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# Physiological and Biochemical Response of the Photosynthetic Apparatus of Two Marine Diatoms to Fe Stress[1]

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**Flavodoxin is a small electron-transfer protein capable of replacing ferredoxin during periods of Fe deficiency. When evaluating the suitability of flavodoxin as a diagnostic indicator for Fe limitation of phytoplankton growth, we examined its expression in two marine diatoms we cultured using trace-metal-buffered medium. *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* were cultured in ethylenediaminetetraacetic acid-buffered Sargasso Sea water containing from 10 to 1000 nM added Fe. Trace-metal-buffered cultures of each diatom maintained high growth rates across the entire range of Fe additions. Similarly, declines in chlorophyll/cell and in the ratio of photosystem II variable-to-maximum fluorescence were negligible (*P. tricornutum*) to moderate (*T. weissflogii*; 54% decline in chlorophyll/cell and 22% decrease in variable-to-maximum fluorescence). Moreover, only minor variations in photosynthetic parameters were observed across the range of additions. In contrast, flavodoxin was expressed to high levels in low-Fe cultures. Despite the inverse relationship between flavodoxin expression and Fe content of the medium, its expression was seemingly independent of any of the indicators of cell physiology that were assayed. It appears that flavodoxin is expressed as an early-stage response to Fe stress and that its accumulation need not be intimately connected to limitations imposed by Fe on the growth rate of these diatoms.**

Fe is an abundant component of the earth's crust yet is believed to be a biolimiting element in the vast HNLC regions of the world's oceans (Martin and Fitzwater, 1988; Martin et al., 1990b, 1991). This apparent paradox results from low, sporadic deposition of Fe from atmospheric aerosols to these remote locations (Duce and Tindale, 1991). Also of consequence may be a low bioavailability of Fe to phytoplankters; Fe speciation in oxic seawater is a dynamic process and is influenced by a variety of factors (Wells et al., 1995; Wu and Luther, 1995). The results of numerous field-based enrichment bioassay experiments lend support to the assertion that Fe is a limiting resource for phytoplankton communities in HNLC regions (Martin and Fitzwater, 1988; Martin et al., 1990a, 1991; de Baar et al., 1990; Coale, 1991; Price et al., 1991, 1994). The addition of Fe (1–10 nM) to water samples from these locales is accompanied by marked increases in phytoplankton biomass compared with unamended control bottles. Further supporting this view, results from two recent, wide-scale fertilization efforts in the HNLC waters of the equatorial Pacific unambiguously demonstrate that mesoscale Fe amendment relieves nutritional constraints imposed on phytoplankton $\mu$, photosynthetic efficiency, and biomass accumulation (Kolber et al., 1994; Martin et al., 1994; Behrenfeld et al., 1996; Coale et al., 1996b).

Problems inherent in the interpretation of enrichment bioassays have led to the application of alternative approaches for the identification of potentially biolimiting nutrients such as Fe. Foremost among these, properties of chl fluorescence diagnostic of nutrient limitation have been characterized and exploited for this purpose (Falkowski et al., 1992; Greene et al., 1994; Kolber et al., 1994; Falkowski and Kolber, 1995; Behrenfeld et al., 1996). However, fluorescence-based biophysical indices are not informative under all circumstances (Cullen et al., 1992; Falkowski et al., 1992; Greene et al., 1992; Geider et al., 1993), and as a complementary approach, several groups have offered the possibility of using molecular and biochemical diagnostics in the assessment of nutrients limiting phytoplankton growth (Falkowski et al., 1992; Scanlan et al., 1993; Geider and LaRoche, 1994; Doucette et al., 1996; LaRoche et al., 1996). Candidate markers are induced subject to stress by a specific nutrient. Moreover, whereas the fluorescence-based biophysical approach is unable to resolve taxon-specific responses (Falkowski et al., 1992), molecular and biochemical markers can be chosen that provide such a resolution.

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Abbreviations: $\alpha^{chl}_{initial}$, slope of P-I curve; ANOVA, analysis of variance; chl, chlorophyll; D1, PSII reaction center protein; $F_o$, initial fluorescence; $F_m$, DCMU-enhanced fluorescence; $F_v$, variable fluorescence; FCP, fucoxanthin chlorophyll light-harvesting protein; Fe′, dissolved inorganic Fe; HNLC, high-nutrient, low-chlorophyll; $I_k$, intercept at which $\alpha$ and $P_m$ converge; $\mu$, growth rate; NR, nitrate reductase; $P_m^{chl}$, chl-specific light-saturated rate of photosynthesis; P-I, photosynthesis versus irradiance; $t$, time; TBS-T, TBS containing 0.05% (v/v) Tween 20; TMB, trace metal-buffered.

Flavodoxin has been identified as a candidate marker of Fe limitation in marine diatoms (LaRoche et al., 1993, 1995; Doucette et al., 1996). Induction of this protein is specific in response to Fe deficiency (LaRoche et al., 1993); its accumulation was observed in cultures in which the $\mu$ was limited by Fe, but not in Fe-replete cultures (LaRoche et al., 1995). Moreover, our immunoprobe is reactive primarily with diatoms (LaRoche et al., 1995, 1996), thereby providing a measure of taxonomic resolution in a mixed phytoplankton assemblage. To assess more fully the potential of flavodoxin as an in situ biochemical indicator of Fe limitation in marine diatoms, we used a TMB culture technique (Brand et al., 1983; Sunda et al., 1991; Sunda and Huntsman, 1995) to more adequately define the relationship between flavodoxin expression and Fe limitation of diatom growth.

## MATERIALS AND METHODS

### Cell Culture

The marine diatoms *Phaeodactylum tricornutum* (CCMP 1327, formerly clone Phaeo) and *Thalassiosira weissflogii* (Gru.) Fryxell et Hasle (clone T-VIC) were cultured in TMB seawater medium, essentially as described by Sunda et al. (1991). Water collected from the Sargasso Sea during an April 1995 research cruise aboard the R/V Cape Henlopen was diluted to 80% full-strength with $H_2O$ (Milli-Q, Millipore) and supplemented with medium f/2 levels of macronutrients and vitamins (Guillard and Ryther, 1962). In addition, the medium was supplemented with an EDTA-buffered trace metal solution providing 0.1 mM EDTA, 0.01 $\mu$M $H_2SeO_3$, 0.1 $\mu$M $CuSO_4$, 0.1 $\mu$M $CoCl_2$, 0.1 $\mu$M $Na_2MoO_4$, 0.25 $\mu$M $ZnSO_4$, and 1.2 $\mu$M $MnCl_2$. The medium was filter-sterilized (0.2 $\mu$M) and dispensed into acid-rinsed polycarbonate bottles (2.5 L) and tubes (35 mL). Iron was provided as $FeCl_3$ and added in concentrations of 10, 25, 50, 100, and 1000 nM. Fe' was calculated to be 4.5, 11.3, 22.7, 45.4, and 240.4 pM, respectively; derived values were based on conditional formation and dissociation rate constants for Fe-EDTA complexes in seawater using the modeling program MINEQL, as described by Taylor et al. (1994).

Cells were cultured in polycarbonate tubes until rates of growth, characteristic of each Fe regimen, became stable. During this period fresh medium was inoculated every 4 d with 0.01 volume of late-log-phase culture. Growth was monitored by in vivo chl fluorescence (model 10–005 R fluorometer, Turner Designs, Mountain View, CA). In several instances, $\mu$ in cultures of *P. tricornutum* was also determined from cell counts. This method consistently yielded $\mu$ values 10% higher than those determined by fluorometry. Reproducible $\mu$ values were typically obtained following three to four inoculations; however, experimental manipulation was performed only after a culture had passed through at least seven successive rounds of inoculation. For experimental work, a late-log-phase culture was used to inoculate 2 L of seawater medium in a 2.5-L polycarbonate bottle. Chl fluorescence was monitored daily, and the cultures were harvested in mid-log phase, by which time they had attained 10 to 20% of maximum yield based on cell counts. All cells were grown in standing batch culture with continuous illumination provided by cool-white fluorescent lamps at a photon fluence rate of 215 $\mu$mol quanta $m^{-2}$ $s^{-1}$. *P. tricornutum* was maintained at 20°C, and *T. weissflogii* was maintained at 24°C.

### Physiological Measurements

Photosynthetic measurements were performed essentially as described by MacIntyre et al. (1996). P-I curves were generated by measuring $^{14}C$ uptake over a light gradient in a temperature-controlled "photosynthetron" (Lewis and Smith, 1983). A 30-mL aliquot of mid-log-phase culture was dark-adapted for 30 min, following which 100 $\mu$L of $NaH^{14}CO_3$ was added, and the culture was dispensed in 1-mL aliquots to glass scintillation vials arranged in the photosynthetron. At this time ($t$ = 0), the photosynthetron light source was activated and samples were incubated for 30 min. The reaction was terminated by the addition of 50 $\mu$L of formaldehyde to each sample. Scintillation vials were transferred to a shaker table, and labile $^{14}C$ was driven off by acidification of the samples with 50 $\mu$L of 6 N HCl. Acid-stable $^{14}C$ was measured by liquid-scintillation counting (5 min) following the addition of 4.5 mL of OptiPhase "HiSafe 3" cocktail (Wallac Scintillation Products, Turku, Finland) to each vial. Total activity of the added $^{14}C$ was determined by adding 20 $\mu$L of the sample at $t$ = 0 to scintillation cocktail containing 200 $\mu$L of $\beta$-phenylethylamine. Background activity was determined at $t$ = 0 by dispensing a sample aliquot directly into formaldehyde prior to adding scintillation cocktail. Experiments were performed on replicate cultures. Rates of photosynthesis were estimated using the nonlinear regression curve-fitting procedure of SigmaPlot, version 2.0 (Jandel Scientific, San Rafael, CA) and the equation:

$$P^{chl} = P_m^{chl}(1 - e[-\alpha^{chl} I / P_m^{chl}])$$

where $P^{chl}$ is the chl *a* normalized rate of photosynthesis at irradiance $I$, $P_m^{chl}$ is the maximum rate of photosynthesis in the absence of photoinhibition, and $\alpha^{chl}$ is the initial slope of the P-I curve.

Measures of in vivo fluorescence were made with a fluorometer (Turner Designs) on samples dark-adapted for 15 min prior to ($F_0$) and following ($F_m$) the addition of DCMU. $F_v$ is defined as the difference between these values; the ratio $F_v/F_m$ is a measure of PSII photochemical efficiency (Butler and Kitajima, 1975). Chl was extracted overnight at −20°C in 90% (v/v) acetone and measured by fluorometry prior to and following sample acidification. The fluorometer was calibrated using spinach chl *a* (Sigma); an internal standard of rhodamine B was used between calibrations. Cell density was determined using a hemocytometer (Thomas Scientific, Philadelphia, PA).

### Protein Analysis

Mid-log-phase cells were concentrated by filtration through a 5-$\mu$m polycarbonate membrane (Poretics, Livermore, CA) and the concentrate was harvested by centrifugation. Cell pellets were flash-frozen in liquid nitrogen and

stored at −80°C. For protein extraction, cells were disrupted by sonicating (Micro Ultrasonic Cell Disruptor; Kontes, Vineland, NJ) twice for 20 s in ice-cold acetone containing 10% (w/v) TCA and 0.07% (v/v) mercaptoethanol, and the extracts were stored at −20°C for 1 h. Precipitated protein was recovered by centrifugation, and the pellet was rinsed with cold acetone and air-dried briefly. Total protein was resuspended in PAGE-reducing sample buffer (3% [w/v] SDS, 17 mM $Na_2CO_3$, 50 mM DTT, 7.5% [v/v] glycerol, 0.025% [w/v] bromphenol blue, 4 mM ϵ-amino-*n*-caproic acid, and 1 mM benzamidine) and analyzed by denaturing PAGE (15% resolving gel) using SDS-Tris-Gly buffer (Laemmli, 1970) and the Mini-PROTEAN II system (Bio-Rad). Protein concentration was determined using a bicinchoninic acid assay (Pierce) and BSA as a standard.

Gels were either silver-stained (Rabilloud et al., 1988) or electrophoretically transferred overnight to nitrocellulose membranes (0.45 μm; Hybond-ECL, Amersham) at 30 V and 4°C in Tris-Gly buffer containing 20% (v/v) methanol (Towbin et al., 1979). Filters containing transferred proteins were blocked for 1 h in TBS (10 mM Tris and 150 mM NaCl) containing 4% (w/v) nonfat powdered milk (Carnation, Nestle, Glendale, CA) and then rinsed briefly in TBS-T. Blots were subsequently incubated with primary antibody for 2 h, rinsed in TBS-T (3 × 15 min), and incubated for 1 to 2 h with affinity-purified goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad) diluted 1:10,000. Immunoreactive proteins were detected by chemiluminescence using the ECL system (Amersham). In some instances film (Hyperfilm-ECL, Amersham) was sensitized with a modified flash unit (Sensitize Pre-Flash Unit, Amersham) prior to exposure. This served to counteract the nonlinear response characteristic of film exposed to low-intensity emissions of light (Laskey and Mills, 1975).

Anti-Rubisco (holoenzyme) and anti-FCP complex were raised from antigens purified from *Isochrysis galbana*. Anti-flavodoxin was raised against antigen purified from *P. tricornutum* (LaRoche et al., 1995); in some experiments an affinity-purified form of this antibody was used. NR antiserum was prepared against antigen isolated from *T. weissflogii* (J.J. Vergara, J.A. Berges, and P.G. Falkowski, unpublished data). Anti-D1 was prepared against a fusion protein comprised of BSA and a synthetic peptide corresponding to a conserved, internal 30-residue sequence, deduced for the D1 PSII reaction center protein of *Solanum nigrum*. Primary antisera were diluted in TBS-T containing 1% (w/v) milk prior to use. Blots prepared in triplicate were probed sequentially with each antibody and were stored in TBS at 4°C between reactions. All reactions were performed at room temperature with nominal agitation. Gels and film were analyzed by laser densitometry (Computing Densitometer model 300B; Molecular Dynamics, Sunnyvale, CA) using ImageQuant version 3.22 software (Molecular Dynamics).

## RESULTS

### Growth Characteristics of TMB Cultures

Both diatoms maintained high rates of growth across the entire range of Fe additions (Fig. 1). In TMB cultures of *P. tricornutum* the μ decreased slightly with diminishing Fe additions, but to no less than 85% of $\mu_{max}$ (1.7 $d^{-1}$). Similarly, TMB cultures of *T. weissflogii* provided 50, 100, or 1000 nM Fe displayed nearly identical rates of growth (1.64–1.74 $d^{-1}$); only cultures provided 25 nM Fe grew at a significantly lower rate (1.21 $d^{-1}$; one-way ANOVA, $P < 0.1$). One exception to this trend was our attempt to culture *T. weissflogii* at 10 nM Fe. Under these conditions this alga was unable to grow, despite the use of inoculum acclimated to low Fe (50 nM Fe) and attempts to culture in two different batches of TMB medium. Because of constraints on culture facilities experienced during the course of the experiment, 25 nM Fe TMB cultures of *T. weissflogii* were grown under a slightly increased photon fluence rate of 315 μmol quanta $m^{-2}$ $s^{-1}$.

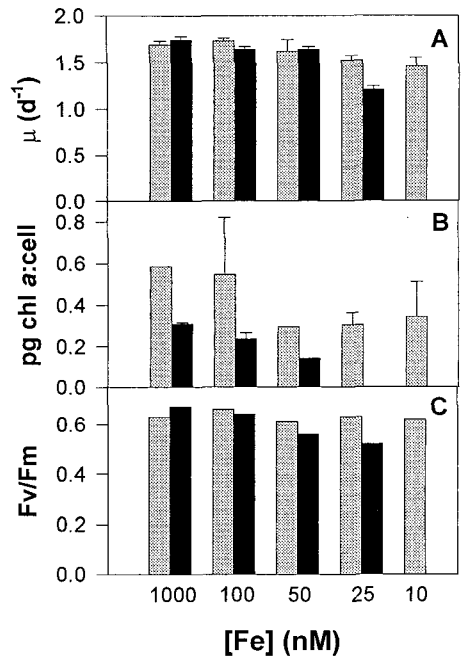


**Figure 1.** Physiological parameters of diatom TMB cultures provided varying levels of Fe. A, μ determined from measures of in vivo fluorescence (means ± SE, $n$ = 4–6). B, Chl *a* extracted in acetone and values normalized to cell numbers determined by direct counts (means ± SE, $n$ = 2). Chl was not determined for TMB cultures of *T. weissflogii* provided 25 nM Fe. C, Ratios of $F_v/F_m$ (means ± SE, $n$ = 2). Gray bars, *P. tricornutum*; black bars, *T. weissflogii*.

### Photosynthetic Physiology

Levels of chl *a* normalized per cell declined with diminishing Fe input (Fig. 1). This decline was significant in TMB cultures of *T. weissflogii*, in which chl was 2-fold higher in cells cultured with 1000 nM Fe compared with 50 nM Fe (one-way ANOVA, $P < 0.1$). Variation in chl levels was not significant in cultures of *P. tricornutum*.

Ratios of $F_v/F_m$ observed for all TMB cultures were remarkably stable. Values typical for Fe-replete diatoms are reported to approach 0.65 (Kolber et al., 1988; Geider et al., 1993) yet they frequently decline to <0.4 in nutrient (including Fe)-limited cultures (Geider et al., 1993), thereby

providing a useful indicator of nutrient limitation. In the present study this ratio did not decline below 0.6 for *P. tricornutum* and decreased to only 0.52 for 25 nM Fe-grown *T. weissflogii* (Fig. 1).

Likewise, photosynthetic parameters varied little between Fe treatments (Table I). Rates of light-saturated photosynthesis ($P_m^{chl}$) and $\alpha$, a measure of photosynthetic efficiency calculated as the initial slope of the P-I curve, did not vary significantly among Fe treatments for either diatom (one-way ANOVA). This trend was also reflected in values of $I_k$ a parameter derived from $P_m^{chl}/\alpha^{chl}$ to estimate the irradiance at which photosynthesis becomes light-saturated. Values for this parameter varied only slightly across treatments.

A photoinhibitory response was observed for cultures of each diatom, regardless of the level of Fe amendment (data not shown). Rates of photoinhibition varied between treatments, with no significant deviation (one-way ANOVA) and with no apparent trend.

## Protein Analysis

Profiles of protein resolved by SDS-PAGE revealed few, if any, differences among TMB cultures of *P. tricornutum* (Fig. 2) and *T. weissflogii* (Fig. 3). Likewise, relative abundance of the large subunit of Rubisco and D1 in *T. weissflogii* did not vary significantly across any Fe regimen tested (Figs. 3 and 4). Similarly, among TMB cultures of *P. tricornutum*, relative levels of the large subunit of Rubisco did not vary significantly, whereas levels of D1 were significantly lower (<2-fold) only for cultures grown at 10 nM Fe (one-way ANOVA, $P < 0.01$; Figs. 2 and 4). Staining by anti-FCP showed 50 and 1000 nM Fe TMB cultures of *P. tricornutum* to contain nearly 2-fold higher levels of the light-harvesting chl protein complex than cultures grown with 10, 25, or 100 nM Fe (Fig. 4; one-way ANOVA, $P < 0.005$). The apparent low levels of FCP in the 100 nM Fe TMB culture are puzzling, although a protein-loading artifact introduced during SDS-PAGE might explain the lower FCP signal. This is supported by qualitative observation of the silver-stained gel (Fig. 2) but not by densitometry of staining for Rubisco and D1 (Fig. 4). Relative levels of NR assessed for TMB cultures of *T. weissflogii* did not vary significantly (Figs. 3 and 4). The NR antiserum used in this particular assessment has limited specificity and does not cross-react with an antigen of an appropriate mass in the extracts of *P. tricornutum* (J. Berges and J. Vergara, personal communication).

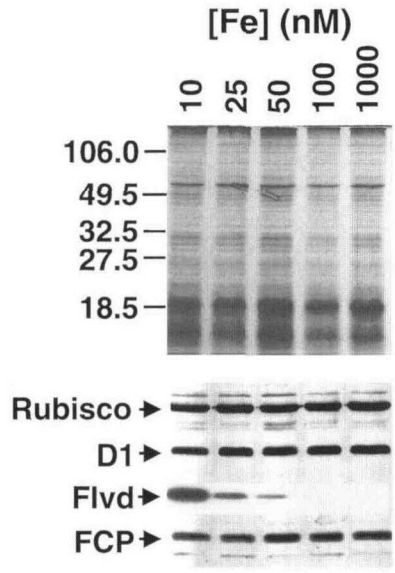


**Figure 2.** SDS-PAGE and immunoblotting of *P. tricornutum* protein extracts. Top, Silver-stained gel. Positions of prestained $M_r$ markers are shown. Bottom, Chemiluminescent detection of immunoreactions. Reactions were performed sequentially on the same blot with chemiluminescent detection following each reaction. Flvd, Flavodoxin.

Despite the apparent lack of heterogeneity evident by silver-staining and immunoblotting as described above, immunodetection of flavodoxin yielded large and reproducible differences among diatom TMB cultures (Figs. 2–4). Relative levels of flavodoxin in 10 nM Fe cultures of *P. tricornutum* were >130-fold higher than in cultures containing 100 nM Fe, >13-fold higher than in cultures containing 50 nM Fe, and nearly 4-fold higher than in cultures containing 25 nM Fe (Fig. 4). Flavodoxin was below the limit of detection offered by enhanced chemiluminescence in *P. tricornutum* cultures containing 1000 nM Fe. Significant variations in the flavodoxin content of TMB cultures of *T. weissflogii* were also evident. Relative levels of flavodoxin

**Table I.** *Photosynthetic parameters of diatom TMB cultures*

Values represent means of replicate determinations from two separate cultures. SEs are shown in parentheses.

| Parameter | Species | [Fe] | | | | |
|---|---|---|---|---|---|---|
| | | 1000 | 100 | 50 | 25 | 10 |
| | | | | nM | | |
| $P_m^{chl}$ (gC $g^{-1}$ Chl $h^{-1}$) | *P. tricornutum* | 3.68 (0.20) | 5.48 (0.27) | 4.32 (0.15) | 5.20 (0.28) | 5.51 (0.30) |
| | *T. weissflogii* | 2.28 (0.08) | 1.89 (0.10) | 2.01 (0.08) | 2.62 (0.15) | n.d.[a] |
| $\alpha^{chl}$ ([gC $g^{-1}$ Chl $h^{-1}$]/ [$\mu$mol $m^{-2}$ $s^{-1}$]) ($\times 10^{-2}$) | *P. tricornutum* | 2.63 ($3.1e^{-3}$) | 3.87 ($4.3e^{-3}$) | 3.38 ($2.7e^{-3}$) | 3.50 ($4.0e^{-3}$) | 3.09 ($3.1e^{-3}$) |
| | *T. weissflogii* | 1.68 ($1.3e^{-3}$) | 1.73 ($1.9e^{-3}$) | 1.95 ($1.6e^{-3}$) | 1.65 ($1.9e^{-3}$) | n.d. |
| $I_k$ ($\mu$mol $m^{-2}$ $s^{-1}$) | *P. tricornutum* | 140 | 141 | 128 | 149 | 178 |
| | *T. weissflogii* | 137 | 110 | 103 | 158 | n.d. |

[a] n.d., Not determined.

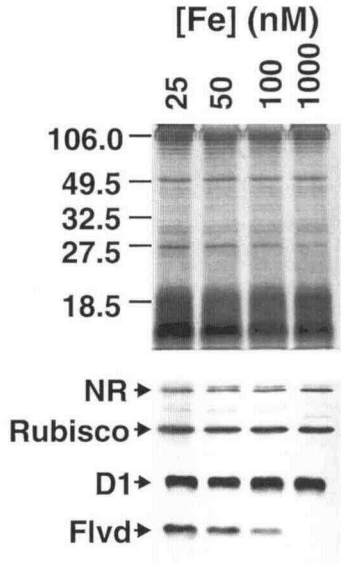


**Figure 3.** SDS-PAGE and immunoblotting of *T. weissflogii* protein extracts. Top, Silver-stained gel. Positions of prestained $M_r$ markers are shown. Bottom, Chemiluminescent detection of immunoreactions. Flvd, Flavodoxin.

in 25 nM Fe cultures were 2-fold higher than in cultures containing 100 nM Fe and 1.3-fold greater than in cultures containing 50 nM Fe. More striking was the >900-fold elevation in levels estimated from 25 nM Fe culture and the faint, yet detectable, signal observed in cultures containing 1000 nM Fe.

## DISCUSSION

One strategy phytoplankton have adopted to counter nutrient deficiency is the expression of specific proteins aimed at alleviating stress. Notable in the case of Fe deficiency is flavodoxin, a small FMN-containing protein in which induction is specific in response to Fe stress and which is capable of functionally replacing Fd, an Fe-containing electron-transport protein (Yoch and Valentine, 1972; Geider and LaRoche, 1994; Straus, 1994).

Diatoms make up a considerable component of the Fe-responsive phytoplankton assemblage of HNLC locales (Boyd et al., 1996; Coale et al., 1996b). Using our diatom-specific probe, we recently demonstrated the potential of flavodoxin as a candidate marker of Fe stress along a transect in the subarctic Pacific stretching from Canadian coastal waters to the open ocean site of former weather station PAPA (LaRoche et al., 1996), the archetypal HNLC locale (Martin and Fitzwater, 1988). This investigation did not assess the relationship between flavodoxin expression and Fe limitation of diatom growth, although it was noted that flavodoxin abundance increased dramatically in phytoplankton sampled from waters containing <1 nM dissolved Fe, a value recently identified as that at which algal (primarily diatom) $\mu$ saturates in HNLC waters of the equatorial Pacific (Coale et al., 1996a). It is the relationship between flavodoxin expression and the onset of $\mu$ limitation that we specifically address in the present investigation. That a biomarker, in this case flavodoxin, might provide an index of Fe limitation of phytoplankton growth is appealing to oceanographers as an in situ diagnostic aid, complementing diagnostic, fluorescence-based biophysical approaches (Falkowski et al., 1992) and avoiding the ambiguities associated with conventional "bottle assays."

In the present study the physiological response to growth in TMB culture medium over a range of Fe additions was assessed for *P. tricornutum* and *T. weissflogii*. Both diatoms exploited for this study are recognized estuarine/coastal isolates and, as such, are presumed to possess high growth requirements for Fe relative to oceanic species (Brand et al., 1983; Brand, 1991; Sunda et al., 1991; Sunda and Huntsman, 1995). Use of a TMB culture technique in the present investigation ensured that Fe, in addition to other trace metal nutrients, remained in a bioavailable form upon addition to the culture medium. Furthermore, in TMB culture, the free-ion concentration of the medium is

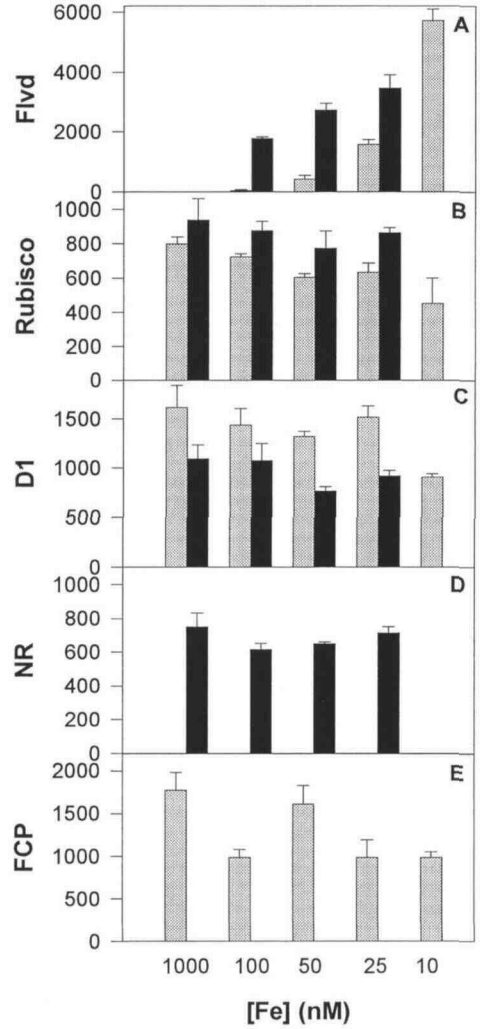


**Figure 4.** Relative levels of protein in diatom TMB culture determined by laser densitometric scans of immunoblots (means ± SE, $n$ = 3). A, Flavodoxin. B, Large subunit of Rubisco. C, D1. D, NR; not determined for *P. tricornutum*. E, FCP; not determined for *T. weissflogii*. Gray bars, *P. tricornutum*; black bars, *T. weissflogii*.

regulated. This approach has been used successfully in discerning differences in the Fe requirement for growth between oceanic and neritic phytoplankton species (Brand et al., 1983; Brand, 1991; Sunda et al., 1991; Sunda and Huntsman, 1995). Unexpectedly, growth and photosynthetic indices remained relatively stable for all of the TMB cultures of *P. tricornutum*. The rate of growth in medium containing 10 nM Fe decreased to no less than 85% of $\mu_{max}$ and maximum rates of photosynthesis did not vary significantly. Despite the apparent indifference of growth and photosynthetic parameters to Fe addition observed for cultures of *P. tricornutum*, our data do not support gross contamination by Fe of the TMB culture medium. This is demonstrated by the more pronounced physiological response to decreasing Fe amendment shown by TMB cultures of *T. weissflogii*. Although cultures provided 50, 100, or 1000 nM Fe exhibited similar, high rates of growth, cultures of *T. weissflogii* amended with 25 nM Fe grew at a significantly reduced rate of 1.21 $d^{-1}$ (70% of $\mu_{max}$), and notably, medium containing 10 nM Fe supported no growth.

Comparison of our data with other published studies (Brand et al., 1983; Sunda and Huntsman, 1995) confirms that the $\mu$ observed in the present investigation are not particularly inflated (Fig. 5). All data in Figure 5 should be comparable, since the composition of TMB medium, and most importantly, the concentration of exogenously added chelator, was the same in each investigation. The normalized $\mu$ reported for both *P. tricornutum* and *T. weissflogii* cluster with other diatoms, both neritic and oceanic, at an addition of 100 nM Fe. With 25 nM total Fe added, the normalized rate reported for our clone of *T. weissflogii* was only marginally higher than that of the clone used by Sunda and Huntsman (1995; Fig. 5A), and medium provided 10 nM Fe supported no growth of our clone. Although *P. tricornutum* clustered with oceanic strains when provided 10 nM Fe (Fig. 5C), its normalized rate of growth was only 20% higher than that of the neritic diatoms *Ditylum brightwellii* and *Bacteriastrum hyalinum* provided this same low level of Fe (Fig. 5B).

Despite the apparent indifference of growth and photosynthetic parameters to Fe addition in the present investigation, the observed pattern of flavodoxin expression indicates that both diatoms responded to the reduction of Fe in the growth medium. Flavodoxin was induced to high levels in low-Fe TMB cultures of each diatom and yet was not observed in cultures amended with 1000 nM Fe. Flavodoxin expression in the absence of attendant reduction in $\mu$ is not without precedent. In fact, there are some reports of constitutive expression of flavodoxin in algae (Fitzgerald et al., 1978; Doucette et al., 1996). Notably, among cyanophytes, the Fe-responsive accumulation of flavodoxin compromises neither $\mu$ (Sandmann and Malkin, 1983) nor rates of photosynthetic $O_2$ evolution (Sandmann et al., 1990). The apparent uncoupling of flavodoxin expression from Fe limitation of diatom growth observed in the present investigation is consistent with these findings and, as such, imposes constraints on the use of flavodoxin as a diagnostic tool in this capacity. It is

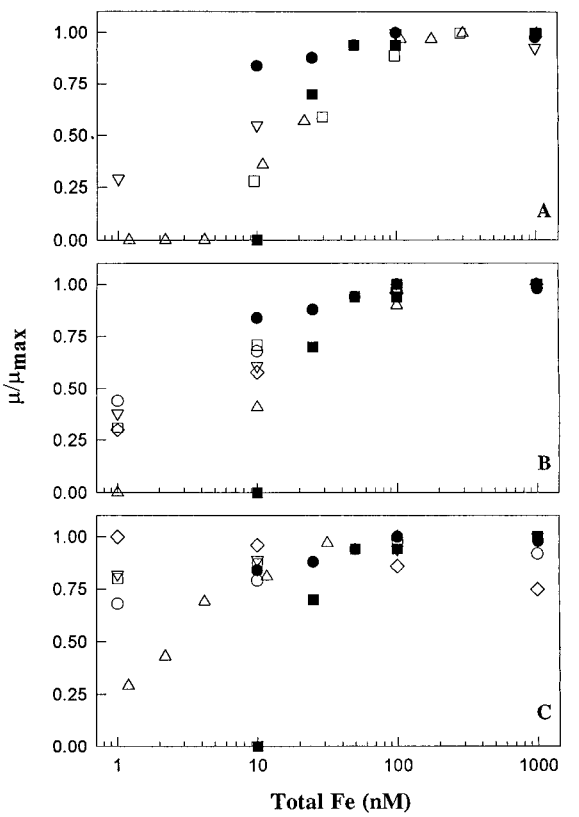


**Figure 5.** Relationship between diatom $\mu$ and total Fe added to the culture medium. Growth is normalized to $\mu_{max}$. Presented is a comparison of available historic data with those determined from the present investigation (■, *T. weisflogii*; ●, *P. tricornutum*). A, Comparison with neritic *T. weissflogii* congeners. □, *T. weissflogii* (S); △, *T. pseudonana* (S); ▽, *T. pseudonana* (B). B, Comparison with other neritic diatoms. ○, *B. hyalinum* (B); □, *D. brightwellii* (B); △, *Lithodesmium undulatum* (B); ▽, *Streptotheca tamesis* (B); ◇, *Skeletonema costatum* (B). C, Comparison with oceanic diatoms. ○, *Rhizosolenia setigera* (B); □, *Hemiaulus sinensis* (B); △, *Thalassiosira oceanica* (S); ▽, *T. oceanica* (B); ◇, *Bacteriastrum delicatulum* (B). (S), Sunda and Huntsman, 1995; (B), Brand et al., 1983.

clear that the mere presence of flavodoxin need not be correlated with Fe-imposed limitation of growth. In addition, under the growth conditions examined, the degree to which cellular flavodoxin is accumulated did not provide a gauge of growth limitation.

Our data are consistent with the finding in *Synechocystis* sp. that flavodoxin is accumulated to high levels prior to the onset of symptoms of severe Fe stress, including reduction in $\mu$ (Sandmann and Malkin, 1983). A similar pattern of expression has been reported for alkaline phosphatase in field populations of phytoplankton prior to onset of severe phosphorus stress (Perry and Eppley, 1981). High levels of alkaline phosphatase activity were measured at reduced, but not growth-limiting, phosphate supply. It follows that induction of flavodoxin might be more accurately viewed as an early stress response, providing a means by which the cell is able to redirect a scant Fe resource to obligate Fe-requiring components. Moreover, photosynthetic electron transport need not be compromised by the replacement of

Fd with flavodoxin, since rate constants for the reduction of both proteins by PSI are of the same order (Sandmann et al., 1990) and both redox catalysts provide comparable rates of NADP+ photoreduction (Razquin et al., 1995). That Fd synthesis is of low priority for cells experiencing Fe stress is further demonstrated by the study of Greene et al. (1992), who showed that early-stage recovery from Fe deprivation in *Phaeodactylum* sp. is characterized by specific synthesis of Fe-containing cytochromes and the Rieske Fe-S protein, but not Fd.

We can conclude from available data that Fe is not a limiting factor when flavodoxin is absent. Flavodoxin is generally not detected in diatoms sampled from Fe-sufficient continental shelf waters of the North Atlantic (LaRoche et al., 1995) and North Pacific (LaRoche et al., 1996). The relationship between flavodoxin and yield was not specifically addressed in the present investigation. Although Fe had little apparent effect on $\mu$, Fe stress imposed significant constraints on accumulation of biomass in our TMB culture system (R. McKay and J. LaRoche, unpublished data).

As part of our assessment of flavodoxin expression, we should also consider the physicochemical context within which cells grow in HNLC regions. Fe is intermittently supplied to most open ocean regions from atmospheric deposition (Duce and Tindale, 1991) and is rapidly sequestered as inorganic precipitates and organic complexes. Total dissolved Fe may not exceed 100 pM (Martin and Gordon, 1988), and Fe′ may be in the subpicomolar range (Rue and Bruland, 1995; Wu and Luther, 1995). Disregarding for the moment the possible, although largely unsubstantiated, exploitation of siderophores by marine diatoms (Trick et al., 1983; Soria-Dengg and Horstmann, 1995), it is commonly accepted that diatoms are capable of assimilating Fe only from the small pool of Fe′ (Hudson and Morel, 1990). Given the intermittent supply and rapid chemical sequestration of Fe, it is likely that phytoplankton are exposed to widely varying concentrations of Fe′ in the field. Thus, traditional batch cultures, which challenge phytoplankton with a rapid decline in Fe′ concentration, may be more representative of conditions in HNLC regions than the TMB cultures used in our study. The TMB culture medium is designed to maintain a nearly constant level of Fe′ through chemical exchange with a vast pool of EDTA-complexed Fe. The conditions of balanced growth achieved in TMB cultures may be less indicative of conditions in the open ocean than the unbalanced growth observed in traditional batch cultures. Thus, the marked relationship of flavodoxin abundance to $\mu$ during unbalanced growth of *P. tricornutum* observed by LaRoche et al. (1995) using traditional batch cultures may more closely mimic conditions experienced by phytoplankton in HNLC regions. It is clear that the use of flavodoxin and Fd to study Fe stress in natural populations of phytoplankton will benefit from studies designed to understand both the subcellular regulation of these proteins by Fe in laboratory culture and more detailed studies of the chemical speciation of Fe in natural seawater.

Furthermore, unless additional experiments show no additional increase in flavodoxin expression at lower Fe-limited balanced $\mu$ beyond levels that we observe at $\mu$ near $\mu_{max}$, we must keep open the possibility that we have not documented the full dynamic range of flavodoxin abundance. However, even if flavodoxin is fully expressed under conditions that fail to elicit a marked reduction of $\mu$, its abundance may still provide an useful index of the cell quota for Fe. Many questions remain to be answered before we can use flavodoxin and/or Fd abundance to unambiguously assess Fe limitation of phytoplankton $\mu$ in the sea. Is synthesis of flavodoxin cued to the external Fe′ concentration or to the cell quota of Fe? To what extent does luxury uptake of Fe allow cells to accumulate intracellular storage pools that can provide a buffer against physiological limitation of $\mu$? What is the relationship between flavodoxin abundance and the cell quota for Fe? How stable are flavodoxin and Fd under highly dynamic conditions such as those expected in nature?

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