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Influence of N substrate on Fe requirements of marine centric diatoms

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ABSTRACT: The interaction between Fe requirements and N metabolism in centric diatoms was investigated to determine whether use of nitrate (NO_3^-) imparts a higher cellular Fe demand for growth than use of ammonium (NH_4^+), and thus reduces fitness under Fe deficiency. Six species of the genus *Thalassiosira* from a variety of habitats were examined. Coastal and central gyre representatives grew faster in Fe-sufficient media containing NH_4^+, but isolates from the equatorial Pacific, an oceanic high-nutrient, low-biomass region, achieved maximum rates with NO_3^-. Iron quotas ranged from 26 to 102 µmol Fe mol^{-1} C and were not affected in a predictable manner by N source or habitat. Relative growth rates were diminished in Fe-deficient media, particularly in coastal species which grew at less than 25% of their maximum rates (μ_{max}). All oceanic species maintained fast rates of growth (0.8 μ_{max}) under the same Fe-limiting conditions, despite having 4 times less intracellular Fe than the coastal species. Fe:C ratios of Fe-deficient *Thalassiosira* spp. ranged from 0.7 to 14 µmol mol^{-1} and were significantly greater (by ~ 1.8 times) in all species when NO_3^- was the N source ($p < 0.05$). Steady-state Fe uptake rates were also faster in NO_3^- dependent cells at low Fe. Nitrogen source had different effects on Fe-limited growth rates. Surprisingly, *T. oceanica* (clone 1003) and *T. weissflogii* grew faster with NO_3^- even though higher Fe requirements for use of oxidized N were expected to reduce division rates relative to NH_4^+-grown cells. When total Fe concentrations in the medium were decreased to 1 nM, growth rates of *T. oceanica* (clone 1003) decreased to 0.2 μ_{max} and were significantly faster (25%) in NH_4^+ than in NO_3^--amended media. Under these more stressful Fe-limiting conditions, Fe quotas were the same in cells cultured in both N-based media. Our results thus demonstrate that phototrophic phytoplankton require significantly more cellular Fe to grow on NO_3^- than NH_4^+. Nitrate-grown cells are able to obtain this extra Fe, even when Fe is limiting, suggesting that Fe acquisition is somehow linked to NO_3^- metabolism. Under severe Fe deficiency, however, NO_3^- utilization reduces division rates compared to NH_4^+, because cells are unable to fulfill their extra Fe requirements.

KEY WORDS: Fe limitation · Biochemical composition · Fe use efficiencies · Diatoms · Nitrate · Ammonium

INTRODUCTION

Surface waters of most oceanic regions contain extremely low dissolved Fe concentrations (Landing & Bruland 1987, Martin et al. 1989, 1991), the cumulative result of low Fe inputs and high chemical and biological reactivity. In parts of the Pacific and Southern oceans, for example, the concentrations of Fe are so low that they limit primary productivity (Martin & Fitzwater 1988, Martin et al. 1989). Evidence for Fe limitation has been obtained from experiments conducted at different spatial and temporal scales, from bottle incubations (Martin & Fitzwater 1988, Martin et al. 1990, 1991, Price et al. 1991, 1994) to *in situ* fertilization experiments (Martin et al. 1994). Some studies, investigating Fe-N interactions in waters rich in NO_3^- but deficient in Fe, have demonstrated that Fe additions not only enhanced net phytoplankton growth and biomass, but also N-specific NO_3^- uptake rates of the phytoplankton community (Price et al. 1991, 1994). In contrast, specific NH_4^+ uptake rates were unaffected by Fe enrichments.

The field results provide indirect evidence of the role of Fe in NO_3^- metabolism. They are consistent with

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theoretical calculations based on Fe-use efficiencies and cellular metabolic Fe demands that predict that phytoplankton growing on NO_3^- need 1.6 times more intracellular Fe than those growing on NH_4^+ (Raven 1988). Ammonium is incorporated directly into amino acids after it is taken up by the cell, in contrast to NO_3^- which must first be reduced to NH_4^+ before assimilation (Syrett 1981). The increased Fe requirement for NO_3^- use arises because the NO_3^- assimilatory enzymes nitrate reductase (NR) and nitrite reductase (NiR) are Fe-containing redox enzymes (with cytochrome$_{557}$- and ferredoxin-prosthetic groups, respectively) (Zumft 1971, Cardenas et al. 1974, Guerrero et al. 1981, Galvan et al. 1986). Additional reductant is also necessary for NO_3^- reduction (8 mol e^- mol^{-1} N) and is derived from Fe-dependent photosynthetic redox reactions. Phytoplankton Fe requirements should thus reflect the N source used for growth: NO_3^- users are predicted to need higher intracellular Fe concentrations. Such high Fe requirements should impede NO_3^- consumption when Fe is scarce so that in NO_3^--rich, Fe-deficient waters, new production will be significantly restrained.

Despite the field investigations (Price et al. 1991, 1994) and a theoretical prediction (Raven 1988), the interdependence of Fe and N metabolism in phytoplankton has received little attention. Activity of the NO_3^- assimilatory enzymes, for example, is inhibited when Fe limits cell growth (Kessler & Czygan 1968, Timmermans et al. 1994) and NO_3^- uptake rates are correspondingly reduced (Rueter & Ades 1987). Consistent with the role of Fe in NO_3^- metabolism, Fe:N ratios in Fe-limited *Gymnodinium sanguineum* grown on NO_3^- were found by Doucette & Harrison (1991) to be higher than in those grown on NH_4^+ . While these studies infer a relationship between Fe and NO_3^- metabolism, direct proof of the higher intracellular Fe concentrations required for phytoplankton growth on NO_3^- compared with those required for growth on NH_4^+ is still lacking.

The main goal of this study was to experimentally confirm the results of Price et al. (1991, 1994) and the theoretical predictions of Raven (1988), which suggest a higher Fe demand for phytoplankton growth on NO_3^- than on NH_4^+. To investigate the generality of a higher Fe requirement for NO_3^- use by phytoplankton, we examined a variety of centric diatoms isolated from coastal and oceanic regions, including the low Fe waters of the equatorial Pacific, an oceanic high-nutrient, low-biomass region (HNLB) (Chisholm & Morel 1991). Six species of the genus *Thalassiosira* from these different habitats were cultivated in Fe-sufficient (8.4 µM Fe and 100 µM EDTA) and Fe-limiting media (12.5 nM Fe and 100 µM EDTA) enriched with either NO_3^- or NH_4^+ as a N source. Experiments tested the effects of N source (NH_4^+ vs NO_3^-) on growth rates, biochemical composition, and cellular Fe requirements as a function of Fe nutritional state.

MATERIALS AND METHODS

Study organisms. Six species of centric diatoms of the genus *Thalassiosira* were examined. Two species, *T. pseudonana* (clone 3H) and *T. weissflogii* (clone Actin), were isolated from coastal waters (coastal isolates), and 2 others, *T. oceanica* (clones 13.1 and 1003), were oceanic species from the Sargasso Sea (oceanic central gyre isolates). These phytoplankton were obtained from the Center for Culture of Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME, USA). *T. subtilis* (clone 50 Ait) and *T. partheneia* (clone Thal 9) were isolated (by N. M. Price) from Fe-enriched (1 nM) water samples from the equatorial Pacific Ocean (0° 01.9' N, 139° 59.2' W) in August 1992 (EQPAC isolates).

Culture media. All phytoplankton were grown in the artificial seawater medium Aquil (Morel et al. 1979, Price et al. 1988/89) containing standard enrichments of phosphate (PO_4^{3-}) and silicate (SiO_3^{2-}), with either 50 µM NO_3^- or 50 µM NH_4^+. Synthetic ocean water (SOW) and nutrient enrichment stock solutions (NO_3^- and NH_4^+) were purified of trace metals using Chelex 100 ion exchange resin (Bio-Rad Laboratories, Richmond, CA, USA) according to the procedure of Price et al. (1988/89). Media were sterilized by microwaving in acid-washed Teflon bottles (Keller et al. 1988), and enriched with filter-sterilized (0.2 µm Acrodisc) EDTA-trace metal and vitamin (B12, thiamine and biotin) solutions. Trace metal concentrations were buffered with 100 µM EDTA so that Cu, Mn, Zn, and Co free-ion concentrations were $10^{-13.79}$ M, $10^{-8.27}$ M, $10^{-10.88}$ M, and $10^{-10.88}$ M, respectively. These concentrations were calculated using the chemical equilibrium program MINEQL (Westall et al. 1976). Total Mo and Se concentrations in the media were 10^{-7} M, and 10^{-8} M, respectively. Premixed Fe-EDTA (1:1) was added separately at a total concentration of 8.4 µM or 12.5 nM to achieve free ferric ion concentrations of $10^{-18.18}$ M (pFe 18) and 10^{-21} M (pFe 21), corresponding to estimated inorganic Fe (Fe') concentrations of 41 nM and 20 pM. The estimated Fe' concentrations were those computed for Aquil seawater medium in the dark using MINEQL, ignoring possible precipitation of Fe hydroxides at high Fe concentrations (Sunda & Huntsman 1995) and photoreduction of Fe-EDTA chelates (Hudson & Morel 1990).

In some experiments Fe was not added to the medium. In these cases, phytoplankton were able to grow using the low levels of Fe contamination in our

medium, estimated from final biomass yield of *Thalassiosira oceanica* 13.1 to be 1 to 2 nM. To minimize contamination, all manipulations were performed in a laminar flow hood. The medium was allowed to equilibrate chemically overnight before use and was stored in sterile, acid-washed polycarbonate bottles. All bottles and tubes used in our experiments were acid-washed (soaked for at least 24 h in 10% HCl solution), followed by repetitive washes with Q-H_2O (Millipore, >18 MΩ cm^{-1}).

Phytoplankton were grown in acid-washed, 28 ml polycarbonate tubes at 20°C under a continuous, saturating photon flux density of 200 µE m^{-2} s^{-1}, using the semi-continuous batch culture technique described by Brand et al. (1981). Although sterile techniques were used for all culture work to minimize bacterial contamination, the cultures were not axenic. Using the Coulter Counter® (model ZM), however, we estimated that bacterial biovolumes in our cultures were negligible compared to those of phytoplankton. Furthermore, in most of the experiments, cultures were collected on filters with pore sizes of 1 µm or 3 µm, which were sufficient to capture all the phytoplankton, but very few bacteria.

Growth rates. Biomass was determined daily by measuring *in vivo* fluorescence of cultures using a Turner Designs model 10-AU fluorometer Specific growth rates were determined from linear regressions of ln *in vivo* fluorescence versus time during the exponential phase of growth. Acclimation was considered complete when growth rates of successive transfers did not vary by more than 10%. Unless otherwise specified, all data reported here are expressed as mean ± standard error.

Fe quota measurements. Iron quotas (µmol Fe mol^{-1} C) were measured using the radiotracer $^{55}FeCl_3$ (specific activity 25 to 40 mCi mg^{-1}, DuPont Canada). After cultures were acclimated, a phytoplankton inoculum was transferred in triplicate to an identical medium in which 10% or 1% of the total Fe was added as the ^{55}Fe-radiotracer solution (pFe 21 and pFe 18, respectively). To ensure complete labelling of cellular Fe, 8 or more cellular divisions were allowed before harvesting. During mid-exponential phase, samples were filtered onto 3 µm or 1 µm polycarbonate Poretics filters (depending on cell size), and allowed to stand for 10 min in Ti (III) EDTA-citrate reducing solution to dissolve Fe hydroxides and deabsorb ferric ions bound to cell surfaces (Hudson & Morel 1989). The filters were washed with 10 ml of SOW before running dry. Fluor (Ecolite) was added to the samples, and then ^{55}Fe was measured by liquid scintillation counting with a Beckman model LKB scintillation counter. Cellular ^{55}Fe values were corrected for filter adsorption using culture media without cells. Total cellular Fe was computed using the specific activity of ^{55}Fe in the medium (dpm mol^{-1}) and the particulate ^{55}Fe (dpm), correcting for quenching and decay. Culture samples were preserved with Lugol's solution and cell density measurements made by microscopic counting (minimum 600 cells) in a Palmer-Maloney chamber. A single sample was counted twice.

Relative Fe quotas of Fe-limited phytoplankton grown in medium with no Fe additions were measured by adding 0.2 nM ^{55}Fe to the medium. These quotas are uncertain because the ^{55}Fe specific activity was calculated using an estimated background Fe contamination of 1 to 2 nM. The relative difference between Fe content of cells in the NO_3^- and NH_4^+ cultures however is not biased, since the same medium was used for both.

To convert the cellular ^{55}Fe values (mol Fe $cell^{-1}$) to Fe:C ratios and intracellular Fe concentrations (M), measurements of mol C $cell^{-1}$ and cell volumes (fl $cell^{-1}$) were determined in parallel cultures with non-radioactive Fe additions.

C and N intracellular concentrations. Cellular C and N were measured in mid-exponential phase cultures. Samples (25 ml) were filtered onto glass fiber filters (Whatman GF/C, pre-combusted at 450°C for 4.5 h), and rinsed with 25 ml of SOW before filtration was completed. The filters were then oven dried at 50°C, pelletized, and analyzed for elemental C and N using a Carlo Erba 1106 elemental analyzer. An aliquot of the phytoplankton culture was reserved for immediate cell volume determinations using a Coulter Counter® (model ZM) previously calibrated with polystyrene beads. An additional subsample was preserved with Lugol's solution for subsequent cell counts.

Habitat classification. For comparative purposes, phytoplankton were grouped according to their specific growth rates in Fe-deficient (pFe 21) medium. Statistical analysis showed that the 6 species formed 3 distinct habitat groups: coastal, oceanic central gyre, and EQPAC. The coastal group, comprising *Thalassiosira pseudonana* (clone 3H) and *T. weissflogii* (clone Actin), grew slowest, the central gyre group, comprising *T. oceanica* (clones 13.1 and 1003), grew fastest, and the EQPAC group, comprising *T. subtilis* (clone 50Ait) and *T. partheneia* (clone Thal 9), grew at intermediate rates (2-way ANOVA, $p < 0.001$; Bonferroni *t*-test, $p < 0.05$).

RESULTS

Fe sufficiency

Under Fe-replete conditions (pFe 18) maximal specific growth rates (regardless of the N source in the

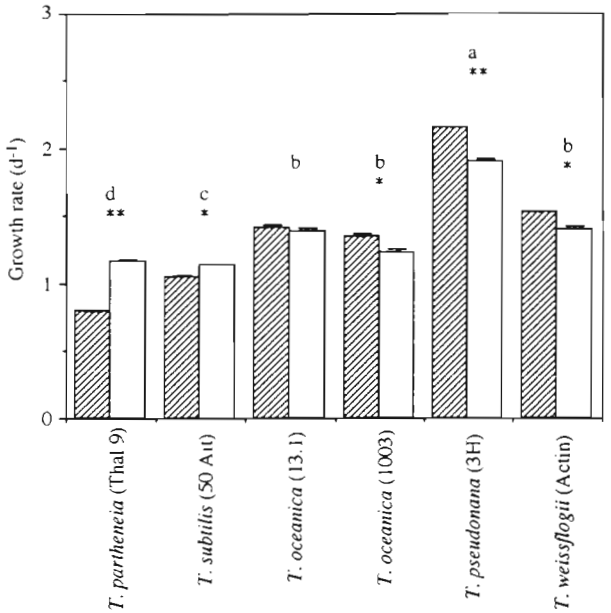

Fig 1. Mean growth rates (d^{-1}) for *Thalassiosira oceanica* 13.1 (n = 9) and 1003 (n = 10), *T. partheneia* Thal 9 (n = 10), *T. subtilis* 50 Ait (n = 13), *T. pseudonana* 3H (n = 15), and *T. weissflogii* Actin (n = 25), grown on Fe-sufficient (pFe 18) Aquil medium enriched with 50 µM NO_3^- (open bars) or NH_4^+ (shaded bars) as N source. Error bars represent the standard error around the mean, and was too small to be resolved in cases where it is not visible. *Significantly different (paired *t*-test: NO_3^- vs NH_4^+ growth rates, $p < 0.01$), **significantly different (paired *t*-test: NO_3^- vs NH_4^+ growth rates, $p < 0.001$). Species growth rates with the same letter above bars were not significantly different from one another [2-way ANOVA (N source and Habitat group), $p < 0.05$; Bonferroni *t*-test, $p < 0.05$]

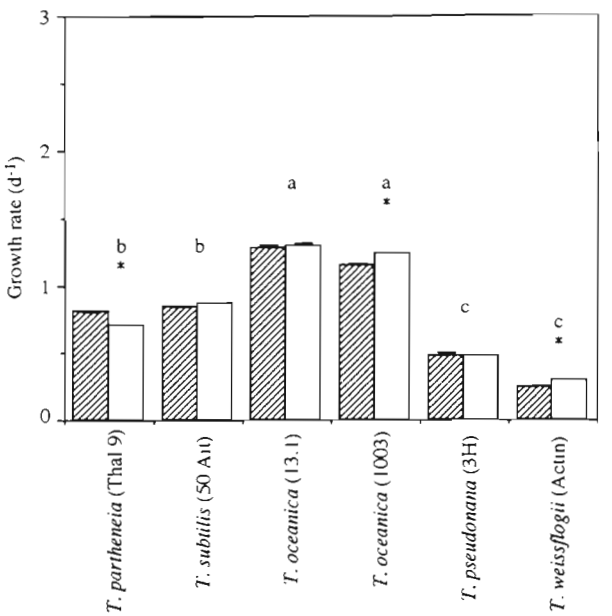

Fig. 2. Mean growth rates (d^{-1}) for *Thalassiosira oceanica* 13.1 (n = 20) and 1003 (n = 25), *T. partheneia* Thal 9 (n = 12), *T. subtilis* 50 Ait (n = 12), *T. pseudonana* 3H (n = 12), and *T. weissflogii* Actin (n = 25), grown on Fe-limiting (pFe 21) Aquil medium enriched with 50 µM NO_3^- (open bars) or NH_4^+ (shaded bars) as N source. Error bars represent the standard error around the mean, and was too small to be resolved in cases where it is not visible. *Significantly different (paired *t*-test: NO_3^- vs NH_4^+ growth rates, $p < 0.05$). Species growth rates with the same letter above bars were not significantly different from one another [2-way ANOVA (N source and Habitat group), $p < 0.05$; Bonferroni *t*-test, $p < 0.05$]

medium) ranged from 1.14 to 2.16 d^{-1} (Fig. 1). *Thalassiosira pseudonana* had significantly faster rates of growth than any of the other species ($p < 0.05$). The other coastal representative, *T. weissflogii*, grew faster on average than the central gyre isolates (*T. oceanica* 1003 and 13.1), but the difference between the rates was not significant ($p > 0.05$). The EQPAC species (*T. subtilis* and *T. partheneia*) had the slowest growth rates at pFe 18 ($p < 0.05$). Growth rates were independent of cell size (Spearman rank correlation, $p > 0.05$).

Nitrogen substrate significantly affected the maximum growth rate attained by the species (Fig. 1) except in *Thalassiosira oceanica* 13.1. The EQPAC isolates grew faster (*t*-test, $p < 0.01$) in NO_3^-- than in NH_4^+-amended media, while the central gyre clone *T. oceanica* 1003 and the coastal isolates *T. pseudonana* and *T. weissflogii* grew significantly faster with NH_4^+ as the N substrate (*t*-test, $p < 0.01$).

Neither N source nor habitat had predictable effects on the intracellular chemical composition of the Fe-replete phytoplankton species. Intracellular Fe, C, and N concentrations ranged from 0.4 to 2.8 mM, 7.8 to 27 M, and 1.16 to 3.32 M, respectively (Table 1). C:N ratios were in good agreement with Redfield proportions, indicative of healthy cells. Fe:C ratios ranged from 26 to 102 µmol Fe mol^{-1} C and in 3 species (*Thalassiosira partheneia*, *T. oceanica* 13.1, and 1003) were similar in NH_4^+- and NO_3^--amended cultures. *T. subtilis* and *T. pseudonana* had higher Fe:C ratios when growing with NO_3^- *T. weissflogii* showed the opposite trend with higher Fe:C ratios in the NH_4^+-grown cells. With the exception of *T. oceanica* 13.1, cell volumes were always greater in NH_4^+- than in NO_3^--grown cells (Table 1).

Fe deficiency

In general, growth rates of all the species were significantly reduced under Fe-limiting conditions (pFe 21) (Fig. 2). The exceptions were *Thalassiosira partheneia* (NH_4^+-amended cultures) and *T. oceanica* 1003 (NO_3^--amended cultures), which had slower rates of growth in pFe 21 than in pFe 18, but the difference

Table 1. Mean cell volumes (fl cell^{-1}) and elemental chemical composition for 6 *Thalassiosira* spp. Phytoplankton were grown on Fe-sufficient (pFe 18) Aquil medium enriched with either 50 µM NO_3^- or NH_4^+ as N source

Phytoplankton species	N substrate	Cell volume (fl cell^{-1})	Cellular Fe (mM)	Cellular C (M)	Cellular N (M)	C:N	Fe:C (µmol Fe mol^{-1} C)
T. partheneia (Thal 9)	NH_4^+	129 ± 8.8	2.8 ± 0.1	27 ± 0.1	3.32 ± 0.13	8.1 ± 0.04	102 ± 0.1
	NO_3^-	111 ± 1.2	2.3 ± 0.03	25 ± 0.04	2.97 ± 0.04	8.4 ± 0.01	90 ± 0.1
T. subtilis (50 Ait)	NH_4^+	1467 ± 42	0.5 ± 0.1	17 ± 0.1	2.6 ± 0.07	6.5 ± 0.03	28 ± 0.1
	NO_3^-	1208 ± 78	1 ± 0.1	18 ± 0.1	2.5 ± 0.11	7.2 ± 0.05	56 ± 0.04
T. oceanica (13.1)	NH_4^+	87 ± 0.9	0.8 ± 0.03	21 ± 0.02	2.2 ± 0.03	9.5 ± 0.02	37 ± 0.03
	NO_3^-	128 ± 1.5	0.5 ± 0.1	13 ± 0.03	1.84 ± 0.07	7.1 ± 0.04	35 ± 0.1
T. oceanica (1003)	NH_4^+	118 ± 0.7	0.42 ± 0.1	16 ± 0.1	2.09 ± 0.04	7.7 ± 0.02	26 ± 0.1
	NO_3^-	77 ± 0.6	0.4 ± 0.2	14 ± 0.02	2.3 ± 0.23	6.1 ± 0.11	29 ± 0.5
T. pseudonana (3H)	NH_4^+	48 ± 0.5	0.71 ± 0.1	19 ± 0.1	2.69 ± 0.09	7.1 ± 0.04	38 ± 0.1
	NO_3^-	30 ± 0.2	0.95 ± 0.1	18 ± 0.02	2.45 ± 0.03	7.4 ± 0.03	56 ± 0.1
T. weissflogii (Actin)	NH_4^+	1431 ± 13	0.47 ± 0.1	7.8 ± 0.02	1.16 ± 0.1	6.7 ± 0.09	60 ± 0.03
	NO_3^-	1269 ± 7	0.41 ± 0.1	10.3 ± 0.02	1.43 ± 0.09	7.2 ± 0.06	40 ± 0.02

Table 2. Mean cell volumes (fl cell^{-1}) and elemental chemical composition for 6 *Thalassiosira* spp. Phytoplankton were grown on Fe-limiting (pFe 21) Aquil medium enriched with either 50 µM NO_3^- or NH_4^+ as N source

Phytoplankton species	N substrate	Cell volume (fl cell^{-1})	Cellular Fe (µM)	Cellular C (M)	Cellular N (M)	C:N	Fe:C (µmol Fe mol^{-1} C)
T. partheneia (Thal 9)	NH_4^+	112 ± 3.0	51 ± 0.03	25 ± 0.04	3.23 ± 0.06	7.74 ± 0.02	2.1 ± 0.03
	NO_3^-	86 ± 4.0	83 ± 0.1	25 ± 0.1	3.11 ± 0.05	8.04 ± 0.02	3.34 ± 0.1
T. subtilis (50 Ait)	NH_4^+	1179 ± 30	14 ± 0.04	21 ± 0.1	2.84 ± 0.04	7.39 ± 0.02	0.65 ± 0.1
	NO_3^-	814 ± 17	31 ± 0.1	19 ± 0.1	2.44 ± 0.08	7.79 ± 0.03	1.7 ± 0.1
T. oceanica (13.1)	NH_4^+	66 ± 0.9	29 ± 0.1	17 ± 0.01	2.48 ± 0.05	6.85 ± 0.02	1.68 ± 0.1
	NO_3^-	63 ± 0.5	32 ± 0.01	16 ± 0.04	1.88 ± 0.03	8.51 ± 0.02	2.03 ± 0.03
T. oceanica (1003)	NH_4^+	87 ± 0.8	73 ± 2.5	22 ± 0.04	3.14 ± 0.07	7.01 ± 0.02	3.26 ± 0.1
	NO_3^-	65 ± 0.2	100 ± 3.9	20 ± 0.1	2.46 ± 0.03	8.13 ± 0.01	4.92 ± 0.1
T. pseudonana (3H)	NH_4^+	14 ± 0.1	115 ± 16	12 ± 0.1	2.8 ± 0.01	4.29 ± 0.01	9.5 ± 0.5
	NO_3^-	16 ± 0.03	154 ± 1.8	11 ± 0.1	2.83 ± 0.02	3.89 ± 0.01	14 ± 0.1
T. weissflogii (Actin)	NH_4^+	550 ± 11	51 ± 8.6	12.6 ± 0.02	1.96 ± 0.74	6.44 ± 0.38	4.1 ± 0.07
	NO_3^-	632 ± 8	110 ± 10	11.3 ± 0.02	1.49 ± 0.12	7.56 ± 0.08	9.79 ± 0.03

was not statistically significant ($p > 0.05$). Coastal species showed the greatest growth rate reduction (mean μ/μ_{max} = 0.21), whereas all oceanic isolates grew at rates near their maximum (mean μ/μ_{max} = 0.8). Regardless of N source, the specific growth rates of the coastal, the central gyre and the EQPAC species were significantly different at pFe 21 (2-way ANOVA, $p < 0.001$; Bonferroni *t*-test, $p < 0.05$) and increased in the order of coastal, EQPAC, and central gyre species.

Intracellular Fe concentrations of cells in Fe-limited cultures ranged from 14 to 154 µM and were greatly decreased by an average of 10-fold compared with those of Fe-sufficient cells. Except for *Thalassiosira pseudonana*, C (11 to 25 M) and N (1.88 to 3.23 M) concentrations, however, were not significantly affected by Fe limitation (Table 2). Fe:C ratios of coastal species (mean = 9.35 ± 4.1 µmol Fe mol^{-1} C) were approximately 4 times those of all other species (mean = 2.46 ± 1.3 µmol Fe mol^{-1} C, *t*-test, $p < 0.05$) (Table 2), a consequence of higher intracellular Fe (mol Fe fl^{-1}), and lower C (mol C fl^{-1}) concentrations. Coastal isolates were smaller when grown on NH_4^+, whereas oceanic isolates were smaller when grown in NO_3^- cultures (*t*-test, $p < 0.05$). Cell volumes of *T. oceanica* 13.1 were the same in both media.

Under Fe-limiting conditions, half of the species examined had similar specific growth rates in NO_3^-- and NH_4^+-amended media (Fig 2). The effects of N source on those species with dissimilar growth rates in the 2 media varied. *Thalassiosira partheneia* achieved faster growth rates in NH_4^+ (*t*-test, $p < 0.05$), while *T. oceanica* 1003 and *T. weissflogii* grew significantly faster in NO_3^--amended media (paired *t*-test, $p < 0.05$). This latter result was particularly surprising given the

Table 3. Steady-state Fe uptake rates of Fe-limited (pFe 21) *Thalassiosira* spp. grown in media enriched with either 50 µM NO_3^- or NH_4^+ (n = 3, mean ± SD)

Species	Steady-state Fe uptake rates ($\times 10^{-20}$ mol Fe µm^{-2} d^{-1})	
	NH_4^+	NO_3^-
T. partheneia (Thal 9)	4.12 ± 0.01	5.38 ± 0.01
T. subtilis (50 Ait)	2.53 ± 0.01	5.29 ± 0.01
T. oceanica (13.1)	3.13 ± 0.01	3.40 ± 0.01
T. oceanica (1003)	7.75 ± 0.01	10.3 ± 0.01
T. pseudonana (3H)	2.63 ± 0.02	3.71 ± 0.01
T. weissflogii (Actin)	2.90 ± 0.02	5.79 ± 0.2

anticipated higher Fe demand for NO_3^- metabolism. Measurement of Fe cell quotas for Fe-limited cultures, however, demonstrated that in all cases Fe:C ratios were significantly higher (1.8 ± 0.56 times) in NO_3^-- than in NH_4^+-grown cells (paired *t*-test, $p < 0.05$) (Table 2).

The consistently greater Fe:C ratios for all NO_3^--grown cells resulted from higher intracellular Fe concentrations (mol Fe fl^{-1}). Under Fe-limiting conditions all species had reduced cell volume, yet absolute cell volumes were not always smaller for NO_3^-- than NH_4^+-grown cells, and thus could not account for their consistently higher Fe:C ratios. Since C concentrations (mol C fl^{-1}) were unaffected by N source (Table 2), higher intracellular Fe quotas could only result from faster steady-state uptake rates.

Steady-state Fe uptake rates of Fe-limited cultures were calculated from growth and quota data, viz.: $\rho^{ss} = \mu Q_{Fe}$, where μ = growth rate (d^{-1}), and Q_{Fe} = amol Fe cell^{-1} (Table 3). Cells grown with NO_3^- had significantly faster Fe transport rates than cells grown with NH_4^+. This result was true regardless of the normalization: Fe uptake rates per unit C biomass (µmol Fe mol^{-1} C d^{-1}, paired *t*-test, mean difference = 1.39, df = 5, $p < 0.01$), per unit volume (µmol Fe l^{-1} cell volume d^{-1}, paired *t*-test, mean difference = 19.27, df = 5, $p < 0.01$), or per unit surface area (amol Fe µm^{-2} d^{-1}, paired *t*-test, mean difference = 1.94, df = 5, $p < 0.01$) were faster in NO_3^--grown cells.

Severe Fe limitation

To test whether more severe Fe limitation would cause cells to grow slower with NO_3^- than with NH_4^+, we grew cells in a medium without added Fe except for the 0.2 nM amount added with the ^{55}Fe-radiotracer solution. *Thalassiosira oceanica* 1003, one of the 2 species that grew faster with NO_3^- than NH_4^+ at pFe 21, was chosen for these experiments. The lower Fe concentrations in this medium decreased growth rates to 20% of those observed under the normal Fe-limiting conditions (pFe21) Phytoplankton in NO_3^--enriched cultures grew significantly slower (0.17 ± 0.09 d^{-1}) than those in NH_4^+-enriched cultures (0.23 ± 0.04 d^{-1}) (*t*-test, $p < 0.05$), and their Fe:C ratios were not significantly different (0.58 ± 0.03 vs 0.67 ± 0.08 µmol Fe mol^{-1} C, $t = 1.8$, $t_{0.05\,(2),\,4} = 2.78$, $p > 0.05$). Nitrate-grown cells were thus unable to acquire the extra Fe needed for NO_3^- metabolism under these very limiting conditions and consequently grew slower than NH_4^+-grown cells.

DISCUSSION

Fe quotas and growth rates

The results of this study show clearly that N substrate influences both Fe uptake and Fe requirements for phytoplankton growth. Under Fe deficiency, all species examined contained significantly more Fe per cell volume and had higher Fe:C ratios when using NO_3^- as opposed to NH_4^+. Carbon and N composition of the cells was unaltered by Fe limitation, consistent with previous studies (Doucette & Harrison 1991, La Roche et al. 1993). The average increase in cellular Fe content (1.6 and 1.8 times, mol Fe cell^{-1} and Fe:C, respectively) agrees remarkably well with the increase in metabolic iron requirements predicted from first principles (Raven 1988, Morel et al. 1991). Thus, the greater Fe quota of NO_3^--grown cells can be accounted for by the iron contained in the NO_3^- assimilatory enzymes and in the redox proteins that supply reductant for NO_3^- reduction.

Higher Fe demand for NO_3^- use might easily be fulfilled when Fe concentrations are saturating for growth, as in the case of pFe 18 medium. Changes in the Fe transport system could accommodate increases or decreases in cellular requirements effected by the N sources. Such modulation of Fe transport is one means by which phytoplankton maintain near maximal rates of growth over a wide range of Fe concentrations. Iron uptake is a function of the rate constant of metal binding to the transport ligand, the transport ligand density and the concentration of reactive Fe (Fe') (Hudson & Morel 1990). When phytoplankton are grown in low Fe media, Fe uptake capacity increases by several-fold (Harrison & Morel 1986). This adaptive strategy compensates for lower Fe concentrations, but it has a limit. The limit is ultimately set by the maximum density of iron transport ligands that can fit in the cell membrane. In *Thalassiosira weissflogii*, this corresponds to about 5 pmol cm^{-2} (Hudson & Morel 1990). As Fe concentrations decrease further, the enhanced transport system can no

longer acquire Fe at a sufficient rate, and cellular growth rates are correspondingly reduced. Thus, under growth rate limiting conditions, both NO_3^-- and NH_4^+-grown cells should equally enhance their Fe transport systems to maximize Fe acquisition. Nitrate-grown phytoplankton should not have a greater ability to take up more Fe than NH_4^+-grown cells.

Another way for phytoplankton to achieve higher Fe quotas under limiting conditions is to decrease cell size. Indeed, all *Thalassiosira* species were observed to be significantly smaller in Fe-limiting than in Fe-sufficient media. The benefit of size reduction exists because transport is proportional to surface area and Fe quotas are proportional to volume. Small cells, which have greater surface area to volume ratios (SA/V) than do large ones, may thus transport Fe at faster rates relative to volume and hence accumulate larger quotas. Nitrate-grown cells could theoretically obtain this same advantage if they were smaller than NH_4^+-grown cells, however, their reduction in size would have to be very substantial for this to be an effective mechanism.

The effect of size reduction is illustrated in the following example by considering 2 phytoplankton cells, one growing on NO_3^- and the other on NH_4^+. At steady state, Fe quota (Q; mol cell^{-1}) is simply the ratio of the uptake rate (ρ; mol cell^{-1} h^{-1}) to the growth rate (μ; h^{-1}):

$$Q = \rho/\mu \quad (1)$$

This equation can also be rewritten as a function of cellular volume (V):

$$Q/V = \rho_{SA}(SA/V)/\mu \quad (2)$$

where ρ_{SA} is the transport rate per membrane surface area. If ρ_{SA} is the same regardless of N source and we assume for the moment that growth rates are also equal, then the relative increase in quota is equal to the relative increase in SA/V of the cell:

$$(Q/V_{NO_3^-})/(Q/V_{NH_4^+}) = (SA/V_{NO_3^-})/(SA/V_{NH_4^+}) \quad (3)$$

To account for a 1.6 times greater Fe quota, NO_3^--grown phytoplankton would thus have to have a 1.6 times greater SA/V than NH_4^+-grown cells. However, the mean $(SA/V_{NO_3^-})/(SA/V_{NH_4^+})$ at pFe 21 for all species examined in this study was only 1.04 ± 0.08, so size reduction alone cannot account for the increased Fe quota in the NO_3^- cultures. If, under Fe limitation, volume reduction were the only means by which NO_3^--grown cells could increase Fe uptake compared to NH_4^+-grown cells, then a hypothetical cell of a 1000 µm^3 would have to reduce its size to 298 µm^3 when grown in NO_3^-. This extent of volume reduction was never observed during our study, and in 2 cases (*Thalassiosira weissflogii* and *T. pseudonana*) cells were actually smaller when growing on NH_4^+, but had higher Fe quotas with NO_3^-.

According to Eq. (1), NO_3^--grown cells must either grow slower and/or transport Fe at a faster rate than NH_4^+-grown cells if they contain more Fe when Fe is limiting. A preferential increase in the Fe transport rate in NO_3^--grown cells is inconsistent with our understanding of Fe transport regulation, as discussed above. The low concentration of Fe in the pFe 21 medium was clearly limiting to all species to some degree so that Fe transport rates should be maximized in both N treatments.

The growth rate results are also unable to account for the differences that were seen in the Fe quotas. In all but one species, NO_3^--grown cells grew as fast or faster than NH_4^+-grown cells (Fig. 2), which should tend to decrease not increase their Fe quotas. This observation and the preceding analyses suggest that our assumptions regarding Fe transport may be incorrect or that NO_3^--grown cells have other means to increase cellular Fe content.

Because of the higher quotas and fast growth rates, calculated steady state uptake rates of NO_3^--grown cells were significantly faster than in NH_4^+-grown cells in pFe 21 medium (Table 3). Under these Fe-limiting conditions NO_3^--grown cells were thus able to take up Fe at faster rates than NH_4^+-grown cells, a result that was true regardless of how the rates were normalized (per unit volume, surface area or cellular C). This observation is surprising, because it suggests that NO_3^--grown cells have a unique ability to increase their uptake rates for Fe.

Experiments that compared the effects of N substrate and Fe requirements used Aquil medium enriched with all nutrients except N. The different treatments were established by adding either NO_3^- or NH_4^+ to subsamples of the same medium so that any variations in Fe concentration in the base medium would be the same for both N substrates. We note that any variation in the levels of Fe contamination in the 2 N-based media cannot simultaneously explain the growth and quota difference that we observed. Preferential Fe contamination of the NO_3^- media, for example, would decrease the measured Fe quota by increasing phytoplankton growth rates (Eq. 1) and by diluting the ^{55}Fe-tracer The most obvious difference between the phytoplankton in the cultures is the additional biochemical pathways required for NO_3^- assimilation and transport. Ammonium produced from NO_3^- or taken up directly from the medium was assumed to be incorporated into amino acids by the same mechanism; although, the use of a glutamate dehydrogenase instead of the glutamine synthetase pathway for NH_4^+ assimilation (Syrett 1981) may possibly have differed between the cultures.

A decrease in Fe concentration in the medium to the background level reduced the growth rate of *Thalassiosira oceanica* 1003 to 20% of the maximum. In this experiment, Fe quotas were not significantly different between the NO_3^-- and the NH_4^+-grown cultures. The growth rates of the NO_3^--grown cultures were slower than those in NH_4^+, indicating that if the NO_3^- cells are unable to acquire the extra Fe they need, they grow slower. Our results show that there is a great deal of variability in phytoplankton response among species. Some species grew slower on NO_3^- even at pFe 21. We predict that all species in this study would eventually grow slower on NO_3^- if the Fe concentration was reduced to still lower levels.

The link between Fe acquisition and NO_3^- metabolism that our results suggest has been observed in other taxa. Bacteria can reduce Fe(III) bound to organic complexes by a process involving NO_3^- reductase (Ottow 1968). Mutants lacking this enzyme are deficient in the Fe reduction pathway (Ottow 1970). Field studies (Sørensen 1982, Jones et al. 1983) also suggest that facultative NO_3^- reducing heterotrophs are involved in iron reduction. Purified NR from higher plants is able to reduce a variety of Fe complexes *in vitro*, such as ferric citrate (Redinbaugh & Campbell 1983), cytochrome *c* (Solomonson & Vennesland 1972), ferricyanide (Solomonson & Vennesland 1972), and Fe bound to siderophores (Castignetti & Smarrelli 1984). Plasmalemma bound NR might be involved in the acquisition of Fe by root cells since it reduces Fe-containing molecules such as cytochromes (Stöhr et al. 1993, Meyerhoff et al. 1994). Although none of these studies demonstrate that NR reduction of Fe could enhance Fe acquisition during Fe-deficient conditions, they strongly support the link between Fe acquisition and NO_3^- metabolism.

Phytoplankton cultures are also able to reduce Cu^{2+} and Fe^{3+} complexes (Anderson & Morel 1980, Jones et al. 1987) by an extracellular reaction that has been hypothesized to be mediated by the diaphorase subunit of plasmalemma bound NR (Jones & Morel 1988). For Fe-limited phytoplankton growing in chelated medium, most of the Fe is present as Fe-EDTA complexes and is not directly available for uptake. Reduction of this organic Fe should result in higher inorganic Fe concentrations and faster phytoplankton growth rates. Nitrate-grown cells may thus have a unique mechanism to increase the concentrations of dissolved inorganic Fe near the cell surface, either by release of soluble reducing compounds (Anderson & Morel 1980, Jones et al. 1987) which reduce Fe bound to organic complexes, or by extracellular reduction of organic Fe by plasmalemma bound redox proteins (Jones et al. 1987).

Iron use efficiencies

Iron-use efficiency (IUE) is defined as the C assimilation rate per unit of cellular Fe (C Fe^{-1} h^{-1}) calculated from the quotient of the specific growth rate and the Fe:C ratio. It thus provides a comparative measure of the ability of phytoplankton to grow under low Fe that Fe quotas alone cannot convey. Oceanic phytoplankton are well known to grow faster than coastal species under low Fe concentrations (Ryther & Kramer 1961, Brand et al. 1983). Their lower Fe requirements are thought to be an evolutionary adaptation to the low ambient levels of Fe that characterize their oceanic habitats. Iron-use efficiencies are considerably higher for oceanic than coastal species (Sunda & Huntsman 1995), a trend that is also apparent in our data (Table 4). The 2 EQPAC isolates are also indistinguishable from the central gyre species in this regard: their average IUE for both N substrates was $(7.0 \pm 4.4) \times 10^5$ mol C mol^{-1} Fe d^{-1}.

The average IUE for coastal and oceanic phytoplankton was (0.44 ± 0.14) and $(5.5 \pm 3.6) \times 10^5$ mol C mol^{-1} Fe h^{-1} (Table 4), respectively, which are in agreement with those we calculated from other studies ($IUE_{coastal}$ = 0.66 ± 0.28 and $IUE_{oceanic} = 4.62 \pm 1.19$, Table 5). Nitrogen substrate had a considerable effect on IUE, as was expected from the large differences in quotas in NO_3^-- and NH_4^+-amended cultures. Theoretical calculations of IUE (Raven 1988, 1990) are considerably lower (by a factor of 6) than the measured values for oceanic phytoplankton. These theoretical IUEs were calculated using maximal growth rates and optimal relative ratios of Fe redox catalysts necessary for the photosynthetic electron transport chain for non Fe-limited algae and plants. These ratios include Fe-containing redox catalysts involved in photosynthetic electron transfer and the Fe containing components of PSI and PSII. Because the relative ratios of these Fe redox catalysts are

Table 4. Fe-use efficiencies (mol C mol^{-1} Fe d^{-1}) of Fe-limited (pFe 21) *Thalassiosira* spp. grown under either 50 µM NO_3^- or NH_4^+ amended media

Species	Fe-use efficiencies ($\times 10^5$ mol C mol^{-1} Fe d^{-1})	
	NH_4^+	NO_3^-
T. partheneia (Thal 9)	3.8 ± 0.05	2.1 ± 0.04
T. subtilis (50 Ait)	13.0 ± 3.6	5.1 ± 0.2
T. oceanica (13.1)	7.6 ± 0.2	6.4 ± 0.05
T. oceanica (1003)	3.6 ± 0.03	2.5 ± 0.01
T. pseudonana (3H)	0.5 ± 0.01	0.34 ± 0.01
T. weissflogii (Actin)	0.6 ± 0.03	0.3 ± 0.01

altered under Fe stress before the rates of photosynthetic electron transport and growth are affected, we have revised the calculation slightly using recently published data on the stoichiometry of Fe-containing redox catalysts in Fe-limited phytoplankton (Table 5). The new estimates are twice as high as the previous values, but still cannot explain the high IUEs that are measured. The greatest uncertainty in our calculation exists in the relative ratios of Fe redox catalysts for oceanic and coastal phytoplankton under Fe-limitation. Even though replacements of Fe-containing redox catalysts by non-metallic molecules and substitution of Fe by other metals within certain catalysts have been documented, phytoplankton might have Fe substitutions unknown to us presently. Further research on possible Fe substitutions by phytoplankton might enlighten the discrepancy between calculated and measured IUE.

EQPAC isolates

The oceanic species of phytoplankton for which we have the best information regarding Fe requirements have been isolated from the central gyres or the Gulf Stream. Iron concentrations in some of these habitats are undoubtedly low and have selected for tolerant genotypes, but the major nutrients (N and P) are believed to be the resources in shortest supply in these regions. The Fe requirements of the EQPAC isolates examined in this paper are the first reported for centric diatoms from Fe-limited waters (equatorial Pacific Ocean). Both of these species were isolated from incubation bottles containing water samples taken from 0° 01.9' N, 139° 59.2' W that had been enriched with 1 nM Fe. Their growth rates under Fe-limitation were indistinguishable from one another, and were significantly slower than those of the *Thalassiosira oceanica*

Table 5. Calculated and measured Fe-use efficiencies (mol C mol^{-1} Fe d^{-1}) of Fe-limited phytoplankton species

	Growth rate (d^{-1})	Fe:C (μmol mol^{-1})	Fe-use efficiency ($\times 10^5$ mol C mol^{-1} Fe d^{-1})	N source
Theoretical				
This study, Fe-limited cells[a]			2.17	NH_4^+
			1.12	NO_3^-
Raven (1988, 1990), Fe-sufficient cells[b]			1.21	NH_4^+
			0.74	NO_3^-
Laboratory measurements				
Coastal species				
Thalassiosira pseudonana (3H)[c]	0.86	12.5	0.66	NO_3^-
Thalassiosira weissflogii (Actin)[c]	0.55	13.1	0.50	NO_3^-
Prorocentrum minimum (Exuv)[c]	0.48	10.7	0.45	NO_3^-
Thalassiosira weissflogii (Actin)[d]	0.69	18	0.38	NO_3^-
Thalassiosira weissflogii (Actin)[e]	1.39	12.5	1.11	NO_3^-
Pleurochrysis carterae (Cocco II)[e]	0.69	7.5	0.924	NO_3^-
Thalassiosira pseudonana (3H)[f]	0.6	9.1	0.66	NO_3^-
Oceanic species				
Emiliania huxleyi (A1387)[c]	1.51	3.1	4.86	NO_3^-
Emiliania huxleyi (A1387)[c]	1.52	4.6	3.32	NH_4^+
Thalassiosira oceanica (13.1)[c]	1.88	3	6.28	NO_3^-
Pelagomonas calceolata (MC-1)[c]	1.56	4.3	3.62	NO_3^-
Thalassiosira oceanica (13.1)[f]	1	2	5	NO_3^-

[a]Our calculated theoretical maxima IUEs for Fe-limited phytoplankton; assuming 8 mol Fe per 'mol' electron transport chain, calculated using chl:PSI:PSII:cyt.*b*-f-FeS:ferredoxin:cyt.*c* molar ratios as 250:0.25:0.5:0.25:0.1:1 (Glover 1977, Sandmann & Malkin 1983, Terry 1983, Sandmann 1985, Green et al. 1992), and inferring that ferredoxin is replaced by flavodoxin and that the ATP necessary for biosynthesis is generated directly from photophosphorylation

[b]Theoretical minima IUE for Fe-sufficient cells as calculated by Raven (1988, 1990) assuming 0.45 g C g^{-1} dry weight, and growth rates of 2.6 d^{-1}

Sources:

[c]Sunda & Huntsman (1995). Specific growth rates were multiplied by 1.71 to adjust their 14 h light:10 h dark cycle to the continuous light cycle used in all the other studies

[d]Anderson & Morel (1982), assuming a C quota of 12 pmol C $cell^{-1}$

[e]Hudson & Morel (1990), assuming a C quota of 12 and 10 pmol C $cell^{-1}$ for *T. weissflogii* and *P. carterae*, respectively

[f]Sunda et al. (1991)

clones from the Sargasso Sea. At first blush, it might appear that the EQPAC species are not as well adapted to living in low Fe waters as the oceanic central gyre species. However, considering that the equatorial Pacific is a region of intermittent upwelling (Murray et al. 1994), the phytoplankton in these waters might encounter sporadic Fe inputs instead of constantly low concentrations. The ability of species to sequester and store large amounts of Fe when concentrations are high (Sunda & Huntsman 1995) would thus seem to be advantageous. We evaluated the ability of phytoplankton to grow under Fe-limiting conditions with sporadically high Fe concentrations (i.e. scarce aeolian Fe deposition in HNLB regions, or intermittent upwelling) by calculating the ratio between Fe-sufficient (pFe 18) and Fe-limiting (pFe 21) Fe:C quotas (µmol Fe mol^{-1} C). This ratio (Q_{Femax}/Q_{Femin}) reflects the ability of the diatoms to store high intracellular Fe concentrations when dissolved Fe concentrations are high, and lower their Fe requirements to a minimum when Fe concentrations are limiting. The ratio of the quotas was significantly higher in the EQPAC species (45.5 ± 3.5) compared to the coastal (9.33 ± 7.54) and central gyre clones (15 ± 9.9) (2-way ANOVA, $p < 0.05$; Bonferroni *t*-test, $p < 0.05$). Thus, *T. partheneia* and *T. subtilis* would be able to maintain positive rates of growth for longer periods of time during Fe starvation than any of the other species. Maximizing Fe storage and minimizing Fe requirements may thus represent a unique strategy for phytoplankton living in low Fe environments with sporadic Fe inputs.

The 2 EQPAC phytoplankton were also unique in their preference for NO_3^- at high Fe. Ammonium was not likely toxic to these species because lower concentrations in the growth media yielded the same slow growth rates (results not shown). If the increase in Fe quota that we observed in the NO_3^--amended cultures is realized in the field, then NO_3^- consumption may not be an obvious disadvantage, although the extent of Fe deficiency will be important. The *in situ* growth rates of *Thalassiosira subtilis* and *T partheneia* can be estimated using the measured Fe-limited quotas. Assuming that the inorganic dissolved Fe concentration is 10% of the total dissolved Fe (Wells et al. 1994) and that phytoplankton can take up 2/3 of the diffusive flux (Hudson & Morel 1993), growth rates can be determined from:

$$\mu = (2/3\ K_d[Fe'])/Q \quad (4)$$

where $K_d = 4\pi r \times$ diffusion coefficient (9×10^{-6} cm^2 s^{-1}), and Fe' = 3 pM. The calculation suggests that both species should grow at about 50 and 75% of their maximum rates in the equatorial Pacific Ocean.

Until now phytoplankton species have been categorized into either coastal or oceanic habitat groups based on their physiology and isolation sites (Ryther & Kramer 1961, Tortell & Price 1996). Our results suggest that phytoplankton from Fe-limited HNLB regions, such as the equatorial Pacific Ocean, may be distinctly different from other oceanic species. This conclusion is based on their unique growth rates under Fe-sufficient and Fe-deficient conditions, their high ratios of Q_{Femax}/Q_{Femin}, and their particular preference for growth in NO_3^--based media.

Conclusion

The results of this study demonstrate that phytoplankton require 1.6 times more Fe for growth on NO_3^- than on NH_4^+. Nitrate-grown cells can obtain their extra Fe even when Fe is limiting, suggesting that Fe acquisition is somehow linked to nitrate metabolism. Under severe Fe stress, nitrate-grown cells grow slower than those using NH_4^+ because they are unable to obtain the extra Fe needed for growth. The degree of Fe limitation of phytoplankton in the ocean may thus strongly influence their use of different N substrates.

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