CHARACTERIZATION OF FERREDOXIN AND FLAVODOXIN AS MOLECULAR INDICATORS OF IRON LIMITATION IN MARINE EUKARYOTIC PHYTOPLANKTON

by

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ABSTRACT

Expression and regulation of the ferredoxin and flavodoxin proteins in marine phytoplankton were investigated to assess their utility as biomarkers of iron limitation. A phylogenetic survey of seventeen microalgal species showed flavodoxin induction, with accompanying ferredoxin repression, to be a common response to iron stress. A minority of organisms examined never expressed flavodoxin, a condition associated with, but not characteristic of, neritic habitats. Antibodies raised against ferredoxin and flavodoxin from Thalassiosira weissflogii proved to be mono- and diatom-specific, respectively.

Flavodoxin induction responded specifically to iron limitation and not to nitrogen, phosphorus, silicate, zinc or light deficiency. In iron-limited $T.$ weissflogii, relative cellular ferredoxin and flavodoxin content (Fd index) varied with growth rates above ~50%μ_{max} and was not affected by growth on either nitrate or ammonium as a sole nitrogen source. Below ~50%μ_{max}, ferredoxin was absent. This variation with severity of stress and specificity to iron limitation make the Fd index an excellent choice as an indicator of iron limitation.

HPLC measurement of ferredoxin and flavodoxin during the IronExII mesoscale enrichment experiment detected a strong flavodoxin signal but no significant ferredoxin synthesis, despite increases in chlorophyll and photosynthetic efficiency (F_v/F_m) observed by others. The absence of ferredoxin and the persistence of flavodoxin suggested that iron addition released the phytoplankton from iron starvation but was insufficient to completely relieve physiological iron limitation. Laboratory experiments demonstrated that a pennate diatom clone isolated from the IronExII bloom expressed both flavodoxin and ferredoxin and could alter its protein expression in about one day, further supporting the conclusion of continued iron limitation during IronExII.

During IronExII, Fd index was uniformly zero while F_v/F_m increased from 0.26 to 0.56. In contrast, a laboratory iron addition experiment showed little change in F_v/F_m when the Fd index increased from 0.5-0.9. A conceptual model of the covariation of F_v/F_m and Fd index describes a complementary relationship between the two measures. Model results suggest that photochemical systems are affected by iron limitation only after cellular adaptive capacity, in the form of ferredoxin, is exhausted.

Thesis supervisor: Donald M. Anderson

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For Donna and Jim

maggie and milly and molly and may
went down to the beach (to play one day)

and maggie discovered a shell that sang
so sweetly she couldn't remember her troubles,and

milly befriended a stranded star
whose rays five languid fingers were;

and molly was chased by a horrible thing
which raced sideways while blowing bubbles:and

may came home with a smooth round stone
as small as a world and as large as alone.

For whatever we lose (like a you or a me)
it's always ourselves we find in the sea

                                  - e.e. cummings

Introduction

Oceanic carbon fixation is one of the largest single fluxes in the global carbon cycle. While oceanic phytoplankton constitute only 1-2% of global plant carbon, they account for a disproportionately large part of global carbon fixation: 35-50 billion metric tons every year, about 40% of the total (Sarmiento & Siegenthaler 1992). Oceanic photosynthesis generates a "biological pump" in which the sinking of phytoplankton serves to enrich the deep ocean with carbon in excess of that due to strictly chemical processes such as gas diffusion. To understand the ways in which oceanic production responds to, and is affected by, increases in atmospheric CO_2, it is essential that we understand the factors that control carbon fixation by marine organisms.

In addition to carbon, phytoplankton require significant amounts of nitrogen and phosphorus for growth. Since concentrations of N and P are essentially undetectable in most of the world's oceans, growth of phytoplankton is thought to be constrained by their availability. However, in three oceanic regions, the Southern Ocean and the Equatorial and Subarctic Pacific, there exists an excess of surface macronutrients. These areas are designated "high nutrient-low chlorophyll" (HNLC) areas because the phytoplankton biomass present is low relative to the amounts of available macronutrients. Concentrations of N and P are high and should not limit growth. These areas have been studied by oceanographers for years in attempts to determine the factor(s) that limit production.

Several hypotheses were proposed to explain this paradox. It was argued that zooplankton grazing could maintain phytoplankton abundance below levels required to deplete all available nutrients (Walsh 1976, Frost

1991, Minas & Minas 1992). A related theory suggested inhibition of nitrate uptake by the ammonium produced as a result of grazing (Wheeler & Kokkinakis 1990). In the Southern Ocean, it was posited that insufficient light due to deep mixing kept phytoplankton biomass low (Mitchell et al. 1991). In addition, low temperatures in Antarctic waters could slow enzyme uptake kinetics (Tilzer et al. 1986, Dugdale & Wilkerson 1990). A parallel hypothesis was that, in general, the uptake kinetics of phytoplankton in HNLC areas were not fast enough to utilize the available nutrients (Dugdale & Wilkerson 1991). Toxicity, due to an excess of a metal micronutrient, was also suggested (Barber & Ryther 1969, Huntsman & Sunda 1980). However, the hypothesis that has received the most attention in recent years was the idea that phytoplankton production in HNLC waters is limited by a lack of iron.

The suggestion by Martin in 1990 that iron-limited phytoplankton could be stimulated to absorb more atmospheric CO_2 by the addition of iron generated considerable controversy and research (Martin et al. 1990). Primary evidence of iron limitation was initially derived from iron addition bioassays. Such bioassays consist of the addition of iron to a natural water sample contained in a bottle, keeping a duplicate sample as a control. The concentrations of nitrate, chlorophyll-a and iron are then monitored throughout on-deck incubations. Similar experiments were attempted years ago (Barber & Ryther 1969), but results are suspect given the discovery of the critical importance of trace metal clean sampling techniques (Fitzwater et al. 1982). Due to the vanishingly low (pM to nM) concentrations of most trace metals in seawater , it is imperative that investigators adhere to strict trace

metal clean protocols when conducting these bottle experiments. Even slight contamination can completely invalidate the results.

Martin's Fe-enriched samples showed an increase in chlorophyll and an accompanying decrease in nitrate compared to control, unenriched bottles after several days of incubation. This seemed to suggest enhancement of phytoplankton growth due to the added iron, leading to increased nitrate uptake and greater biomass. These results led to the "Iron Hypothesis" (Martin 1990), which linked increased primary production, presumably due to enhanced iron inputs, to enhanced uptake of atmospheric CO_2 and hence climatic change. Further support for this hypothesis was derived from ice core analyses which show an inverse correlation between CO_2 and Al (a proxy for Fe) concentrations in the Vostok ice core (Barnola et al. 1987).

The bottle bioassays provided compelling circumstantial evidence of iron limitation, but were widely criticized for a number of reasons. Execution of the bioassays is difficult at best because of metal contamination problems, and interpretation of the results is plagued by uncertainties (e.g. Venrick et al. 1977). Changes in chlorophyll and nitrate concentrations in the bottles are indirect measures of iron limitation and their conversion to growth rates is not straightforward (Banse 1990, Martin et al. 1990). Bottles are an unnatural environment that introduces several possible artifacts. Large zooplankton are often excluded, thus reducing grazing pressure. The bottles also remove sinking effects, as larger cells that might normally fall from surface waters will be able to grow, especially if the larger grazers are not present.

A prominent observation from the bottle incubations is that there was typically a change in phytoplankton species composition in enriched samples

compared to the controls (Martin et al. 1989, Buma et al. 1991). Initially, the samples are dominated by picoplankton, but iron enrichment tends to favor the growth of large diatoms and coccolithophorids. This could have several causes. It is possible that only the larger cells, present initially in low numbers, are iron-limited, and they then bloom upon addition of iron. Alternatively, the entire community could be iron-limited, but the lack of large grazers allows the larger diatoms to outcompete the picoplankton and dominate the sample.

The confusing and controversial experimental evidence collected prior to 1991 was reconciled in the "Ecumenical Hypothesis" (Price et al. 1991). In this scenario, the phytoplankton community in HNLC waters was iron-stressed. Picoplankton dominated, growing chiefly on NH_4^+ in a tight coupling to grazers in a microbial loop. Iron addition allowed the larger phytoplankton, which were iron limited, to utilize nitrate (nitrate reductase contains iron) and grow, thus altering the species composition, increasing biomass, and reducing nitrate levels to zero.

All of the above hypotheses are difficult to prove, due to complex interactions involving grazers, nutrients, sinking, etc. It seemed necessary to uncouple the different putative limiting factors and examine them separately. It was at this point that this dissertation was initiated, in an attempt to create better tools for the unambiguous assessment of iron limitation in the ocean. The goal was to develop an assay for the diagnosis of iron limitation in natural samples that was rapid, specific to iron limitation, and did not require incubation or manipulation of the phytoplankton community. This could be achieved through the use of so-called "diagnostic indicators", measures of

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cellular processes that are directly influenced by a particular limiting factor. Such diagnostics use the cell as a reporter of the environmental conditions that it "perceives". This project aimed to identify a specific protein, gene, or transcript that was specifically affected by iron limitation, for use as a diagnostic molecular probe in situ, allowing separation of the effects of different limiting factors.

The search for an iron-stress indicator began in the literature, where a likely candidate was soon identified. One particularly well-studied response to iron limitation is the replacement of the common iron-sulfur protein ferredoxin with the functionally equivalent, but non-iron-containing, protein flavodoxin. This response had been identified first in 1965 (Smillie 1965). Since then, it had been widely studied in the laboratory, primarily in bacteria, the green alga Chlorella and the freshwater cyanobacterium Anabaena.

Ferredoxin is a catalytic iron-sulfur protein containing a 2Fe-2S cluster in algae and plants, and from 3Fe-3S to 8Fe-8S in bacteria (for review see Buchanan & Arnon 1971). Algal ferredoxins are small, usually ca. 12 kDa, and extremely acidic with low isoelectric points. They perform a number of common redox functions in cells, e.g. serving as the terminal electron donor to NADPH in photosynthetic electron transport and participating in nitrite reduction, pyruvate reduction and nitrogen fixation. Ferredoxin is highly water-soluble, being located in the stroma of chloroplasts. In marine diatoms, the ferredoxin gene is located in the chloroplast genome (Kowallik et al. 1995).

Flavodoxin, the functional equivalent of ferredoxin, is almost twice as large, ca. 22 kDa in algae (for review see Tollin & Edmondson 1980). Instead of Fe, it contains one molecule of flavin mononucleotide (FMN) as a co-

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factor. Flavodoxin is also very acidic, with a low isoelectric point, and is capable of substituting for ferredoxin in vitro for most reactions that have been tested. It has been reported to be both more and less efficient than ferredoxin in electron transfer, depending upon the reaction being studied (e.g. Smillie 1965, Sandmann et al. 1990, Razquin et al. 1995). Like ferredoxin, it is water soluble and present in the chloroplast stroma. In contrast to ferredoxin, there is no evidence of the flavodoxin gene on the chloroplast genome, thus it is presumably nuclear-encoded.

Laboratory studies of the ferredoxin-flavodoxin switch indicate that its role as an iron-stress response is not completely straightforward. There exists some heterogeneity in response, as not all organisms examined produce flavodoxin while some induce it under iron limitation and others express it constitutively. Various strains of the freshwater cyanobacterium Anabaena show three different modes of induction: Anabaena ATCC29413 induces flavodoxin when iron-limited (Fillat et al. 1988), Anabaena ATCC29211 does not produce flavodoxin (Pardo et al. 1990), while Anabaena ATCC29151 produces flavodoxin constitutively in its heterocysts (Sandmann et al. 1990). In addition, some cyanobacteria possess different ferredoxin and/or flavodoxin isoforms that are dedicated to nitrogen fixation (Schrautemeier & Böhme 1985, Klugkist et al. 1986, Böhme & Schrautemeier 1987). Still other studies have found an influence of other types of stress on flavodoxin expression. Flavodoxin is induced by light in both wheat and pea (Dobres et al. 1987, Bringloe et al. 1995). Salt and heat shock, as well as iron stress, induce flavodoxin expression in Synechocystis (Fulda & Hagemann 1995). In the green alga Chlorella, flavodoxin is expressed during heterotrophic growth in

both iron-replete and -deplete conditions (Inda and Peleato, pers. comm.). These uncertainties made it necessary to characterize the use and regulation of this switch in algae before it could be properly used as an ecological indicator in the field.

In spite of the uncertainties regarding flavodoxin use and regulation, there were a few isolated attempts to use it as a diagnostic of iron limitation in natural populations. In 1986, Entsch et al. purified ferredoxin and flavodoxin from cyanobacteria and dinoflagellates collected from the Great Barrier Reef (Entsch et al. 1983). The cyanobacteria, which contained both flavodoxin and ferredoxin, were judged to be iron-limited whereas the lack of flavodoxin in the symbiotic dinoflagellates was taken as evidence of iron sufficiency. A subsequent study by Jones examined the relative proportions of ferredoxin and flavodoxin in the nitrogen-fixing colonial cyanobacterium Trichodesmium collected from the Caribbean (Jones 1988). Increases in the ferredoxin:flavodoxin ratio of this organism were correlated with storm events, which were presumed to provide iron-rich aeolian dust. Both of these analyses must be considered preliminary, as there were no published studies regarding flavodoxin induction in marine microalgae at that time. Entsch et al. relied on information from the freshwater literature and the techniques used by Jones were based on the freshwater cyanobacterium Anabaena. There remained a clear need for information regarding the ferredoxin-flavodoxin system in marine phytoplankton.

That was the state of our knowledge in 1991 when this dissertation was initiated. In the six years since then, much has been learned, both about iron

limitation in the oceans and about ferredoxin and flavodoxin. Most notably, the hypothesis that iron limits primary productivity in the Equatorial Pacific was tested in 1993 and 1995 by direct in situ fertilization of the ocean. At the same time, the concept of flavodoxin expression as an indicator of iron limitation has gained considerable prominence.

The IronEx experiment, performed in October of 1993, enriched a 64 km^2 patch of the equatorial Pacific ocean with ~4 nM iron (Martin et al. 1994). Scientists detected a significant biological change but small chemical changes (nutrients and CO_2) during the four days that the patch was tracked prior to its subduction beneath a low-salinity front. These results demonstrated not only a direct iron-mediated response of the ecosystem but, more importantly, the feasibility of mesoscale open-ocean manipulations. The knowledge gained during IronEx was applied in an expanded fertilization experiment, dubbed IronEx II, which was conducted in May and June of 1995 (Coale et al. 1996). This second experiment added iron in three infusions, spaced three days apart, whereas IronEx utilized a single iron addition. The fertilization triggered a tremendous phytoplankton bloom which removed large quantities of both nitrate and CO_2 during the nineteen days that it was monitored. These two experiments demonstrated unequivocally that iron does indeed limit primary production in the Equatorial Pacific. In the process they revolutionized the way we think about ecological studies of the ocean. While mesoscale ocean enrichments provide invaluable data about ocean processes at the ecosystem scale, they are not a useful and practical method for the routine assessment of iron limitation in the environment. They do not

obviate the need for diagnostic indicator systems such as ferredoxin and flavodoxin.

The ferredoxin and flavodoxin proteins, and their potential for use in the detection of iron limitation, did not escape notice in the intervening years. LaRoche et al., when searching for proteins induced in response to nutrient starvation, identified a major iron-stress induced protein from Phaeodactylum tricornutum as flavodoxin (LaRoche et al. 1993). They subsequently developed a polyclonal antibody to Phaeodactylum flavodoxin, which reacts with flavodoxin from a number of diatom species (LaRoche et al. 1995). This antibody has been used to demonstrate the insensitivity of flavodoxin expression to nitrogen and phosphorus stress. In addition, LaRoche et al. used this antibody to detect flavodoxin expression in Rhizoselenia mats from the equatorial Pacific and diatoms from the northeast subarctic Pacific (LaRoche et al. 1996). The work of LaRoche et al. has done much to popularize flavodoxin's use as an indicator of iron limitation. Their antibody techniques are, however, presently restricted to use in diatoms, and detect flavodoxin qualitatively on the basis of its presence or absence. The aim of this dissertation was more broad: to develop a quantitative, universally applicable assay for the assessment of iron limitation of marine phytoplankton in the environment.

The characterization of the ferredoxin and flavodoxin system presented here followed the reasoning of Falkowski et al. (1992), who stated that "useful diagnostic tools must identify those processes that

1) impose a truly physiological limitation,

2) are uniquely affected by a specific limiting factor,

3) are broadly applicable across phylogenetic lines, and

4) can be used in the field."

Beginning with the knowledge that iron stress is a truly physiological limitation, the remaining three characters were systematically addressed.

The phylogenetic generality of flavodoxin induction was assessed by screening seventeen different marine phytoplankton isolates, representing four classes, for their ability to produce flavodoxin when iron limited. Results of this phylogenetic survey are given in Chapter 1. Chapter 2 describes the regulation of ferredoxin and flavodoxin expression by nitrogen, phosphorus, silicate, zinc and light as well as iron in a model species, Thalassiosira weissflogii. Potential interacting effects of iron and nitrogen growth substrate on ferredoxin and flavodoxin expression are also investigated in Chapter 2.

Verification of the utility of ferredoxin and flavodoxin as diagnostic indicators in the field is provided in Chapter 3, which details their use during the IronEx II in situ iron fertilization experiment. Results of ferredoxin-flavodoxin analyses during IronEx II were quite different from those obtained by others using biophysical techniques. These differences were examined in detail in the laboratory, and this comparison of biochemical and biophysical methods for the detection of iron limitation is presented in Chapter 4. Chapter 5 concludes with a summary of this work.

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Chapter 1

A Phylogenetic Survey of Flavodoxin Induction in Marine Phytoplankton

Abstract

Under conditions of iron stress, many organisms are able to replace the common iron-sulfur redox protein ferredoxin with flavodoxin, a functionally equivalent, non-iron-containing protein. These proteins have been proposed to be indicators of iron nutritional status in marine phytoplankton, but relatively little is known of their existence in algae other than diatoms. In this study, seventeen marine phytoplankton isolates were tested using high pressure liquid chromatography (HPLC) for their ability to induce flavodoxin when their growth was limited by iron. The most common response (12 of 17 isolates) was an absence of flavodoxin under iron-sufficient conditions, with induction of flavodoxin and suppression of ferredoxin expression when the cells were iron-stressed. Flavodoxin expression was never observed in the remaining five organisms, a phenomenon that has been observed mainly in neritic organisms. This lack of flavodoxin induction correlates with high intrinsic Fe requirements for growth in these same organisms. The prevalence of the flavodoxin response supports its use as an indicator of iron limitation in open-ocean regions; non-inducing species are unlikely to occur in such waters due to their high iron requirements.

All 17 isolates were also screened using antibodies raised against ferredoxin and flavodoxin purified from Thalassiosira weissflogii . The anti-ferredoxin antibody detected ferredoxin only from Thalassiosira weissflogii. The anti-flavodoxin antibody was more cross-reactive, detecting most diatom flavodoxins but not those from other algal classes. Comparison of the antibody results to those obtained by HPLC illustrates the strengths and limitations of each technique. Antibodies have low detection limits and can

provide species- or class-specific results. However, they have varying affinities for their target protein in different organisms, making quantitation difficult for natural plankton communities. HPLC with diode array detection can quantify ferredoxin and flavodoxin in individual cultures or entire communities, though its higher detection limits make it unsuitable for class- or species-specific analyses. Both methods should nonetheless prove useful for the assessment of iron limitation in marine phytoplankton.

Introduction

Achieving an understanding of the factor(s) that limit primary production in the oceans has been a goal of biological oceanographers for decades. In most regions it is thought to be controlled by the availability of macronutrients such as nitrate or phosphate. Persuasive evidence for the concept of micronutrient, particularly trace metal, limitation has come about only recently, with the advent of trace metal-clean techniques (Martin & Fitzwater 1988, Martin et al. 1990, Coale 1991, Martin et al. 1993). While elimination of metal contamination has greatly improved the accuracy of nutrient limitation studies (Fitzwater et al. 1982), the basic assay methods have changed little since their development decades ago.

The principal technique employed in studies of micronutrient limitation is the bottle bioassay (Ryther & Guillard 1959). Natural water samples are placed into bottles, now using clean techniques, where they are enriched with the nutrient in question. Enhanced growth in experimental bottles as compared to unamended control bottles provides evidence of nutrient limitation. For example, nutrient addition bioassays of this type were used by Martin and Fitzwater (1988) to make a case for iron limitation of phytoplankton growth in the Subarctic Pacific. Their Fe-enriched samples showed an increase in chl $\underline{a}$ and an accompanying decrease in nitrate after several days of incubation relative to the controls. This suggested enhancement of phytoplankton growth due to the added iron, leading to elevated nitrate uptake and greater biomass. Results such as these formed the basis of the "iron hypothesis", which links changes in iron availability and oceanic primary production with variation in atmospheric CO_2 and glacial-

interglacial transitions (Martin 1990). This proposal touched off a vigorous debate about the relationship between iron and oceanic primary production.

Resolution of this debate is hindered in part by a lack of direct methods for assessing iron limitation. Bottle bioassays entail manipulation of the natural community, are difficult to perform in a clean fashion and require days of incubation. In addition, interpretation of bioassay results is confounded by so-called "bottle effects" - potential artifacts introduced by enclosing natural populations in a small volume (e.g. Venrick et al. 1977). Large zooplankton are often excluded, thus reducing grazing pressure. The bottles also eliminate sinking effects as larger cells that might normally fall from surface waters will be able to grow, particularly if larger grazers are not present. The methodological difficulties inherent in the traditional bioassay method highlight the need for better, more efficient ways to study nutrient limitation.

In a recent attempt to address the specific question of iron limitation, scientists performed an open-ocean nutrient enrichment (Coale et al. 1996). During the IronEx II experiment, iron was added to a large patch of the Equatorial Pacific Ocean and its response as well as that of a control area was tracked for over two weeks. The results provided conclusive evidence for limitation of primary production in the Equatorial Pacific by iron. The IronEx II experiment was an unconventional approach to the study of iron limitation and a truly revolutionary accomplishment in oceanography. Unfortunately, large open-ocean manipulations such as this are time-consuming, prohibitively expensive and far beyond the scope of the average field study.

In the continued search for innovative ways to assess phytoplankton nutrient limitation, some oceanographers have turned to the tools of molecular biology (Ward 1990, Falkowski & LaRoche 1991). Most attention has focused on molecular "diagnostics" of various physiological states (Laudenbach & Straus 1988, Scanlan et al. 1989, LaRoche et al. 1993, Doucette et al. 1996). This approach is based upon the concept that an organism's response to environmental conditions often involves the synthesis or expression of molecules that are unique to that condition. By detecting alterations in the abundance of such proteins or nucleic acids, the cell may be used as a reporter of the environmental conditions that it "perceives". Such molecular indicators could provide a rapid assay of the cell's physiological state without the need for excessive manipulation. The challenges thus far have been the identification of appropriate diagnostic molecules and the development of methods for their measurement.

The primary candidate for a diagnostic indicator of iron limitation is the redox protein flavodoxin. Flavodoxin is induced in response to iron stress as a functional replacement for the iron-sulfur protein ferredoxin, which cells utilize when iron is not limiting growth. This adaptation has been studied for decades, primarily in freshwater algae and bacteria (e.g. Vetter & Knappe 1971, Zumft & Spiller 1971, Fillat et al. 1988). Some variability in response has been observed, as not all organisms employ this strategy (Pardo et al. 1990). Before flavodoxin can be used as an indicator of iron limitation in the ocean, however, it must be better characterized in marine phytoplankton. Criteria for such a characterization were detailed by Falkowski et al (1992): "To be useful, diagnostic tools must identify those

processes that 1) impose a truly physiological limitation, 2) are uniquely affected by a specific limiting factor, 3) are broadly applicable across phylogenetic lines, and 4) can be used in the field."

This study addresses the third of these criteria - the generality of flavodoxin induction as a response to iron limitation. A few laboratory studies to date have documented flavodoxin induction in a small number of unicellular marine organisms (LaRoche et al. 1995, Doucette et al. 1996), all of which were diatoms. Here a variety of marine phytoplankton cultures representing different taxonomic groups were examined to determine the generality of the flavodoxin substitution strategy. These same organisms were also used to characterize the cross-reactivity of antibodies developed as a potential detection method for flavodoxin and ferredoxin. Used as immunological probes, antibodies have the potential to reveal iron limitation at the level of individual cells or species.

Materials and Methods

Cultures Organisms used and their clone designations are listed in Table I. The organisms listed as "EqPac pennate diatom sp." were isolated by E. Mann from the iron-fertilization-induced pennate diatom bloom during the IronEx II experiment. They were rendered clonal via single-cell isolation by D. Erdner. While sterile techniques were employed and bacteria were not apparent in cultures viewed under the light microscope, the cultures were likely not axenic.

Growth of phytoplankton All cultures, except Synechococcus DC2, were grown in a modification of ESNW medium as described previously

(Doucette et al. 1996). Cultures of <u>Synechococcus</u> DC2 were not grown by the authors, but were a gift from the University of Warwick and supplied as freeze-dried cell pellets. Cultures were grown in 2.8 l Fernbach flasks containing 2 l of medium. Organisms were transferred twice into the appropriate medium (iron-replete or iron-limited) in 25 ml tubes before inoculation into experimental flasks.

Cultures were grown at 15°C, 20°C or 25°C under a 14:10 light:dark cycle with illumination from "cool white" fluorescent lights at approximately 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ as measured by a photometer (Biospherical Instruments model QSP-100). Growth was monitored by microscope cell counts or Coulter counter (Coulter Instruments, Hialeah, FL, USA), and cultures were harvested by centrifugation when they reached late exponential phase. Cell pellets were frozen at -80°C until analysis.

Initially, trace metal-clean culturing techniques were employed for growth of phytoplankton. All sterilization was performed by microwaving, except for certain nutrient stocks (see below) (Keller et al. 1988). Cultures were grown in sterile, acid-washed polycarbonate Fernbach flasks. Nutrient stocks were kept in acid-cleaned teflon (PTFE) bottles. Chelex-treated Sargasso seawater, sterilized by microwaving, was used as a base for the ESNW medium (Price et al. 1989). All nutrient stocks were prepared using milli-Q distilled deionized water. Nitrate, phosphate and silicate stocks were Chelex-treated and sterilized by microwaving. Iron, EDTA, trace metal, vitamin and selenium stocks were filter sterilized (0.2 μm).

After it became evident that strict trace metal-clean methods were unnecessary for our purposes, "standard" culturing techniques were

employed. Acid-washed polycarbonate or glass Fernbach flasks and nutrient stock bottles were sterilized by autoclaving. Media were prepared using 0.45 µm filtered and autoclaved Vineyard Sound seawater as a base (31‰). Macronutrient and micronutrient stocks were sterilized by autoclaving and sterile-filtering, respectively.

Iron-replete cultures grown in trace-metal clean medium contained 1×10^{-6} M Fe/10×10^{-6} M EDTA. In Vineyard Sound water medium, Fe addition to replete cultures was 5×10^{-6} M Fe/50×10^{-6} M EDTA for all organisms except for T. weissflogii, which was grown at 10×10^{-6} M Fe/ 100×10^{-6} M EDTA. The replete Fe addition for T. weissflogii was subsequently increased to 60×10^{-6} M Fe/ 100×10^{-6} M EDTA. Iron-limited cultures in both Sargasso and Vineyard Sound water contained ESNW concentrations of all nutrients except Fe, which was decreased according to the Fe requirements of individual clones. EDTA was added to iron-limited cultures at 10 times the added Fe concentration or 1×10^{-6} M, whichever was higher.

Confirmation of iron limitation Iron limitation at late exponential phase in limited cultures was determined by several complementary methods: decrease in growth rate compared to replete culture grown in tandem, presence of chlorosis (assessed visually by comparison t replete cultures), and Fe-addition bioassay (for trace metal-clean cultures). For bioassays, 30 ml aliquots of iron-limited culture were transferred to acid-cleaned polycarbonate tubes after which the remaining culture volume was harvested. One tube was kept as a control, a second was enriched with Fe to replete levels and the third was enriched with all nutrients except Fe. The

tubes were incubated as above and growth was tracked by in vivo fluorescence.

HPLC analysis Protein extraction and high performance liquid chromatography (HPLC) were performed as previously described (Doucette et al. 1996).

Antibody production Cell extracts of T. weissflogii were precipitated in 90% acetone, dried and resuspended in extraction buffer (20 mM phosphate, 100 mM EDTA, 100 mM NaCl, 0.013 M ß-mercaptoethanol, 1 mM PMSF pH 7.0). Ferredoxin and flavodoxin were purified from this aqueous extract by semi-preparative anion-exchange HPLC. Polyclonal antibodies were prepared by East Acres Biologicals (Southbridge, MA, USA). Hens were immunized with either ferredoxin or flavodoxin after collection of pre-immune serum. Primary immunization and boosts occurred at three week intervals and consisted of 100 µg of native protein with incomplete Freund's adjuvant. Antibody solutions for Western blotting were derived both from serum and from the yolks of eggs laid by immunized hens.

Western blotting Cells were disrupted by sonication in 20 mM phosphate buffer pH 7.0 with 1% SDS. Protein concentrations were measured using the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA), and extracts were diluted to 1 µg total protein·μl^{-1} in sample buffer (4% SDS, 12% w/v glycerol, 50 mM Tris, 2% v/v β-mercaptoethanol, 0.01% Serva blue pH 6.8). Proteins were separated on 10%T, 3%C tricine gels according to Schägger and von Jagow (1987) and transferred to nitrocellulose. The blots were challenged with chicken primary antiserum at 1:500 dilution followed by anti-chicken-horseradish peroxidase conjugated secondary antibody at 1:2500

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dilution (Jackson Immunoresearch, West Grove, PA, USA).

Chemiluminescent ECL reagents (Amersham, Cleveland, OH, USA) were used as the detection system.

Results

Modes of expression

In total, seventeen isolates representing four phytoplankton classes were examined for their ability to induce flavodoxin when limited by iron. Many of the organisms were grown and tested several times to ensure the accuracy of determinations. The most common response (12 of 17 isolates) was an absence of flavodoxin under iron-sufficient conditions, with induction of flavodoxin and suppression of ferredoxin expression occurring when the cells were iron-stressed. The remaining five organisms were never observed to express flavodoxin under either iron-replete or -limited conditions. The results of the HPLC analysis are summarized in Table I.

Absence of flavodoxin expression

Amongst the 17 isolates used in this study, a minority (5 of 17) were never observed to express flavodoxin. With this group of organisms, a logical concern was that the cultures examined were not iron-limited. However, in iron-limited medium all exhibited both decreased growth rates compared to replete cultures and visible chlorosis, which are consistent with iron stress (Synechococcus was grown by others for this analysis, so similar growth rate information is not available). Further evidence of iron limitation was obtained from nutrient addition bioassays (Figure 3). Syracosphaera elongata

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(Fig. 3a), Pleurochrysis carterae (Fig. 3b) and Minutocellus polymorphus (Fig. 3c) exhibited positive growth responses when iron was added to aliquots of the iron-limited culture; addition of other nutrients elicited no change. These cultures were indeed iron-limited. Cultures of Alexandrium fundyense , however, showed no clear pattern of response in iron-addition bioassays (Fig. 3d) when grown repeatedly under a variety of iron additions from 0 nM to 1 µM. Nevertheless a marked decrease in specific growth rate ($\mu=0.325 \cdot d^{-1}$ replete vs. $0.073 \cdot d^{-1}$ limited) was observed for A. fundyense cultured in low-iron medium. As all other culturing conditions were identical, the lack of iron must be responsible for the reduced growth rate for this dinoflagellate.

Antibody cross-reactivity

These same 17 isolates analyzed by HPLC were used to determine the specificity of antibodies developed against the ferredoxin (Figure 4) and flavodoxin (Figure 5) proteins from Thalassiosira weissflogii. The Western blot in Figure 4 clearly shows that the ferredoxin antibody exhibits a high degree of specificity, showing reaction only against T. weissflogii. No cross-reactions were seen, even with members of the same genus (Fig. 4, lane f). The flavodoxin antibody exhibited broader cross-reactivity, detecting flavodoxin from 4 of the 6 diatoms which were shown by HPLC to produce flavodoxin. A seventh diatom, M. polymorphus , did not induce flavodoxin nor react with the anti-flavodoxin antibody.

In addition to cross-reaction with diatom flavodoxins, the anti-flavodoxin antibody seems to exhibit a lesser reaction against flavodoxins

from other algal classes: <u>Alexandrium fundyense</u> (Fig. 5h), <u>Symbiodinium microadriaticum</u> (Fig. 5i), <u>Amphidinium carterae</u> (Fig. 5j) and <u>Chrysochromulina ericina</u> (Fig. 5m). In organisms other than diatoms, there seems to be increased non-specific reaction, as evidenced by the dark background in lanes i-o, with lane k being an extreme example. The blotted samples contained total protein, not purified flavodoxin. Thus it is possible that the flavodoxin antibody non-specifically recognizes a protein in a similar size range. This can only be resolved by screening purified flavodoxin proteins from the various organisms. Because of this uncertainty, it cannot be stated definitively that the anti-flavodoxin antibody reacts with the organisms in lanes h, i, j and m in Figure 5.

Discussion

This study constitutes the first comprehensive screening of flavodoxin expression in a diverse group of marine phytoplankton, in an attempt to determine the generality of flavodoxin induction as a biomarker of iron limitation. Amongst the 4 classes of organisms surveyed, encompassing 17 different isolates, most exhibited flavodoxin induction when iron-limited. Flavodoxin expression was never observed in several species (5 of 17), a phenomenon that is observed mainly in neritic organisms. This implies that non-induction should not present problems for ferredoxin/flavodoxin measurements made in low-iron open-ocean environments, but may complicate studies in coastal areas.

This phylogenetic survey also included characterization of the reactivity of two antibodies developed as analytical tools for ferredoxin and

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flavodoxin detection. The antibodies exhibit different levels of specificity which determine, and also limit, their usefulness in natural populations. The immunological method and HPLC technique used in this paper differ in several respects, including phylogenetic specificity as well as the qualitative and quantitative nature of the results. These differences are often complementary and may be considered as advantages or disadvantages to their use in the field, depending upon the ecological question being addressed. It is therefore necessary to carefully consider the requirements of a particular experiment in order to choose the analytical method most appropriate to the task. These and other concepts relating to the use of ferredoxin and flavodoxin as indicator proteins are discussed below.

Phylogenetic survey

From the data presented in Table I, it is evident that flavodoxin induction is a common, but not universal, response to iron limitation in marine phytoplankton. This heterogeneity in response is similar to that documented in freshwater algae, where ferredoxin and flavodoxin have been most extensively studied. Various strains of the freshwater cyanobacterium Anabaena show three different modes of induction: Anabaena ATCC29413 induces flavodoxin when iron-limited (Fillat et al. 1988), Anabaena ATCC29211 does not produce flavodoxin (Pardo et al. 1990), while Anabaena ATCC29151 produces flavodoxin constitutively in its heterocysts (Sandmann et al. 1990). The cyanobacteria Synechocystis (Bottin & Lagoutte 1992) and Anacystis nidulans (Laudenbach et al. 1988), and the green alga Chlorella fusca are all known to induce flavodoxin when iron-limited. Given our

results and those in the literature, flavodoxin induction in response to iron limitation seems to be the most typical and frequently observed response.

Absence of flavodoxin expression

For organisms that did not express flavodoxin it is important to confirm that the cultures were truly iron-limited. Nutrient addition bioassays were used as one test of limitation. While growth of primary cultures was monitored by cell counts, the bioassays were monitored by measurement of _in vivo_ fluorescence. Positive bioassays are characterized by an increase in fluorescence in Fe-enriched samples. However, _in vivo_ chlorophyll fluorescence is known to be affected by iron stress, and its use may seem to confound interpretation of the bioassay results. The increase in fluorescence following iron addition can be accounted for in a number of ways. First, higher fluorescence may be due to increases in chlorophyll per cell rather than an increase in cell number. Second, it may be due to increased biomass due to growth of cells in the culture. Finally, increases in fluorescence are observed when cells become iron-limited as iron stress causes damage to the photochemical apparatus and impairs electron transfer capability (Terry 1983). In a sample that has been enriched with iron it is unlikely that the cells would experience further iron stress. Therefore, the enhanced fluorescence must be due to either increases in cell number, cell volume or fluorescence per cell, all of which imply that the original culture was indeed limited by iron.

While a positive bioassay provides additional evidence for iron limitation, it is not a required proof of iron limitation when using controlled

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laboratory cultures. One non-inducing organism, <u>Alexandrium fundyense</u>, consistently gave inconclusive results in the iron addition bioassays (Fig. 3d), yet exhibited lower growth rates when iron limited growth. Iron-enriched samples of low-iron <u>A</u>. <u>fundyense</u> cultures showed a bioassay response similar to that of both control samples and samples enriched with all nutrients except iron. This was true for cultures grown with iron levels ranging from 0 nM to 1 μM in which no flavodoxin signal was ever observed using HPLC. All deplete cultures were grown in tandem with a replete culture, such that the two flasks were identical in all respects except for iron concentration. <u>A</u>. <u>fundyense</u> showed a 75% decrease in growth rate in low-iron cultures, consistent with limitation of its growth rate by iron.

Flavodoxin expression in Thalassiosira weissflogii

Results obtained from the centric diatom <u>Thalassiosira weissflogii</u> during these analyses are somewhat different than those reported previously (Doucette et al. 1996). Like most others, this species induces flavodoxin in response to iron limitation, with an accompanying suppression of ferredoxin expression. The response of <u>T</u>. <u>weissflogii</u> under iron-replete conditions, however, is not as straightforward. At 1 μM Fe, <u>T</u>. <u>weissflogii</u> produces similar amounts of both ferredoxin and flavodoxin (Figure 2a). In comparison, the other organisms utilized in this study contain only ferredoxin when grown at this iron level. When Fe additions are subsequently increased to 10 μM, the level used in Doucette et al. (1996), <u>T</u>. <u>weissflogii</u> contains primarily ferredoxin (Figure 2b). This is slightly more iron than the highly defined medium Aquil (8.32 μM)(Price et al. 1988/1989)

but identical to the Fe level in the common f/2 medium (Guillard & Ryther 1962). However, a low level of flavodoxin expression persists, which we now know to be the result of exceedingly high Fe requirements in T. weissflogii. This low-level expression is suppressed by increasing the added Fe in the culture to 60 μM, six times that of f/2 medium (Figure 2c). Thus, T. weissflogii does not express flavodoxin when iron-replete (i.e., it is not constitutive), but the Fe addition necessary for "replete" culture is far above that required for the other phytoplankton examined here.

Flavodoxin as a diagnostic indicator of iron limitation

The existence of both flavodoxin-inducing and non-inducing organisms suggests caution in the application of flavodoxin alone as a biomarker of iron limitation in marine phytoplankton. Lack of flavodoxin expression under iron-limiting conditions would yield a "false-negative" in an assay for iron limitation if the presence of flavodoxin is used as the sole criterion. Organisms that do not induce flavodoxin will not appear to be iron-limited even when they are. Non-induction does not seem to be a typical mode of expression, but it could potentially interfere with interpretation of field measurements. Some of this uncertainty could be reduced if some correlate of expression mode could be identified.

Accordingly, the data in Table 1 were examined for potential patterns in the modes of expression. Results indicate that a lack of flavodoxin is not confined to a particular taxonomic group; examples are seen amongst the dinoflagellates, prymnesiophytes and diatoms. It does, however, seem to occur primarily in coastally-derived clones within a class. Of the five non-

inducing organisms in this study, three were isolated from neritic habitats while a fourth, Syracosphaera elongata, is of unknown origin. Phytoplankton from neritic vs. oceanic habitats are known to exhibit different responses to trace metals. Neritic species generally suffer iron limitation at Fe levels far above those at which oceanic species show little or no impairment (Brand et al. 1983, Brand 1991). However, this tendency for non-inducing organisms to be coastally derived does not mean that coastal species in general are non-inducers. In fact, the majority of the neritic clones examined in this study expressed flavodoxin.

The results presented here agree well with published data on phytoplankton iron requirements. Brand (1991) measured high minimum iron requirements for the coastal coccolithophorids Syracosphaera elongata and Pleurochrysis carterae, and the cyanobacterium Synechococcus DC2, all of which do not produce flavodoxin. Doucette et al. (1989) calculated extremely high minimum iron requirements for the red tide dinoflagellate Alexandrium fundyense, another non-inducer. Organisms that are unable to express flavodoxin seem to consistently exhibit elevated cellular requirements for iron.

Sunda et al. (1991) argue that the increased success of oceanic organisms at low metal concentrations as compared to neritic isolates is not due to more efficient uptake systems, but to decreased cellular Fe requirements for growth. The substitution of flavodoxin for ferredoxin permits a reduction in cellular Fe quotas, since the former contains no iron while the latter requires two iron atoms per molecule. Thus, organisms that cannot express flavodoxin should be unable to persist in chronically low-iron

oceanic environments, and therefore would be constrained in their distributions to more iron-rich coastal areas. In the analysis of samples from open ocean, low iron environments, non-expression of flavodoxin should therefore not be a large complication. Caution should be exercised, however, with samples derived from more coastal environments. Iron availability in nearshore areas, as measured chemically, seems to vary widely on small temporal and spatial scales (Wells 1988/1989). When iron availability is very low, non-inducing organisms are unlikely to constitute a significant portion of biomass, due to their poor growth at low iron levels. In situations of high iron stress, interference from non-inducing organisms may be negligible. When iron stress is moderate, the situation becomes more complex. Communities may contain a mix of organisms in varying proportions: non-inducers which experience different levels of growth impairment and inducing organisms that may or may not be expressing flavodoxin depending upon their particular cellular iron requirements. In a scenario such as this, results of flavodoxin assays may be uninterpretable. Further research may elucidate some pattern or relationship that would facilitate ferredoxin/flavodoxin studies in coastal areas.

Antibodies

The antibodies characterized here were developed as tools for studying ferredoxin and flavodoxin in natural samples. Before they could be effectively utilized, their phylogenetic range of reactivity needed to be determined. The two antibodies show quite distinct levels of specificity. The anti-ferredoxin antibody is monospecific - it reacts only against Thalassiosira

weissflogii, the species from which the original antigen was purified. It will be useful for studies of ferredoxin regulation in T. weissflogii, but its use in natural waters is otherwise limited. The flavodoxin antibody, on the other hand, is less specific, reacting with 4 of the 6 diatoms that were tested. It too will be useful for laboratory studies but also shows promise for use in natural samples which would contain a variety of species.

Comparison of flavodoxin detection methods

An antibody-based method for measurement of flavodoxin and ferredoxin is an attractive concept because of the low detection limits of immunoassays. In contrast, the HPLC technique has detection limits that are approximately 3 orders of magnitude higher (picomolar as compared to femtomolar sensitivity). The implications for sample collection in the field are obvious. The two methods also differ in other respects, such as phylogenetic applicability and qualitative vs. quantitative nature of the results.

The HPLC method with diode array detection (Doucette et al. 1996) carries no bias with respect to phylogeny, whereas antibodies are generally reactive towards particular subset(s) of plankton. Ferredoxin and flavodoxin, regardless of source, will be measured chromatographically while antibodies can only detect the response of certain organisms. For example, the anti-flavodoxin antibody described here could be used to assess the iron status of marine diatoms. This could be done either on a single cell basis, or on the diatom sub-population, if used in conjunction with Western blotting (e.g. LaRoche et al. 1995). In contrast, the HPLC technique is most useful for

examining total community response (see Chapter 3). In order to study sub-populations, it would be necessary to first separate the cells by a method such as size fractionation or flow cytometric sorting. Each fraction can then be analyzed separately, to determine the iron status of the various size classes or sub-groups. While it is theoretically possible to apply the HPLC method in this manner, available techniques for sample collection and cell sorting make it impractical.

While antibodies are useful for dissecting community response, it is extremely difficult to employ them in a quantitative manner. Antisera generally exhibit varying binding affinity for target proteins from different species (e.g. Orellana & Perry 1992). In a natural sample containing several species of diatoms, the strength of the antibody reaction with our diatom-specific antibody does therefore not depend solely upon the amount of diatom flavodoxin present. It varies with binding affinity for flavodoxins from different diatoms, the proportion of those species within the diatom population, and the amount of diatom biomass relative to total biomass. For these reasons, it is difficult to quantify proteins from a mixed sample based upon antibody reaction strength. The HPLC method, in contrast, relies upon spectrophotometric detection of flavodoxin and ferredoxin, which allows calculation of protein quantity regardless of its source.

At a very basic level, the antibody and HPLC techniques measure different substances. Chromatographic detection is based upon light absorption at specific wavelengths. For ferredoxin and flavodoxin, absorbance depends upon the presence of appropriate cofactors such that only functional (holo-) protein is detected. Antibodies often to react with both apo- and

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holoproteins, so that even inactive forms will contribute to the signal. For certain applications, it may be necessary to know the abundance of both forms but for ecological purposes the amount of functional protein is most relevant. The differences in the HPLC and antibody methods may be considered as either advantages or disadvantages depending upon the problem to be answered. The two techniques exhibit a complementary set of strengths and limitations.

Field Applications

Several attempts have been made to utilize flavodoxin and/or ferredoxin as indicators of iron stress in natural populations. Over a decade ago, Entsch et al. (1983) argued for iron limitation of cyanobacteria (but not the co-occurring symbiotic dinoflagellate Symbiodinium microadriaticum) from the Great Barrier Reef based on presence or absence of flavodoxin purified using chromatography. The clone of S. microadriaticum used in our study was isolated as a symbiont from Tridacna clams, and as such is very similar to the organism examined by Entsch et al. S. microadriaticum induces flavodoxin when iron limited, supporting their conclusion that the clam symbionts were not experiencing iron stress.

More recently, the presence of flavodoxin, detected by HPLC (Erdner & Anderson 1996) and antibodies (LaRoche et al. 1995, LaRoche et al. 1996), has been used as a qualitative indicator of iron stress in marine diatoms. The only quantitative assessment of changes in nutritional status in natural communities to date was by Jones (Jones 1988), who documented variation in the flavodoxin:ferredoxin ratio over time in natural samples of

Trichodesmium. Those differences could be correlated with changes in atmospheric iron inputs. All of these studies, however, must be considered preliminary, as little is known about the regulation of ferredoxin and flavodoxin in marine algae save for its induction in response to iron stress. Further characterization is needed before these biomarkers may be effectively applied in the field.

This phylogenetic survey of the flavodoxin response is the first step in the characterization of the ferredoxin/flavodoxin system in marine phytoplankton. While this analysis included multiple representatives of the diatoms, dinoflagellates and prymnesiophytes, only one marine cyanobacterium, Synechococcus clone DC2, was examined. Marine Synechococcus are a physiologically diverse group (Waterbury et al. 1986) and might be expected to exhibit intragroup variability similar to that observed for freshwater cyanobacteria and marine eukaryotes. As they are a prominent component of the natural phytoplankton community in low-iron areas, Synechococcus, and also Prochlorococcus, merit additional study with respect to their flavodoxin response. In general, the phylogenetic survey of flavodoxin expression should be extended to include as many organisms from different classes and environments as possible.

To further evaluate the use of flavodoxin as a diagnostic indicator, it is necessary to determine if it is induced by other types of limitation. It does not seem to be affected by macronutrient (N or P) stress (LaRoche et al. 1993) but other possible limiting factors need to be considered. While it is not tractable to examine all potential limiting factors, it is essential to test those which organisms are most likely to experience in the environment. These should

include trace metals which are present in low levels in natural waters, such as zinc and manganese, as well as metals that in excess can cause toxicity, such as copper. It is also advisable to examine the effects of irradiance levels, since both flavodoxin and ferredoxin are involved in photosynthetic electron transport. There are numerous reports of light regulation of ferredoxin expression in higher plants (Dobres et al. 1987, Vorst et al. 1993, Bovy et al. 1995, Bringloe et al. 1995), so it is possible that irradiance may play a similar role in algae. Only if we establish that ferredoxin and flavodoxin are uniquely regulated by iron and recognize the phylogenetic and geographic variability in induction described above can we begin to intelligently apply these biomarkers to ecological questions in natural systems.

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TABLE I: List of organisms examined in this study and their taxonomic class, clone designation, isolation locale (classified as N-neritic or O-oceanic) and presence (+) or absence (-) of flavodoxin induction in response to iron limitation.

organism	class	clone	location	habitat	Flv[a]
Alexandrium fundyense	dino.	GTCA29	Gulf of Maine 43°00'N, 70°19'W	N	-
Amphidinium carterae	dino.	CCMP1314	Great Pond Falmouth, MA USA	N	+
Symbiodinium microadriaticum	dino.	CCMP829	Great Barrier Reef, Australia	N	+
Thalassiosira weissflogii	diatom	CCMP1336	Gardiners I. Long Island, NY USA	N	+
Thalassiosira oceanica	diatom	CCMP1005	Sargasso Sea 33°11'N 65°15'W	O	+
Minutocellus polymorphus	diatom	CCMP499	Raritan Bay, Sandy Hook NJ USA	N	-
Chaetoceros gracilis	diatom	unknown	unknown	?	+
EqPac pennate diatom	diatom	7-47B	E. Equatorial Pacific Ocean	O	+
EqPac pennate diatom	diatom	10-40A	E. Equatorial Pacific Ocean	O	+
EqPac pennate diatom	diatom	A3-30	E. Equatorial Pacific Ocean	O	+
Emiliania huxleyi	prym.	CCMP370	Oslofjord, North Sea	N	+
Emiliania huxleyi	prym.	CCMP373	Sargasso Sea 32°10'N 64°30'W	O	+
Syracosphaera elongata	prym.	CCMP874	unknown	?	-
Pleurochrysis carterae	prym.	CCMP645	Woods Hole, MA USA	N	-
Phaeocystis sp.	prym.	CCMP1528	Gardiner Bay, Galapagos I.	N	+
Chrysochromulina ericina	prym.	CCMP281	N. Pacific 49°36'N 140°37'W	O	+
Synechococcus DC2	cyano.	CCMP1334	Sargasso Sea 33°44.9'N 67°29.8'W	O	-

a   Determination was by HPLC (Doucette et al 1996).

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TABLE II: List of organisms used in this study to test reactivity of antibodies raised against flavodoxin and ferredoxin from <u>Thalassiosira weissflogii</u>. A "+" indicates positive reaction with the antibody.

| <u>organism</u>	<u>anti-Fd</u>	<u>anti-Flv</u>
Alexandrium fundyense	-	-
Amphidinium carterae	-	-
Symbiodinium microadriaticum	-	-
Thalassiosira weissflogii	+	+
Thalassiosira oceanica	-	+
Minutocellus polymorphus	-	-
Chaetoceros gracilis	-	+
EqPac pennate diatom clone 7-47B	-	-
EqPac pennate diatom clone 10-40A	-	-
EqPac pennate diatom clone A3-30	-	+
Emiliania huxleyi	-	-
Emiliania huxleyi	-	-
Syracosphaera elongata	-	-
Pleurochrysis carterae	-	-
Phaeocystis sp.	-	-
Chrysochromulina ericina	-	-
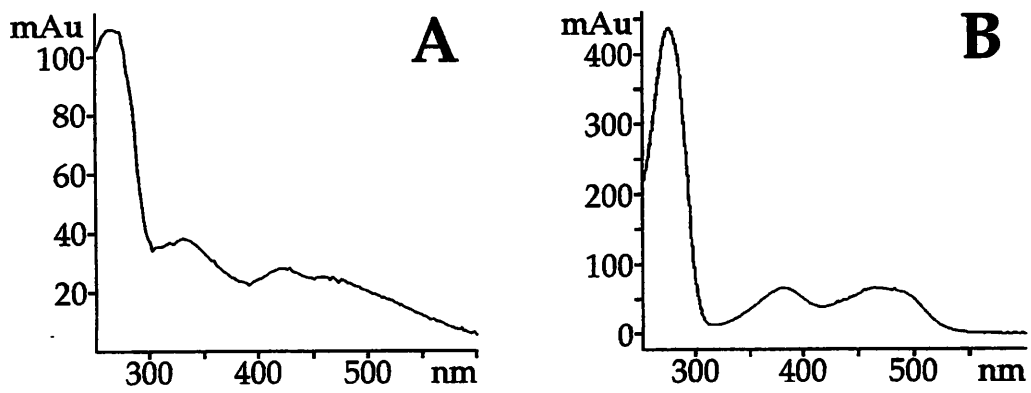
FIGURE 1 - UV-visible absorption spectra of (A) ferredoxin and (B) flavodoxin from Thalassiosira weissflogii, for comparison with the spectra in Figure 2.
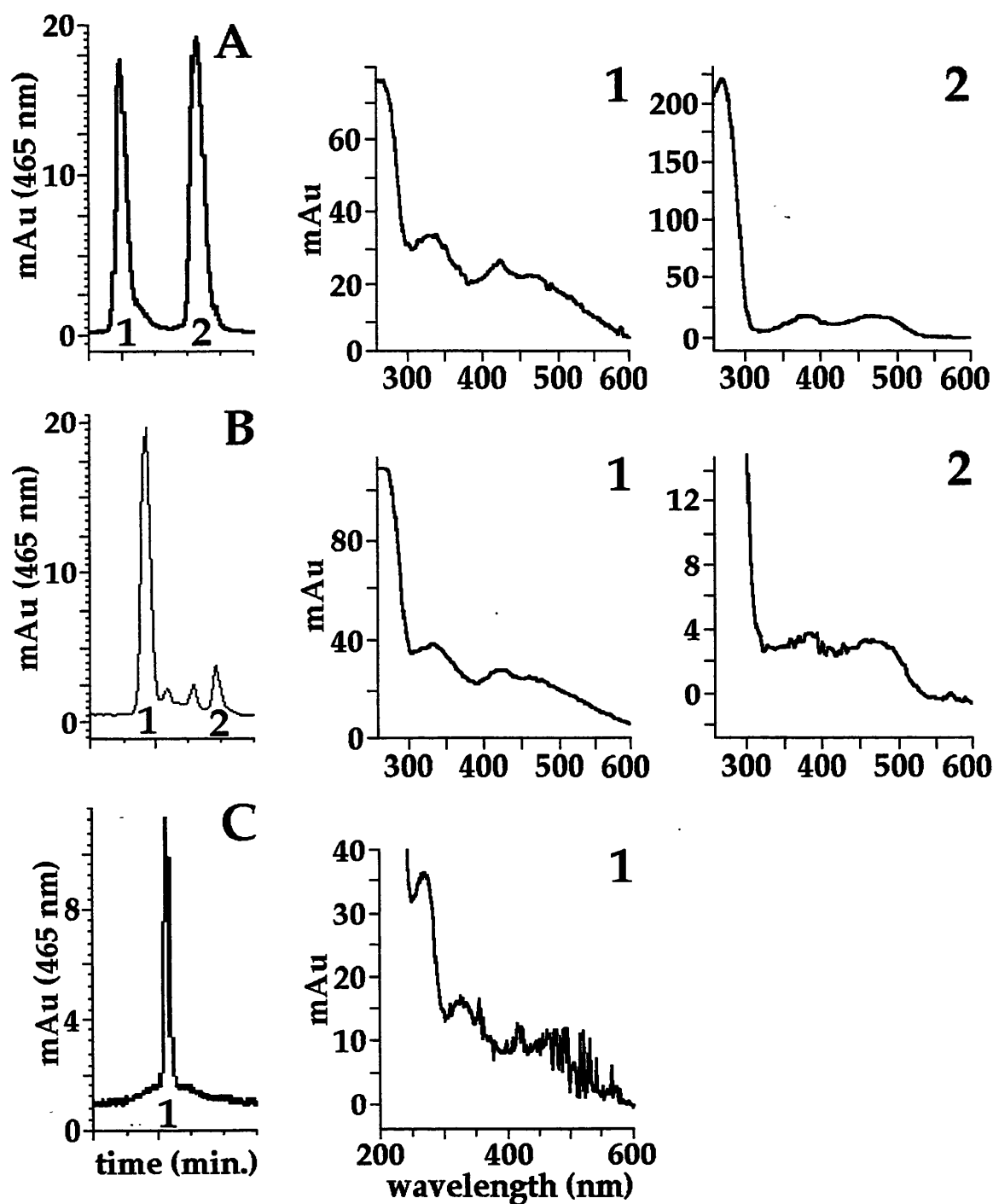
FIGURE 2 - HPLC chromatograms of Thalassiosira weissflogii grown with (A) 1μM, (B) 10μM or (C) 60μM added Fe. Absorbance (vertical axis) was measured at 465 nm. Length of the horizontal axis is 10 minutes in all cases. The UV-visible absorption spectrum of the corresponding chromatographic peak(s) are shown beside the chromatograms and designated by (1) or (2).
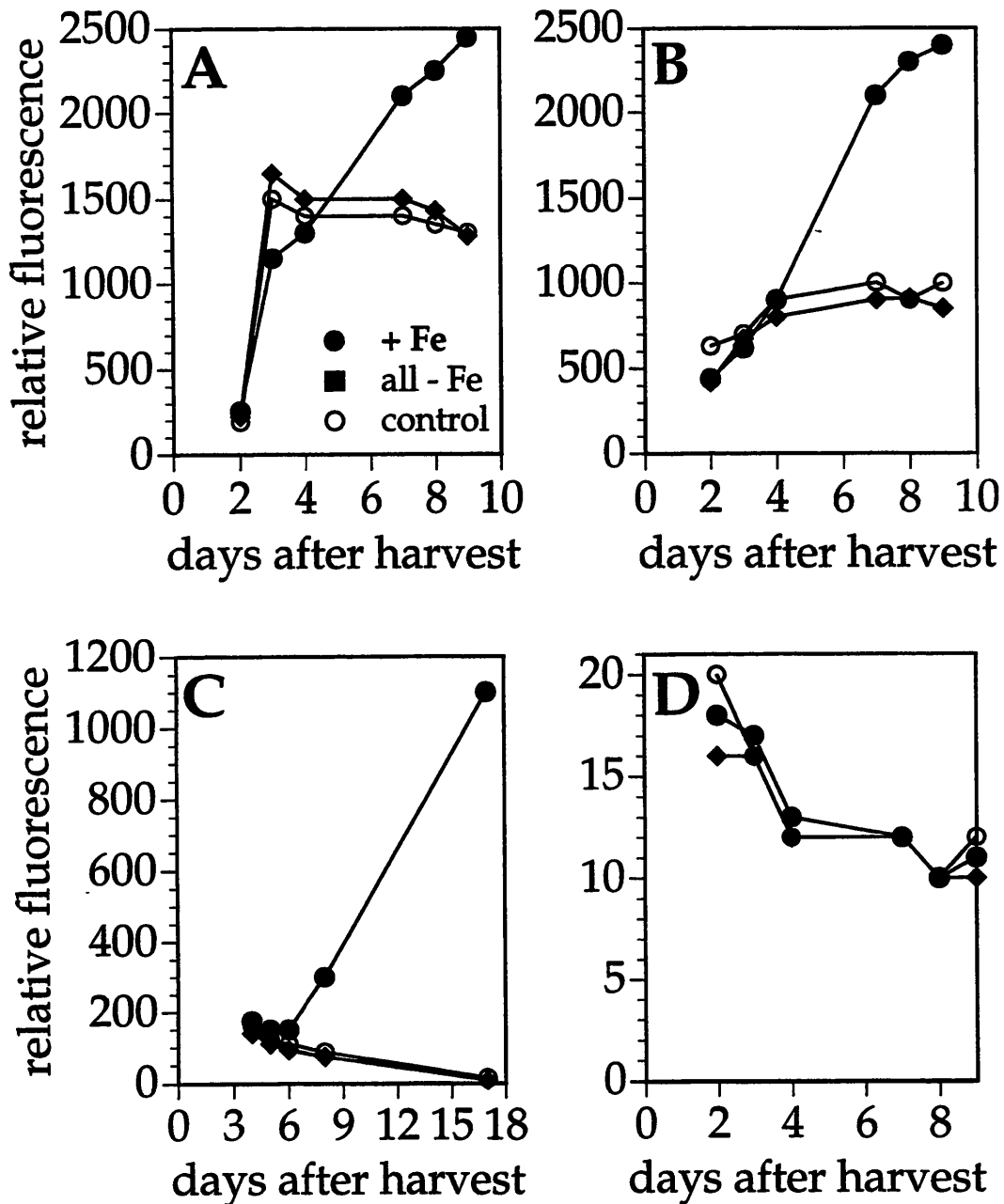
FIGURE 3 - Bioassay results from iron-limited cultures of species that do not express flavodoxin. Culture aliquots removed on the day of harvest were amended with either Fe (●), all nutrients except Fe (■), or no addition (O) as a control. Growth was monitored by fluorescence. (A) Syracosphaera elongata (B) Pleurochrysis carterae (C) Minutocellus polymorphus (D) Alexandrium fundyense.
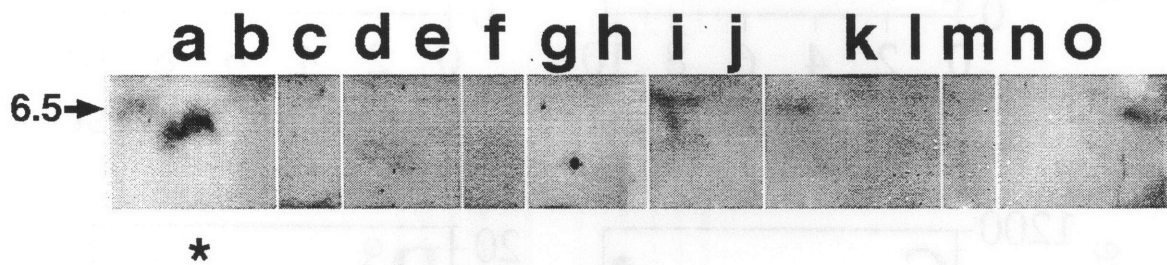
FIGURE 4 - Results of Western blots challenged with antiserum raised against ferredoxin from <u>Thalassiosira</u> <u>weissflogii</u>. Each lane contains 30µg of total protein from: (A) <u>Thalassiosira</u> <u>weissflogii</u>, (B) <u>Minutocellus</u> <u>polymorphus</u>, (C) clone 7-47B, (D) clone A3-30, (E) clone 10-40A, (F) <u>Thalassiosira</u> <u>oceanica</u>, (G) <u>Chaetoceros</u> <u>gracilis</u>, (H) <u>Alexandrium</u> <u>fundyense</u>, (I) <u>Symbiodinium</u> <u>microadriaticum</u>, (J) <u>Amphidinium</u> <u>carterae</u>, (K) <u>Emiliania</u> <u>huxleyi</u> CCMP370, (L) <u>Phaeocystis</u> CCMP1528, (M) <u>Chrysochromulina</u> <u>ericina</u>, (N) <u>Syracosphaera</u> <u>elongata</u>, (O) <u>Pleurochrysis</u> <u>carterae</u>, Unlabeled lanes - molecular weight standards. Positive reactions are indicated with *.
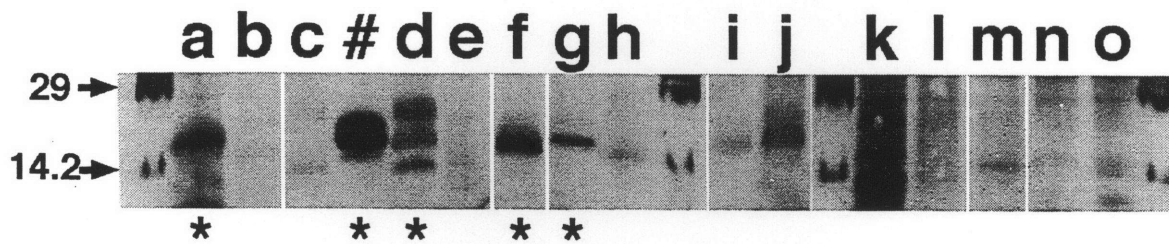
FIGURE 5 - Results of Western blots challenged with antiserum raised against flavodoxin from <u>Thalassiosira weissflogii,</u> as in Figure II, except (#)150 ng of purified <u>Thalassiosira weissflogii</u> flavodoxin.

Chapter 2

Ferredoxin and Flavodoxin Regulation: The Effect of Fe, N source and Other Potential Limiting Factors

Abstract

Both flavodoxin and the ferredoxin:flavodoxin ratio have been proposed as molecular markers of iron stress. While flavodoxin induction is a common response to iron stress in marine phytoplankton, little else is known about its regulation. This study addresses the potential for both direct and indirect regulation of ferredoxin and flavodoxin by iron and other environmental factors. The marine centric diatom Thalassiosira weissflogii was grown in nutrient- or light-deficient batch culture to determine the effects of ecologically relevant limiting factors on flavodoxin expression. In T. weissflogii, flavodoxin is induced specifically by iron stress, and not by nitrate, phosphate, silicate, zinc or light deficiency. This insensitivity to other limiting factors makes it an excellent choice as a diagnostic of iron limitation.

The ability of N substrate to modulate ferredoxin and flavodoxin abundance via changes in intracellular iron requirements was also examined using T. weissflogii grown over a range of limiting iron concentrations with either nitrate or ammonium as a nitrogen source. These results show no evidence of indirect regulation of ferredoxin/flavodoxin abundance (Fd index) by N substrate. The data describe, nonetheless, the relationship between Fd index and growth rate, which is composed of two distinct regions. In the first region, at low growth rates, ferredoxin is undetectable and the Fd index is uniformly zero. In the second region, at moderate-to-high growth rates ($55\text{-}90\%\mu_{max}$), ferredoxin and flavodoxin co-occur in the cells. The substitution of flavodoxin for ferredoxin is a gradual process, not a simple "on-or-off" response. In addition, flavodoxin expression is very sensitive to iron limitation, occurring even at relatively high growth rates ($80\text{-}90\%$ μ_{max}).

When ferredoxin and flavodoxin are both present, the Fd index varies with relative growth rate. Thus, variation in the Fd index has the potential to indicate changes in the severity of iron stress on varying temporal and spatial scales.

Introduction

The potential for iron limitation of oceanic primary productivity has generated considerable controversy since it was proposed by Martin and colleagues (Martin & Fitzwater 1988, Martin et al. 1989, Martin 1990, Martin et al. 1990, Martin et al. 1990). Resolution of this debate has been hindered in part by a lack of suitable analytical techniques for the assessment of iron limitation. The bulk of the evidence in support of iron limitation is derived from nutrient addition bioassays, in bottles (e.g. Buma et al. 1991, Coale 1991) and in the environment (Martin et al. 1994, Coale et al. 1996). The interpretation of bottle bioassays is complicated by the potential for contamination and confinement effects (e.g. Venrick et al. 1977, Cullen 1991) and mesoscale enrichments are expensive and logistically difficult. The methodological problems associated with these methods could be circumvented through the use of a diagnostic indicator - a specific cellular response that is directly regulated by iron availability. Such an indicator would allow rapid diagnosis of iron limitation without manipulation of the natural community.

Both the flavodoxin and/or ferredoxin proteins have been proposed as markers of iron limitation (LaRoche et al. 1995, Doucette et al. 1996). Under conditions of iron stress cells are able to functionally replace ferredoxin, an abundant iron-sulfur redox protein, with flavodoxin, which contains no iron. This response is widespread among the freshwater algae, in which it has been studied for decades (e.g. Smillie 1965, Zumft & Spiller 1971, Peleato et al. 1994). Flavodoxin induction has been shown to occur in marine phytoplankton as well (LaRoche et al. 1993, LaRoche et al. 1995, Doucette et al. 1996). This

adaptation appears to be a common response to iron stress across algal classes, making it a promising candidate as a molecular assay of iron limitation (see Chapter 1). To be useful as a diagnostic, however, the response must be regulated uniquely by changes in iron availability. Sensitivity to other factors, such as nutrients or light, would invalidate their intended use in studies of iron limitation in the environment.

To date, the study of flavodoxin regulation in marine algae has primarily concerned its response to iron (LaRoche et al. 1993, LaRoche et al. 1995, Chapter 1). It has been demonstrated that neither nitrogen nor phosphorous limitation induces flavodoxin expression in the marine diatom Phaeodactylum tricornutum (LaRoche et al. 1993). That study, however, represents the sum total of the present knowledge of non-iron flavodoxin regulation in marine phytoplankton. Regulation of ferredoxin and flavodoxin by factors other than iron has been observed in other organisms. For example, light is able to modulate ferredoxin gene expression in both pea and wheat (Dobres et al. 1987, Bringloe et al. 1995). In the freshwater cyanobacterium Synechocystis, flavodoxin seems to function as a general stress response, accumulating under conditions of salt stress, heat shock and iron limitation (Fulda & Hagemann 1995).

In many potentially iron-limited regions of the ocean, there is also the possibility of limitation by other factors. Nitrogen or phosphorous stress is unlikely in the high-nutrient-low-chlorophyll areas of the ocean where iron limitation is suspected, but it may be a factor in coastal regions where macronutrient levels are often low and iron availability may vary on both temporal and spatial scales (Wells et al. 1991). Another macronutrient,

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silicate, may be the 'next' limiting nutrient for diatoms in iron-limited areas, whose growth seems to be favored by iron enrichment (Coale et al. 1996). Oceanic regions with low surface water iron concentrations also typically exhibit low levels of other biologically important micronutrients such as zinc and manganese (Bruland et al. 1978, Landing & Bruland 1980, Gordon et al. 1982). In the Southern Ocean, where iron limitation is one possible explanation for persistent HNLC conditions, light is also thought to constrain primary production (de Baar et al. 1990, Mitchell et al. 1991). Induction of flavodoxin by any of the aforementioned factors would generate "false positives" for iron limitation. To be a truly diagnostic indicator, a marker must be able to discriminate between iron and other types of limitation.

In addition to the direct effect of various limiting factors, there is potential for indirect regulation of ferredoxin and flavodoxin expression. Cellular ferredoxin and flavodoxin content is undoubtedly linked to the cell's iron quota, which is in turn thought to be affected by the nitrogen source available for growth. Theoretical calculations by Raven (1988) predict that nitrate assimilation requires 1.6 times more cellular iron than ammonium utilization. Variations in cellular iron requirements for growth on different N substrates may then be reflected in the relative abundance of ferredoxin and flavodoxin. This is the case for the freshwater cyanobacterium Anabaena 7120 which exhibits varying flavodoxin:ferredoxin ratios when grown on different nitrogen sources at a constant iron concentration (Fish & Sanders-Loehr 1987). Such a response could be likened to a "co-limitation" by iron and nitrogen, such that low iron availability would restrict nitrate use.

The goal of this study was to determine the effect of nutrients and light upon ferredoxin and flavodoxin regulation, utilizing Thalassiosira weissflogii as a model. The data presented here are derived from two related studies. The first, a "Nutrient Limitation" study, measured the ferredoxin and flavodoxin content of T. weissflogii grown in batch culture under non-limited conditions and nitrate-, phosphate-, silicate-, iron-, light- or zinc-deficient conditions. The second study ("N/Fe Interaction") explored the potential indirect effect of nitrogen substrate on the relative ferredoxin /flavodoxin content of T. weissflogii grown over a range of iron levels. The two analyses together comprise the first comprehensive study of both direct and indirect regulation of ferredoxin and flavodoxin expression by ecologically relevant limiting factors.

Materials and Methods

Cultures The marine eukaryotic phytoplankton species used for all experiments in this study was the centric diatom Thalassiosira weissflogii (Grun.) Fryxell et Hasle (clone ACTIN).

Growth of phytoplankton - Nutrient limitation All Thalassiosira weissflogii cultures, except for zinc-limited cells, were grown in 0.2 µM filtered Vineyard Sound (MA, USA) seawater (31‰) enriched with ESNW nutrients according to Harrison et al. (1980) with several modifications. Na_2HPO_4 was substituted in equimolar amounts for $Na_2glyceroPO_4$. and selenium (as H_2SeO_3) was added to a final concentration of $10^{-8}M$. Trace metal additions were made according to Brand et al. (1983). Seawater was sterilized by autoclaving then enriched with sterile nutrients. Macronutrient

(nitrate, phosphate and silicate) stocks were sterilized by autoclaving while iron, trace metal, selenium, EDTA and vitamin stocks were sterile-filtered (0.2 μm).

Nitrate-, phosphate-, silicate- and light-deficient cultures all contained 10 μM Fe and 100 μM EDTA. The control culture was enriched with 50 μM Fe and 500 μM EDTA and iron-limited cultures contained 100 nM Fe and 1 μM EDTA. Nitrate-, phosphate- and silicate-deficient cultures contained the limiting nutrient at 1/50 the concentration of full medium, 11.0 μM, 0.42 μM and 2.11 μM respectively. Zinc-limited cultures were not grown by the authors, but were provided by Dr. Jenny Lee as cells harvested onto 3 μm polycarbonate filters and frozen in liquid nitrogen. Zinc-limited cells were grown in Aquil medium (Price et al. 1989) with a calculated pZn=11.6.

Cultures (2 l volume) were grown in acid-washed glass or polycarbonate 2.8 l Fernbach flasks. They were maintained at 20°C on a 14:10 hour light:dark cycle at an irradiance of ca 175 μE m^{-2} s^{-1} as measured inside the culture flasks with a photometer (Biospherical Instruments model QSP-100). Light-limited cultures were covered with three layers of neutral density screening, resulting in a measured irradiance of ca 20 μE m^{-2} s^{-1}.

Control, Fe-limited and light-limited cultures were harvested during the exponential phase of growth. Nitrogen-, phosphorous- and silicate-deficient cultures were allowed to reach stationary phase before harvest. At the appropriate stage, subsamples were removed for chlorophyll determinations and cell counts. The remaining volume was collected onto 3 μm polycarbonate filters (47 mm diameter), except for approximately 100 ml. This volume was used as an inoculum for the next culture, which was

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initiated by the addition of two liters of fresh culture medium. Three sequential cultures were grown in this manner for all treatments except the control culture which was grown once. After harvest, cells were frozen at -80°C until analysis. While triplicate cultures were grown for all treatments except the control, data from only one round of culturing is presented here.

Growth rates - Nutrient limitation   Cell densities were determined by four replicate microscopic counts of Utermohl's preserved samples in a Fuchs-Rosenthal hemacytometer. Growth rates during exponential phase were calculated from linear regressions of the natural log of cell density versus time.

Chlorophyll a determinations - Nutrient limitation   Chl a was measured on triplicate samples per flask. Cells were collected by gentle filtration onto Millipore SSWP membranes (3 μm, 25 mm diameter) and frozen in liquid nitrogen until analysis. Filters were extracted in 100% acetone for 24 hours at 4°C in the dark. Before measurement, samples were diluted to 90% acetone and allowed to warm to room temperature. Chlorophyll was measured fluorometrically using a Turner Designs fluorometer (model 10-AU) that had been calibrated with pure chlorophyll a (Sigma) using the extinction coefficients from Jeffrey and Humphrey (1975).

Growth of phytoplankton - N/Fe interaction   Cultures of Thalassiosira weissflogii were grown by Dr. Neil Price's laboratory, using the procedure detailed in Maldonado and Price (1996). Cultures were grown in the artificial culture medium Aquil (Morel et al. 1979, Price et al. 1989) with minor modifications. Media contained standard enrichments of phosphate and silicate, with either 50 μM nitrate or ammonium. Premixed Fe-EDTA (1:1)

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was added separately at a range of concentrations from 10 nM to 8.4 µM, to achieve free ferric ion concentrations of $10^{-21.1}$ M (pFe 21.1) to $10^{-18.8}$ M (pFe 18.8). Synthetic ocean water was sterilized by microwaving in acid-washed teflon bottles (Keller et al. 1988), and enriched with sterile nutrients. Trace-metal-clean technique was employed for all cultures, as outlined by Price et al. (1989).

Cultures were grown in acid-washed polycarbonate flasks. They were maintained at 20°C under continuous irradiance of 200 µE m^{-2} s^{-1}, using semi-continuous batch culture technique described by Brand et al. (1981). Although sterile techniques were used to minimize bacterial contamination, the cultures were not axenic. Cells were harvested by filtration, stored in liquid nitrogen and subsequently lyophilized prior to analysis.

Growth rates - N/Fe interaction Growth rates were determined by measurements of in vivo fluorescence using a Turner Designs fluorometer (model 10-AU). Growth rates were calculated from linear regressions of the natural log of in vivo fluorescence versus time. Cultures were considered to be acclimated when growth rates of successive transfers did not vary by more than 10%.

Protein extraction Initially, cell extracts were prepared by an acetone precipitation technique. Briefly, cells were washed from filters using extraction buffer and lysed by the addition of 9 volumes of acetone. The resultant acetone precipitate was collected by centrifugation, dried and then resuspended in extraction buffer. The resulting aqueous extract was analyzed by HPLC. Half of the N/Fe interaction samples were extracted using this method.

The remaining N/Fe samples and all of the nutrient limitation samples were extracted using a more simplified method. Cells and their filters were placed in a 2 ml tube with extraction buffer and zirconium beads. Cells were ruptured by three, fifty second cycles in a mini-beadbeater (Biospec Instruments, Bartlesville, OK, USA). The cell lysate was centrifuged for one hour at 105,000 x g and the supernatant was filtered (0.45 μm) and injected into the HPLC. Prior to use of the simplified extraction method, it was compared to the acetone powder method using laboratory cultures of Thalassiosira weissflogii. The extraction efficiency using the simplified method is slightly better, and the same ratio of ferredoxin:flavodoxin is obtained from the two methods when using identical samples (data not shown).

HPLC of ferredoxin and flavodoxin   All cultures were analyzed using a previously described HPLC method (Doucette et al. 1996). Ferredoxin and flavodoxin in cell extracts were separated by anion-exchange HPLC. Detection was performed with a Hewlett-Packard model 1050 diode array detector (Hewlett-Packard Co., Andover, MA, USA), which also allowed identification of the proteins by their absorption spectra. Quantification of peak areas was performed by HP ChemStation software (Hewlett-Packard) in autointegration mode. The zinc-limited samples, which were analyzed prior to the other cultures, are one exception to this procedure. The same chromatographic method was employed, but a Hewlett-Packard Model 1040A diode array detector was used instead of the model 1050. The model 1040A records wavelength data only in the range from 250 nm to 600 nm, whereas the 1050 covers the range from 200 nm to 600 nm. This is reflected in Figure 4, where

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the spectrum of the chromatographic peak of the zinc sample begins at 250 nm. The data collected by the 1040A was analyzed using the HP ChemStation program, thus quantification of peak areas was by the same method as for the other samples.

Results

Growth of cells in light- or nutrient-deficient culture

Growth curves for all cultures are displayed in Figure 1. For the nutrient- or light-deficient cultures (Fig. 1B-F), data from the control culture are included for comparison. The control culture was grown in complete medium and harvested during mid-exponential phase and was considered to be non-limited. The low-light (Fig. 1E) and low-iron (Fig. 1F) cultures exhibited slowed but exponential growth rates relative to the control (μ=0.009, 0.023 and 0.038 hr^{-1}, respectively). The low-nitrate (Fig. 1B), -phosphate (Fig. 1C) and -silicate (Fig. 1D) cultures exhibited reduced final cell densities as compared to the control, which was harvested in exponential stage before it achieved maximum density. In both the N and P starved cultures, a decrease in specific growth rates during exponential phase relative to the control was also observed (μ=0.019, 0.019 and 0.038 hr^{-1}, respectively). The Si-starved culture, however, grew slightly faster than the control during exponential (μ=0.042 vs. 0.038 hr^{-1}) although the final cell yields were markedly lower.

Effect of nutrients and light on cellular chlorophyll levels

Nutrient or light deficiency also affected the chlorophyll content of Thalassiosira weissflogii (Figure 2). Chlorosis was evident in nitrate-,

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phosphate- and iron-deficient cultures, whose cellular chlorophyll content was only 29%, 70% and 59% of the level in control cells, respectively. In contrast, cellular pigment content of the light-limited and silicate-starved cultures increased to 170% and 260% of the control level, respectively.

Effect of nutrients and light on ferredoxin and flavodoxin expression

All cultures exhibited only one peak on their chromatograms that could be attributed to either ferredoxin or flavodoxin (Figure 4A-G). Peaks were identified by their absorption spectra, which are displayed beside the corresponding chromatograms in Figure 4. For reference, absorbance spectra of ferredoxin and flavodoxin from Thalassiosira weissflogii are shown in Figure 3. Flavodoxin was present only in iron-limited cells, and was not expressed in response to limitation by nitrate, phosphate, silicate, light or zinc.

Influence of N substrate on ferredoxin and flavodoxin expression

In a related experiment, ferredoxin and flavodoxin abundance was measured in cells grown over a range of iron concentrations using either nitrate or ammonium exclusively as a nitrogen source (Figure 5A). Nitrogen was added at sufficient levels (50 µM), so that growth rate was controlled by iron availability. The "Fd index" is a modification of the equation 1 of Doucette et al. (1996), which relates the flavodoxin abundance to the total ferredoxin/flavodoxin pool on the basis of percentages. The Fd index used here expresses the proportion of ferredoxin in the ferredoxin/flavodoxin pool and varies from 1 (only ferredoxin, no flavodoxin) to 0 (only flavodoxin, no

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ferredoxin). The Fd index is shown as a function of growth rate, which is expressed as a percentage of the known maximum rate and serves as a proxy for iron availability.

At the highest growth rate examined here, 93% of maximum, cells express only ferredoxin (Fd index = 1). As iron availability and therefore growth rate decreases, cellular ferredoxin content begins to decrease relative to flavodoxin (0 < Fd index < 1). Eventually, ferredoxin expression ceases and cells contain only flavodoxin (Fd index = 0). The relationship between Fd index and growth rate is composed of two different regions. In the first region, below 55% of maximum growth rate, only flavodoxin is expressed and the Fd index is uniformly zero. In the second region, at growth rates higher than 55% of maximum, ferredoxin and flavodoxin are both present in the cells. In this second region, the Fd index increases as relative growth rate (and iron availability) increases, and is therefore inversely related to the severity of iron stress.

The results for the ammonium- and nitrate-grown cultures were examined separately to assess the effect of N substrate on ferredoxin and flavodoxin expression (Figure 5A). The data points for nitrate- and ammonium-grown cells do not separate into two distinct groups. In fact, there is considerable overlap in the region between 55-65% μ_{max}, where many of the nitrate and ammonium points cluster together. The data do not therefore provide evidence of N substrate-dependent differences in the ferredoxin/flavodoxin response.

Discussion

Both flavodoxin alone (LaRoche et al. 1995, LaRoche et al. 1996) and the cellular ratio of ferredoxin:flavodoxin (Doucette et al. 1996, Chapter 1) have been proposed as molecular markers of iron limitation in marine phytoplankton. The effect of iron on expression of these two proteins is well established, but little else is known regarding their regulation. Before this system can be used confidently as an indicator in the field, it is essential to determine that it is regulated uniquely by iron. While it may be impractical to examine all possible limiting factors for their effect on ferredoxin and flavodoxin expression, it is advisable to test at least those factors which may be ecologically relevant.

This study examines the influence of several common limiting factors - nitrogen, phosphorous, silicate, zinc and light - on ferredoxin and flavodoxin expression. Regulation was also examined in nitrate- and ammonium-grown cells over a broad range of limiting iron levels, to investigate the potential modulating effect of N substrate on ferredoxin/flavodoxin content. These data describe the relationship between growth limitation by iron and relative cellular abundance of ferredoxin and flavodoxin. Together, this information provides a comprehensive picture of ferredoxin and flavodoxin regulation by various nutrients and light, information that is essential if we are to confirm their use as diagnostics of iron limitation in natural populations.

Nutrient- and light- deficient growth

Although all cultures used during the nutrient limitation study were technically batch cultures, there are subtle but significant differences in the manner in which limitation was achieved. The control, nitrogen-, phosphorous- and silicate-deficient cultures were true batch cultures. In such cultures cells will grow exponentially until the medium is depleted of the nutrient present at the lowest relative concentration, at which point growth ceases. It is therefore the final biomass yield of the culture that is determined by the quantity of limiting nutrient initially added to the medium. During early-to-mid exponential growth, all nutrients should be saturating and cells should be essentially "unlimited". Once they reach stationary phase, however, they have been effectively starved of the limiting nutrient.

This type of growth was evident in the control (Fig. 1A), N- (Fig. 1B), P- (Fig. 1C) and Si-deficient (Fig. 1D) cultures. All four exhibited an exponential phase of growth of varying duration. The control culture was harvested in mid-exponential, before it became limited for any particular factor but also before it reached maximum cell density. In the N-, P- and Si-deficient cultures, the exponential period was followed by a plateau where cell density did not change. For all three, this biomass level was below that achieved by the control culture, a hallmark of nutrient limitation in batch culture. The exponential growth rates for N- and P-starved cultures were also lower than the control culture, which suggests that the cells experienced some limitation by low nutrient levels throughout their growth. The cultures were harvested for analysis during stationary phase, however, and are therefore considered to be nutrient-starved.

The situation for the iron-, zinc-, and light-limited cultures is somewhat different. In all of these cultures it is not the absolute quantity of the limiting nutrient, but its rate of supply, that affects growth. In the light-limited culture, the irradiance is controlled by the use of neutral density screening. Growth is therefore limited by the rate at which cells can acquire photons. In trace-metal-buffered cultures, metal availability is controlled through the use of chelators. A relatively constant supply of metal can be maintained in the culture through chemical equilibrium between free metal, free chelator and the metal-chelator complex. The magnitude of the supply is determined by relative chelator and metal concentrations.

Under conditions such as these, cells are able to grow exponentially but at a reduced level dictated by the supply rate of the limiting nutrient. In a batch culture, the cells will eventually run out of a particular nutrient and cease growing. In exponential phase, during which these cells were harvested, growth approximates that of a continuous culture. This type of growth is illustrated by the light- (Fig. 1E) and iron-limited (Fig. 1F) cultures. Their exponential growth rates are lower than that of the control, which is indicative of light or iron limitation.

Chlorophyll content of limited/starved cells

Further evidence of limitation or starvation is provided by cellular chlorophyll measurements (Figure 2). Chlorosis is a common effect of nitrogen, phosphorous and iron stress (e.g. Glover 1977, Laws & Bannister 1980), and the N-, P-, and Fe-deficient cells all contained less chlorophyll than

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the control. The low-light grown cells exhibited increased pigment content, a characteristic adaptation to limiting light levels (e.g. Laws & Bannister 1980).

The highest chlorophyll per cell, however, was measured in the Si-starved cells, which also showed striking morphological changes evident upon microscopic examination, becoming elongated and visibly darker. Unfortunately, no cell volume data was collected for the Si-starved cells. The dramatically increased chlorophyll content is likely due to a combination of increased cell size due to elongation and increased cellular chlorophyll. This is similar to the results reported by Harrison et al. (1977), who examined the effect of silicate limitation in diatom chemostat cultures. They also observed elongated cells with increased pigment content which resulted in elevated chlorophyll per cell values. In their case, the thinning of the cells compensated for the effects of elongation, resulting in a relatively unchanged cell volume. The increased chlorophyll content of the low-silicate-grown cells is thus consistent with silicate starvation.

Ferredoxin and flavodoxin content of limited/starved cells

The primary goal of this study was to determine if ferredoxin and flavodoxin were regulated by any limiting factor other than iron. In particular, we examined some common forms of limitation that cells are likely to encounter in natural waters. It has been shown previously that flavodoxin in Phaeodactylum tricornutum is not induced in response to nitrogen or phosphorous starvation (LaRoche et al. 1993). This present study provides an independent replication of those results for another species and

extends our knowledge of ferredoxin and flavodoxin regulation to include the effects of silicate-, zinc- and light-deficiency and nitrogen substrate.

The ferredoxin and flavodoxin content of cells grown under light or nutrient deficiency as well as non-limiting conditions (control) were determined using HPLC. Both ferredoxin and flavodoxin were identified by their characteristic absorption spectra (Figure 3). The spectra allow unambiguous identification of ferredoxin and flavodoxin in spite of the slight variation in the elution times of ferredoxin due to such factors as slight batch-to-batch differences in buffer composition or variations in column packing. The total range of the elution times is less than one minute, with the exception of the zinc-limited sample. It was analyzed some months prior to the other samples and exhibits a shifted elution time as well as a truncated UV-visible spectrum (see Materials and Methods).

In all cases, only one chromatographic peak was observed during the appropriate range of elution time (Fig. 4 A-G). In Figure 4, the UV-visible absorption spectra of each HPLC peak is displayed beside its corresponding chromatogram. Flavodoxin was observed only in iron-limited cells, and was not induced in response to nitrate, phosphate, silicate, zinc or light stress. The insensitivity of flavodoxin to other potential limiting factors makes it an excellent candidate for a marker of iron limitation.

Effect of N substrate on relative ferredoxin/flavodoxin abundance

In addition to the examining the direct effects of alternative limiting factors, this study was extended to include potential indirect regulation of ferredoxin and flavodoxin expression by nitrogen substrate. Nitrate-grown

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cells are thought to require more iron than those utilizing ammonium (Raven 1988) and may compensate for this increased cellular demand through changes in relative ferredoxin/flavodoxin content. To test this hypothesis, ferredoxin and flavodoxin abundances were measured in cells that had been grown over a range of iron levels and provided with either nitrate or ammonium exclusively as a nitrogen source (Figure 5).

Relative ferredoxin/flavodoxin abundance is expressed in Figure 5 as the Fd index, defined as the proportion of ferredoxin in the combined ferredoxin+flavodoxin pool:

$$\text{Fd index} = [\text{ferredoxin}]/[\text{ferredoxin} + \text{flavodoxin}]$$

This index is calculated from the integrated HPLC peak areas, which are directly proportional to moles of ferredoxin or flavodoxin. The Fd index is shown as a function of culture growth rate, expressed as a percentage of the known maximum rate. When working with very low culture iron levels, small amounts of contamination can have a large effect on medium iron content. The culture growth rate is therefore a more accurate measure of the amount of iron actually present in the culture, rather than moles of iron added or calculated free ion activities. The growth rate is normalized to the maximum observed growth rate, which is slightly different for nitrate- ($0.057 \cdot \text{hr}^{-1}$) and ammonium- ($0.058 \cdot \text{hr}^{-1}$) grown cells.

In Figure 5A, data from nitrate- and ammonium-grown cells is displayed with different symbols, to highlight any potential differences between the two N substrates. The data do not separate into clearly distinct groups, and as such provide no evidence for modulation of cellular ferredoxin/flavodoxin content by the nitrogen source used for growth.

Indeed, the recent results of Maldonado and Price (1996) suggest that this may very well be the case. Their data indicate that nitrate-grown cells have elevated cellular iron quotas but are able to compensate by acquiring more iron. Thus, the cells seem to satisfy their increased iron demands through uptake of extracellular iron rather than via reapportionment of internal iron pools.

If the data from nitrate- and ammonium-grown cells are considered together, the results provide a description of changes in cellular ferredoxin/flavodoxin abundance in response to iron availability (Figure 5B). Flavodoxin expression seems to be very sensitive to iron limitation, occurring at relatively high growth rates (80-90% μ_{max}). The sensitivity of flavodoxin induction is in agreement with the recent results of McKay et al., who observe increases in flavodoxin·protein[-1] when cells are only mildly limited by iron (%μ_{max} ~ 80%) (McKay et al. 1997). As growth limitation increases, ferredoxin is replaced gradually by flavodoxin, not as a simple "on-or-off" response.

The relationship between Fd index and growth rate in T. weissflogii is composed of two distinct regions. In the first region, at very low relative growth rates (i.e. severe iron stress) ferredoxin is undetectable and the Fd index is uniformly zero. In the second region, at moderate to high relative growth rates, ferredoxin and flavodoxin co-occur in the cells. In the latter region the Fd index increases with increasing growth rate, and is therefore inversely related to the severity of iron stress. Unfortunately, the data are insufficient to define precisely the relationship between Fd index and iron availability, e.g. linear, exponential, sigmoidal, etc. The ability to

mathematically describe such data would allow statistical testing of the differences between nitrate- and ammonium-grown cells, an analysis that it not presently possible. The shape of the Fd index/growth rate relationship may also provide insight into possible cellular regulation mechanisms of ferredoxin and flavodoxin expression. More data is needed, however, to accurately describe the effect of iron availability on relative cellular abundance of ferredoxin and flavodoxin.

The data presented here nonetheless support an inverse relationship between Fd index and the severity of iron stress. Flavodoxin, used alone, can detect the presence or absence of iron limitation. The combination of flavodoxin and ferredoxin abundance measurements may extend the utility of this system to the detection of changes in the severity of iron limitation. Thus, variation in the Fd index may allow determination of both temporal and spatial changes in the severity of iron stress e.g. over the course of a phytoplankton bloom or on a transect from coastal to oceanic waters.

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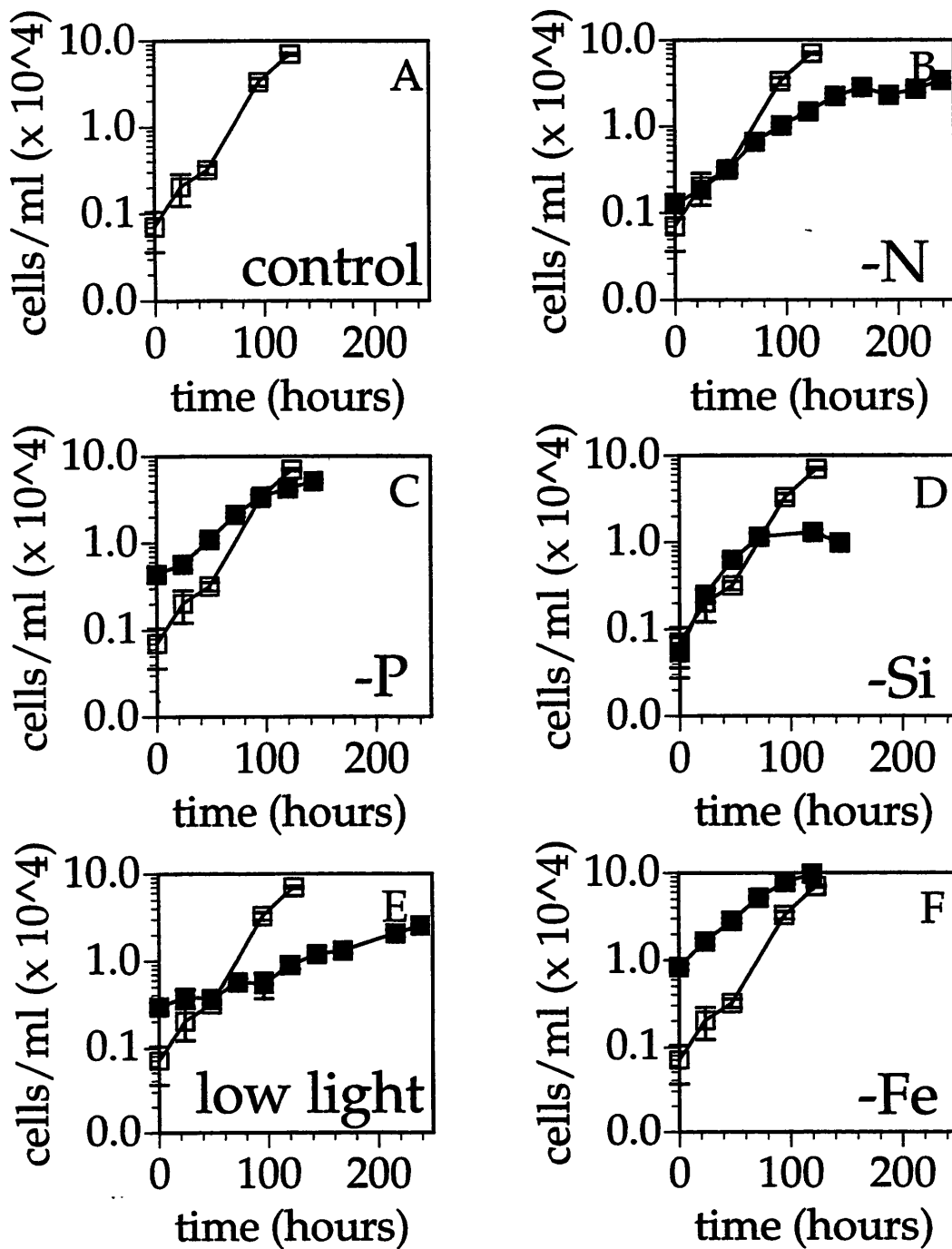
FIGURE 1 - Growth of (A) control (B) phosphate- (C) nitrate- (D) silicate- (E) iron- and (F) light-deficient cultures of Thalassiosira weissflogii. Cell numbers for treated cultures are shown as filled squares (■), whereas control cell numbers are displayed as open squares (□). For nutrient- and light-deficient cultures, cell numbers of the control culture are included for comparison.
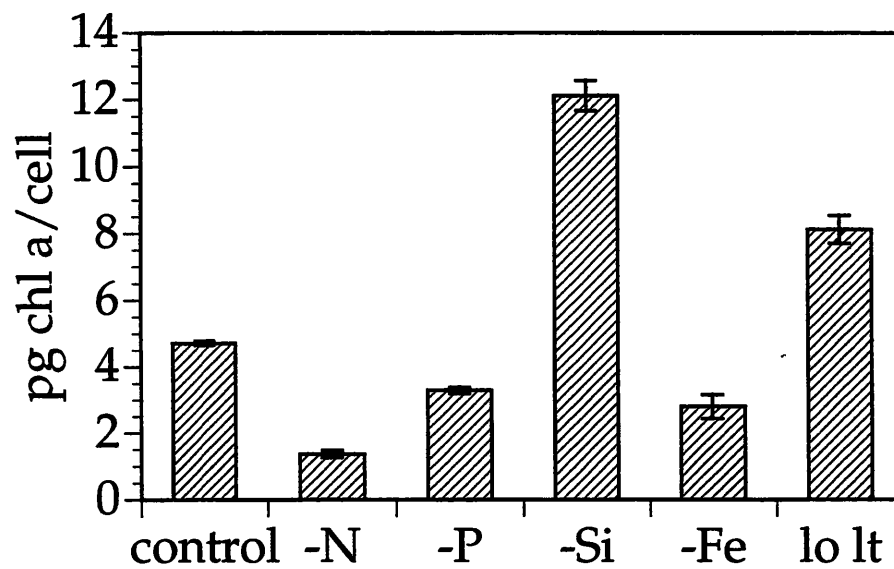
FIGURE 2 - Chlorophyll a in pg·cell^{-1} for the various nutrient treatments. Chl a in the control was determined from triplicate subsamples of one culture. Values from all other conditions are pooled measurements of triplicate samples from each of three replicate cultures. Values shown are mean ± 1 S.E.
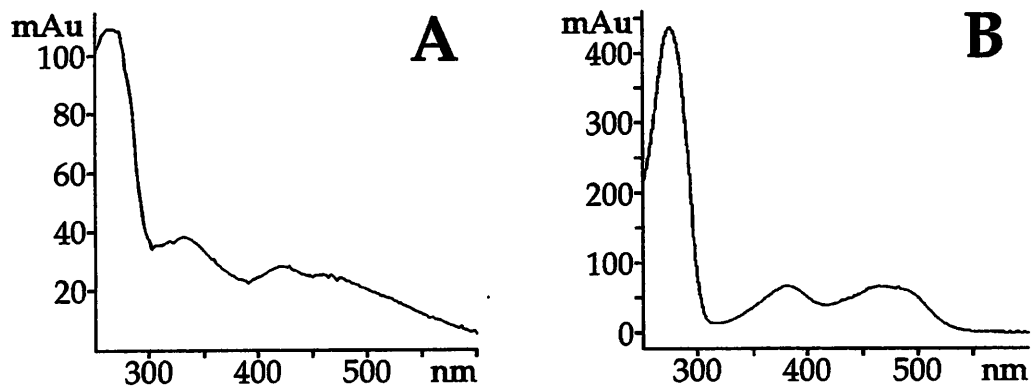
FIGURE 3 - UV-visible absorbance spectra of (A) ferredoxin and (B) flavodoxin from Thalassiosira weissflogii. In addition to the 280 nm absorbance maximum common to all proteins, ferredoxin exhibits secondary maxima at ca. 330 and 430 nm and a shoulder at 465 nm. Flavodoxin, in contrast, has secondary maxima at ca. 365 and 465 nm, giving it a distinctive "camel hump" appearance.
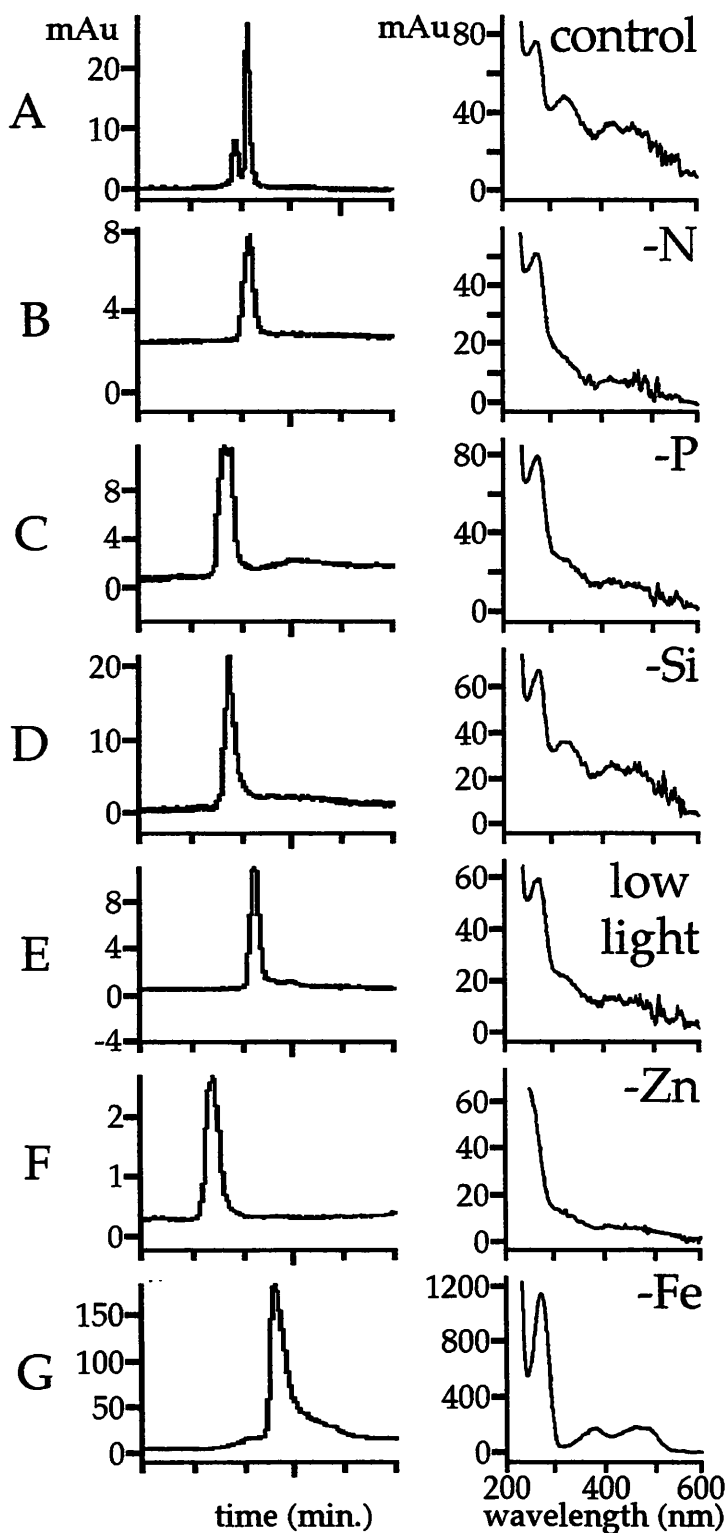
FIGURE 4 - HPLC chromatograms of cell extracts of cultures grown under various limiting conditions (A-G). Compounds were detected by their absorbance at 465 nm (in mAu, vertical axis). The chromatograms have been cropped to show the relevant 10 minute window during which ferredoxin and flavodoxin both elute. The UV-visible absorption spectrum of the peak is shown next to its corresponding chromatogram. (A) control, (B) N-starved, (C) P-starved, (D) Si-starved, (E) light-limited, (F) zinc-limited and (G) iron-limited. Whereas A-F all exhibit the ca 330 and 430 nm maxima of ferredoxin, only G has the characteristic flavodoxin "camel hump".
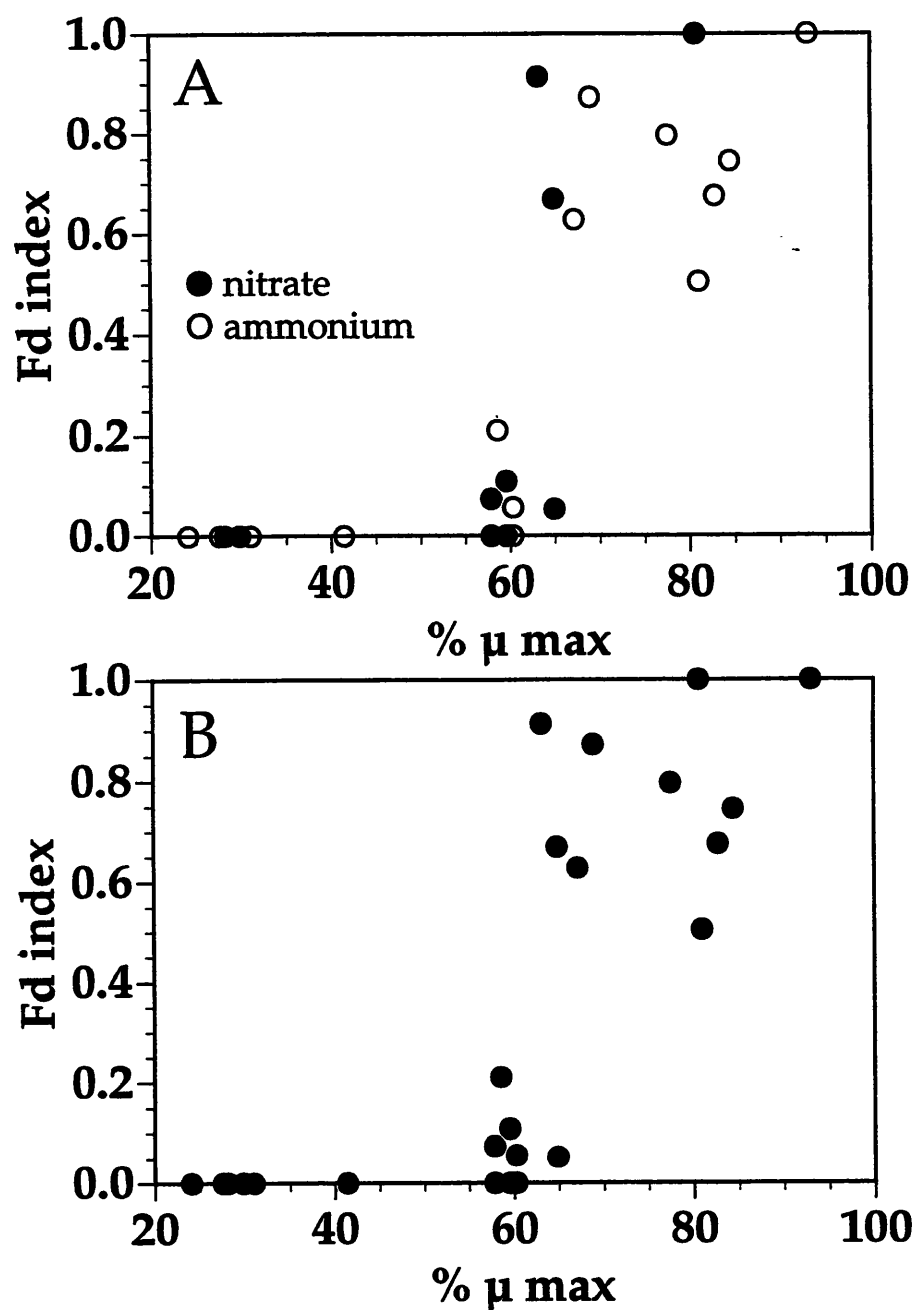
FIGURE 5 - (A) Cellular ferredoxin/flavodoxin content over a range of iron-limited growth rates separated according to the N source for growth. Cells were grown on (●) nitrate or (O) ammonium. (B) The data as in (A), but combined.

Chapter 3

Ferredoxin and Flavodoxin as Biochemical Indicators of Iron Limitation During Open-Ocean Iron Enrichment

Abstract

Substitution of the non-iron protein flavodoxin for the iron-sulfur protein ferredoxin is an iron-stress response employed by a variety of unicellular organisms, including many phytoplankton. The relative abundance of these two proteins has been shown to vary with the severity of growth limitation by iron in marine diatoms. The IronEx II experiment, during which a 64 km^2 patch of the equatorial Pacific ocean was enriched with iron and monitored for several weeks, provided an unprecedented opportunity to test both the ferredoxin-flavodoxin biomarker system and its HPLC detection method in a well-characterized field situation. Large volume (100-600 liter) phytoplankton samples were collected for analysis of community ferredoxin and flavodoxin abundance using HPLC. In addition, three pennate diatom species isolated from the fertilization-induced phytoplankton bloom were used for follow-up laboratory experiments which examined their iron physiology.

HPLC results track the growth of the fertilization-induced phytoplankton bloom. Prior to enrichment, biomass levels were insufficient to obtain any ferredoxin or flavodoxin signals. The first chromatographic peaks were evident on the fifth day following enrichment, coincident with large increases in chlorophyll concentrations. The same HPLC signal, characterized by one large main chromatographic peak, persisted throughout the experiment before it declined and eventually disappeared following the last iron infusion, in parallel with declining chlorophyll concentrations in the enriched patch. The primary chromatographic peak was identified as

flavodoxin by its absorption spectrum; there was no evidence of ferredoxin in any of the samples.

Pennate diatom clones isolated from the fertilization-induced bloom and grown in the laboratory retain the ability to make ferredoxin when iron-replete and induce flavodoxin when iron-stressed. When iron-limited, they are able to completely repress flavodoxin expression in about one day in response to iron resupply. Thus, the absence of ferredoxin during IronEx II is indicative of continuing iron limitation of the phytoplankton population despite the increases in biomass and photosynthetic efficiency observed during the experiment. The persistence of flavodoxin suggests that the iron additions were insufficient to completely relieve iron limitation. These results demonstrate the utility of the HPLC method for detection of ferredoxin and flavodoxin in natural samples and the potential for use of the ferredoxin-flavodoxin ratio as an indicator of iron stress in the field.

Introduction

Since Martin proposed the "iron hypothesis", considerable effort has been invested in the study of the relationship between iron availability and primary production (Martin 1990). Initial evidence for iron limitation of phytoplankton growth was derived primarily from the results of shipboard nutrient addition bottle bioassays (e.g. Buma et al. 1991, Coale 1991). More recently, two mesoscale iron fertilization experiments performed in the eastern equatorial Pacific provided direct proof of iron limitation of production in that area (Martin et al. 1994, Coale et al. 1996). Interpretation of bottle bioassay results is complicated by potential artifacts due to manipulation and enclosure of the natural population (e.g. Venrick et al. 1977), and mesoscale enrichments are impractical and prohibitively expensive. The difficulties inherent in these nutrient addition methods illustrate the need for a sensitive test for iron limitation that does not entail excessive manipulation or incubation of the phytoplankton community.

One of the most promising candidates for a specific assay of iron stress is the ferredoxin/flavodoxin system of proteins (LaRoche et al. 1995, Doucette et al. 1996). Under conditions of iron stress, many organisms are able to replace the iron-sulfur redox protein ferredoxin with it's non-iron-containing functional equivalent, flavodoxin. Whereas this adaptation has been studied in freshwater algae and bacteria for more than thirty years (Smillie 1965, Zumft & Spiller 1971, Peleato et al. 1994), it has only recently been definitively identified in marine phytoplankton (LaRoche et al. 1993, Doucette et al. 1996).

Results of laboratory studies of the ferredoxin/flavodoxin response support its use as an indicator of iron stress. Induction of the flavodoxin

protein is a common response to iron limitation in a diversity of marine

phytoplankton (LaRoche et al. 1995, Chapter 1). Flavodoxin expression is also

specific to iron stress, observed only in iron-limited cells and not in those

growing under nitrate, phosphate, silicate, zinc or light stress (LaRoche et al.

1993, Chapter 2). Furthermore, flavodoxin protein expression is extremely

sensitive to iron limitation, evident in cells growing at only 10-20% less than

their maximum rate (Chapter 2). These characteristics make flavodoxin alone

an excellent diagnostic of the presence or absence of iron limitation, but the

comparative abundance of flavodoxin and ferredoxin together can potentially

indicate not only the presence but also the severity of iron stress. In

Thalassiosira weissflogii grown under iron-limiting conditions, the relative

proportion of ferredoxin and flavodoxin varies with the extent of growth

impairment by iron (Chapter 2).

The aforementioned laboratory studies aimed to characterize this

method for detection of iron limitation in natural waters. The true test of

this system, however, is its application in the environment. The use of

relative flavodoxin/ferredoxin abundance to assess temporal changes in iron

stress is not without precedent. Jones (1988) used fast protein liquid

chromatography (FPLC) to measure ferredoxin and flavodoxin in

Trichodesmium collected near Barbados using a plankton net. Increases in

the ferredoxin:flavodoxin ratio of this species tracked wind events associated

with increased aeolian dust inputs. The Trichodesmium samples used by

Jones were basically monospecific and could be collected in large quantities.

For routine use in the oceans, however, a technique must be able to measure

ferredoxin/flavodoxin content in much smaller quantities of phytoplankton cells.

The IronEx II mesoscale iron enrichment experiment (Coale et al. 1996) offered a matchless opportunity to perform such a test of our analysis and detection methods for ferredoxin and flavodoxin. During the experiment, a patch of the eastern equatorial Pacific Ocean was enriched with iron and monitored for several weeks. Timing and magnitude of iron addition were controlled, allowing direct correlation with changes in phytoplankton physiology. The experimental patch was also intensively sampled for a variety of physical, chemical and biological parameters, allowing comparison of the ferredoxin/flavodoxin results with those obtained via other independent methods.

During the experiment, large volume phytoplankton samples were collected to monitor changes in the total community ferredoxin and flavodoxin abundance. If the resident phytoplankton were indeed limited by iron, iron addition was expected to stimulate growth with a corresponding increase in the ferredoxin to flavodoxin ratio of the phytoplankton community. Proteins were extracted from filters containing a mixed phytoplankton assemblage (0.7-63 μm particles) and analyzed using an HPLC detection method (Doucette et al. 1996). In addition, three pennate diatom species isolated during IronEx II were used for follow-up laboratory experiments which examined their iron physiology. The results of the IronEx II analysis alone report the physiological response of the phytoplankton to iron addition. The combined results of IronEx II and laboratory studies clarify

the role of ferredoxin and flavodoxin in cellular adaptation to iron stress in the environment.

Materials and Methods

Sample collection - IronEx II A detailed description of the IronEx II in situ fertilization experiment and its results is given by Coale et al. (1996). Briefly, a 75 km^2 patch of the equatorial Pacific ocean near 3.5°S, 104°W was enriched with iron and monitored for 19 days. Three separate iron infusions of ca 2 nM, 1 nM and 1 nM occurred on days 0, 3 and 7 of the experiment, respectively. For analysis of ferredoxin and flavodoxin, phytoplankton were collected from large volumes of seawater (100-600 liters) using a pump and filter system. An air-powered double-diaphragm pump (The Aro Corp., Bryan, OH, USA) was used to draw water through a length of reinforced PVC tubing (0.75 inch I.D.) whose intake was positioned approximately four meters away from the ship at a depth of about three meters. Seawater was pumped through a 142 mm diameter filter stack (Oceanic Industries, Buzzards Bay, MA, USA) containing a 63 μm mesh Nitex screen and a Whatman GF/F glass fiber filter in series. The glass fiber filters were then frozen in liquid nitrogen for later analysis in the laboratory. For some samples, considerable autotrophic biomass collected on the Nitex screens as well. In these cases, the cells were washed from the Nitex and frozen in liquid nitrogen as cell pellets. Otherwise, the contents of the Nitex screens (mainly larger heterotrophs) was discarded.

Protein extraction - IronEx II samples Filters were removed from liquid nitrogen and allowed to thaw partially on ice. While still frozen, the

glass fiber filters were minced with a razor blade. The minced filter was placed in a fifty-ml chamber with 35 ml of ice-cold 0.5 mm diameter glass beads, 25 ml of ice-cold chloroform and 25 ml of ice-cold extraction buffer (0.1 M sodium phosphate, 0.1 M NaCl, 1 mM EDTA, pH 7.0 with 13 mM beta-mercaptoethanol, and 1 $\mu g \cdot ml^{-1}$ each pepstatin, leupeptin and aprotinin). The filters were homogenized using a bead-beater (Bio-Spec Products, Bartlesville, OK, USA) for two, one-minute cycles in an ice and water bath with a one-minute cooling period in between. The filter slurry was transferred to a Pyrex glass bottle and centrifuged for ten minutes at 2000 x g, during which the organic and aqueous phases separated. After centrifugation, the aqueous phase was removed and concentrated to approximately two milliliters in a 3000 MW cutoff ultrafilter (Centricon-3, Amicon Inc., Woburn, MA, USA). The concentrated samples were filtered (0.2 μm) prior to analysis by HPLC.

Ferredoxin and flavodoxin analysis Samples were analyzed using a previously described HPLC method (Doucette et al. 1996). Ferredoxin and flavodoxin in cell extracts were separated by anion-exchange HPLC. Detection was performed with a Hewlett-Packard model 1050 diode array detector (Hewlett-Packard Co., Andover, MA, USA), which also allowed identification of the proteins by their absorption spectra (Figure 1). Quantification of peak areas was performed by HP ChemStation software (Hewlett-Packard) in autointegration mode.

Pennate diatom isolates Three pennate diatom clones were isolated from the iron-fertilization-induced bloom during IronEx II by E. Mann. They were rendered clonal by D. Erdner via plating (ESNW seawater medium with

1% agar) and single-cell isolations. They are identified here by their clone numbers, A3-30, 10-40A and 7-47B, pending further taxonomic identification.

Iron-replete and -deplete culturing of diatom isolates Cultures were grown in 0.2 µM filtered Vineyard Sound (MA, USA) seawater (31‰) enriched with ESNW nutrients according to Harrison (1980) with several modifications. Na_2HPO_4 was substituted in equimolar amounts for $Na_2glyceroPO_4$. and selenium (as H_2SeO_3) was added to a final concentration of 10^{-8} M. Trace metal additions were made according to Brand et al. (1983). Seawater was autoclave sterilized then enriched with sterile nutrients. Macronutrient (nitrate, phosphate and silicate) stocks were sterilized by autoclaving while iron, trace metal, selenium, EDTA and vitamin stocks were sterile-filtered (0.2 µm). Iron replete cultures contained 5 µM added Fe and 50 µM EDTA. Iron-deplete cultures contained no added Fe and 1 µM EDTA.

Cultures (2 l volume) were grown in acid-washed polycarbonate 2.8 l Fernbach flasks, maintained at 26°C on a 14:10 hour light:dark cycle at an irradiance of ca 200 $\mu E\ m^{-2}\ s^{-1}$ as measured with a photometer (Biospherical Instruments model QSP-100). Cells were harvested by filtration onto 3 µm polycarbonate filters (47 mm diameter). Despite numerous attempts, it was not possible to obtain growth rates from any of the pennate diatom clones, as the cells formed large sticky aggregates that could not be dispersed.

Protein extraction - pennate diatom cultures The polycarbonate filters containing the cells were minced and placed in a two-ml screw-capped eppendorf tube with one ml of extraction buffer (recipe above) and ice-cold 0.5 mm diameter zirconium beads. Cells were lysed by three, fifty-second cycles

in a mini-beadbeater (Bio-Spec Products, Bartlesville, OK, USA), and cooled on ice for at least one minute between cycles. The cell lysate was centrifuged for one hour at 105,000 x g and the resulting supernatant was filtered (0.45 μm) before analysis by HPLC as described above for the field samples.

Ferredoxin induction kinetics One of the three pennate diatom clones, 7-47B, was chosen for analysis of the time course of ferredoxin induction. A one liter culture of clone 7-47B in iron-deplete medium (as described above) was inoculated into nineteen liters of fresh iron-deplete medium in an acid-washed glass carboy. The cells were allowed to acclimate for two days prior to the start of the experiment. At time zero, 5 μM Fe and 50 μM EDTA were added to the carboy. Samples for ferredoxin and flavodoxin analysis were removed at six hour intervals for the next 48 hours, then again at 60 and 72 hours. Cells were harvested by filtration then extracted and analyzed by HPLC as described above.

Results

Open ocean iron enrichment

Neither ferredoxin or flavodoxin could be detected in samples collected prior to fertilization and outside of the iron-enriched patch during the experiment. HPLC chromatograms of extracts from samples collected inside the iron-fertilized patch are presented in Figure 2. During the first two days of the experiment, following iron infusion #1, no ferredoxin or flavodoxin peaks were evident. Identifiable protein signals emerged following the second iron infusion and persisted through Day 14 of the enrichment. Samples collected on Day 17 again show no chromatographic peaks.

The chromatograms from in-patch samples are characterized by one large peak accompanied by one to three much smaller peaks. This large primary peak exhibited a consistent retention time between samples and dominated the community profiles throughout the experiment. It was identified as flavodoxin by comparison of its UV-visible absorption spectrum (Figure 3) with the spectra of ferredoxin and flavodoxin from a marine diatom (Figure 1). No ferredoxin signals were observed during the course of the experiment.

Ferredoxin and flavodoxin in equatorial Pacific pennate diatom cultures

Three pennate diatom clones, A3-30, 10-40A and 7-47B, were isolated from the iron-fertilization-induced phytoplankton bloom. All three isolates were grown under iron-replete and -deplete conditions to assess their ability to synthesize ferredoxin and flavodoxin. The three organisms express only ferredoxin in high-iron medium and only flavodoxin in iron-deficient culture (Figure 4).

Ferredoxin and flavodoxin kinetics in an equatorial Pacific pennate diatom

Iron was resupplied to an iron-limited culture of clone 7-47B to determine the time required for synthesis of ferredoxin and suppression of flavodoxin expression. Changes in relative ferredoxin and flavodoxin abundance, expressed as the Fd index ($[ferredoxin]/[ferredoxin + flavodoxin]$) are shown in Figure 5. At the time of iron resupply, the culture was moderately iron-limited, with a Fd index of approximately 0.5. Over the next thirty hours, the Fd index steadily increased until flavodoxin expression was

completely absent (Fd index = 1). Flavodoxin was undetectable after 30 hours of incubation.

Discussion

Laboratory studies suggest that relative cellular ferredoxin/flavodoxin content, as measured by HPLC, is a sensitive and reliable indicator of iron limitation in marine phytoplankton (Chapters 1 and 2). The IronEx II mesoscale iron enrichment experiment provided an unprecedented opportunity to test both this biomarker system and its HPLC detection method in a well-characterized field situation. During IronEx II, HPLC analysis was successfully used to monitor the iron nutritional status of the phytoplankton community over the course of the experiment. Several follow-up laboratory studies utilizing pennate diatom cultures isolated from the fertilization-induced phytoplankton bloom further substantiate our interpretation of the IronEx II data. The results of this analysis tell us not only about the response of the phytoplankton community to iron fertilization, but also about the adaptive role of the ferredoxin and flavodoxin proteins in the environment.

HPLC of ferredoxin and flavodoxin in natural samples

The HPLC detection method worked well for samples collected from the IronEx II phytoplankton bloom, provided that sufficient biomass could be collected. Samples obtained prior to fertilization and outside of the patch during the experiment were analyzed but showed no chromatographic peaks. This complete lack of a signal indicates that the ferredoxin and/or flavodoxin

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levels in these samples were below the detection limits of the method, even though 300-600 liters had been filtered for each. The detection limit for this method is determined by the amount of protein that can be reliably identified by its UV-visible absorption spectrum using the diode array detector. This requires about 200 and 300 pmol of flavodoxin and ferredoxin, respectively, although smaller amounts will yield detectable (but not identifiable) peaks. Use of the absorption spectrum of a peak rather than retention time for identification is preferable when analyzing mixed samples such as those from IronEx II or in situations where no appropriate standards are available.

Large amounts of biomass are therefore needed to overcome the detection limits of the instrument, a problem which is exacerbated by the inefficiency of protein extraction from the filters. The glass fiber filters, which allow the filtration of large volumes of seawater, also hinder cell breakage and protein extraction. Calculations based on laboratory data for cellular flavodoxin and ferredoxin content estimate that only about 5-10% of these proteins were successfully extracted (see Appendix I). At first glance, it may seem that the use of the HPLC technique for analysis of environmental samples is hampered by the relative insensitivity of detection. However, its utility could be easily be extended through improvements in extraction techniques, reducing the required sample size by a factor of ten to twenty.

Ferredoxin and flavodoxin during IronEx II

The iron fertilization induced a "massive phytoplankton bloom" that was dominated primarily by pennate diatoms (Coale et al. 1996). Thus, the HPLC results are presumed to reflect the cellular response of these organisms.

The HPLC data track the growth of the fertilization-induced phytoplankton bloom. Peak chlorophyll concentrations in the experimental patch doubled by Day 3, before increasing to more than ten times their initial value on Day 5. Values remained elevated and peaked on Day 9, then declined to about twice their initial concentration before subsiding to background levels on Day 17 (Coale et al. 1996). Protein peaks in the HPLC chromatograms show a similar pattern (Figure 2). The first two in-patch samples were below the detection limit because phytoplankton biomass was still too low. The first identifiable peaks appeared on Day 5, coincident with huge increases in chlorophyll concentrations. A large signal persisted throughout the experiment, but then, like chlorophyll, declined then disappeared between Days 14 and 17.

The chromatograms from in-patch samples are characterized by one large main peak, identified as flavodoxin by its absorption spectrum (Figure 3). There is no clear evidence of ferredoxin induction in any of the samples. This result is somewhat unexpected, as we anticipated that ferredoxin would be resynthesized when the iron-starved cells were supplied with iron. There are several potential explanations for the observed lack of ferredoxin during the IronEx II enrichment despite the tremendous increase in biomass generated by fertilization:

1)   ferredoxin may not be extractable or detectable in natural communities

2)   the endemic phytoplankton, having evolved in a chronically low-iron environment, may lack the ability to make ferredoxin

3)   there was insufficient time for ferredoxin resynthesis

4)   there was no significant induction of ferredoxin despite iron addition

Each of these possibilities is explored below.

HPLC detection of ferredoxin in natural samples

It is possible that ferredoxin, unlike flavodoxin, is difficult to extract from natural samples. Alternatively, it may be unstable once isolated and thus not detectable. This is not the case, however, as ferredoxin has been observed in both estuarine and coastal marine phytoplankton using this same HPLC technique (Figure 6).

Ferredoxin and flavodoxin in equatorial Pacific pennate diatoms

Several pennate diatom clones isolated from the IronEx II bloom were cultured under iron-replete and -deplete conditions to assess their ability to synthesize ferredoxin and flavodoxin (Figure 4). Ferredoxin expression was evident in all three species when grown in high-iron medium. When iron-limited, all of the organisms expressed only flavodoxin. These results are consistent with those obtained from a number of other marine phytoplankton (see Chapter 1). Flavodoxin expression is somewhat variable amongst species, but all marine phytoplankton species examined to date are able to express ferredoxin. Thus, the lack of ferredoxin induction during IronEx II cannot be attributed to the inability of the native phytoplankton to synthesize ferredoxin.

Kinetics of ferredoxin and flavodoxin protein expression

One of the three diatom isolates, clone 7-47B, was used to determine the time-scale in which cells can alter their ferredoxin and flavodoxin expression. Iron was resupplied to an iron limited culture of clone 7-47B and its relative ferredoxin and flavodoxin content was measured every six hours

afterward using HPLC. Within thirty hours of iron addition, this organism was able to up-regulate its ferredoxin synthesis and degrade any residual flavodoxin (Figure 5). These findings are consistent with the results of LaRoche et al (1995), who find that natural populations of diatoms can switch on flavodoxin synthesis in about a day . Thus, the IronEx II experiment, which was monitored for nineteen days, provided ample time for cells to respond to iron addition by modifying their protein expression.

Absence of ferredoxin induction during IronEx II

The final, and most plausible, explanation for the absence of ferredoxin expression during IronEx II is that the cells simply did not synthesize ferredoxin to any significant degree. Although no ferredoxin peaks were identified in any of the samples, it is possible that ferredoxin was present below the identification limit of about 300 pmol per sample. The largest IronEx II sample analyzed contained the biomass from 1063 liters of water collected during the height of the phytoplankton bloom (chromatogram not shown). The area of its flavodoxin peak corresponds to approximately 1100 pmol of flavodoxin (using a standard curve for flavodoxin from Chlorella fusca). Thus, during IronEx II, there was less than three molecules of ferredoxin for every eleven molecules of flavodoxin. If ferredoxin was present, it was at an extremely low level.

Persistence of iron limitation during IronEx II

There is no doubt that phytoplankton growth in the equatorial Pacific is limited by iron, based upon the massive biological response to iron

fertilization (Coale et al. 1996). The biochemical data presented here support this conclusion, but also indicate that the iron addition was insufficient to completely relieve physiological iron limitation. The presence of flavodoxin has been shown to be a sensitive and specific indicator of iron limitation (see Chapters 1 and 2). Flavodoxin expression in the absence of ferredoxin is symptomatic of severe iron limitation, a conclusion that is consistent with the relatively low growth rates observed during the experiment. From the results of dilution experiments, Constantinou et al. (1996) calculated a maximal diatom growth rate during IronEx II of 1.8 divisions·day^{-1}. This is slow relative to the 3.3 divisions·day^{-1} measured by Fryxell and Kaczmarska (1994) for similar pennate diatoms in iron enrichment bottles.

The apparent contradiction between the lack of ferredoxin resynthesis and the substantial biological response observed during IronEx II emphasizes the role of ferredoxin and flavodoxin in cellular adaptation to iron stress. Flavodoxin substitution provides a significant decrement in cellular iron requirements. Iron contained in ferredoxin may account for approximately 30-40% of the Fe quota of an iron replete Thalassiosira weissflogii cell (Appendix II). Thus, replacement of ferredoxin with flavodoxin allows cells to easily and significantly lower their iron requirements. For diatoms, which exhibit extremely high maximum growth rates, utilization of flavodoxin allows rapid growth under sub-optimal iron supply.

Cellular ferredoxin and flavodoxin content is also extremely sensitive to iron stress. In Thalassiosira weissflogii, flavodoxin synthesis is induced when cells are only mildly limited by iron. Ferredoxin content decreases and flavodoxin levels rise when cells are grown at 90% of their maximum rate

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(Chapter 2). Thus, ferredoxin may be one of the first cellular components to respond to iron limitation, representing an internal labile iron pool for phytoplankton. Conversely, it may be one of the last components to be reconstituted when iron is resupplied. More essential compounds such as light harvesting pigments and photosynthetic reaction centers, which do not have equivalent substitutes, are apparently resynthesized before ferredoxin. This is evident from the increases in cellular chlorophyll content (Coale et al. 1996) and photochemical efficiency (Behrenfeld et al. 1996) observed during IronEx II in the absence of ferredoxin induction.

The results presented here also indicate that the adaptive role of flavodoxin that has been observed in the laboratory is relevant to conditions in natural waters. Flavodoxin expression is an extremely sensitive indicator of iron limitation, responding immediately when iron availability drops below that required for optimum growth. This may be a moot point for populations in areas such as the Equatorial Pacific; it is unlikely that they receive iron inputs that are even a fraction of that required for fully replete growth. The IronEx II iron additions of 2, 1 and 1 nM were slightly more than what would be expected from natural inputs, which suggests that organisms in the study area may always be reliant upon flavodoxin. For these organisms, flavodoxin substitution is an essential adaptation that allows them to persist in iron-poor environments, respond rapidly to periodic inputs, and grow quickly on very small amounts of iron.

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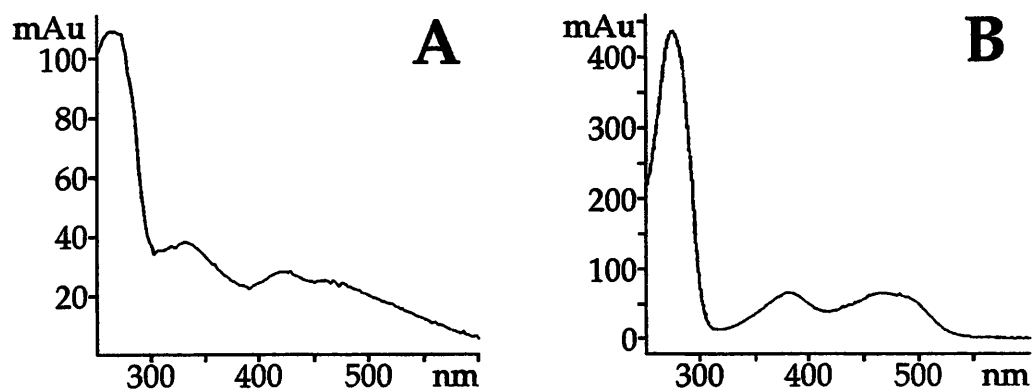
FIGURE 1 - UV-visible absorption spectra of (A) ferredoxin and (B) flavodoxin from the marine diatom Thalassiosira weissflogii. These spectra are representative of those obtained from ferredoxin and flavodoxin in a variety of marine eukaryotic phytoplankton.
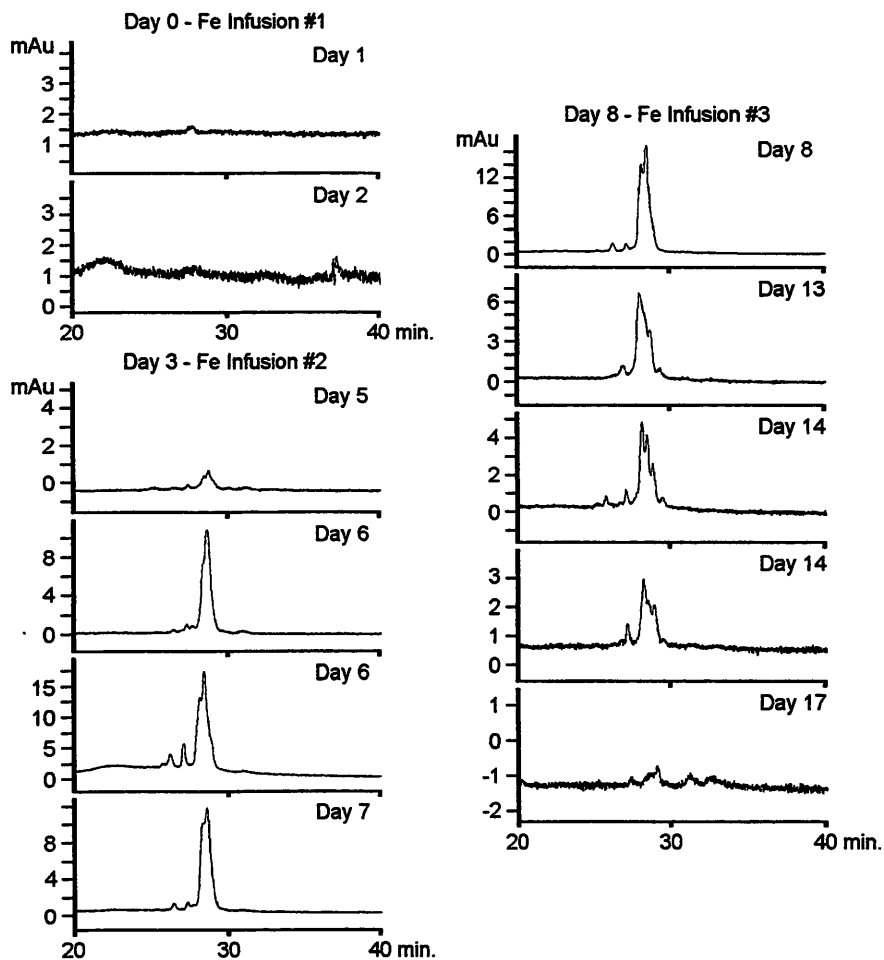
FIGURE 2 - HPLC chromatograms of samples collected in the IronEx II iron-enriched experimental patch. Collection date is given as days after the first iron infusion. On Day 6, two samples were collected, the first at dawn and the second at dusk. On Day 14, two samples were again collected, one before dawn and another at mid-morning. Ferredoxin and flavodoxin from marine phytoplankton elute between 25-30 minutes in this system, and the chromatograms have been formatted to cover the relevant temporal region.

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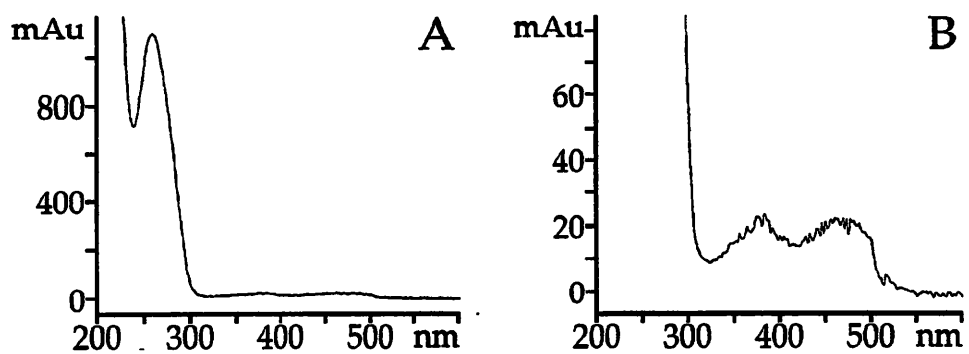
FIGURE 3 - Representative spectrum of the major peak in the IronEx II chromatograms shown (A) full scale and (B) enlarged to show features in the 300-600 nm range. This component is clearly identified as flavodoxin (see Figure 1).
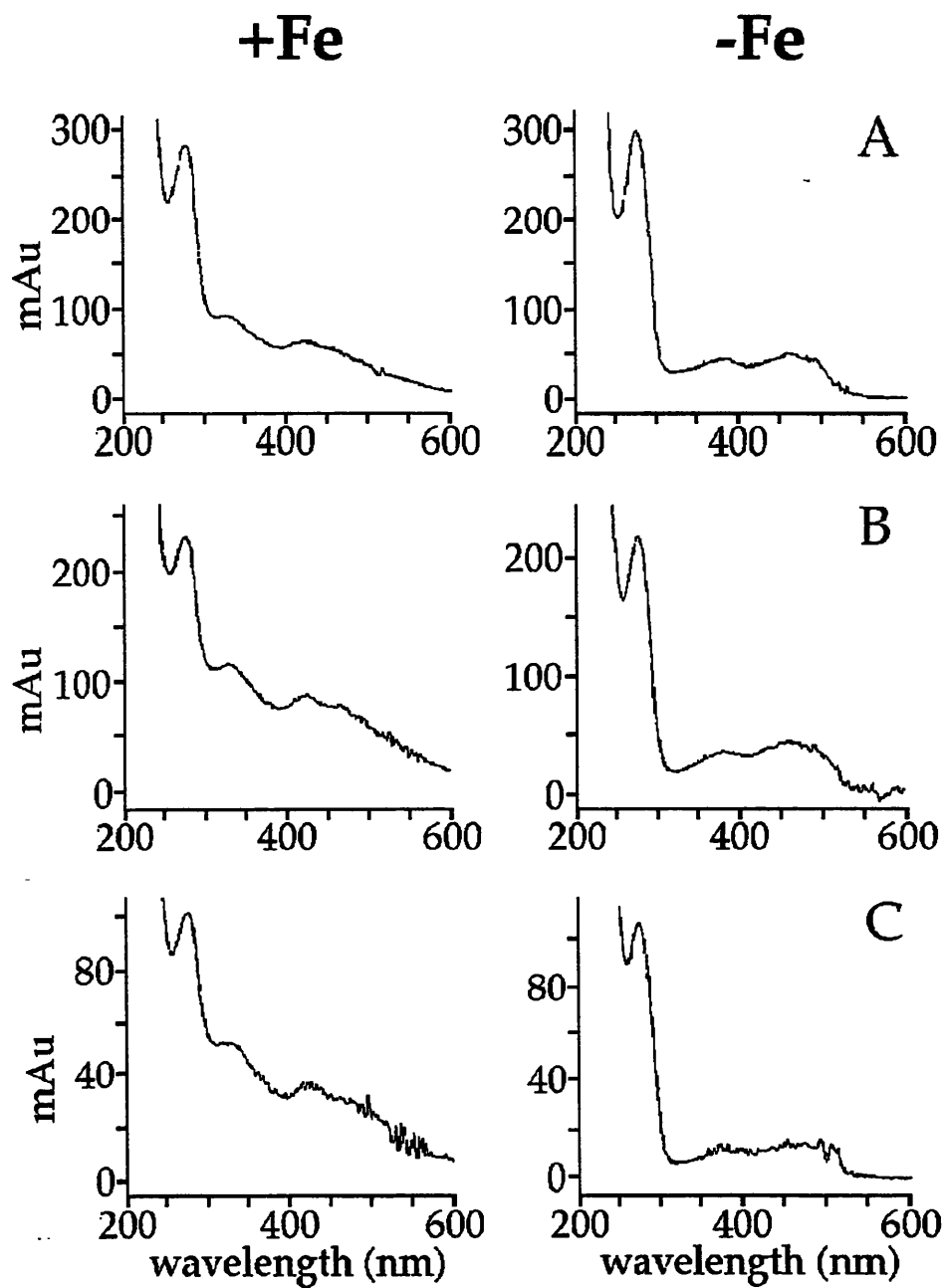
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FIGURE 4 - UV-visible absorption spectra from HPLC analysis of pennate diatom clones (A) 10-40A, (B) 7-47B and (C) A3-30 grown under iron-replete (+Fe) and -deplete (-Fe) conditions.
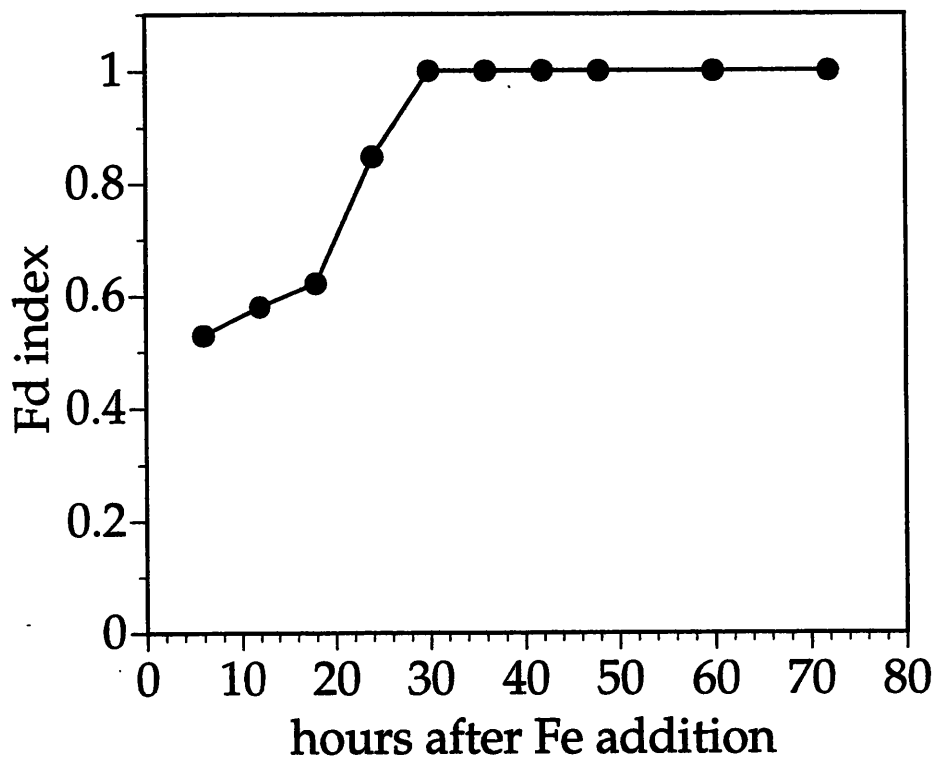
FIGURE 5 - Changes in the relative ferredoxin and flavodoxin abundance in iron-limited culture of clone 7-47B after resupply of iron. Ferredoxin and flavodoxin content is expressed as the Fd index, a ratio of HPLC peak areas (Fd index = ferredoxin peak area/sum of ferredoxin+flavodoxin peak areas)
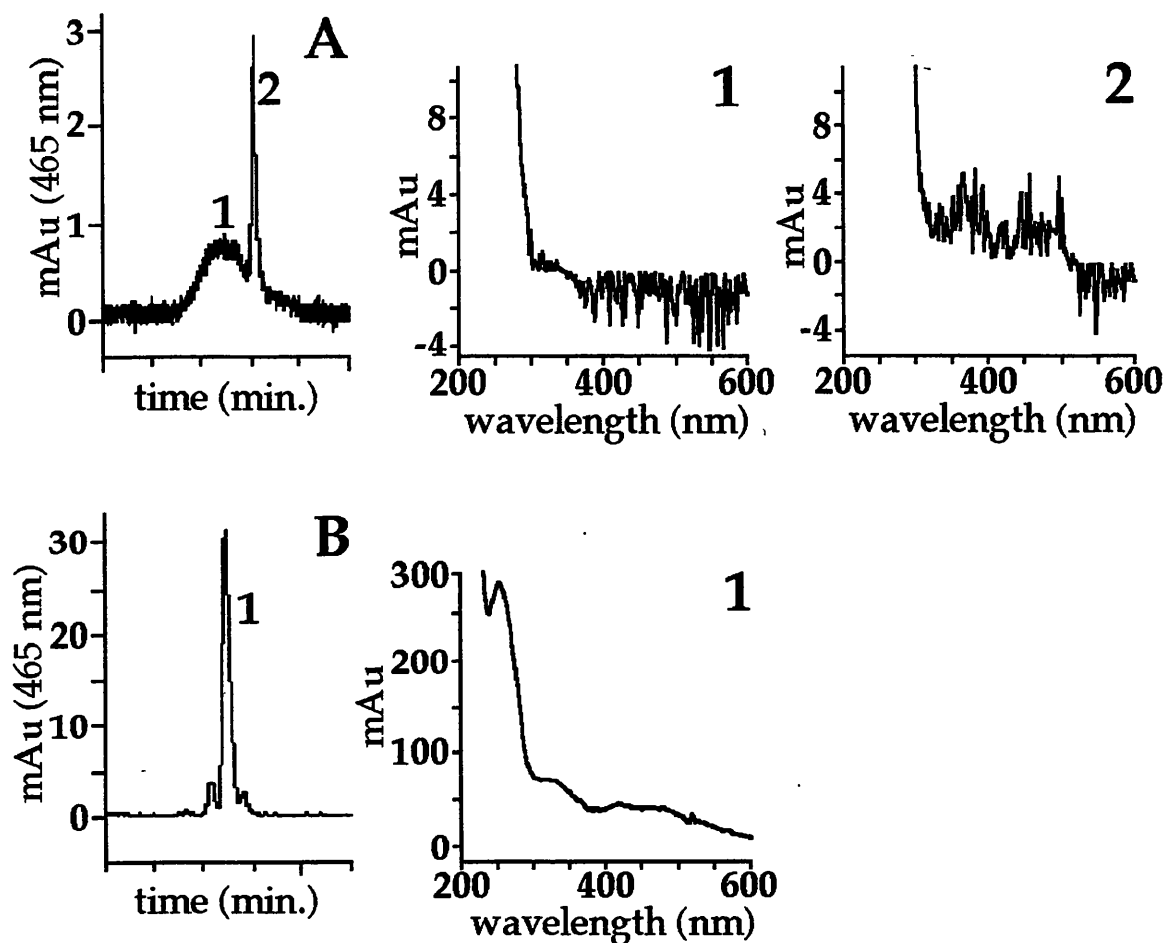
FIGURE 6 - Chromatograms and corresponding peak absorption spectra of plankton samples collected from (A) coastal marine and (B) estuarine environments. Samples were collected with a 20 μm plankton net from (A) Eel Pond, Woods Hole, MA USA in March 1997 and (B) Parker River, Ipswich, MA USA in September 1996. The chromatograms have been formatted to show the relevant 10 minute temporal region of ferredoxin/flavodoxin elution.

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Chapter 4

Preliminary Comparison of Biochemical (Fd index) and Biophysical (F_v/F_m) Indices of Iron Limitation

Abstract

A laboratory analogue of the IronEx II mesoscale iron enrichment experiment was conducted to compare changes in biochemical (Fd index) and biophysical (F_v/F_m) indicators of iron stress during recovery from iron limitation. Both the Fd index, which relates the abundance of the iron-regulated ferredoxin and flavodoxin proteins, and F_v/F_m, a nutrient-sensitive measure of the photochemical yield, were used during IronEx II to monitor the phytoplankton community response to fertilization. During IronEx II, F_v/F_m increased to near maximal values after iron addition, while the Fd index remained uniformly zero. Thus, results obtained with the two methods seem contradictory with respect to the physiological state of the phytoplankton following iron addition.

During the laboratory experiment, a large volume iron-limited culture of the marine centric diatom Thalassiosira weissflogii was monitored at six-hour intervals for changes in pigments, growth rate, F_v/F_m and Fd index for 72 hours following iron resupply. Measurements of F_v/F_m were made with both a pump-during-probe flow cytometer (PDP-FCM) and a pulse amplitude modulated (PAM) fluorometer. Whereas both measures of F_v/F_m showed significant differences after iron addition as compared to a non-iron-enriched control culture, the magnitude of the measured increase in F_v/F_m was greater with the PAM fluorometer than with PDP-FCM. This is likely due to differences in the time-scale of the two measurements (100 μs in PDP-FCM vs. 600 ms in PAM), which leads to differences in the assessment of F_m between the two methods.

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The results of the Fd index and PDP-FCM analyses also show similar pattern but varying magnitude in response to iron resupply. After iron addition, the cells traversed half of the total range of Fd indices (0.5 to 0.9) but showed only a 10% increase in F_v/F_m (0.63 to 0.68). In contrast, during IronEx II, F_v/F_m increased from 0.26 to 0.56 while the Fd index was uniformly zero. The laboratory and field data were combined to develop a conceptual model of the covariation of F_v/F_m and Fd index which describes a complementary relationship between the two measures; F_v/F_m changes little as the Fd index decreases from 1 to 0 (this study), but declines sharply in the range where Fd index = 0 (IronEx II). These results suggests that photochemical systems are affected by iron limitation only after the cell's adaptive capacity, in the form of ferredoxin, is exhausted. If this is the case, flavodoxin induction represents a more sensitive indicator of iron stress than F_v/F_m. However, F_v/F_m will respond to changes in iron stress in the region where the Fd index is uniformly zero. Thus, a combination of the two measures should reveal changes in the severity of iron stress across the full response range. Further studies are nonetheless required to test and validate this model of biophysical and biochemical response.

Introduction

The debate over potential iron limitation of oceanic productivity was finally resolved, at least in the case of the equatorial Pacific, by the success of the IronEx II mesoscale iron enrichment experiment in 1995 (Coale et al. 1996). During that experiment, a tremendous phytoplankton bloom resulted from the enrichment of a 72 km^2 patch of the eastern equatorial Pacific ocean with nanomolar quantities of iron. Chlorophyll concentrations increased thirty-fold and photosynthetic efficiency, which was very low prior to fertilization, reached near-maximum levels. The tremendous biological response to fertilization unequivocally demonstrated iron limitation of the extant phytoplankton community. Both IronEx II and its predecessor IronEx I (Martin et al. 1994) were performed in part to address criticisms regarding the interpretation of in vitro enrichment experiments or "bottle bioassays" (e.g. Buma et al. 1991, Coale 1991). Such assays are difficult to perform in a trace-metal-clean fashion (Fitzwater et al. 1982) and suffer from potential artifacts due to containment of the natural community (e.g. Venrick et al. 1977). Due to their unavoidably small scale, translation of bottle bioassay results to whole ecosystem response is problematic at best.

The IronEx I and IronEx II iron enrichments provided invaluable information on the relationship between iron, primary productivity and carbon dioxide uptake - data difficult to obtain using conventional techniques. While large-scale fertilizations such as these are the most direct way to understand ecosystem response, they are not a practical method for assessing iron limitation. Mesoscale fertilizations are controversial, expensive, logistically difficult and not possible on small temporal or spatial scales.

There is still a need for rapid, non-invasive analytical methods that can assess the physiological state of the phytoplankton in situ. This need has provoked the search for so-called "diagnostic indicators", defined by Falkowski et al. (1992) as "signals (or procedures) that empirically identify the symptoms of an environmental constraint on phytoplankton growth rates". Two proposed diagnostics of iron limitation are the ferredoxin (Fd) index and the ratio of variable to maximum fluorescence (F_V/F_m).

The Fd index uses the relative cellular abundance of two proteins, ferredoxin and flavodoxin, as a measure of the severity of iron stress (Chapters 2 and 3). When iron becomes limiting, some organisms are able to functionally replace ferredoxin, a common iron-sulfur redox protein, with the non-iron-containing flavodoxin (e.g. Smillie 1965, Vetter & Knappe 1971, Peleato et al. 1994). This response is common amongst oceanic phytoplankton (Chapter 1) and seems to be specific to iron limitation (Chapter 2). Thus, these two proteins serve as reporters of the cells iron nutritional history. The presence or absence of flavodoxin in a phytoplankton cell or community serves as a qualitative indicator of iron stress. The relative abundance of both ferredoxin and flavodoxin, expressed here as the Fd index= $[ferredoxin]/[ferredoxin + flavodoxin]$, varies with the extent of growth limitation by iron. Changes in the Fd index should therefore be indicative of alleviation or exacerbation of iron stress.

Both the "presence/absence" and "relative abundance" approaches have been utilized in the field. The presence of flavodoxin alone has been used as an indicator of iron stress on the Great Barrier Reef (Entsch et al. 1983)and in the subarctic Pacific (LaRoche et al. 1996). The ratio of ferredoxin

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and flavodoxin has been correlated with changes in iron stress in Trichodesmium colonies in the Caribbean (Jones 1988), and also used to monitor the response of the phytoplankton community to iron addition during IronEx II (Chapter 3). The techniques developed for detection of these proteins, antibodies (LaRoche et al. 1995) and FPLC (Jones 1988) or HPLC (Doucette et al. 1996), are rapid and require no incubation or manipulation of the cells.

Another proposed measure of the cellular response to iron stress is the ratio of variable to maximum fluorescence (F_v/F_m). Since the time course of fluorescence induction was first elucidated (Kautsky & Hirsch 1931), it has become one of the most important tools in photosynthesis research. Fluorescence induction describes the increase in fluorescence upon illumination of a dark-adapted sample; it varies from F_o, the value observed immediately upon illumination, to a maximum value, F_m. Almost all chlorophyll fluorescence originates in photosystem II (PSII), and the rise from F_o to F_m reflects increased energy dissipation via fluorescence as PSII reaction centers become progressively "closed". Closure of PSII reaction centers occurs when the primary electron acceptor pool (Q) becomes reduced, effectively blocking further electron flow until Q is reoxidized. The ratio of variable fluorescence ($F_v = F_m-F_o$) to maximum fluorescence (F_m) is a measure of the maximum potential quantum yield of photochemistry (Butler 1978). As such, it is sensitive to environmental factors that affect the components of the photosynthetic electron transport. Both nitrogen and iron limitation cause a loss of certain proteins or cofactors in PSII reaction centers, with corresponding reductions in F_v/F_m proportional to the severity of nutrient

stress (e.g. Greene et al. 1992, Falkowski et al. 1994). Iron and nitrogen stress can be distinguished from each other by their differential effects on the turnover time of electrons, which can also be calculated from the fluorescence induction data (Falkowski et al. 1992). The practical applications of this method have been greatly extended by the design of instruments for the measurement of F_v/F_m in the field (Kolber et al. 1988, Falkowski & Kolber 1993). To date, it has been used to assess photosynthetic efficiency in areas as diverse as the Gulf of Maine (Kolber et al. 1990) and the equatorial Pacific (Kolber et al. 1994).

Both the Fd index and F_v/F_m methods were utilized during the IronEx II mesoscale iron enrichment experiment to monitor the phytoplankton community response to fertilization. Increases in photosynthetic efficiency, measured using a fast repetition rate (FRR) fluorometer (Falkowski & Kolber 1995), were detected inside the experimental patch within one day of iron addition (Behrenfeld et al. 1996). F_v/F_m values remained elevated during fertilization, achieving levels near the theoretical maximum, then declined after the last iron infusion. These results demonstrate that the extant community was physiologically limited by iron.

Parallel measurements of ferredoxin and flavodoxin, measured using HPLC, support the assertion of iron limitation of the phytoplankton population (Chapter 3). However, all samples collected from the fertilization-induced phytoplankton bloom had a Fd index of 0, i.e. the cells contained no ferredoxin. This implies that the phytoplankton were still severely limited by iron, despite three iron infusions that triggered an immense bloom and enhanced the photosynthetic efficiency of the phytoplankton. Results

obtained with the two methods seem contradictory with respect to the physiological state of the phytoplankton after fertilization. By the standards of F_V/F_m, the cells were functioning at near maximum levels. Measurements of ferredoxin and flavodoxin, however, suggest that the cells were still severely limited.

This study aims to compare these Fd index and F_V/F_m methods in a controlled laboratory situation, to better understand the relationship between biochemical and biophysical responses to iron limitation. This was achieved using a laboratory analogue of the IronEx II experiment. A twenty liter, iron-limited culture was enriched with iron and monitored at six hour intervals for three days. The iron-amended culture was analyzed for cellular chlorophyll content, growth rates and ferredoxin and flavodoxin content. Photosynthetic measurements of both a control culture and the iron-enriched culture were performed using a pump-during-probe flow cytometer (PDP-FCM) and also a pulse amplitude modulated (PAM) fluorometer. While the experiment did not proceed exactly as planned, it provides compelling preliminary data on the differences between the biochemical and biophysical techniques. These results also highlight the need for further characterization of this relationship, through experiments of similar design but greater scope.

Materials and Methods

Cultures   The marine centric diatom Thalassiosira weissflogii (Grun.) Fryxell et Hasle (clone ACTIN) was used for this study.

Growth of phytoplankton   Cultures were grown in 0.2 μM filtered Vineyard Sound (MA, USA) seawater (31‰) enriched with ESNW nutrients

according to Harrison et al. (1980) with several modifications. Na_2HPO_4 was substituted in equimolar amounts for $Na_2glyceroPO_4$. and selenium (as H_2SeO_3) was added to a final concentration of 10^{-8} M. Trace metal additions were made according to Brand et al. (1983). To yield iron-limited cultures, iron and EDTA were added to 100 nM and 1 µM, respectively. Seawater was autoclave- or filter- (0.2 µm) sterilized then enriched with sterile nutrients. Macronutrient (nitrate, phosphate and silicate) stocks were sterilized by autoclaving while iron, trace metal, selenium, EDTA and vitamin stocks were sterile-filtered (0.2 µm). All cultures were maintained at 20°C on a 14:10 hour light:dark cycle at an irradiance of ca 175 µE m^{-2} s^{-1} as measured with a photometer (Biospherical Instruments model QSP-100).

Experimental procedures The inoculum culture (one liter volume) was grown in an acid-washed glass 2.8 l Fernbach flask as described above. When cell densities reached approximately 3×10^3 cells·ml^{-1}, the inoculum was added to 19 liters of fresh medium in a 20 liter glass carboy. The cells were acclimated for two days in the carboy, during which time the cell densities increased to approximately 10^4 cells·ml^{-1}. Immediately prior to iron addition, samples were withdrawn for cell counts, ferredoxin and flavodoxin analysis, chlorophyll determinations and photosynthetic measurements (time zero). Two liters of culture were removed and placed in a sterile glass Fernbach flask, to serve as the control culture for the photosynthetic rate measurements.

The experiment was initiated by the addition of 10 µM iron and 100 µM EDTA (as a premixed FeEDTA solution) to the experimental flask. Iron addition, and thus time "zero", was timed to occur one hour after the onset of

the 14 hour light cycle in the incubator. Every 24 hour light:dark cycle included 3 "daytime" samples and one during the "night", all spaced at 6 hour intervals. Samples were taken every 6 hours for the first 48 hours then again at 60 and 72 hours. The experimental (iron added) carboy was sampled for cell counts, ferredoxin and flavodoxin analysis, chlorophyll determinations and photosynthetic measurements, whereas the control flask, because of its small volume, was only sampled for cell counts and photosynthetic rate measurements.

Growth rates Cell densities were determined by four replicate microscopic counts of Utermohl's preserved samples in a Fuchs-Rosenthal hemacytometer. Growth rates during exponential phase were calculated from linear regressions of the natural log of cell density versus time.

Chlorophyll a determinations Chl a was measured in triplicate samples. Cells were collected by gentle filtration onto Millipore SSWP membranes (3 µm, 25 mm diameter) and frozen in liquid nitrogen until analysis. Filters were extracted in 100% acetone for 24 hours at 4°C in the dark. Before measurement, samples were diluted to 90% acetone and allowed to warm to room temperature. Chlorophyll was measured fluorometrically using a Turner Designs fluorometer (model 10-AU) that had been calibrated with pure chlorophyll a (Sigma) using the extinction coefficients from Jeffrey and Humphrey (1975).

Protein extraction For ferredoxin and flavodoxin measurements, approximately one liter of culture was collected onto a 3 µm polycarbonate filter (47 mm diameter) and frozen at -80°C until analysis. Cells and their filters were placed in a 2 ml tube with extraction buffer and zirconium beads.

Cells were ruptured by three, fifty second cycles in a mini-beadbeater (Biospec Instruments, Bartlesville, OK, USA). The cell lysate was centrifuged for one hour at 105,000 x g and the supernatant was filtered (0.45 μm) and injected into the HPLC.

HPLC of ferredoxin and flavodoxin Ferredoxin and flavodoxin abundance was determined by a previously described HPLC method (Doucette et al. 1996). Ferredoxin and flavodoxin in cell extracts were separated by anion-exchange HPLC. Detection was performed with a Hewlett-Packard model 1050 diode array detector (Hewlett-Packard Co., Andover, MA, USA), which also allowed identification of the proteins by their absorption spectra. Quantification of peak areas was performed by HP ChemStation software (Hewlett-Packard) in autointegration mode. The relative abundance of ferredoxin and flavodoxin is expressed as the "Fd index", a ratio of their HPLC peak areas (see also Chapter 2):

$$\text{Fd index} = {}^{[\text{ferredoxin}]}/_{[\text{ferredoxin} + \text{flavodoxin}]}$$

Pump-during-probe flow cytometry The design of the pump-during-probe flow cytometer is described in Olson et al (1996). Cells were dark-adapted for 2-5 minutes prior to analysis. As with traditional flow cytometry, cells were injected into a stream of sheath fluid which carried them in a single file through the assay region. As a cell entered the assay region, it passed through an infrared laser beam which triggered the excitation laser (488 nm light) pulse. The focus width of the laser beam and the sample flow rate were controlled such that each cell was exposed to the blue excitation light for 100 μs. Fluorescence induction rise times of 30-100 μs were obtained by changing the laser output power. The fluorescence emitted by the cell was

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normalized to fluorescence data obtained from 9.3 μm fluorescent latex microspheres and plotted as a function of time. The resultant time-course of fluorescence yield was fit to a theoretical model using least-squares linear regression (see Olson et al. equation 7). Values for the maximum (F_m) and minimum (F_o) fluorescence were derived from the model equation and used to calculate the maximum potential quantum yield of photochemistry as:

$$(Fm - Fo)/Fm = Fv/Fm$$

PDP-FCM measurements were not performed for cells collected at "night" when incubator lights were off. Measurements were also not conducted at the 72 hour time point for control or experimental cells, or at the 60 hour time point for control cells.

PAM fluorometry A Pulse Amplitude Modulated (PAM) fluorometer (Heinz Walz, Effeltrich, Germany) was used to measure photosynthetic parameters in both the control and iron-amended cultures. The fluorometer was equipped with a high sensitivity cuvette, which allowed detection of adequate fluorescence signals when using a low intensity measuring light beam (Schreiber et al. 1986). The minimum fluorescence yield (F_o) was measured after 15 minutes of dark adaptation, to alleviate any photochemical or non-photochemical quenching. The maximum fluorescence level (F_m) was then determined using a 600 ms flash from a Schott saturation flash lamp. Increases in flash duration and/or intensity led to detectable decreases in the maximum fluorescence level. The maximum quantum yield for stable charge separation was calculated as:

$$(Fm - Fo)/Fm = Fv/Fm$$

PAM fluorometer measurements were not conducted on samples collected during the "night", when the incubator lights were off.

Results

Cell growth with and without added iron

Both the control and iron-amended cultures exhibited a period of exponential growth from about 0 to 48 hours followed by a plateau in cell numbers (Figure 1). Growth rates in the experimental and control cultures were similar during this exponential phase ($\mu = 0.45 \cdot d^{-1}$ vs. $0.41 \cdot d^{-1}$), although the iron-enriched culture achieved a higher final cell density.

Changes in chlorophyll and ferredoxin index after iron addition

Both chlorophyll per cell and the Fd index showed an overall increase during the course of the experiment (Figure 2). Cellular pigment content nearly doubled between 0 and 72 hours, from 4.01 to 7.72 $pg \cdot cell^{-1}$. There was also a diel pattern evident in the chlorophyll data, with decreases in chlorophyll per cell during the dark period. The Fd index, in contrast, did not seem to be sensitive to the light:dark cycle. Changes in the Fd index paralleled cell growth (Figure 3). The Fd index increased from 0.52 to 0.89 in the first 48 hours of the experiment, during the exponential phase of cell growth. After 48 hours, growth rate slowed with a corresponding decrease in the Fd index.

Photosynthetic efficiency in control and iron-amended cultures

The maximum quantum yield of photochemistry (F_v/F_m) was determined in the control and experimental cultures using both pump-during-probe flow cytometry (PDP-FCM) and pulse-amplitude-modulated (PAM) fluorometry. Both methods measured a significantly greater F_v/F_m in response to iron addition as compared to the control (Figure 4). The maximum value measured by PDP-FCM (t=48) was 0.68 in the experimental culture compared to 0.58 in the control. Using PAM fluorometry, maximum values of 0.73 and 0.54 were measured for the iron-amended and control cultures, respectively, also at t=48.

The results of the two methods at the initial and final time points diverged somewhat. F_v/F_m values measured with the PDP-FCM are lower at 60 hours (0.56 +Fe, 0.57 control) than at time zero (0.63) for both the control and experimental cultures . The data obtained from the PAM fluorometer, however, showed similar F_v/F_m for control and +Fe cells at the beginning (0.58) and end (0.61 +Fe, 0.57 control) of the experiment.

The absolute values of F_v/F_m obtained using the two methods were somewhat different. To eliminate this effect and facilitate comparison of the two data sets, F_v/F_m measurements were normalized to their initial values for both control and iron-amended cultures (Figure 5). The magnitude of the change observed upon iron addition is greater with the PAM fluorometer as compared to the PDP-FCM. The maximum value obtained with PDP-FCM (t=36 hours) was 108% of the initial value, as opposed to a maximum of 127% of initial using the PAM fluorometer (t=48 hours). The final values of F_v/F_m measured with the PDP-FCM and the PAM fluorometer were 89% and 106%

of initial values, respectively. In the control culture, the final value of F_V/F_m measured with PDP-FCM was slightly lower than that obtained from PAM fluorometry, 90% vs. 99% of the initial values.

Changes in Fd index and F_V/F_m in response to iron addition

The response of the Fd index to iron addition showed a pattern similar to that of F_V/F_m as measured by both PDP-FCM and PAM fluorometry (Figure 6). The Fd index, F_V/F_m (PDP) and F_V/F_m (PAM) all increased throughout the first 48 hours following iron addition, which was also the exponential growth phase of the culture. Between 48 and 72 hours, all three measures decreased, although F_V/F_m declined more rapidly than the Fd index. The maximum increase in F_V/F_m was also proportionately smaller than the corresponding change in Fd index. F_V/F_m changed by 8 to 27% of its initial value while the Fd index almost doubled.

Discussion

This study was devised to investigate the differences in the photochemical and biochemical responses of the phytoplankton observed during IronEx II. The goal was to characterize the photosynthetic response of iron-limited *Thalassiosira weissflogii* to iron resupply over the full range of Fd indices. At the start of the experiment, however, the cells were only mildly iron-stressed, as evidenced by a Fd index of 0.5. The results nonetheless provide a detailed description of the relationship between F_V/F_m and the Fd index as the latter varies from 0.5 to 0.9, over about half of the intended range, following iron addition. In contrast, Fd indices measured

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during the IronEx II experiment, the inspiration for the present study, remained uniformly zero. By combining the results of this analysis with those of the IronEx II enrichment, a model of the inferred variation in F_v/F_m over the full range of Fd indices was constructed. Further study is necessary to test and verify this model; suggested modifications and improvements on the present experimental design are discussed below.

Iron limited growth

The magnitude of the iron addition used during this experiment (10 μM) is much greater than that of IronEx II (1-2 nM). A higher iron level was chosen for a number of reasons. A secondary aim of the study was to determine the time scale of changes in protein expression, thus we wanted the cells to switch from flavodoxin to ferredoxin expression. It was also desirable to examine the biophysical response over the entire range of Fd index values. The organism used for this study, Thalassiosira weissflogii, has high iron requirements relative to equatorial Pacific phytoplankton (Chapter 1). Therefore, it was necessary to add sufficient iron to not only relieve limitation but also to make the cells entirely iron-replete.

Unfortunately, the cells were not fully iron-limited at the start of the experiment, thus the analysis did not cover the full range of Fd indices as planned. Nonetheless, results of the biophysical and biochemical assays suggest that the cells responded positively to iron addition. Significantly higher F_v/F_m values were measured both by PDP-FCM and PAM fluorometry in the experimental culture as compared to the control (Figure 4). In addition, the iron-enriched culture exhibited nearly a doubling in its Fd index, from

~0.5 at the beginning of the experiment (indicative of moderate iron stress) to ~0.9 by the end of exponential growth (Figure 3).

The experiment encompassed not only the exponential phase of growth (t=0-48 hours) but also a plateau stage (t=48-72 hours). The decline in growth rate between 48 and 72 hours coincided with a drop in the Fd index, suggesting that the cells were once again becoming iron-stressed. Thus, the period from 0 to 48 hours represented the response of the cells to iron resupply. After 48 hours, the process was reversed, and the cells adapted to the shortage of iron. During the 48 hour period of exponential growth when cells were becoming more iron-replete, about half of the intended range of the Fd index was examined.

Comparison of fluorometric methods

The biophysical response to iron addition observed with the PDP-FCM and the PAM fluorometer showed similar patterns (Figure 4A). Both exhibit a general increase in the maximum quantum yield of photosynthesis during the first 48 hours (exponential growth). The primary difference between the results of the two methods is the magnitude of the change in F_v/F_m observed in response to iron resupply. In Figure 5, all F_v/F_m measurements have been normalized to their initial values to facilitate comparisons between the two methods. The increase in F_v/F_m observed with the PAM fluorometer is ~30% over initial, as compared to ~10% with the PDP-FCM (Figure 5A). The design of the two instruments is similar in that they both utilize a single, saturating flash of light to induce fluorescence. The length of this flash, however, is quite different: 100 μs in PDP-FCM and 600 ms in PAM fluorometry. It is this

flash-duration difference that leads to the observed differences in the magnitude of F_V/F_m.

The higher F_V/F_m measured by the PAM fluorometer compared to the PDP-FCM arises from differences in the assessment of maximum fluorescence F_m. When a dark-adapted cell is exposed to light, the increase in fluorescence with time shows a polyphasic rise (e.g. Strasser et al. 1995). This so-called "induction curve" begins with a fast, or photochemical, rise from the initial fluorescence F_o (or O) to an elevated level, termed J by Strasser and Govindjee, which occurs in less than 2 ms. This is followed by a slow or thermal rise to a yet higher level I within 100 ms. The final rise to the maximum value, F_m or P, occurs on the time scale of less than 1 s. The fast phase of fluorescence induction is attributed to saturation of photochemical quenching i.e. closure of PSII reaction centers via reduction of the primary stable acceptor Q_A to Q_A-. The subsequent fluorescence rise from the J to I levels is well-documented but not as well understood. Several hypotheses have been presented to explain this slow phase of fluorescence induction (see e.g. Dau 1994, Strasser et al. 1995, Schreiber & Krieger 1996).

Both the PDP-FCM and the PAM fluorometer record F_o similarly. The PDP-FCM, however, records the F_m value obtained during 100 µs of continuous illumination, the J level. This corresponds to the strictly photochemical component of the fluorescence induction curve. It requires more than 100 µs for electron transfer to proceed beyond Q_A, thus the light flash during PDP-FCM provides enough time for each PSII to "fill-up" once (Olson et al. 1996). The PAM, in contrast, measures F_m after 600 ms, at the I or P level. During this time, electron flow proceeds past Q_A, and a given PSII

center can fill-up or "turnover" more than once. The PDP-FCM measurement records only fluorescence associated with the saturation of photochemical capacity. The fluorescence measured by the PAM fluorometer may include that arising from suppression of both photochemical and non-photochemical quenching.

There is considerable debate in the photosynthesis community regarding the "proper" level for the assessment of maximal fluorescence. It can be argued that J is an underestimate due to the presence of nonphotochemical quenching (Schreiber et al. 1986). On the other hand, measurements made on long time scales lead to multiple turnovers of PSII and incorporate effects other than photochemistry which occur downstream of PSII (Ting & Owens 1992, Büchel & Wilhelm 1993). Despite theoretical differences in measurement, the two methods yield results which are qualitatively similar, both here and in the literature (Schreiber & Krieger 1996). Thus the lack of true mechanistic understanding of F_v/F_m does not interfere with the goal of this study, which was to compare the time course of changes in F_v/F_m relative to Fd index during recovery from iron limitation. In this context, it is possible to note the observed differences in the magnitude of the response measured by PDP-FCM and PAM fluorometry and leave the discussion of causes and mechanisms to those more familiar with the subtleties of the methods.

Biochemical vs. biophysical methods

Changes in the Fd index of the iron-amended culture followed the same general pattern as F_v/F_m (Figure 6). All of the indices increased during

the first 48 hours, corresponding to exponential growth of the culture. Between 48 and 72 hours, cell growth slowed and the Fd index and both F_v/F_m measures declined as the cells presumably ran out of iron and entered plateau phase. PAM fluorometric measurements of F_v/F_m, however, tracked the variation in Fd index more closely than those obtained using PDP-FCM. Both Fd index and F_v/F_m (PAM) gradually increased over the first two days, reaching a maximum at 48 hours. The F_v/F_m (PDP) reached a maximal value within the first 12 hours after addition and maintained essentially that level until 48 hours. The magnitude of the observed changes also showed better correlation between the Fd index and F_v/F_m (PAM) as opposed to F_v/F_m (PDP). During this study, the cells traversed half of the total range of Fd indices, yet F_v/F_m changed by only ~30%, in the case of the PAM, and a mere ~10% with the PDP-FCM.

The results of Fd index and F_v/F_m analyses also showed a similar pattern but varying magnitude during the period from 48 to 72 hours, when the cells again became iron limited. Growth rate, Fd index and F_v/F_m (both PAM and PDP) all decreased during this time. The decline in F_v/F_m was much more drastic than that in Fd index. Thus, the relative response of the biochemical and biophysical indices was quite different during the transition from iron-limitation to iron-sufficiency than during the onset of iron limitation. Any further comparisons of the Fd and F_v/F_m measures should then investigate both the response to iron addition and also the adaptation to iron starvation.

Synthesis of laboratory and field data

The data from this study cover only the range of Fd indices from ~0.5 to 0.9. However, by combining these results with those obtained during the IronEx II experiment, it is possible to construct a "hybrid" model of the responses of the biochemical and biophysical indicators over nearly the entire range of Fd indices. The relationship between relative growth rate and Fd index determined in Chapter 2 for <u>Thalassiosira weissflogii</u> serves as the basis for this model (Figure 7). Only the PDP-FCM data from this analysis are used in the model, as it most closely resembles the fast repetition rate (FRR) fluorometer used during IronEx II. The FRR utilizes a series of subsaturating blue-light flashes to close PSII reaction centers and induce maximal fluorescence (Falkowski & Kolber 1995), whereas both the PDP-FCM and the PAM fluorometer use only one saturating flash. However, the measurement time scale of the FRR (ca. 150 μs) is very close to that of the PDP-FCM (100 μs) and much shorter than that of the PAM fluorometer (600 ms). The matching of measurement time seems particularly important, considering the effect of flash length on the determination of F_m.

In the present study, cellular Fd indices ranged from 0.5-0.9 and values of F_v/F_m varied between a minimum of 0.63 and a maximum of 0.68. The measured Fd indices were used to calculate %μ_{max} for the corresponding F_v/F_m measurement using the linear fit of the Fd index-growth rate data from Chapter 2. During IronEx II, pennate diatom division rate increased from a minimum of ~1 division·day^{-1} to a maximum of ~1.8 divisions·day^{-1} (Constantinou et al. 1996). Using the value of 3.3 divisions·day^{-1}, measured by Fryxell and Kaczmarska (1994) for similar pennate diatoms in iron

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enrichment bottles, as a conservative maximum, diatom growth rates during IronEx II increased from 30 to 55% of μ_{max}. The minimum and maximum F_V/F_m measured during IronEx II were 0.26 and 0.56 (Behrenfeld et al. 1996). The data from this study and the IronEx II experiment is summarized in Table I.

In Figure 7, the calculated %μ_{max} and measured F_V/F_m from this study are plotted along with the minimum and maximum values of measured F_V/F_m and estimated %μ_{max} from IronEx II. The solid line is the best linear curve fit determined in Chapter 2 and the broken lines are linear fits of the study and IronEx II data. The broken lines are not intended to imply a linear relationship between the F_V/F_m and growth rate date, they are merely for illustration and discussion.

The F_V/F_m response inferred from the model seems to be the reverse of that of the Fd index. In the range above ~60% μ_{max} (this study), the Fd index ranges from 0 to 1 but there is little change in F_V/F_m. At growth rates below ~55% of μ_{max} (IronEx II), the Fd index is uniformly zero while F_V/F_m increases with increasing growth rate. Thus, it seems that cells may first respond to iron stress by reducing their ferredoxin pools (Fd index → 0). Only after this adaptive capacity is exhausted (Fd index = 0) do they start to experience impairment in their photochemical apparatus, evidenced by the resultant decrease in photochemical yield (F_V/F_m).

If this is the case, flavodoxin induction represents a more sensitive indicator of iron stress than F_V/F_m. It will be induced before significant decreases in F_V/F_m are detected. However, in the region where the Fd index is uniformly zero, e.g. during IronEx II, F_V/F_m will respond to changes in iron

stress that are not apparent using the Fd index. Thus, the two methods together may provide the best information about changes in the severity of iron stress over the entire range of iron limited growth.

Suggestions for further study

The preliminary results of this study, in combination with those observed during IronEx II, present an incomplete but thought-provoking picture of the relationship between biochemical and biophysical indices of iron limitation. To clarify this relationship, this comparison should be repeated, with several important modifications. First and foremost, it is essential to cover the entire range of Fd indices from 0 to 1. While it is necessary to add a large quantity of iron to ensure that the cells cover the entire range of Fd indices, it would also be useful to examine the effects of smaller iron additions, a design more similar to that of IronEx II. In addition to iron resupply experiments, further studies should also examine the inverse case of iron starvation. The patterns of Fd index and F_v/F_m observed during exponential and plateau growth phases in this experiment (Figure 6) imply that the time course of response for the two conditions may be very different.

The variety of measurements should also be expanded. Estimates of cell volume would greatly aid in the analysis of diel changes in pigment·cell^{-1} as well as comparison of pigment and F_v/F_m data. The PDP-FCM is currently unable to perform the traditional flow cytometric analyses of forward light scatter and red fluorescence. It would be informative to compare the results of traditional flow cytometric measures with those obtained for the same cells

using the PDP-FCM. Finally, this study characterized only the response of the iron-amended culture in detail. Any further comparisons should include complete analysis of control cells as well as experimentally treated cultures.

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TABLE I: Summary of F_v/F_m, Fd index and relative growth rate data from the present study and the IronEx II experiment.

source	Fd index	F_v/F_m	$\%\mu_{max}$
IronEx II	0[a]	0.26-0.56[b]	30-55[c]
this study	0.5-0.9	0.63-0.68	72-86[d]

[a] from Chapter 3
[b] from Behrenfeld et al. (1996)
[c] actual growth rates from Constantinou et al. (1996)
     maximum growth rates according to Fryxell and Kaczmarska (1994)
[d] calculated from measured Fd indices using linear fit of Chapter 2 data
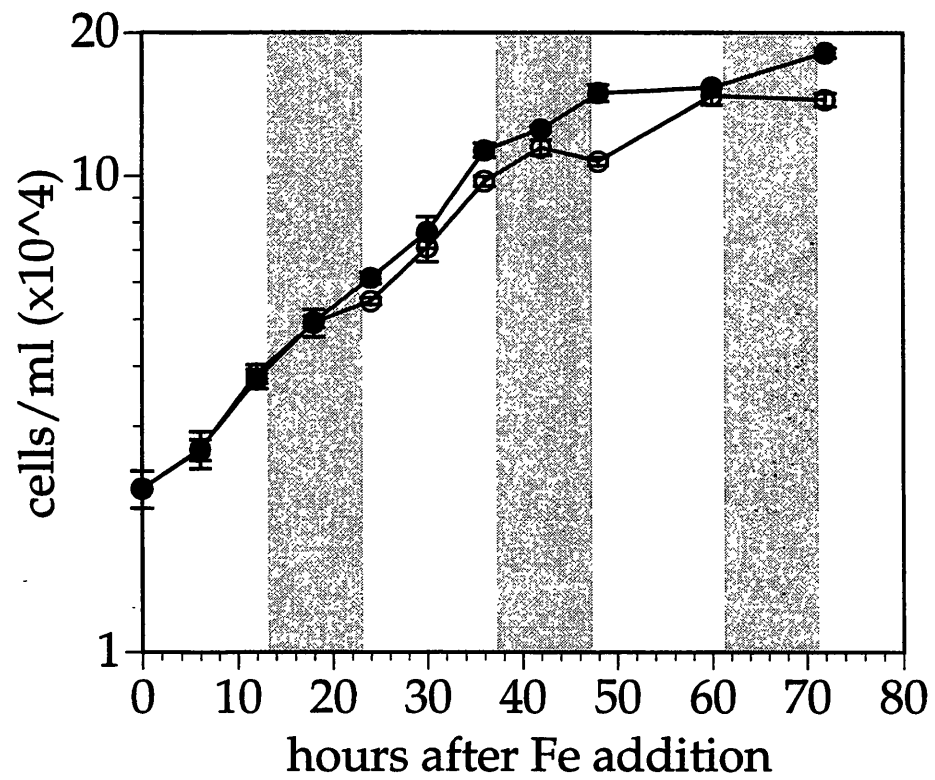
FIGURE 1 - Increase in cell density with time in (O) control cells and (●) cells with 10 µM added Fe.
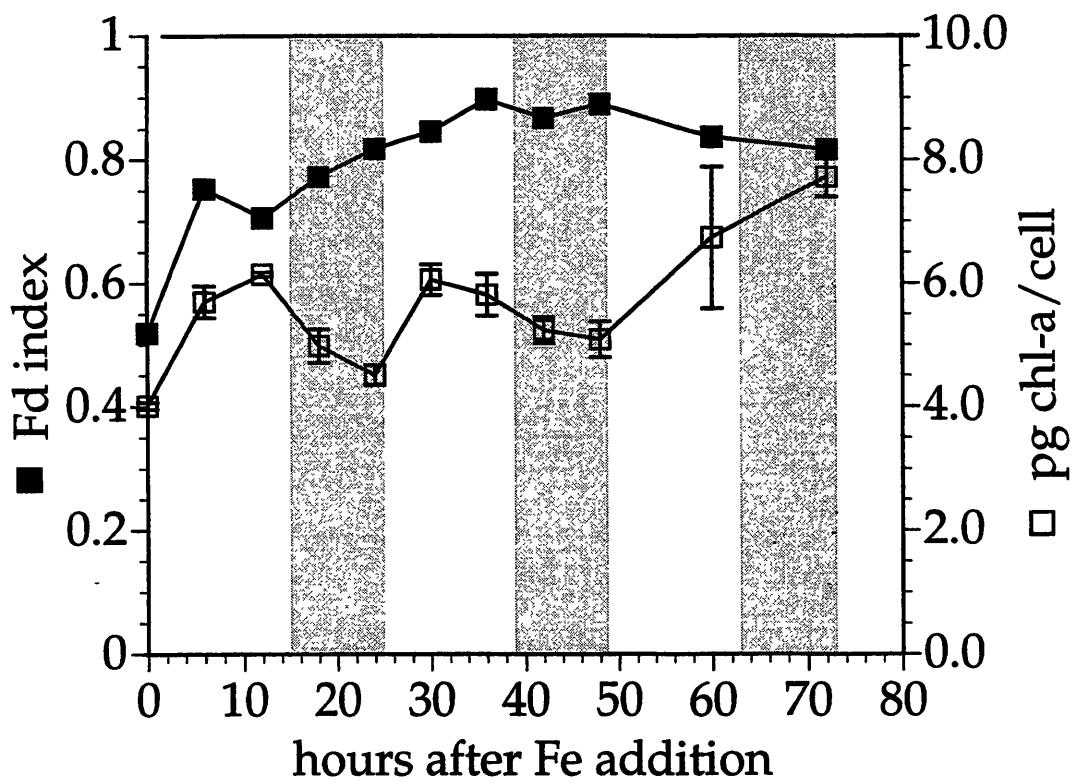
FIGURE 2 - Time course of changes in (■) Fd index and (□) chlorophyll·cell^{-1} following iron addition to Fe-limited cells.
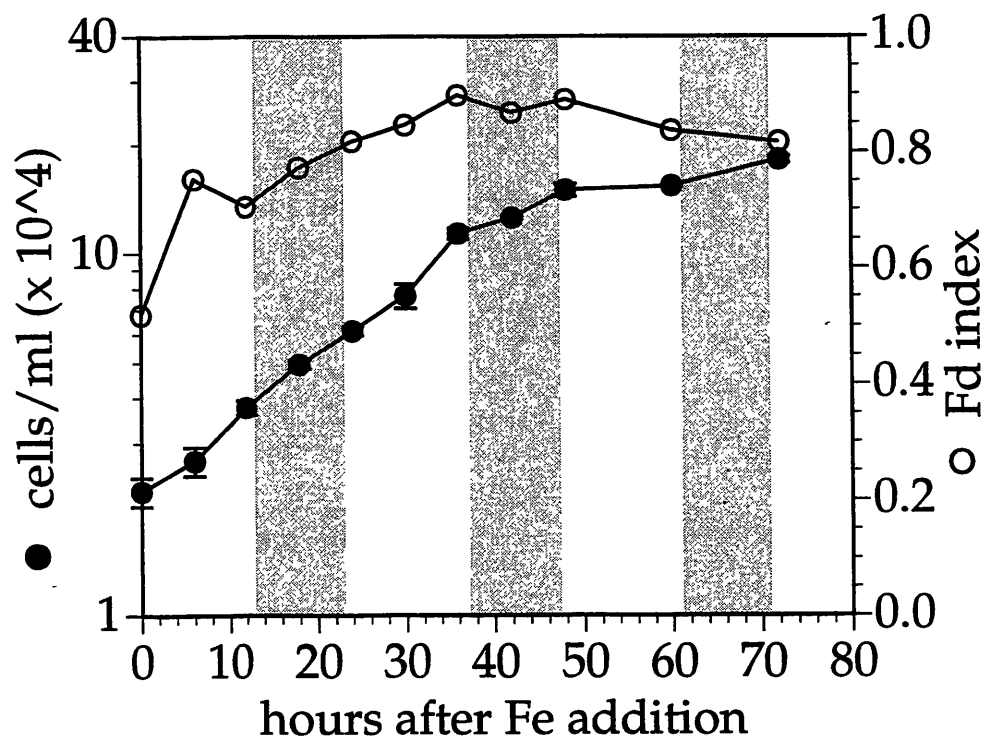
FIGURE 3 - Changes in (●) cell density and (O) Fd index following iron addition to Fe-limited cells.
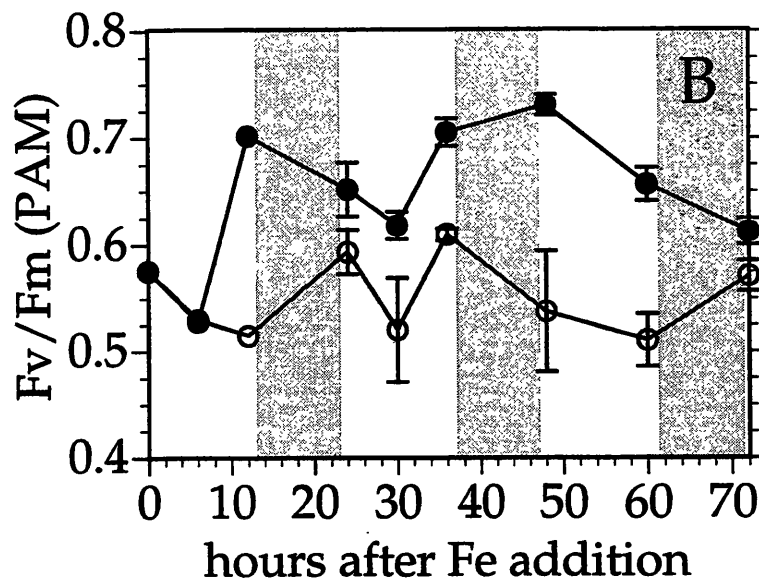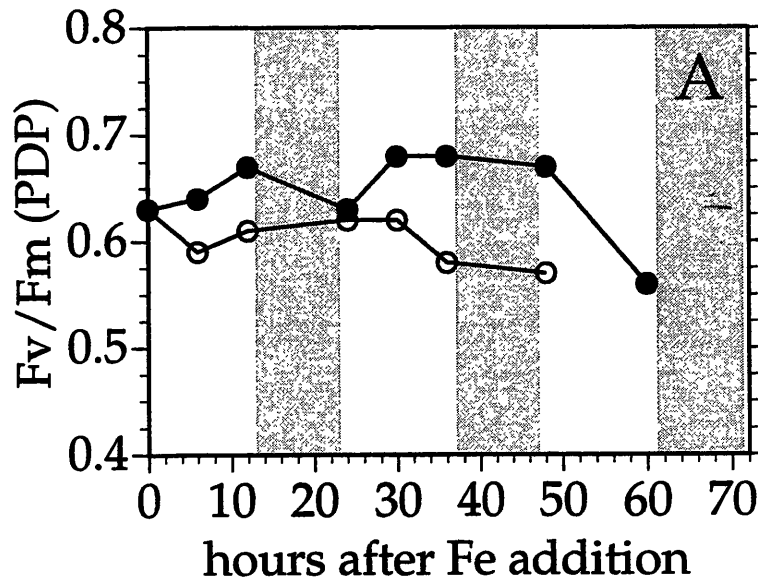
FIGURE 4 - Changes in the maximum potential quantum yield of photochemistry (F_v/F_m) as measured by (A) pump-during-probe flow cytometry and (B) pulse amplitude modulated fluorometry in cells with (●) and without (O) added iron.
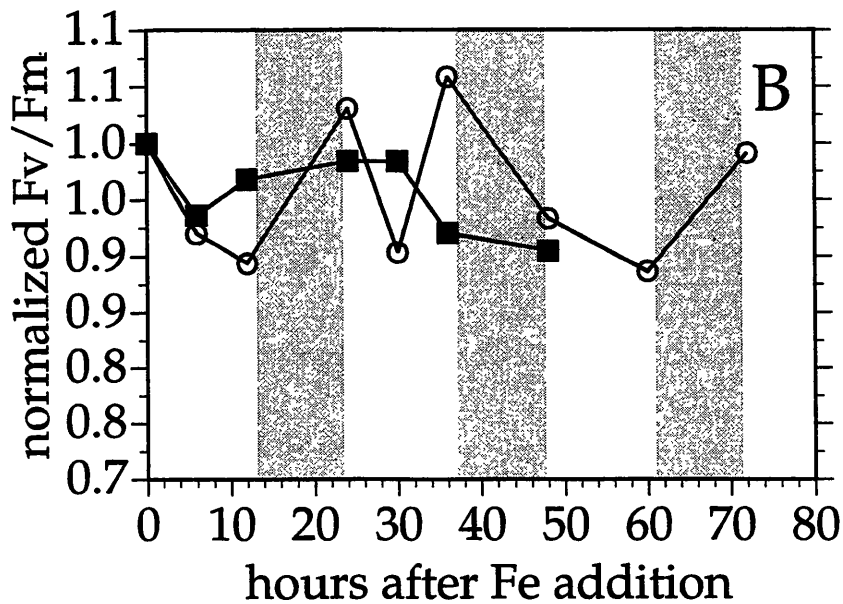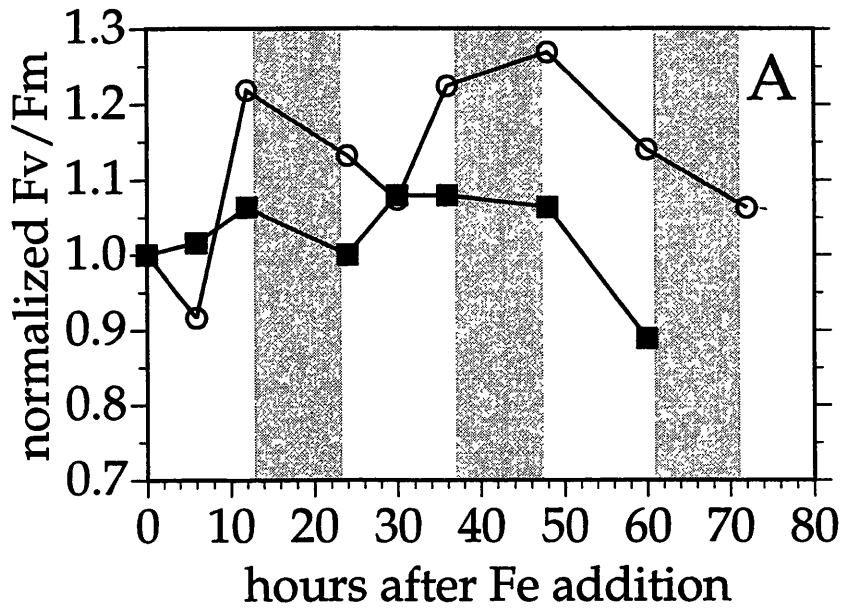
FIGURE 5 - Comparison of (■) PDP flow cytometric and (O) PAM
fluorometric measurements of F_v/F_m in (A) cells with added iron and (B)
control cells. All measurements have been normalized to their respective
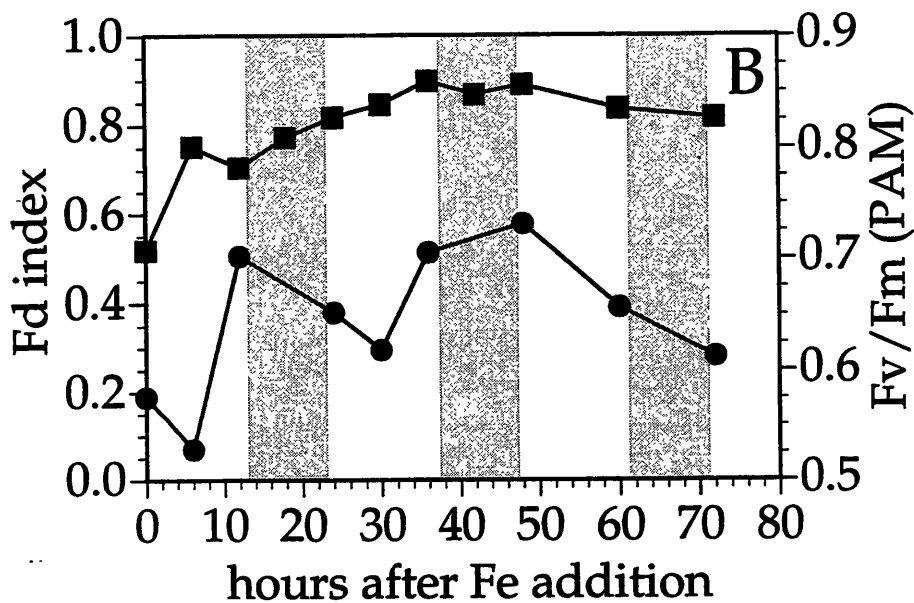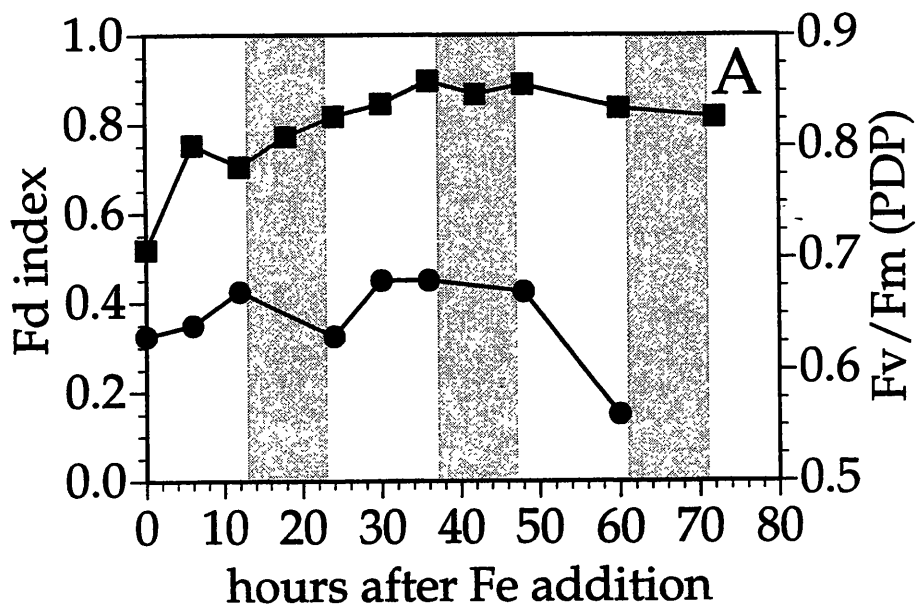initial F_v/F_m value.

FIGURE 6 - Comparison of changes in (■) Fd index and (●) F_v/F_m following addition of iron to Fe-limited cells. F_v/F_m was measured with (A) PDP flow cytometer and (B) PAM fluorometer.
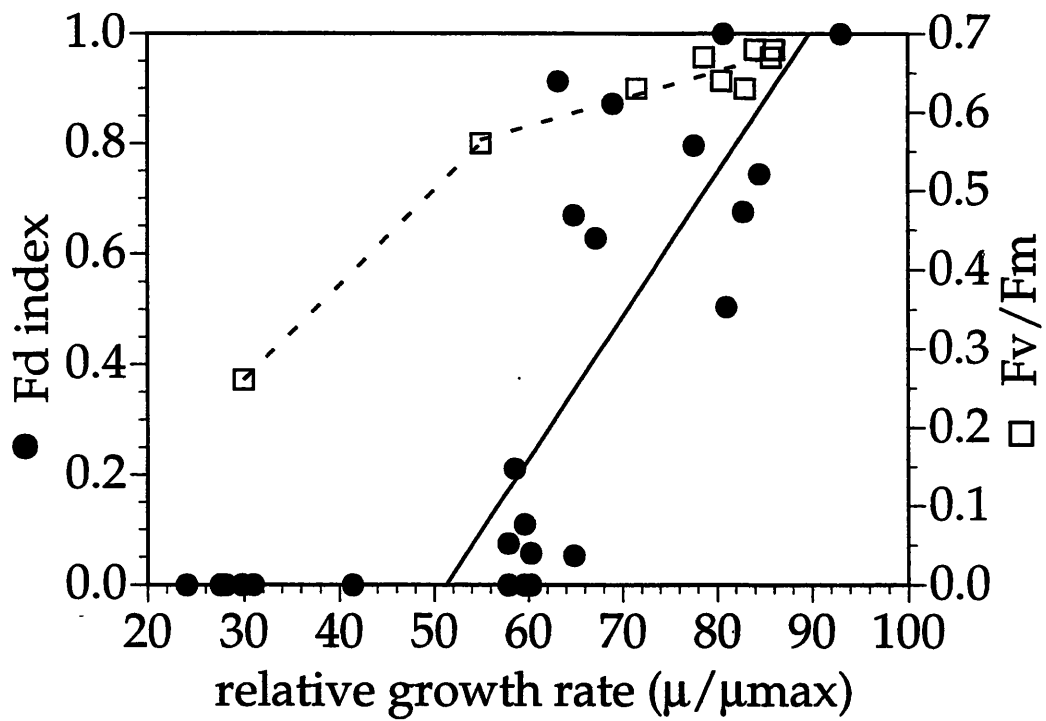
FIGURE 7 - Conceptual model of the relationship between (●) Fd index and (□) Fv/Fm and iron-limited growth rate inferred from laboratory and field studies.

Chapter 5

Conclusions

The expression and regulation of the proteins ferredoxin and flavodoxin were investigated to assess their utility as biomarkers of iron stress in marine phytoplankton. Previous studies of iron limitation of phytoplankton growth have been hindered by the lack of suitable analytical techniques. Thus, the goal was to develop these two proteins as a tool to facilitate ecological studies of iron limitation. Ideally, the ferredoxin-flavodoxin biomarker system would permit the unambiguous identification of physiological iron limitation on varying temporal and spatial scales. This dissertation addressed several key questions:

1)   Do marine phytoplankton employ the switch from ferredoxin to flavodoxin in response to iron stress?

2)   If so, is this switch specific to limitation by iron?

3)   Can ferredoxin and flavodoxin be used to detect iron limitation in natural populations of phytoplankton?

Chapter 1 presents the results of a comprehensive screening of flavodoxin expression in a diversity of marine phytoplankton, in an attempt to determine the generality of flavodoxin induction as a biomarker of iron limitation. Most of the organisms examined (12 of 17) express flavodoxin when iron-limited, while the remaining 5 species were never observed to express flavodoxin. This variability in flavodoxin expression is similar to that observed in freshwater algae. The phenomenon of non-expression is relatively uncommon and seems to be restricted to, but not characteristic of, organisms from neritic habitats. This implies that non-induction should not

confuse the interpretation of ferredoxin and flavodoxin measurements made in open-ocean areas.

Chapter 1 also summarizes the reactivity of two polyclonal antibodies developed as analytical tools for ferredoxin and flavodoxin detection. The antibodies, raised against proteins from <u>Thalassiosira weissflogii</u>, exhibit differing levels of specificity which determine, and also limit, their usefulness in natural populations. The immunological and chromatographic methods for ferredoxin and flavodoxin detection employed in Chapter 1 differ in several respects, including phylogenetic specificity and qualitative and quantitative nature of the results. While both techniques should prove useful for analysis of ferredoxin and flavodoxin in natural samples, their differences make it necessary to consider the requirements of a particular study in order to choose the most appropriate analytical tool.

Chapter 2 addresses the specificity of flavodoxin expression in <u>Thalassiosira weissflogii</u> with respect to several common limiting factors other than iron: nitrogen, phosphorous, silicate, zinc and light. Flavodoxin induction proves to be specific to iron stress and insensitive to limitation by the alternative limiting factors examined. This study also investigates potential indirect regulation of cellular ferredoxin and flavodoxin content by N substrate in iron-limited <u>T</u>. <u>weissflogii</u>. Use of either nitrate or ammonium as the sole N source does not affect the relative abundance of ferredoxin and flavodoxin despite the well-known effect of N substrate on cellular iron quota. The insensitivity of flavodoxin expression to light and nutrients other than iron makes it an ideal indicator of iron limitation.

The data in Chapter 2 also describe the relationship between ferredoxin and flavodoxin abundance, expressed by the "Fd index", and iron-limited growth rate. The Fd index, a concept introduced in this chapter, relates the amount of ferredoxin to the combined ferredoxin and flavodoxin pool, or:

$$[\text{ferredoxin}]/[\text{ferredoxin} + \text{flavodoxin}]$$

This relationship between Fd index and growth rate is comprised of two distinct regions. In the first region, at low growth rates, ferredoxin is undetectable and the Fd index is uniformly zero. In the second region, at moderate-to-high growth rates, ferredoxin and flavodoxin co-occur in the cells. The substitution of flavodoxin for ferredoxin proves to be a gradual process, not a simple "on-or-off" response. In addition, flavodoxin expression is very sensitive to iron limitation, occurring even at relatively high growth rates (80-90% μ_{max}). When the two proteins co-occur in cells, the Fd index varies according to changes in the severity of iron stress. While more data is needed to establish the exact form of the relationship (e.g. linear, exponential, etc.), the results do support an inverse relationship between Fd index and severity of iron limitation. The data presented in Chapter 2 provide a comprehensive picture of ferredoxin and flavodoxin regulation by various nutrients and light - information that is essential to verify the use of these proteins as diagnostics of iron limitation in natural populations.

Results detailed in Chapters 1 and 2 suggest that relative cellular ferredoxin/flavodoxin content, as measured by HPLC, is a sensitive and reliable indicator of iron limitation in marine phytoplankton. Chapter 3 describes the use of this biomarker system and its HPLC detection method in the field during the IronEx II open-ocean iron enrichment experiment.

During IronEx II, HPLC analysis was successfully used to monitor the iron nutritional status of the phytoplankton community over the course of the experiment. The results of this analysis were somewhat surprising, in that no ferredoxin expression was observed in response to iron fertilization. Laboratory studies with clonal cultures of equatorial Pacific pennate diatoms confirmed the ability of these organisms to synthesize ferredoxin when iron-replete and to modulate their cellular ferredoxin-flavodoxin content in about one day. The lack of ferredoxin resynthesis during IronEx II must therefore represent continued iron limitation of the phytoplankton community, despite the tremendous increases in chlorophyll and photosynthetic efficiency following iron addition. The results of the IronEx II analysis describe not only the response of the phytoplankton community to iron fertilization, but also illustrate the adaptive role of the ferredoxin and flavodoxin proteins in the environment.

The results of the ferredoxin/flavodoxin analysis from IronEx II were quite different from those obtained using biophysical methods. The relationship between biophysical (F_v/F_m) and biochemical (Fd index) measures of iron limitation was examined in the laboratory using T. weissflogii, the results of which are presented in Chapter 4. The laboratory data provide a detailed description of the relationship between F_v/F_m and the Fd index as the latter varies from 0.5 to 0.9, over about half of the total range, following iron addition. These laboratory data were combined with the results from IronEx II to construct a conceptual model of the covariation of F_v/F_m and Fd index. The model describes a complementary relationship in which F_v/F_m changes little as the Fd index decreases from 1 to 0, but shows a

steep decline in the range where Fd index = 0. Thus, photochemical systems seem to be spared the ill effects of iron limitation until the cell's adaptive capacity, in the form of ferredoxin, is exhausted. Further studies are required, however, to test and validate this model of biophysical and biochemical response.

In summary, the ferredoxin and flavodoxin proteins should prove to be a useful indicators of iron stress in marine eukaryotic phytoplankton. The presence or absence of flavodoxin serves as a sensitive qualitative indicator of growth limitation by iron. Measurements of ferredoxin and flavodoxin abundance together should allow assessment of the severity of iron stress. The use of ferredoxin and flavodoxin as indicators in the field, however, is not without limitations. Flavodoxin induction is not a universal response to iron stress, but it does seem to be the rule for phytoplankton from open-ocean, low-iron habitats, where issues of iron limitation are most relevant. Analyses made in coastal areas may be more difficult to interpret because of the potential for flavodoxin non-induction. The relative abundance of ferredoxin and flavodoxin should provide an indication of the severity of iron stress when both proteins are present in the cell. This measure is not informative when the Fd index is uniformly zero, as in IronEx II. Based on the model data in Chapter 4, a combination of ferredoxin/flavodoxin measurements in parallel with biophysical data may provide the most reliable information on the severity of iron limitation across the full range of possible growth rates.

Appendix I

Estimation of ferredoxin and flavodoxin
extraction efficiency from cells collected on
glass fiber filters

CELL VOLUME CALCULATION:

Thalassiosira weissflogii	970 femtoliters

IronEx II pennates:

7-47B	138 fl
A3-30	213 fl
10-40A	272 fl
average=	208 fl

T. weissflogii cell volumes from Latasa (1995) and Maldonado & Price (1996).

Cell volumes for pennates calculated as 0.5 (length x width x depth).
Dimensions of pennate clones (estimated from SEM micrographs):

7-47B	3.5μm x 22.5μm x 3.5μm
A3-30	4.5μm x 21μm x 4.5μm
10-40A	4.5μm x 11μm x 11μm

FLAVODOXIN·CELL^{-1} CALCULATION:

Estimate of flavodoxin·cell^{-1} for _Thalassiosira weissflogii_ growing at 55-60% of μ_{max} (possible growth rate during IronEx II - see Chapter 3): 50 amol·cell^{-1}

Average pennate cell volume (208 fl) is 0.21 _T. weissflogii_ volume (970 fl).
Scaling flavodoxin·cell^{-1} by cell volume for pennates:
$$10.5 \text{ amol·cell}^{-1}$$

THEORETICAL FLAVODOXIN CONCENTRATIONS DURING IRONEX II:

Cell density on JD154-155 was approximately 1.59×10^3 cells·ml^{-1} (pennates)
If each cell contained 10.5 amol flavodoxin, the seawater contained:
$$16695 \text{ amol flavodoxin·ml}^{-1}$$

EXTRACTED vs. THEORETICAL FLAVODOXIN DURING IRONEX II:

Sample collected on JD155 (243 mm filter) contained cells from 1063 liters. Its flavodoxin HPLC peak area was 1173, corresponding to
$$1.09 \text{ nmol of flavodoxin total.}$$
Theoretically, 1063 liters should contain 16695 amol·ml-1 x 1,063,000 ml =
$$17.7 \text{ nmol flavodoxin total}$$

$$\text{Estimated recovery: } 1.09 \text{ nmol}/17.7 \text{ nmol} = 6.2\%$$

Smaller sample collected on JD155 (142 mm filter) contained cells from 280 liters. It's flavodoxin HPLC peak area was 418.4, corresponding to
$$0.39 \text{ nmol of flavodoxin total.}$$
Theoretically, 280 liters should contain 16695 amol·ml-1 x 280,000 ml =
$$4.67 \text{ nmol flavodoxin total}$$

$$\text{Estimated recovery: } 0.39 \text{ nmol}/4.67 \text{ nmol} = 8.4\%$$

NOTES ON POTENTIAL IMPROVEMENTS:

The small filters, which had a more dense layer of cells, seemed to extract slightly better than the lightly-covered filter from the larger in situ pump. Thus, the amount of glass fiber seems to hinder extraction, likely by interfering with cell breakage. There are two basic ways to improve upon ferredoxin and flavodoxin recovery from field samples for analysis by HPLC: improved collection methods or improved extraction processes.

Collection methods: It appears that the necessary volume of water filtered for a sample are less than that collected during IronEx II, because of extraction problems.. Reasonably large volumes of water could be filtered using alternative filtration techniques such as cross-flow filtration, Nitex meshes arranged in series or membrane filter cartridges followed by backflushing. This would allow cells to be collected as a cell pellet, thus eliminating glass fibers that are likely to hinder cell breakage.

Extraction methods: If cells are to be collected on filter, extraction could be improved by increasing cell breakage. The addition of detergents to extraction buffer would aid cell lysis, but may also require alterations in the HPLC protocol to accommodate detergents during chromatography. Processes that would reduce the size of glass fiber fragments might also enhance cell breakage. This could possibly be achieved through mechanical means (crushing of glass fibers) or chemical means (dissolution of glass fibers). It would be necessary to check that the mechanical or chemical procedures do not affect protein stability or structure.

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Appendix II

Estimation of cellular ferredoxin and flavodoxin content of Thalassiosira weissflogii

Materials and Methods

Cultures   This study utilized the marine centric diatom Thalassiosira weissflogii (Grun.) Fryxell et Hasle (clone ACTIN).

Growth of phytoplankton   Cultures were grown in 0.2 µM filtered Vineyard Sound (MA, USA) seawater (31‰) enriched with ESNW nutrients according to Harrison et al. (1980) with several modifications. Na_2HPO_4 was substituted in equimolar amounts for $Na_2glyceroPO_4$. and selenium (as H_2SeO_3) was added to a final concentration of 10^{-8} M. Trace metal additions were made according to Brand et al. (1983). Seawater was autoclave-sterilized then enriched with sterile nutrients. Macronutrient (nitrate, phosphate and silicate) stocks were sterilized by autoclaving while iron, trace metal, selenium, EDTA and vitamin stocks were sterile-filtered (0.2 µm).

All cultures were maintained at 20°C on a 14:10 hour light:dark cycle at an irradiance of ca 175 µE m^{-2} s^{-1} as measured with a photometer (Biospherical Instruments model QSP-100). Cultures (2 L volume) were grown in acid-washed 2.8 L Fernbach flasks in medium containing either 500 nM, 1 µM, 10 µM or 50 µM added iron. EDTA was added to ten times the iron concentration. A 10 ml aliquot of culture was collected by filtration onto a 25 mm diameter GF/F filter for determination of protein per cell. The remaining culture volume was harvested by filtration onto 3 µm pore size polycarbonate filters (47 mm diameter) and frozen in liquid nitrogen prior to analysis. Cells were collected onto a total of four polycarbonate filters for the 500 nM, 1 µM and 50 µM cultures and three filters for the 10 µM culture.

Cell counts Cell densities were determined by four replicate microscopic counts of Utermohl's preserved samples in a Fuchs-Rosenthal hemacytometer.

Determination of protein per cell Cells harvested onto glass fiber filters were placed in a 2 ml tube (with the filter) containing extraction buffer (20 mM phosphate, 100 mM EDTA, 100 mM NaCl, 0.013 M ß-mercaptoethanol, 1 mM PMSF pH 7.0) with 1% SDS and zirconium beads. Cells were ruptured by three, fifty second cycles in a mini-beadbeater (Biospec Instruments, Bartlesville, OK, USA). The volume of supernatant was measured, aliquots were diluted five- and ten-fold with sterile distilled water and protein content was measured by BCA assay (see below). "Protein per cell" was calculated as:

(supernatant volume * protein concentration) ÷ cells harvested

Protein extraction for HPLC To prepare extracts for HPLC, cells and their filters were minced and placed in a 2 ml tube with extraction buffer and zirconium beads and homogenized in a bead-beater as described above. Both the volume and protein content of this crude lysate was measured. The crude cell lysate was then centrifuged for one hour at 105,000 x g. The supernatant was transferred to a clean tube, after which its volume and protein content measured. This supernatant was then filtered (0.45 μm) and a 2 ml volume was injected into the HPLC.

Protein determinations Protein concentrations were determined using the BCA Protein Assay (Pierce Co., Rockford, IL USA).

HPLC of ferredoxin and flavodoxin All cultures were analyzed using a previously described HPLC method (Doucette et al. 1996). Ferredoxin and flavodoxin in cell extracts were separated by anion-exchange HPLC. Detection

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was performed with a Hewlett-Packard model 1050 diode array detector (Hewlett-Packard Co., Andover, MA, USA). Quantification of peak areas was performed by HP ChemStation software (Hewlett-Packard) in autointegration mode.

Calculation of ferredoxin and flavodoxin content Standard curves relating HPLC peak area to injected protein were determined using purified ferredoxin and flavodoxin from Porphyra umbilicalis and Anabaena, respectively. HPLC peak areas were converted to "ferredoxin or flavodoxin injected" using these standard curves.

The number of "cells extracted for HPLC" was calculated as:

(crude lysate volume * crude lysate protein concentration) ÷ "protein per cell" After centrifugation of the crude cell lysate, the volume of supernatant was measured (to account for volume lost to insoluble matter in the pellet). This supernatant was assumed to contain the soluble protein from the total number of "cells extracted for HPLC". Two milliliters of this supernatant was injected for analysis. The number of "cells injected" was calculated as:

(2.0 ml/volume of supe after centrifugation) * "cells extracted for HPLC" and used for calculations of ferredoxin and flavodoxin per cell and per protein.

Ferredoxin or flavodoxin per cell was calculated as:

"ferredoxin or flavodoxin injected" ÷ "cells injected"

Ferredoxin or flavodoxin per cell was converted to ferredoxin or flavodoxin per protein using the protein per cell values.

Results

Fe added	μ (div·day^{-1})	Fd amol per cell*	Flv amol per cell*	Fd pg per cell	Flv pg per cell	Fd per prot.	Flv per prot.
500 nM	1.20	39.72 (9.42)	16.64 (2.36)	0.496	0.374	0.009	0.007
1 μM	1.21	65.49 (18.56)	-	0.819	-	0.013	-
10 μM	1.19	83.77 (3.97)	-	1.047	-	0.013	-
50 μM	1.29	94.26 (11.58)	-	1.178	-	0.013	-

*   Mean amol·cell^{-1} values, standard deviation given in parentheses.

For comparison, the cellular iron quota of <u>Thalassiosira weissflogii</u> has been calculated to be from 100-200 amol·cell-1 (Harrison & Morel 1986). Here, <u>T. weissflogii</u> cells grown at 1 to 50 μM added Fe contain 65.49-94.26 amol of ferredoxin, each molecule of which contains 2 Fe atoms, yielding 131-188.5 amol Fe contained in ferredoxin - a significant portion of the cell's total iron quota.
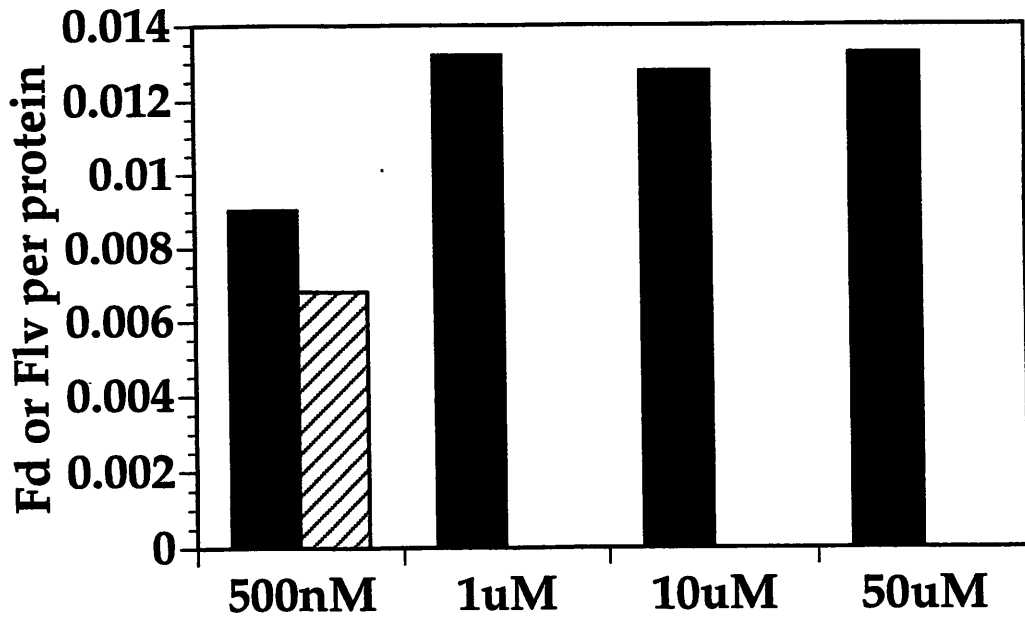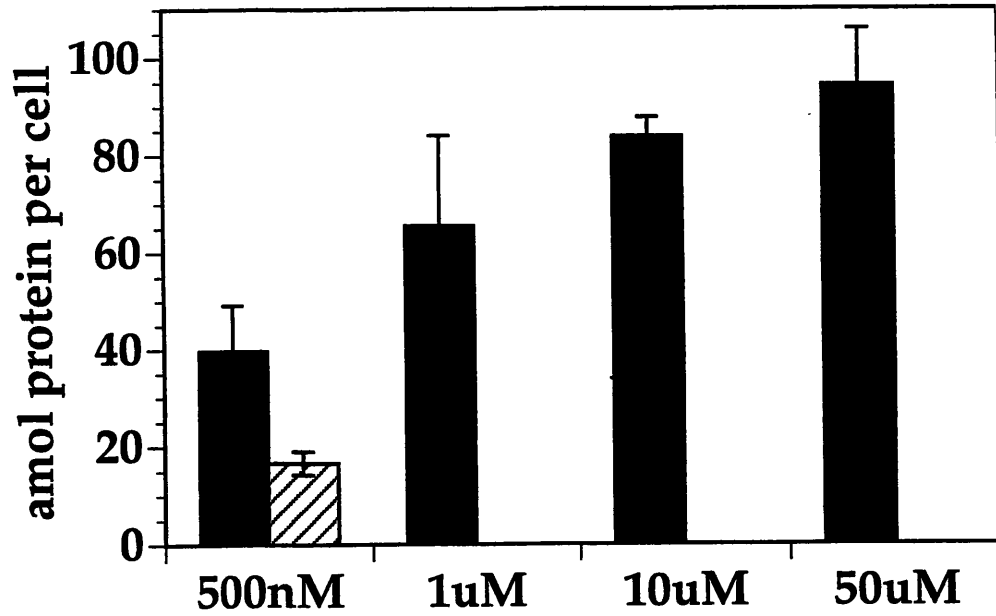
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