsummer to midautumn. During the bloom, pH values of 9.0 are typically observed. Our sampling was conducted in August and September, 1985 at either the Algoma Road overpass at the southern end of the lake or at Wocus Bay in the SW section of the lake.

McKay Reservoir is a 533 ha impoundment lake in Umatilla County (45°36'28"N, 118°47'30"W). The reservoir is in a semi-arid region, receives high nutrient loading and experiences Aphanizomenon flos-aquae blooms throughout the summer. Morgan Lake in Union County (45°18'05"N, 118°08'05"W) is a small reservoir (24.3 ha) with elevated phosphorus levels and strong thermal stratification in the summer. The lake is subject to algal blooms in mid- to late-summer but has generally lower chlorophyll levels than would be expected for the high degree of P-loading (Johnson et al. 1985).

FIELD COLLECTION

Algal samples were collected using 20-mesh plankton nets. Concentrated samples were placed in acid-cleaned polypropylene bottles and stored on ice for transport back to the laboratory. In the laboratory they were further concentrated by a fine nylon mesh and either frozen or processed immediately. Aliquots of unconcentrated lake water were taken near the surface and preserved with Lugol's solution (I_2/KI) for species analysis and cell counts.

PROTEIN EXTRACTION AND ANALYSIS

Acetone powders of concentrated cyanobacterial samples were prepared by resuspending the cell mass in an equal volume of cold 0.15 M buffer (all buffers were Tris/HCl, pH 7.8) and slowly adding the slurry to a well stirred, tenfold excess of acetone kept at -20°C. The suspension was stirred vigorously for 30 min., filtered, resuspended in fresh -20°C acetone, and stirred for another 30 min. The resulting powder was filtered, washed with cold acetone and cold ether, washed with room temperature acetone, and dried under high vacuum for several hours. This treatment removed lipids and lipophilic pigments, leaving a fine, bright blue cell powder that retained the water soluble proteins. Cell powders were stored under N_2 at -20°C.

Soluble proteins were extracted by adding a preweighed quantity of cell powder to 0.15 M buffer using 25 mL buffer per gram of powder for samples greater than 500 mg, and using 100-200 mL buffer per gram of powder for samples less than 500 mg. About 1-2 mg of ribonuclease and deoxyribonuclease were added per 10 mL of buffer, along with the protease-inhibitor phenylmethyl-sulfonyl fluoride (50 μg/mL-buffer). The extraction beaker was covered and stirred overnight at 4°C. The resulting suspension was centrifuged at 4°C at 37,000 x g for 1 h. The deep blue supernatant was decanted and the pellet rinsed gently with buffer. In initial studies using low pressure liquid chromatography, a preliminary protein purification was effected by bringing the supernatant to 60% saturation with ultrapure $(NH_4)_2SO_4$ (0.390 g mL-1 at room temperature). This mixture was centrifuged at 37,000 x g for 30 min at 25°C. The blue phycobiliproteins precipitated and the supernatant was a pale reddish or straw-colored solution. The supernatant was dialyzed against 0.05 M buffer until the conductivity of the dialysate was less than that of a 0.1 M NaCl standard. In later work, the excellent resolution of high-performance chromatography obviated the need for ammonium sulfate precipitation.

In initial experiments, the dialyzed protein mixture was applied to a 1 cm x 7 cm DEAE-cellulose gravity-feed column (Whatman DE-52). A concentrated band of colored proteins was visible at the top of the column which was then washed with one bed volume of 0.05 M buffer followed by four bed volumes of 0.1 M NaCl in 0.05 M buffer. The flavodoxin and ferredoxin were eluted using a linear salt gradient of 0.1 M to 0.5 M NaCl in 0.05 M buffer and 1 mL fractions collected. Optical absorbances and spectra of fractions were recorded on a Perkin-Elmer

Lambda 9 spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer MPF-66 spectrofluorometer.

For most of the experiments, proteins were separated using a Pharmacia fast protein liquid chromatograph (FPLC) equipped with a Mono-Q 5/5 (DEAE-type) anion-exchange column and a UV absorbance detector set to 280 nm. The FPLC is essentially an HPLC system specifically designed for protein work and has all inert, non-steel components. The FPLC was operated at a flow rate of 1.0 mL/min with 0.05 M Tris/HCl buffer at pH 8.0 as the eluant. A protein solution volume of 100-500 uL was injected onto the column (depending upon the concentration of protein) and eluted with a linear salt gradient of 0% to 100% 1.0 M KCl in Tris/HCl buffer. The gradient was developed over a 25 min period with a 3 min starting equilibration of 0% salt and a 3 min post-gradient wash with 100% 1 M KCl. The column was calibrated using ferredoxin and flavodoxin standards previously purified on the low-pressure DE-52 column and with solutions of commercially prepared spinach-chloroplast and red-algal ferredoxins (Sigma). Protein concentrations were determined from integrated peak areas obtained with a Hewlett-Packard integrator connected to the UV monitor.

3. RESULTS AND DISCUSSION

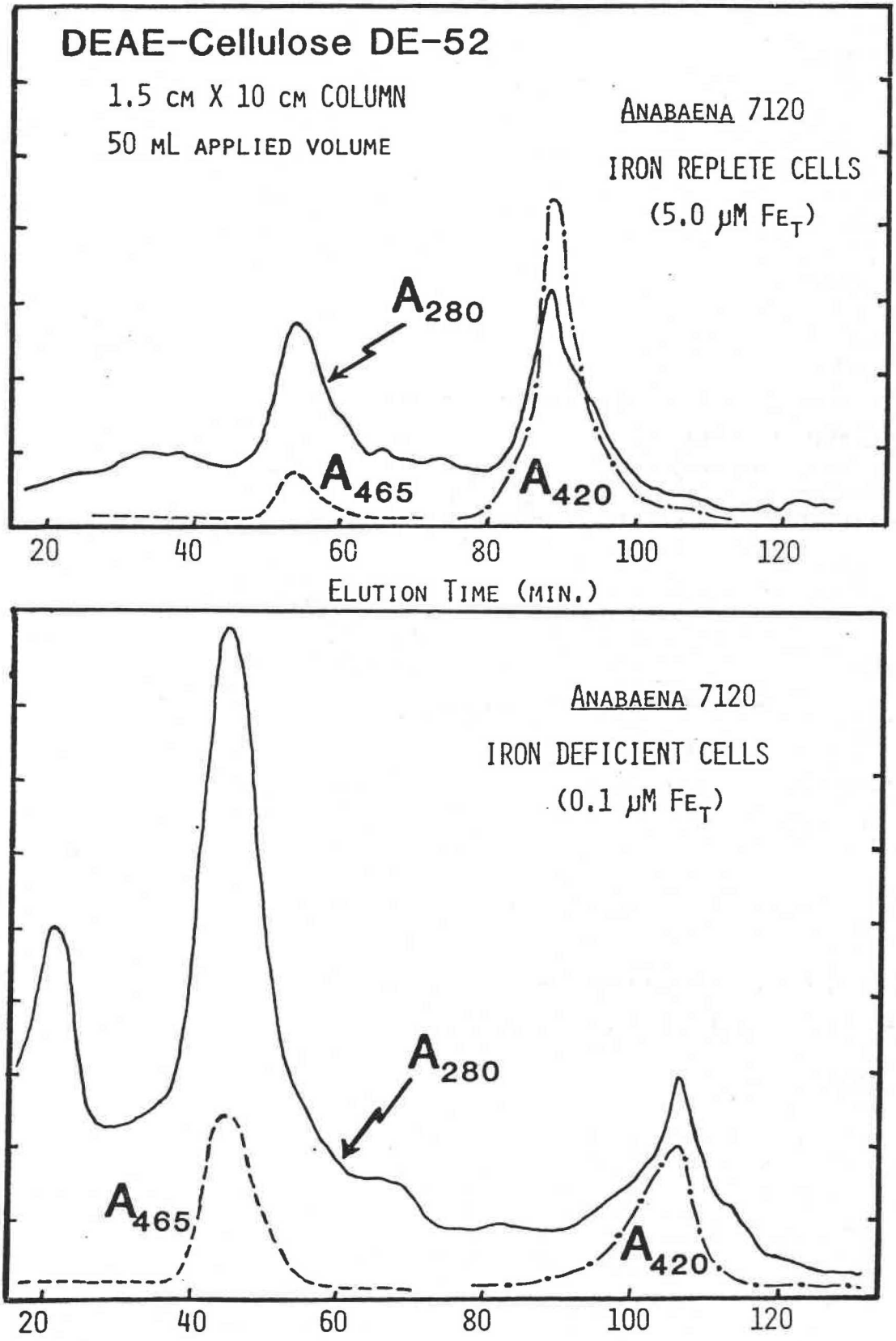
LABORATORY CULTURES

Experiments with laboratory cultures proceeded in four successive stages. First, Anabaena sp. 7120 was grown in nitrate-replete medium at high and low levels of iron and the protein analyses performed by conventional large-column liquid chromatography (LC). Next, these experiments were repeated using FPLC to analyze the protein. After establishing the validity and superiority of FPLC analyses, all subsequent experiments used this method. Then, cultures were grown on medium free of fixed nitrogen (i.e., inducing N_2-fixation) and in medium containing ammonium instead of nitrate. These experiments documented the effects of N-source on Fe-stress levels. Finally, cultures with various concentrations of iron, and growing on either nitrate or N_2, were grown to higher cell densities than previously to determine the effects of culture density on the Fe-stress assay results obtained in earlier experiments.

The initial experiments using LC protein analysis were essentially a replication of the studies of Hutber et al. (1977) except that we scaled down the procedure by tenfold and used only 1.0 g of acetone cell powder rather than the 14.5 g used by Hutber. This enabled us to greatly reduce the volume of the cultures; six 1-L cultures were grown to 25 klett-unit density ("25 klett"). Our results (Fig. 1) were virtually identical to those of Hutber and co-workers: at 5.0 μM added Fe the cells contained ferredoxin at 11.8 μmol/100g dry wt and flavodoxin at only 1.6 μmol/100g dry wt. When the cultures were grown with only 0.1 μM added Fe the ferredoxin concentration decreased to 6.0 μmol/100g dry wt while the flavodoxin increased to 6.6 μmol/100g dry wt. Materials obtained from peak fractions were pooled and examined in a spectrophotometer. The spectra obtained for the putative flavodoxin and ferredoxin peaks were identical to those of authentic specimens of these proteins (Fig. 2).

To test the FPLC protein analysis, these experiments were repeated using even smaller culture volumes of only 50 or 250 mL. The smaller cultures allowed replicates to be prepared conveniently and two or three replicate cultures were grown for each medium condition. Cell powders in these experiments were only 10-50 mg but the high resolution of the FPLC allowed ferredoxin and flavodoxin to be quantified easily (see Fig. 3).

Figure 1.


DEAE-Cellulose DE-52
1.5 cm X 10 cm COLUMN
50 mL APPLIED VOLUME
ANABAENA 7120
IRON REPLETE CELLS
(5.0 μM Fe$_T$)
A$_{280}$
A$_{465}$
A$_{420}$
20
40
60
80
100
120
ELUTION TIME (MIN.)
ANABAENA 7120
IRON DEFICIENT CELLS
(0.1 μM Fe$_T$)
A$_{280}$
A$_{465}$
A$_{420}$
20
40
60
80
100
120

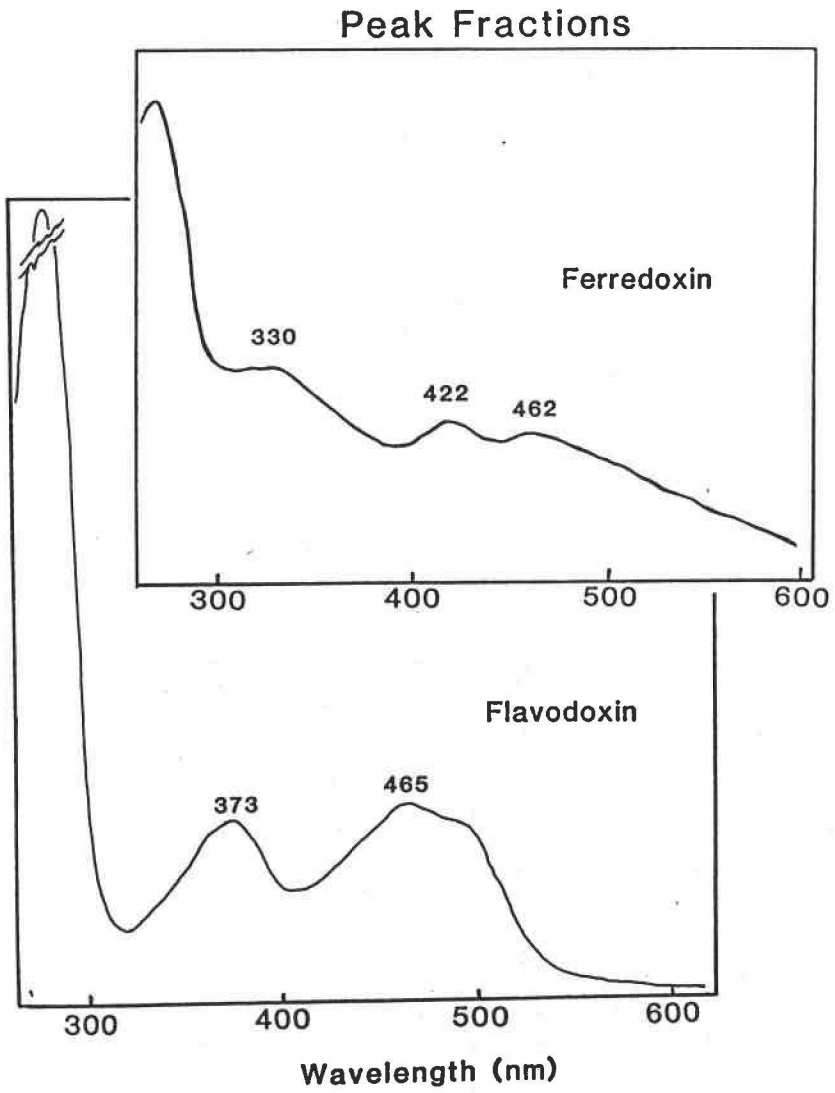
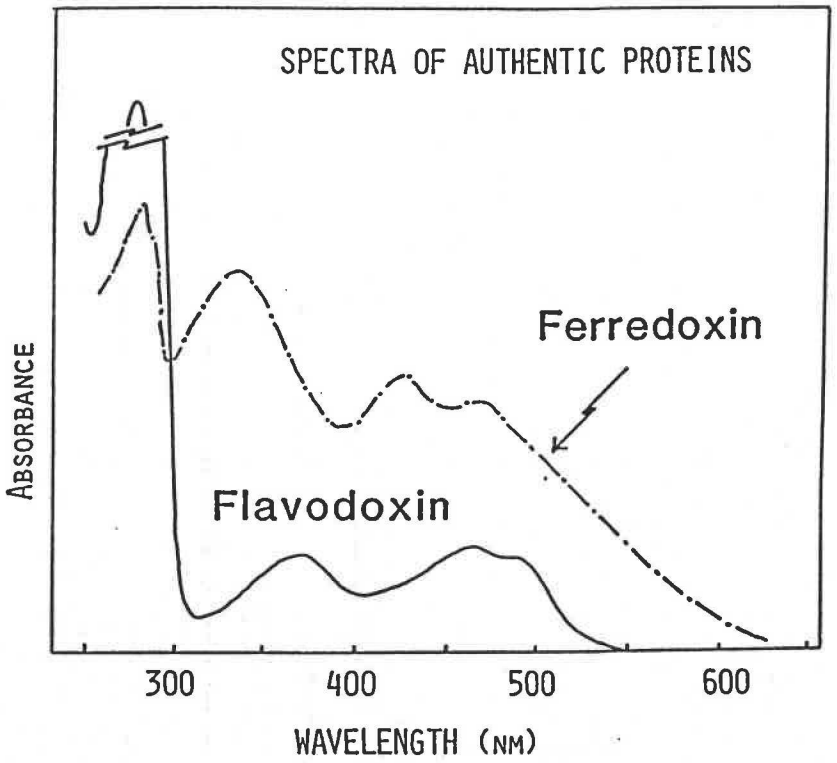


Fig. 2

Uv-visible spectra of peak fractions collected from the DE-52/LC column corresponding to ferredoxin and flavodoxin. Comparison to authentic spectra.



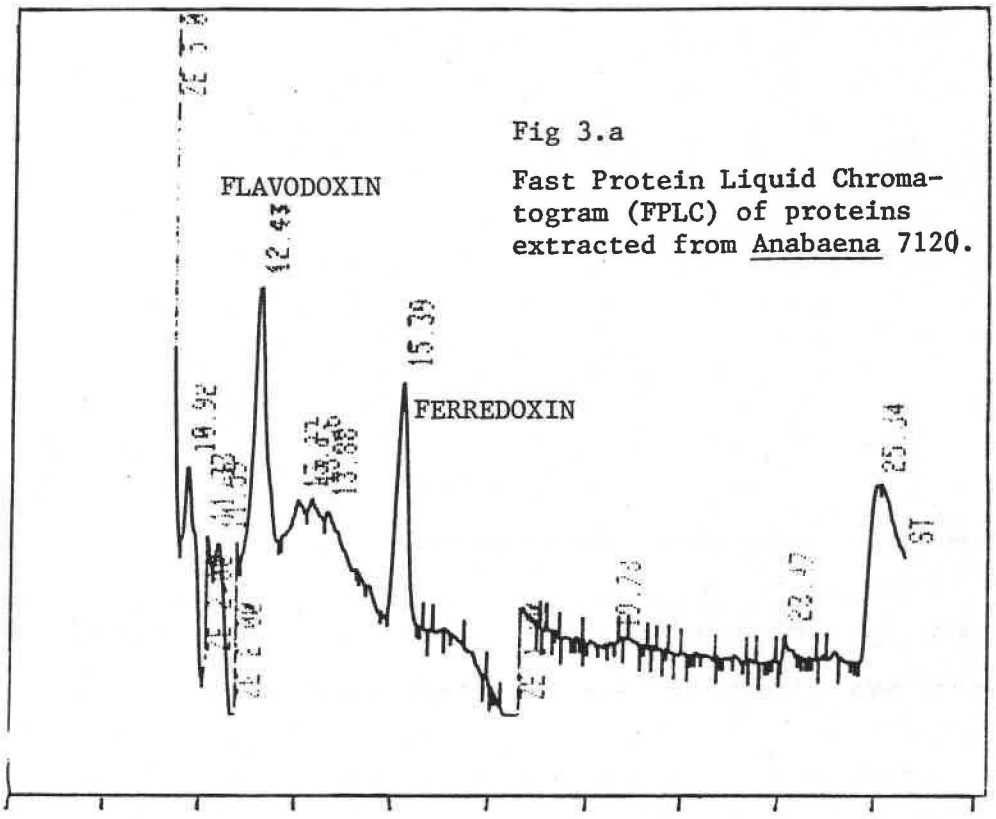
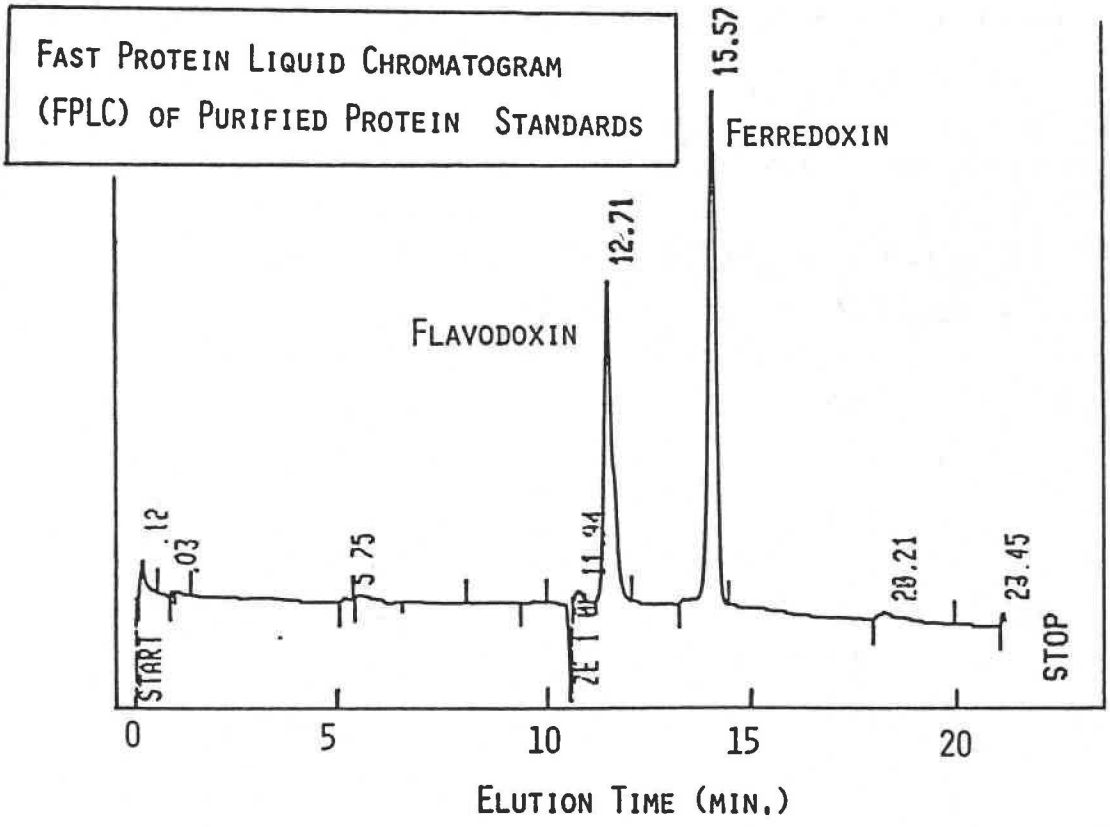


Fig 3.a

Fast Protein Liquid Chromatogram (FPLC) of proteins extracted from Anabaena 7120.

Fig 3.b



As will be discussed in detail below, the cell density (klett number) to which the culture was grown was an important factor in the amount of Fe stress observed. This was a simple consequence of the progressive depletion of nutrient iron in a batch culture. Thus, direct comparisons can be made only between cultures grown to approximately the same cell density. To replicate the conditions used in the DE-52/LC experiments, cultures were again grown to a relatively low density of about 25-30 klett. In most subsequent experiments, cultures were grown to approximately 60 klett to obtain somewhat larger amounts of cell material for analysis. In a final set of experiments (described below), cells were grown to 120-150 klett. The klett density of the cultures will be specified for all experiments and in the resulting figures.

At 5.0 μM added Fe, cells grown to ~25 klett contained ferredoxin at 21.0 μmol/100 g dry wt whereas the flavodoxin concentration was 6.8 μmol/100 g dry wt. At 0.1 μM added Fe the ferredoxin and flavodoxin were nearly equal at 7.8 and 7.9 μmol/100 g dry wt, respectively. Although the relative values paralleled the earlier experiments the levels of ferredoxin and flavodoxin were measured at consistently higher levels by FPLC than by the DE-52/LC column. This pattern was always observed and we believe that the conventional LC method has a negative bias due to losses in the ammonium sulfate precipitation clean-up step, and because of irreversible losses on the large column.

The nitrogen metabolism of cells was shown to have a significant effect on their iron stress levels. Cells were grown to ~60 klett at 0.1, 1.0, and 5.0 μM added Fe in media that were N-replete with nitrate, N-replete with ammonium, and N-deficient (no fixed N added). The induction of N_2-fixation in N-deficient cultures was verified by the presence of heterocysts (specialized N_2-fixing cells) at concentrations of at least one heterocyst for every thirty vegetative cells. In the N-replete cultures, heterocysts were either absent or observed at concentrations of one to every 200-400 vegetative cells.

As depicted in Figs. 4 and 5, flavodoxin increased and ferredoxin decreased in all cultures as added Fe was diminished from 5.0 to 1.0 to 0.1 μM. But, the absolute and relative concentrations of these proteins varied depending on the N-metabolism. Cells raised on ammonium exhibited large concentrations of ferredoxin and always contained the lowest proportion of flavodoxin at any added Fe. Nitrate-grown cells showed moderate levels of both proteins but generally had higher relative levels of flavodoxin at any iron concentration, compared to

the ammonium-grown cells. N_2-fixing cells consistently had the greatest relative concentrations of flavodoxin and relatively high amounts of both proteins.

These results indicate that, for a specified amount of added Fe, cells utilizing ammonium are the least likely to be stressed for iron, cells using nitrate have somewhat greater stress levels, and N_2-fixing cells are the most stressed for iron. The experiments demonstrate that a single strain of cyanobacteria, grown at a single concentration of added Fe, can exhibit three distinct levels of Fe-stress depending only on the source of nutrient nitrogen. Although these experiments do not document the cause of this nitrogen-linked Fe-stress dependency, we believe that the simplest explanation is that the proteins required for nitrogen assimilation contain significant amounts of iron. Ammonium-N is in the proper oxidation state for incorporation into amino acids and requires no redox proteins for uptake or assimilation. In contrast, nitrate must be reduced to nitrite and ultimately to ammonium before assimilation (Syrett 1962). Nitrite reductase, the enzyme that reduces nitrite to ammonium, contains 5-7 iron atoms (Hewitt et al. 1976). In addition, the enzyme system must be supplied with reducing equivalents via ferredoxin or some similar redox protein (Ramirez et al 1966; Zumft et al. 1969, 1970). Thus nitrate assimilation imposes greater iron requirements on the cell than does ammonium assimilation (Pechek 1979). N_2-fixing cells, in principle, have even a greater need for iron than cells utilizing other sources of nitrogen. Nitrogenase, the enzyme complex that fixes and reduces N_2, contains 32-44 atoms of Fe per enzyme complex and also requires auxiliary redox proteins such as ferredoxin (Mortenson and Thornely 1979). Thus, differences in Fe-stress levels among cultures grown on different N-sources can be attributed logically to the relative Fe requirements of the nitrogen assimilatory proteins.

The bar graphs in Figs. 4 and 5 represent mean values of replicate experiments. The trends in ferredoxin and flavodoxin concentrations are fairly clear but there was significant variability in the redox protein concentrations measured for any set of conditions (the ranges of the data are presented in Table 1). As a result, some anomalies to the general pattern were observed such as diminished flavodoxin in nitrate-replete cell grown at 0.5 μm Fe compared to the

Fig. 4. Ferredoxin and flavodoxin in cells grown to 60 klett as a function of Fe added.

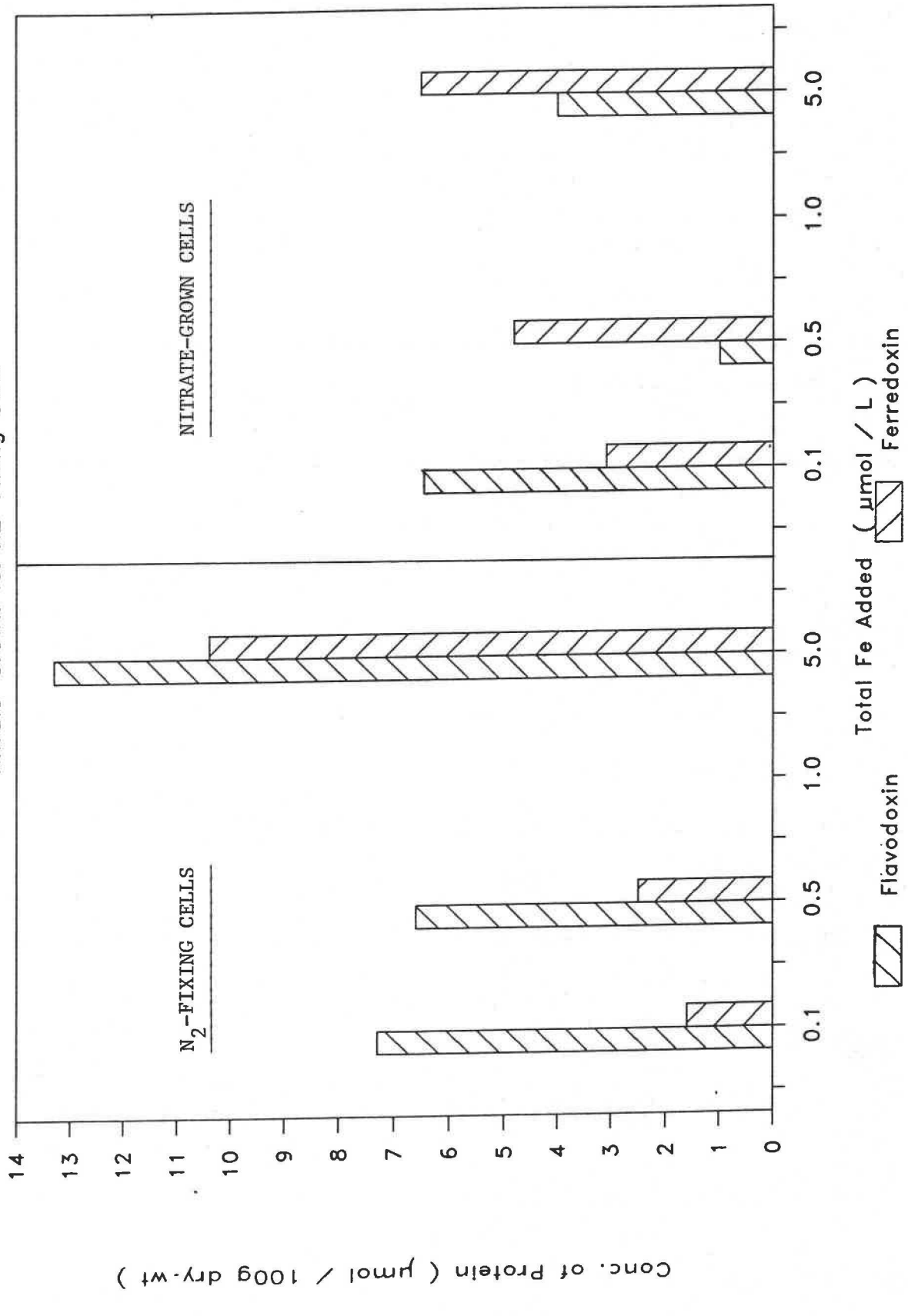


Fig. 5. Flavodoxin and ferredoxin in cells grown to 120 klett with 5.0 uM Fe added.

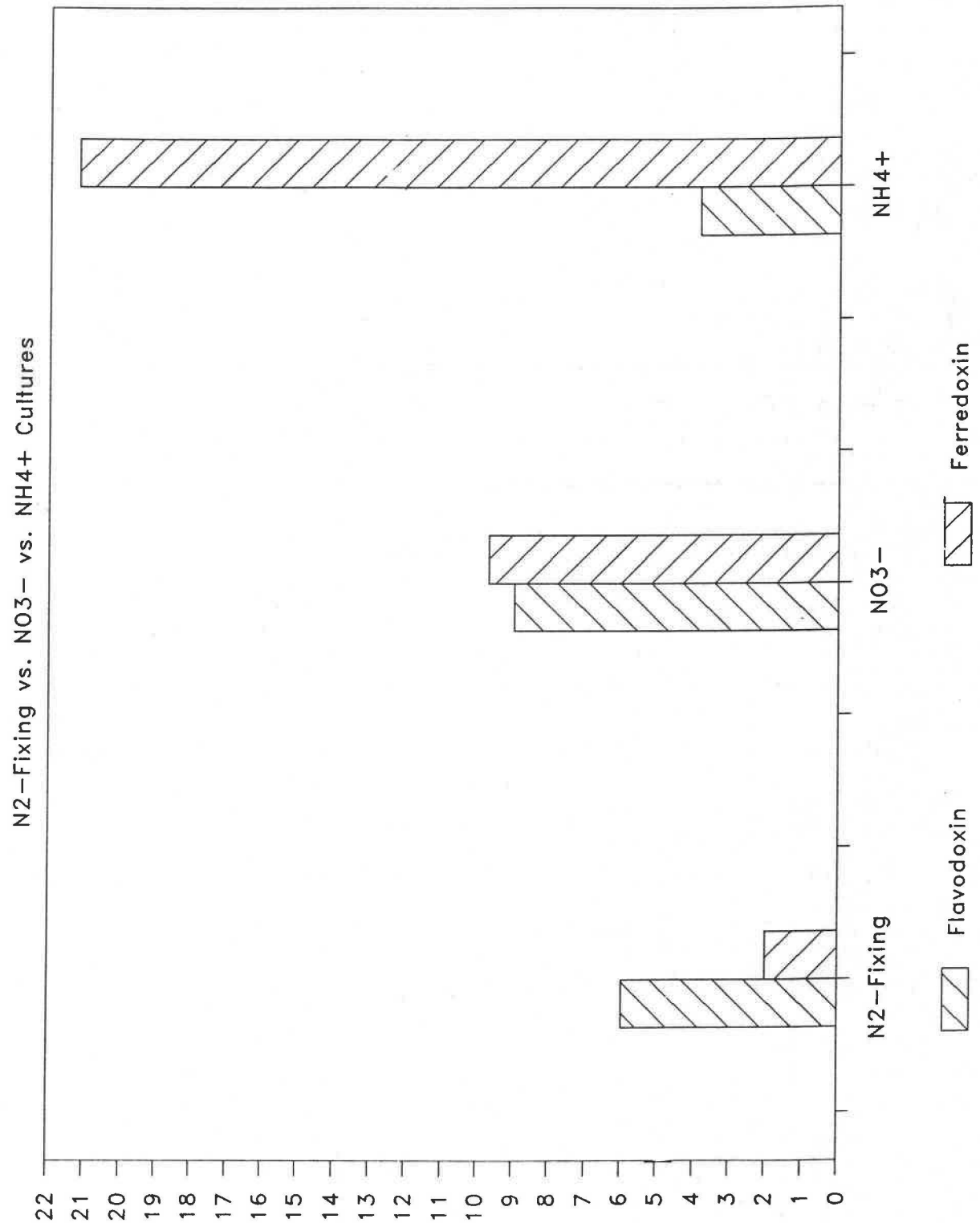


TABLE 1. Ferredoxin and Flavodoxin Data from Laboratory Cultures

Nitrogen Source	Tot. Fe Added (µmol/L)	Final Cell Density			
		~60 klett units		~120 klett units	
		Flavodoxin (µmol/100g-dry wt)	Ferredoxin (µmol/100g-dry wt)	Flavodoxin (µmol/100g-dry wt)	Ferredoxin (µmol/100g-dry wt)
N2	0.1	7.3 + 1.0	1.6 + 0.5	-	-
	0.5	6.6 + 0.2	2.5 + 1.1	-	-
	1.0	-	-	8.2 + 0.1	0.7 + 0.1
	5.0	13.3 + 3.2	10.4 + 2.4	6.0 + 1.0	2.0 + 1.0
NO3-	0.1	6.5 + 3.3	3.1 + 1.5	-	-
	0.5	1.0 + 0.1	4.8 + 0.2	-	-
	1.0	-	-	13.0 + 0.8	9.5 + 0.2
	5.0	4.0 + 1.5	6.5 + 1.2	9.0 + 2.5	9.7 + 1.5
NH4+	0.1	5.4 + 1.6	4.1 + 1.1	-	-
	5.0	-	-	3.9 + 0.3	21.2 + 4.3

flavodoxin in cells grown at 5.0 uM Fe (Fig. 4). Further experimentation is necessary to quantify and understand the variability in redoxprotein concentrations. One important cause of variability identified in our studies was the cell density to which the cultures were grown.

All experiments were performed on batch cultures in which the total Fe is fixed. Because other nutrients were provided in relative excess, all cultures could be grown into a state of relative iron starvation if given enough time. For example, a culture containing 1.0 μM Fe grown to a density of 200 klett might be expected to have the same Fe per cell as a culture containing 0.1 μM Fe grown to only 20 klett. Of course, "luxury uptake" of Fe at low cell density would complicate this simple proportionality. In any case, cell density in batch culture is a variable among the causes of apparent Fe stress. In all comparative experiments we avoided this variability by growing all cultures to approximately the same density. However in the last set of experiments we explicitly documented the effects of cell density on Fe stress.

Nitrate-replete and N_2-fixing cells were grown on 1.0 and 5.0 μM added Fe, as before, except that they were grown to approximately twice the cell density (averaging about 120 klett with a range of 99 klett to 185 klett). The ratio of flavodoxin to ferredoxin is taken as a measure of Fe stress (high ratio values correspond to high Fe stress; see Fig. 6a). The increase in this ratio as a function of cell density for a fixed amount of added iron is shown in Fig 6b. As shown in Fig. 6b, N_2-fixers and nitrate-replete cells showed the same relative pattern of flavodoxin/ferredoxin as in the lower-density experiments (greater flavodoxin in the N_2-fixers). But the dense cultures exhibited higher absolute levels of Fe stress than the less dense cultures for both nitrate-replete and N_2-fixing cells. That is, the N_2-fixing cells always showed greater Fe stress than nitrate-grown cells. This phenomena can be better seen by plotting the flavodoxin/ferredoxin ratio against the ratio of (klett density)/(added Fe) for low- and high-Fe cultures at low and high cell densities (Fig. 7). The great importance of N_2-fixation in aggravating Fe stress is reflected in the steeper slope of the regression line fitted to the N_2-fixing data.

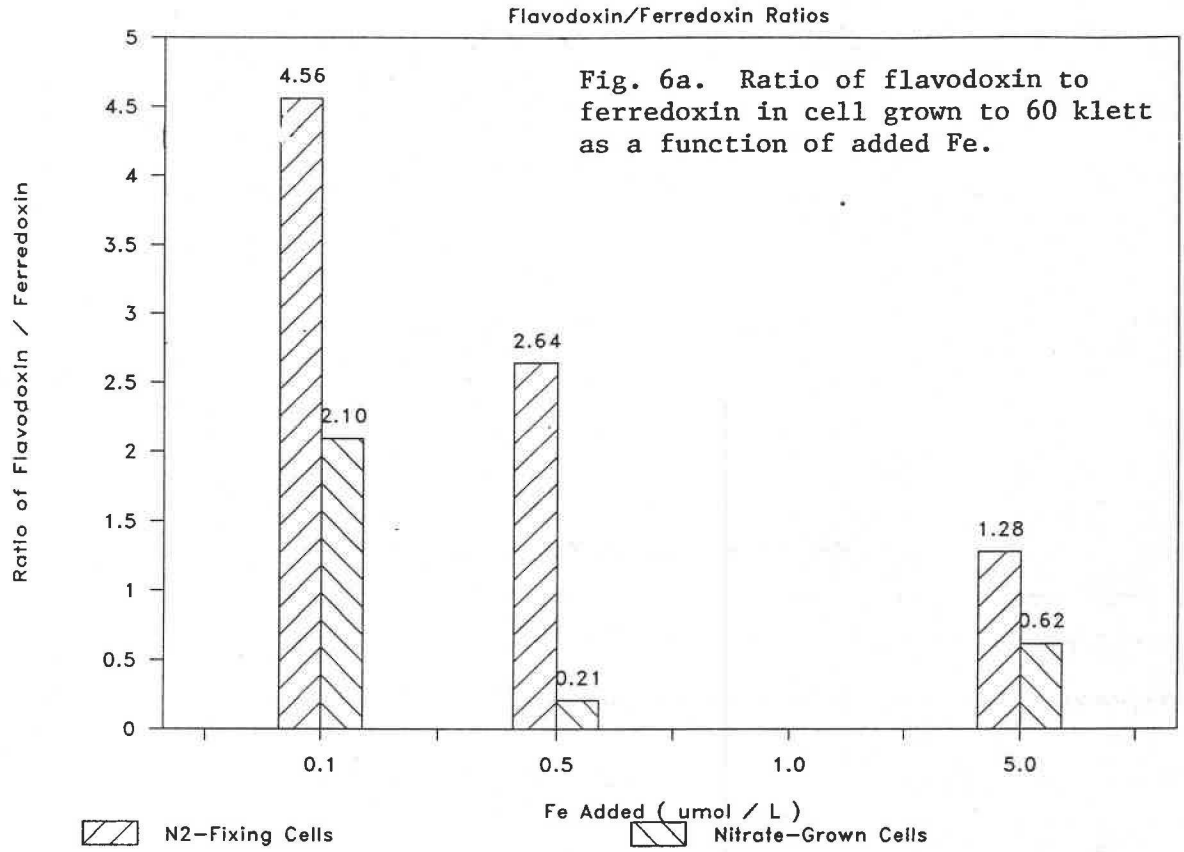


Fig. 6a. Ratio of flavodoxin to ferredoxin in cell grown to 60 klett as a function of added Fe.

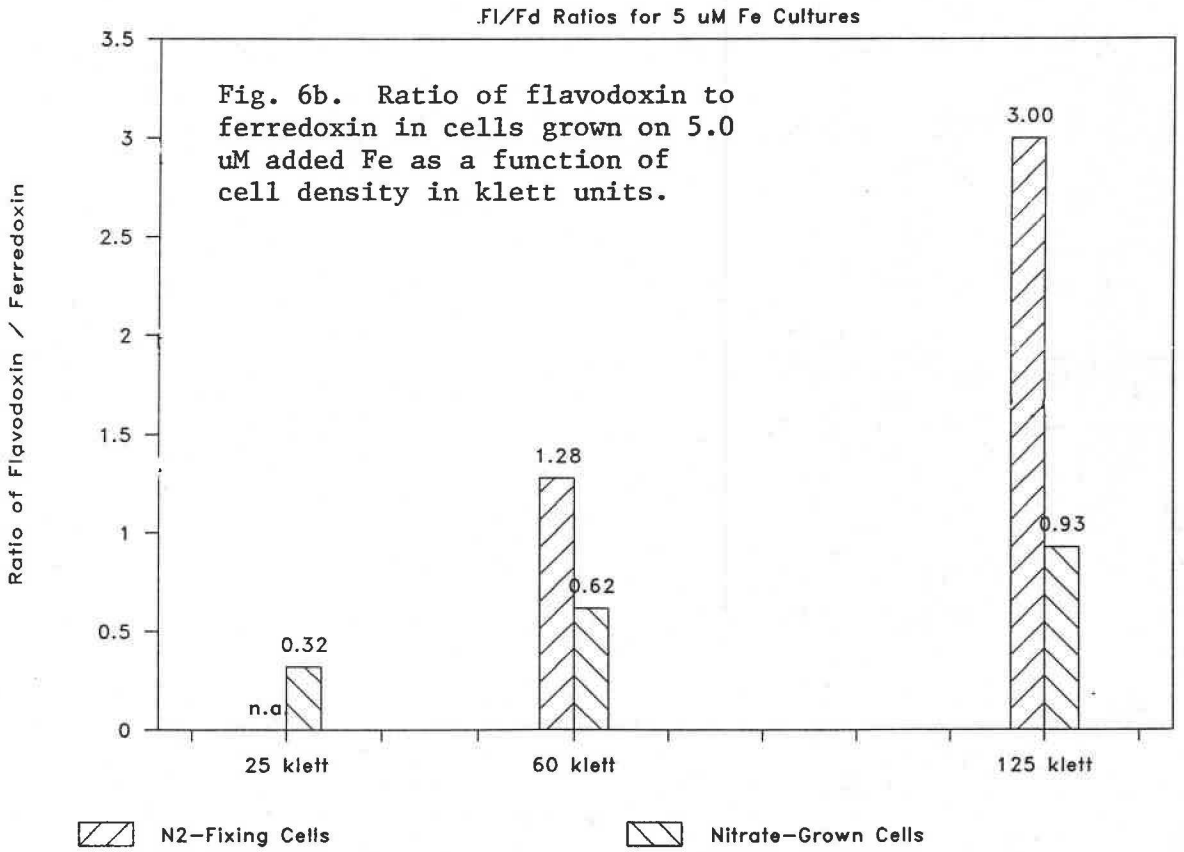


Fig. 6b. Ratio of flavodoxin to ferredoxin in cells grown on 5.0 uM added Fe as a function of cell density in klett units.

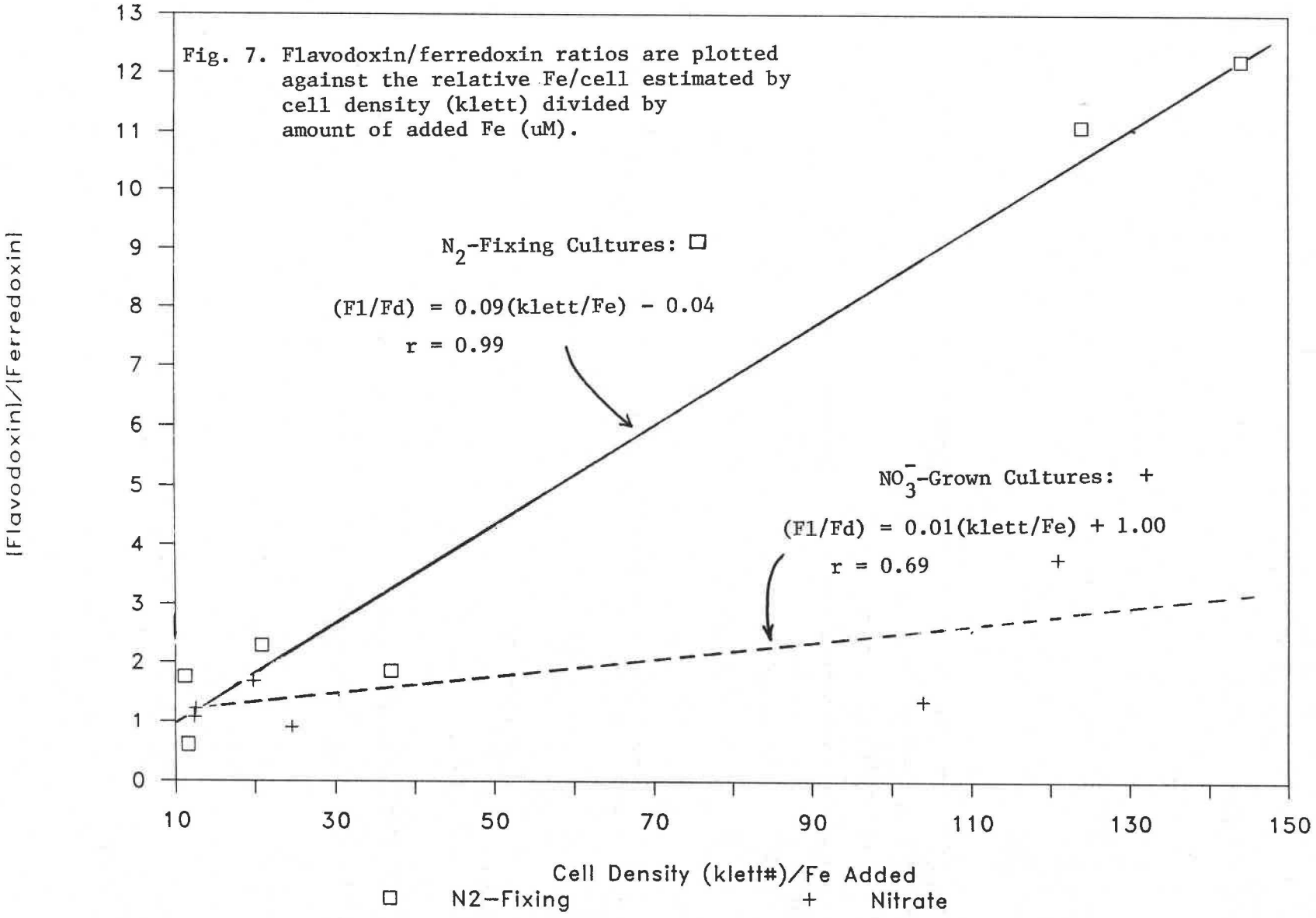


Fig. 7. Flavodoxin/ferredoxin ratios are plotted against the relative Fe/cell estimated by cell density (klett) divided by amount of added Fe (uM).
N_2-Fixing Cultures: □
(F1/Fd) = 0.09(klett/Fe) - 0.04
r = 0.99
NO_3^--Grown Cultures: +
(F1/Fd) = 0.01(klett/Fe) + 1.00
r = 0.69
[Flavodoxin]/[Ferredoxin]
0
1
2
3
4
5
6
7
8
9
10
11
12
13
10
30
50
70
90
110
130
150
Cell Density (klett#)/Fe Added
□ N2-Fixing
+ Nitrate

These experiments provided empirical confirmation of the intuitive notion that "available iron" (as with any nutrient) is not an absolute measure of the Fe stress or potential Fe limitation of a population. The Fe stress level depends on the number of cells competing for the available Fe resources. The batch experiments performed here are roughly analogous to a summer algal bloom in which the population is growing in a stable epilimnion with little or no additional nutrient inputs. The Fe stress, hence its potential for limiting growth, increases during the course of the bloom. Whether the productivity is ultimately limited by Fe availability depends on the amount and form of Fe initially present, the form of nitrogen available, and density that the cells achieve before another nutrient or physical factor becomes growth-limiting. Our results suggest that actual Fe limitation is more likely to occur, or will occur more quickly, if the cells must fix atmospheric nitrogen. Cells utilizing nitrate or nitrite should be able to grow to greater densities before Fe limits growth and cells growing on ammonium will be the least dependent on Fe.

A numerical synopsis of laboratory results is presented in Table 1.

FIELD SAMPLES

Single samples of cyanobacteria were obtained from Cullaby, McKay, and Morgan lakes. The primary purpose of these samples was to test the general feasibility of measuring ferredoxin and flavodoxin in wild populations of phytoplankton. A secondary intent was a simple survey of cyanobacterial ferredoxin and flavodoxin levels in a small but diverse set of Oregon lakes.

A series of three samples was taken during the latter half of the annual bloom in Upper Klamath Lake. The purpose of this time series was to ascertain whether ferredoxin and flavodoxin levels changed during the growth cycle of a bloom and to determine whether these changes corresponded to changes predicted by the laboratory cultures. A corollary purpose was to determine whether a time series of samples provided more useful information about a bloom than did a single sample.

Species Composition of Samples

The species compositions of samples from Cullaby and Upper Klamath Lakes were determined in detail by microscopic examination. The samples from McKay

Reservoir and Morgan Lake were simply analyzed qualitatively for the predominant organisms.

Upper Klamath Lake

The first sample (UK-1) was taken on 7 August 1985 from the Algoma Road overpass when a dense cyanobacterial population was already well established. Microscopic analysis of the sample revealed that 97.4% of the biovolume was the cyanobacterium Aphanizomenon flos-aquae (151,000 cells/mL). Another cyanobacterium, Anabaena sp., constituted 1.4% of the biovolume (1260 cells/mL), and miscellaneous eukaryotic algae (mostly diatoms and green algae) accounted for the remaining 1%. Cyanobacterial heterocysts were observed in the sample at a concentration of 1602 heterocysts/mL, yielding a mean ratio of heterocysts to vegetative cells (h:c) of 1:94. Concentration of the sample in the laboratory resulted in a wet cell volume of 20 mL which yielded 1.6 g of acetone cell powder.

A second sample (UK-2) was taken at Wocus Bay on 19 September 1985. At that time the bloom was extremely dense and algal masses were piling up along the shoreline. Due to an unexpectedly high cell density, the standard addition of Lugol's solution was insufficient to properly preserve the aliquot taken for species analysis, but the sample was estimated to be approximately 99% Aphanizomenon flos-aquae. The bulk sample was processed promptly for flavodoxin/ferredoxin analysis and was not subject to deterioration.

A third Upper Klamath Lake sample was taken from Wocus Bay on 26 September 1985 (UK-3), at which point the bloom was still exceptionally dense. Aphanizomenon flos-aquae constituted 93.5% of the biovolume (210,240 cells/mL) and the remainder was made up of miscellaneous eukaryotic algae. A density of 565 heterocysts/mL was observed for a mean h:c ratio of 1:370. The second and third samples each yielded concentrated wet volumes of 160 mL. About 10 g of cell powder was obtained from each of these.

The consistently low values of the h:c ratios from all three samples indicated that the Aphanizomenon population was fixing little or no N_2.

Cullaby Lake

Cullaby Lake was sampled on 26 August 1985. The cyanobacterial composition at the surface of the lake by biovolume was 46.2% Anabaena planktonica (95,400

cells/mL), 12.2% Aphanizomenon flos-aquae (225,000 cells/mL), and 0.5% Synechocystis sp. (formerly Microcystis aeruginosa) (4770 cells/mL). The biovolume proportion of a eukaryotic phytoplankter, Melosira ambigua was relatively high (36.8%) compared to all other lake samples, which consisted primarily of cyanobacteria. Aphanizomenon flos-aquae in this sample had a density of 19,486 heterocysts/mL and an h:c ratio of 1:20. Anabaena planktonica had a density of 11,389 heterocysts/mL and an h:c of 1:21. The relatively high h:c ratio values indicated the population was actively fixing N_2.

Morgan Lake and McKay Reservoir

Morgan Lake and McKay Reservoir were sampled on 16 August 1986. The Morgan Lake sample contained several phytoplankton species and qualitative microscopic examination indicated these were predominantly Anabaena spp. A relatively small percentage (about 20%) of the cells appeared to be eukaryotic algae, primarily green algae and diatoms. Qualitative microscopic examination of the McKay Reservoir sample revealed almost a pure (>90%) population of Aphanizomenon flos-aquae with small number of diatoms.

Biochemical Analysis of Lake Samples

In Cullaby Lake the ferredoxin concentration was measured at 3.6 μmol/100 g dry wt (Table 2) using the DE-52 method. No peak was observed in the distinctive 465 nm absorbance characteristic of flavodoxin. McKay Reservoir and Morgan Lake samples were analyzed using the FPLC. Replicate samples were prepared and analyzed for each. A large and distinctive ferredoxin peak was observed in the chromatogram of McKay Reservoir sample corresponding to 63 μmol/100g dry wt. The elution region of the chromatogram where flavodoxin should appear revealed a substantial peak at 13.25 min corresponding to 14.1 μmol/100g dry wt. The chromatogram of the Morgan Lake sample indicated a moderate concentration of ferredoxin (14.4 μmol/100g dry wt) and a somewhat indistinct peak eluting at 12.8 min which corresponds to flavodoxin at a concentration of 2.1 μmol/100g dry wt. The chromatograms from lake samples were generally less "clean" than those of the pure, laboratory cultures, but in most cases the ferredoxin and flavodoxin peaks were still readily interpreted.

Cullaby Lake, with abundant humic material and a well buffered, near-neutral pH, was expected to provide an environment of high available iron. The

algal sample collected is consistent with this hypothesis in that no flavodoxin was observed, even though several of the species present are known to produce it under iron stress. Note also that Cullaby Lake cells were fixing N_2, a condition which was shown in the laboratory to enhance iron stress. Thus we conclude that Cullaby Lake, at the time of sampling, did in fact contain levels of available iron high enough to easily support the requirements of the phytoplankton community.

In contrast to coastal Cullaby Lake the two Central Oregon lakes, McKay and Morgan, contained populations of phytoplankton that exhibited significant flavodoxin production. These lakes are more alkaline than Cullaby and have summer pH ranges in excess of 8.0. Situated in arid watersheds, they also contain relatively little dissolved organic carbon. The combination of high pH and low metal-complexing potential means that iron is likely to be relatively unavailable for phytoplankton uptake. The conditions therefore militate for a comparatively high level of iron stress in the algal population. The presence of flavodoxin in the Aphanizomenon and Anabaena collected from these lakes appears to confirm this hypothesis.

It is particularly interesting to note that Morgan Lake is described by Johnson et al. (1985) as follows: "...the chlorophyll concentrations are low. The concentration of phosphorus is surprisingly high and indicates that the lake should be classified as eutrophic." The apparent paradox of low chlorophyll and high phosphorus loading in a shallow, warm lake (summer temp. = ca. 24°C) may be partly explained by iron limitation of productivity. The data from a single sample cannot confirm or refute this explanation, and more detailed, future studies will be necessary. One improvement over the single grab sample is a time series of samples, such as those taken at Upper Klamath Lake.

For Upper Klamath Lake, an equal mass of cell powder (1.56 g) was used from each of the three samples. Determinations of cellular ferredoxin and flavodoxin in the lake samples initially were made using the DE-52 column. The ferredoxin concentration in the first Upper Klamath Lake sample (UK-1) was 4.2 μmol/100 g dry wt, decreasing slightly in UK-2 to 3.5 μmol/100 g dry wt, although this decline probably is not statistically significant. UK-3, however, showed a more pronounced decrease in ferredoxin, to 1.6 μmol/100 g dry wt.

The characteristic flavodoxin absorbance at 465 nm did not exhibit a distinctive peak in the DE-52 column elution profiles of any of the Upper Klamath

or Cullaby Lake samples nor could the visible spectrum of flavodoxin be discerned in any of the column fractions. In UK-3, a small peak of flavin fluorescence was detected in acidified fractions corresponding to the same elution region as the flavodoxin peak in the laboratory samples (at approx. 0.2 M NaCl in the elution gradient). When these fractions were pooled and concentrated eightfold by ultrafiltration a weak but distinctive visible absorption spectrum was obtained that closely resembled flavodoxin. Although the fluorescence and absorbance data suggested the presence of flavodoxin in the sample, the concentration was too low to be accurately quantified by this method.

A great improvement on the initial DE-52 column analyses was achieved by analyzing samples with the Pharmacia FPLC. Pairs of replicate protein extracts were prepared from the cell powders of UK-2 and UK-3. (UK-1 was used entirely in the DE-52 analysis and could not be retested.) Because of the higher sensitivity of FPLC, only 0.5 g of cell powder was used in each extract and replicate analyses could be performed. Ferredoxin concentrations paralleled those measured with the DE-52 column but were substantially higher. UK-2 contained ferredoxin at 8.4 (±0.2) μmol/100 g dry wt. and UK-3 contained 6.8 (± 0.2) μmol/100 g dry wt (Fig. 8). A very small peak was observed in the chromatogram of UK-3 at a retention time of 13.3 min, corresponding to flavodoxin at a concentration of approx. 0.6 μmol/100 g dry wt. A similar but smaller peak at this retention time was observed in UK-2, but the area was too small to be integrated above the noise level. All the field data are in Table 2.

The Upper Klamath Lake series of samples indicated that ferredoxin levels declined in the cell population during the bloom and that small amounts of flavodoxin appeared near the end of the bloom. This pattern suggests that the iron stress level of the population increased during the latter stage of the bloom, presumably because the large cell mass consumed available iron resources. The absence of heterocysts among the cells, and the high concentration of nitrate reported for this lake, indicate that the Aphanizomenon was not fixing nitrogen. Thus these results can be compared to laboratory experiments in which the cells were grown in nitrate-replete media. Recall that in those lab studies, cells exhibited clear iron-stress responses only when grown to high cell densities on low iron concentration. We surmise from the field results that iron availability in Upper Klamath Lake is sufficient to support a very dense population of nitrate-replete cells, but that this abundant population eventually depletes the

available iron. More detailed field studies are necessary to document the actual effect of the resulting iron stress on the growth dynamics of the population.

Fig. 8. FPLC chromatograms of samples taken from Upper Klamath Lake. Top figure is UK-2, lower figure is UK-3 (see text).

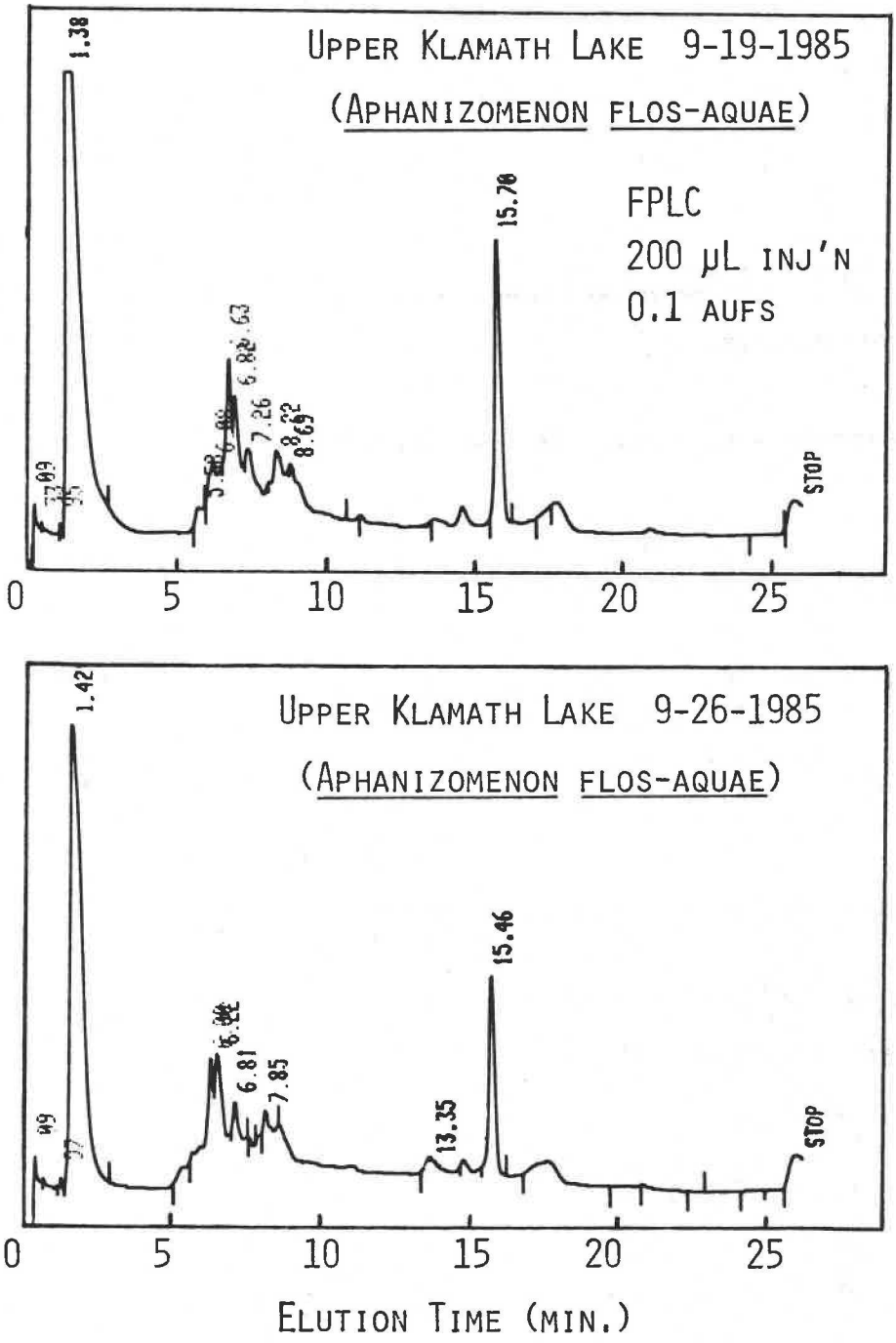


Table 2: Summary of ferredoxin and flavodoxin data for all lake samples.

Sample Name	Collection Date	Cell Powder Mass (mg)	Flavodoxin (umol/100g dry wt)	Ferredoxin (umol/100g dry wt)	Analysis Method
Klamath 1	7 Aug 1985	1,560	~0	4.2	DE-52
Klamath 2	19 Sep 1985	1,560	~0	3.5	DE-52
Klamath 3	26 Sep 1985	1,560	~0	1.6	DE-52
Klamath 1	7 Aug 1985	500	n.a.	n.a.	-
Klamath 2	19 Sep 1985	500	~0	8.2	FPLC
Klamath 3	26 Sep 1985	500	0.6	6.8	FPLC
Cullaby	26 Aug 1985	1,430	~0	3.6	DE-52
McKay	16 Aug 1986	48	14.1	63.0	FPLC
Morgan	16 Aug 1986	49	2.1	14.4	FPLC

4. CONCLUSIONS

Laboratory experiments with the cyanobacterium Anabaena sp. strain 7120 have proven the utility of measurements of cellular flavodoxin and ferredoxin as indicators of iron stress. The method appears to be a useful probe for isolating the effects of iron stress that are induced in a population by any of a number of conditions. The level of iron stress in a given population of organisms is expected to be the result of both the supply of available iron and the internal, physiological requirements for iron. Our experiments confirm this notion. A relative iron deficiency was induced in three ways: by restricting the amount of iron added to the medium, by growing the cultures to high cell densities, and by manipulating the nitrogen metabolism, which affects the cellular iron requirements. In all three cases, relative iron deficiency resulted in enhanced flavodoxin levels and diminished ferredoxin levels.

The generality of this response to iron stress is not yet known. The substitution of flavodoxin for ferredoxin has been documented for a small number of other species and strains of cyanobacteria and one (eukaryotic) green alga, Chlorella. Flavodoxin was observed in the majority of organisms in which it was specifically sought, suggesting that the response is quite general. Most of these studies were relatively qualitative in nature and only the study by Hutber et al. (1977) quantitatively documented the effects of iron limitation on flavodoxin synthesis. However, all of these studies provide a useful basis for anticipating the generality of the technique, and in particular, as a starting point for future, detailed studies of this phenomenon in nature. A compendium of results of previous investigations of flavodoxin and ferredoxin is presented in Table 3. It is hoped that this Table will be useful and stimulating to other investigators.

The substitution of flavodoxin for ferredoxin presumably liberates iron for use in enzyme systems that have an absolute requirement for iron. Examples of the latter are the nitrite reductase complex and the nitrogenase complex. Neither of these systems is required for growth on ammonium, so it is not surprising that ammonium-grown cells showed the least degree of flavodoxin substitution. However, ammonium is relatively uncommon in surface waters except during spring or fall turnover. In well mixed, oxygenated surface waters (such as in the epilimnion of a lake in summer) nitrite and nitrate are the predominant

TABLE 3. Comparison of Results of Studies of Flavodoxin and Ferredoxin in Cyanobacteria.
(n.a.=not analyzed; n.d.=not detected)

Organism	Source	Total Fe in Medium (uM)	Ferredoxin (umoles/100g dry weight)	Flavodoxin (umoles/100g	Reference
Synechocystis sp. (formerly Microcystis aeruginosa)	Lake Kegonsa, Wisconsin	n.a.	n.d.	n.a.	Ho et al. (1979)
Aphanizomenon flos-aquae	Lake Okoboji, Iowa	n.a.	n.d.	n.a.	Ho et al. (1979)
Spirulina maxima	Lake Texcoco, Mexico	n.a.	"low"	n.a.	Ho et al. (1979)
Phormidium sp.	Davies Reef, Australia	<0.03	1.3	0.95	Entsch et al. (1983)
Anabaena planktonica. Aphanizomenon flos-aquae Melosira ambigua (mixed sample)	Cullaby Lake, Oregon	n.a.	3.6	n.d	Entsch et al. (1983)
Synechococcus PCC 6301 (formerly Anacystis nidulans)	laboratory culture	2.5	1.8	2.0	Smillie and Entsch (1971)
Synechococcus PCC 6301 (formerly Anacystis nidulans)	laboratory culture	25.0	15.0[b]	n.d.	Bothe, et al. (1971)
		2.5	5.0[b]	1.5[b]	Bothe, et al. (1971)
Nostoc MAC	laboratory culture	15.0	Type I 15.0 Type II 7.5 [a]	n.d.	Hutber et al. (1977)
		2.0	I 11.7 II 6.2 [a]	1.0	
		1.0	I 6.5 II 1.0 [a]	3.5	
		0.5	I 4.0 II n.d.	5.0	

Continued.

TABLE 3. Comparison of Results of Studies of Flavodoxin and Ferredoxin in Cyanobacteria. (n.a.=not analyzed; n.d.=not detected)

Organism	Source	Total Fe in Medium (uM)	Ferredoxin (umoles/100g dry weight)	Flavodoxin (umoles/100g	Reference
Synechococcus lividus	laboratory culture	n.a.	1.0- 3.0[c]	0.6- 1.8[c]	Crespi et al. (1972)

a: Mean values of several samples.

b: The original data were given as umoles/100g wet weight. To place these in the context of data normalized to dry weights, the original values have been multiplied by a factor of ten.

c: The original paper gives only the ranges of concentrations observed.

forms of inorganic nitrogen. Utilization of these nutrients requires additional iron for algal cells in the form of nitrite reductase and ferredoxin as a source of reducing equivalents. Our experiments indicate that nitrate-grown Anabaena sp. 7120 compensates for this by readily synthesizing flavodoxin at the onset of iron stress. As iron becomes more depleted in the medium ferredoxin levels decrease, freeing iron for use in nitrite reductase and other iron proteins. Again, other species of cyanobacteria should be examined to document the generality of this strategy.

A key ecological advantage possessed by cyanobacteria is their ability to fix atmospheric nitrogen when all other sources of nitrogen are depleted. The substantial iron content of the nitrogenase enzyme complex, coupled with observations of greater iron-content per cyanobacterial cell compared to non-N_2-fixing algae, have led several investigators to conclude that N_2-fixing cyanobacteria may be more likely than other phytoplankton to be limited by iron. So far, no definitive study has proven or refuted this hypothesis but our results clearly indicate that N_2-fixing cells show signs of greater iron stress than do cells supplied with fixed nitrogen under comparable conditions of available iron. Flavodoxin and ferredoxin measurements may be very useful tools in further evaluating the interrelationship between iron limitation and nitrogen limitation in natural ecosystems.

Our preliminary applications of this technique to cyanobacterial populations in four Oregon lakes was encouraging in that we verified that ferredoxin and flavodoxin could be readily quantified by FPLC from relatively small and convenient samples. Ferredoxin was identified in all samples in cellular concentrations comparable both to laboratory cultures and the field samples of other workers. Our analytical refinements are especially important for field studies. Previously published techniques required hundreds of grams of wet cells, making the procedure impractical for all but the most hypereutrophic lakes.

The techniques described here require only a gram or less of wet cells and should be useful in a variety of ecological studies of iron nutrition. Investigations of the connections between iron and nitrogen nutrition of the sort described above are an obvious application. It is hoped that the work presented in this report will encourage further advances in the understanding of the biochemical underpinnings of phytoplankton ecology.

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