

## Copper requirements for iron acquisition and growth of coastal and oceanic diatoms

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### Abstract

Centric diatoms isolated from open ocean environments require higher concentrations of Cu for growth than their coastal counterparts. In artificial seawater medium containing  $<1 \text{ nmol L}^{-1}$  Cu, three coastal species maintained near maximum rates of growth, but the oceanic clones were unable to survive. Copper limitation was more severe in the diatoms grown in low- than in high-Fe seawater, suggesting that Cu and Fe were interacting essential resources. The interactive effect was in part the result of a Cu requirement for Fe transport. *Thalassiosira weissflogii* and *Thalassiosira oceanica* had lower Fe quotas and slower rates of Fe uptake when [Cu] was reduced in the medium. Brief exposure of Cu-limited cells to  $10 \text{ nmol L}^{-1}$  Cu increased the instantaneous Fe uptake rate by 1.5 times in *T. oceanica*. Steady-state uptake rates of both species at high, growth-saturating concentrations of Fe were also Cu dependent. Oceanic species appeared to have an additional Cu requirement that was independent of Fe acquisition and likely responsible for their higher requirements compared to coastal species. Evidence for the importance of Cu in natural communities of phytoplankton was obtained from an incubation experiment performed in the Fe-limited basin of the Bering Sea. Addition of  $2 \text{ nmol L}^{-1}$  Cu doubled the phytoplankton net growth rate compared to the untreated controls and, in the presence of extra Fe, increased the growth rate compared to the samples amended with Fe alone. The results suggest that Cu may be an important micronutrient for phytoplankton growth in low-Fe regions of the sea because of its role in Fe acquisition. Paradoxically, oceanic diatoms may be more susceptible to the effects of low Cu concentrations than coastal species.

Low concentrations of Fe in the open sea are impeding autotrophic production (Tsuda et al. 2003) and must exert strong selection pressure on the Fe-deficient phytoplankton to economize the use of this limiting resource. Laboratory measurements have documented the results of this selection in showing a large difference in the Fe requirements of neritic and oceanic species. In offshore waters, where Fe concentrations are 10 to 100 times more dilute than in coastal seas, phytoplankton have reduced their need for Fe and are able to grow at levels well below those required for reproduction of coastal species (Ryther and Kramer 1961; Brand et al. 1983; Maldonado and Price 1996). Thus, oceanic phytoplankton have adapted to survive in Fe-impooverished waters. A similar habitat-related pattern of phytoplankton requirements is observed for other bioactive metals, including Zn and Mn (Brand et al. 1983; Tortell and Price 1996), which mirrors their availabilities in these environments like Fe (Bruland et al. 1991).

The majority of cellular Fe in algae is used in proteins required for light energy transduction (Raven 1990; Raven et al. 1999), so most attention has focused on these pathways to identify adaptive Fe-sparing strategies. Changes in the

photosynthetic apparatus, ranging from the replacement of ferredoxin with flavodoxin (La Roche et al. 1996) to a general remodeling of the stoichiometry of photosynthetic components (Greene et al. 1991; Moseley et al. 2002), occur in response to Fe deficiency. Recent work by Strzepek and Harrison (2004) shows that an oceanic diatom has adjusted the architecture of its photosynthetic apparatus to reduce Fe-rich components compared to a coastal species. This work has provided the first biochemical explanation of how phytoplankton from the open ocean have reduced their dependence on Fe. In what other ways might oceanic and coastal phytoplankton differ in their adaptations to Fe availability in the environment?

One important physiological response of phytoplankton to low Fe involves their utilization of Fe organic complexes, which are the dominant chemical species in the sea. Under Fe-limiting conditions the marine diatom *Thalassiosira oceanica* develops the ability to reduce Fe bound to chelates and subsequently transports this Fe and utilizes it for growth (Maldonado and Price 2001). Field measurements confirm the importance of Fe(III) reduction by showing that natural communities also use Fe bound to strong organic complexes (Hutchins et al. 1999; Maldonado and Price 1999; Maldonado et al. 2001) and that the rates of Fe uptake are dependent on the Fe nutritional state of the phytoplankton (Maldonado and Price 1999). In other eukaryotes that possess a high-affinity Fe(III) reductive pathway, Cu is a necessary cofactor of an oxidase that is involved in the Fe transport reaction (Stearman et al. 1996). This Cu-containing oxidase (a multicopper oxidase, MCO) oxidizes the Fe(II) produced by the ferric chelate reductase and the Fe(III) thus produced is internalized by a permease. Sequence analysis of the genome of *Thalassiosira pseudonana* has identified a gene with homology to the MCOs of other well-characterized organisms (Armbrust et al. 2004), suggesting that a Cu requirement for Fe transport may also exist in diatoms.

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Whether Fe nutritional state affects the Cu requirement of phytoplankton is presently unknown. We previously observed that diatoms adjust their requirements for Mn under Fe deficiency and hypothesized that the same may be true of Cu. In the case of Mn, Fe-deficient growth increased the requirements of diatoms for Mn because it induced oxidative stress and the need for Mn-containing superoxide dismutase (Peers and Price 2004). Iron limitation may likewise increase the need for Cu if high-affinity Fe transport systems are up-regulated in Fe-limited phytoplankton.

Although Cu-containing proteins such as the MCO (and others, such as plastocyanin) could be potentially important parts of the adaptive responses of phytoplankton to Fe deficiency (Raven et al. 1999), their biosynthesis may present another problem because dissolved Cu concentrations in the ocean are very low. Indeed, total dissolved Cu in surface waters of the open ocean are in the nanomolar range and organic ligands maintain  $\text{Cu}^{2+}$  concentrations at picomoles per liter (Coale and Bruland 1988; Moffett et al. 1990). Such concentrations may be inadequate for phytoplankton with an elevated Cu demand induced by Fe deficiency.

Here we report physiological data from a number of diatom species that suggest that natural selection in the metal-impooverished open ocean has paradoxically resulted in diatoms that require higher ambient concentrations of Cu for growth than their coastal counterparts. Part of the diatom requirement for Cu is for efficient Fe acquisition. Enrichment experiments in the Bering Sea confirm a stimulatory effect of Cu on net phytoplankton growth that can be interpreted as a direct result of Cu limitation of Fe acquisition or possibly primary Cu deficiency.

## Methods

**Culture**—Six centric marine diatoms were obtained from the Provasoli–Guillard Culture Collection of Marine Phytoplankton (CCMP), West Boothbay Harbor, Maine. Strains were assigned to coastal or oceanic provenance according to isolation information provided by the CCMP. The coastal strains were: CCMP 1335, *T. pseudonana* (3H); CCMP 1336, *Thalassiosira weissflogii* (ACTIN); and CCMP 1093, *Thalassiosira minuscula* (WTSIO) and the oceanic strains were: CCMP 1003, *T. oceanica* (35-81); CCMP 1005, *T. oceanica* (13-1); and CCMP 1006, *T. oceanica* (B1SA). These diatoms were maintained at 20°C under constant illumination of  $180 \mu\text{mol m}^{-2} \text{s}^{-1}$  quanta provided by Phillips cool white fluorescent bulbs except where noted. Cells were grown in acid-cleaned 28-ml polycarbonate tubes and biomass was monitored by in vivo chlorophyll fluorescence using a Turner Designs 10-AU fluorometer. Specific growth rates (per day) were determined in fully acclimated, semi-continuous batch cultures (Maldonado and Price 1996).

**Media**—Diatoms were grown in artificial seawater medium (Aquil, Price et al. 1988/89) containing the synthetic chelator ethylenediaminetetraacetic acid (EDTA) at a concentration of  $100 \mu\text{mol L}^{-1}$ . The Cu and Fe salts,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , were of the highest chemical purity (Analar grade, BDH) and were added to the media bound to EDTA (1:1.05). All other trace metals were added as out-

lined in Price et al. (1988/89). Media were allowed to equilibrate for at least 24 h before use. The total concentration of inorganic Fe species ( $\text{Fe}' : \text{Fe}(\text{OH})_2^+$ ,  $\text{Fe}(\text{OH})_3$ ,  $\text{Fe}(\text{OH})_4^-$ ) was calculated using MINEQL and as described (Sunda and Huntsman 1997). Both methods gave similar results. Addition of  $1.29 \mu\text{mol L}^{-1}$ ,  $40.8 \text{ nmol L}^{-1}$ , and  $4 \text{ nmol L}^{-1}$  Fe to media that contained a background concentration of  $5 \text{ nmol L}^{-1}$  resulted in a  $[\text{Fe}']$  of  $5.66 \text{ nmol L}^{-1}$ ,  $90.4 \text{ pmol L}^{-1}$ , and  $24.2 \text{ pmol L}^{-1}$ , respectively. The background concentration of Cu contaminants in our media (nutrient-replete Aquil minus Cu) was undetectable by inductively coupled plasma mass spectrometry (ICP-MS). Media containing no Cu additions are thus reported to contain  $0 \text{ nmol L}^{-1}$  Cu. In Cu-enriched media, Cu was added at concentrations of 1, 3, 5, 8, and  $21.4 \text{ nmol L}^{-1}$ , corresponding to concentrations of  $\text{Cu}'$  of 12, 37, 61, 98, and  $260 \text{ fmol L}^{-1}$  as determined using the conversion of Sunda et al. (2005).

**Fe quotas**—Fully acclimated *T. oceanica* (CCMP 1005) and *T. weissflogii* (CCMP 1336) were inoculated into media containing  $^{55}\text{FeCl}_3$  (10% of total Fe; all isotopes supplied by Perkin Elmer) and grown for at least 12 generations in the radioactive media. In these experiments, the growth irradiance was  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  quanta. Cells were harvested in the mid-exponential phase of growth by gentle filtration onto 25-mm-diameter,  $2 \mu\text{m}$  pore size polycarbonate filters (all membrane filters supplied by Poretics) and extracellular Fe was removed using the Ti-citrate protocol (Hudson and Morel 1989). Filters were placed in scintillation fluor (Ultima Gold, Beckman) for 2 d before counting on a Packard Tri-Carb scintillation counter. Cell density and volume were determined by microscopy as described (Peers and Price 2004).

**Fe:C experiment**—*T. oceanica* (CCMP 1005) was acclimated to  $4 \text{ nmol L}^{-1}$  Fe and  $1 \text{ nmol L}^{-1}$  Cu until the growth rates of successive transfers varied by less than 10%. A subsample of these acclimated cells was then inoculated into media containing  $^{55}\text{FeCl}_3$  ( $78.2 \text{ mCi mg}^{-1}$ ) and  $\text{H}^{14}\text{CO}_3$  ( $5.0 \text{ mCi mmol}^{-1}$ ) and cultivated for at least 12 generations. Cells were subsequently harvested and rinsed as described above. Inorganic  $^{14}\text{C}$  was removed by exposing the filters to fuming HCl for 24 h, after which scintillation fluor was added and the samples counted.

**Short-term Fe uptakes**—*T. oceanica* (CCMP 1005) and *T. weissflogii* (CCMP 1336) were acclimated to Fe and Cu concentrations as described above. Only cultures in the mid-exponential phase of growth were used for uptakes. Cells were harvested by gentle filtration onto acid-cleaned (10% HCl, trace metal grade, Fisher Scientific) 25-mm-diameter,  $2\text{-}\mu\text{m}$  pore-size polycarbonate filters and resuspended into media containing  $50 \text{ nmol L}^{-1} \text{ }^{55}\text{FeCl}_3$  (10% radiolabel, 90% cold stock) complexed with  $100 \mu\text{mol L}^{-1}$  EDTA. This suspension was immediately subsampled for time-zero measurements and uptake was allowed to continue for 2–4 h. The experiments were terminated by filtration and the cells rinsed to remove surface-bound Fe as described above.

The role of Cu in Fe transport was also assessed in short-term Cu addition experiments in which acclimated cells were initially resuspended in Aquil with or without  $10 \text{ nmol L}^{-1}$

Cu complexed with  $100 \mu\text{mol L}^{-1}$  EDTA. After 1 h of treatment, the cells were then refiltered, rinsed with metal-free Aquil, and resuspended in radioactive uptake media. Iron uptake was measured as described above.

**Metal enrichment of Bering Sea plankton communities**—Seawater samples from 5 m depth were collected sequentially as the ship slowly steamed across a distance of  $\sim 20$  km in the basin region of the Bering Sea ( $55.0^\circ\text{N}$ ,  $178^\circ\text{E}$ ) in August 2003. A trace-metal-clean pumping system was used to dispense the water into 48 1-liter low-density polyethylene cubitainer bottles with Teflon-lined caps. The cubitainer bottles were cleaned before use with the following protocol: ethanol rinse, Micro-10 detergent soak (Fisher Scientific, overnight), and 10% HCl soak (trace metal grade, Fisher Scientific, 48 h at  $50^\circ\text{C}$ ). Each bottle was rinsed extensively with  $18.2 \text{ m}\Omega$  Milli-Q- $\text{H}_2\text{O}$  (Millipore) between each cleaning step. The cleaned cubitainers were subsequently filled with 0.1% HCl (baseline grade, Seastar Chemicals) for at least 2 weeks and finally rinsed and filled with clean seawater for 1 d before use. The 48 seawater samples were randomized and amended with either  $2 \text{ nmol L}^{-1}$  Fe,  $2 \text{ nmol L}^{-1}$  Cu, both metals together, or no addition (control) in a class-100 flow hood. There were 8–10 independent replicates of each treatment. Cubitainers were wrapped in polyvinyl chloride screens to supply approximately 30% of incident irradiance and incubated on deck with flowing surface seawater. Duplicate cubitainers were sacrificed at each sampling time and an aliquot of each was filtered onto 25 mm GF/F filters (Whatman) for determination of chlorophyll *a* (Chl *a*, Coale 1991).

**Statistics**—All statistical calculations were performed using Systat v. 10.2 (Systat Software).

## Results

The requirements for Cu of the oceanic and coastal diatoms were assessed during steady-state growth in Aquil medium in which the total Cu concentration was varied from 0 to  $21.4 \text{ nmol L}^{-1}$ , corresponding to a maximum of  $260 \text{ fmol L}^{-1}$  Cu'. An unexpected and surprising result was that all the oceanic strains required much higher Cu concentrations to achieve maximum rates of growth than the coastal species (Fig. 1). This result was generally true regardless of the Fe concentration in the medium. At the lowest Cu concentration, which was below the detection limit of the ICP-MS analysis, none of the oceanic strains was able to divide, whereas the coastal species grew at near maximum rates. Even with  $5 \text{ nmol L}^{-1}$  Cu in the medium, growth of *T. oceanica* was reduced compared to Cu-replete conditions. In the high-Fe medium, when all resources were in excess, the reduction in diatom growth rate was most simply interpreted as a primary limitation by Cu. The growth reduction by Cu under limiting Fe, however, illustrated a multiplicative effect indicative of a Cu and Fe interaction that was inconsistent with single resource limitation. This interaction was clearly evident in *T. oceanica* (CCMP 1003) at  $1 \text{ nmol L}^{-1}$  Cu, which grew at rates of roughly  $0.5 \text{ d}^{-1}$  in Fe-rich medium, but was unable to grow in the Fe-limiting medium. Chemical

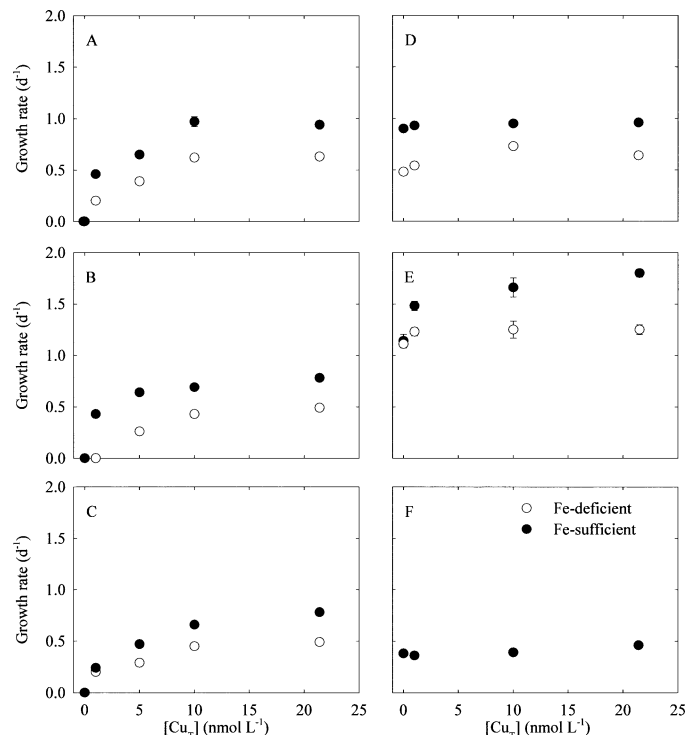


Fig. 1. Steady-state growth rates (per day) of (A–C) oceanic and (D–F) coastal strains of diatoms in Fe-sufficient and -deficient media containing different concentrations of Cu. The oceanic diatoms *T. oceanica* CCMP 1005 (panel A), CCMP 1003 (B), and CCMP 1006 (C) were grown with  $1.29 \mu\text{mol L}^{-1}$  Fe<sub>T</sub> (Fe-sufficient) and  $4 \text{ nmol L}^{-1}$  Fe<sub>T</sub> (Fe-deficient). The coastal diatoms *T. weissflogii* CCMP 1336 (panel D), *T. pseudonana* CCMP 1335 (E), and *T. minuscula* CCMP 1036 (F) were grown with  $1.29 \mu\text{mol L}^{-1}$  (Fe-sufficient) and  $40.8 \text{ nmol L}^{-1}$  Fe<sub>T</sub> (Fe-deficient). No Fe-deficient data were collected for CCMP 1036. Data points represent means and error bars the standard error ( $n = 6$ –14). Error bars are smaller than symbol when not visible.

analysis showed no significant or detectable Cu contamination of the Fe-replete medium compared to the Fe-deplete and Fe-free media (data not shown). The other two oceanic strains showed the same type of response under low Fe with proportional reductions in growth rate as Cu declined.

Within the oceanic and coastal groups the diatoms responded similarly to Cu and Fe so we focused on one representative from each group for further study. *T. oceanica* (CCMP 1005) and *T. weissflogii* (CCMP 1336) were chosen as the oceanic and coastal species, respectively. Iron quotas ( $Q_{\text{Fe}}$ ) of these diatoms were measured under low and high Fe in the presence of varying amounts of Cu as a first step to elucidate the interaction (Table 1). Iron quotas of the coastal species under Fe-sufficient conditions were reduced when Cu was omitted from the medium, a treatment that also significantly reduced growth (ANOVA,  $F_{2,18} = 10.44$ ,  $p = 0.001$ , Tukey; and ANOVA,  $F_{2,18} = 5.67$ ,  $p = 0.012$ , Tukey, respectively). At 1 and  $21.4 \text{ nmol L}^{-1}$  Cu, there were no significant differences in either growth rate or  $Q_{\text{Fe}}$ . *T. weissflogii* appeared to be more sensitive to changes in Cu concentration in the medium under Fe-deficient than Fe-suf-

Table 1. Volumetric Fe quotas,  $Q_{\text{Fe}}$  (mmol Fe L<sup>-1</sup> cell-volume), and specific growth rates,  $\mu$  (d<sup>-1</sup>), of a coastal (*Thalassiosira weissflogii*, CCMP 1336) and an oceanic (*T. oceanica*, CCMP 1005) diatom grown at different concentrations of Fe and Cu complexed with 100  $\mu\text{mol L}^{-1}$  EDTA. Values reported are means  $\pm$  standard errors.

Species	[Fe] (nmol L <sup>-1</sup> )	[Cu] (nmol L <sup>-1</sup> )	<i>n</i>	$Q_{\text{Fe}}$ (mmol Fe L <sup>-1</sup> cell-V)	$\mu$ (d <sup>-1</sup> )
<i>T. weissflogii</i>	1250	21.4	9	0.78 $\pm$ 0.05	0.94 $\pm$ 0.04
	1250	1.0	6	0.88 $\pm$ 0.09	0.87 $\pm$ 0.03
	1250	0	6	0.48 $\pm$ 0.05	0.75 $\pm$ 0.03
	40.8	21.4	6	0.20 $\pm$ 0.01	0.62 $\pm$ 0.03
	40.8	1.0	6	0.13 $\pm$ 0.00	0.46 $\pm$ 0.03
	40.8	0	3	0.16 $\pm$ 0.01	0.27 $\pm$ 0.03
<i>T. oceanica</i>	1250	21.4	9	0.48 $\pm$ 0.06	0.89 $\pm$ 0.02
	1250	1.0	6	0.42 $\pm$ 0.07	0.58 $\pm$ 0.04
	40.8	21.4	3	0.15 $\pm$ 0.01	0.90 $\pm$ 0.02
	40.8	1.0	3	0.073 $\pm$ 0.004	0.59 $\pm$ 0.01
	4.0	21.4	6	0.023 $\pm$ 0.001	0.69 $\pm$ 0.03
	4.0	1.0	9	0.025 $\pm$ 0.002	0.39 $\pm$ 0.05

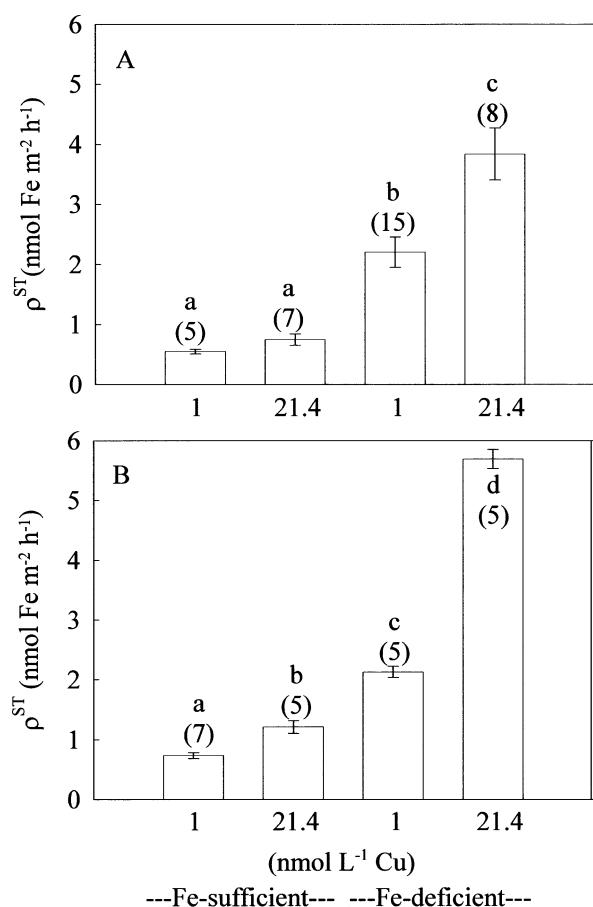


Fig. 2. Short-term Fe uptakes ( $\rho^{\text{ST}}$ ) of a coastal and oceanic diatom, *T. weissflogii* (CCMP 1336) (A) and *T. oceanica* (CCMP 1005) (B), acclimated to different concentrations of Fe and Cu. Uptake rates were measured using 50 nmol L<sup>-1</sup> Fe equilibrated with 100  $\mu\text{mol L}^{-1}$  EDTA. Values are reported as means  $\pm$  standard error. Treatments marked with different letters are significantly different ( $p < 0.05$ , ANOVA, Tukey) and the numbers in parentheses represent the number of independent replicates.

efficient conditions, but  $Q_{\text{Fe}}$  did not decrease in proportion to growth rate. As shown in Fig. 1, a reduction in ambient Cu at all Fe levels reduced growth rate in *T. oceanica* (Table 1). Volumetric Fe quotas, however, were only different between Cu treatments when cells were grown in mildly Fe-deficient conditions ( $t$ -test,  $p = 0.006$ ,  $t = 5.41$ ,  $df = 4$ ). Despite slower growth under Cu deficiency, which might promote Fe accumulation if transport rates remained constant,  $Q_{\text{Fe}}$  did not increase in either species. Thus, Cu appeared to influence the accumulation of Fe by both the oceanic and coastal species.

To evaluate independently the Cu dependence of Fe accumulation in *T. oceanica* and *T. weissflogii*, we measured short-term Fe uptake rates in cells acclimated to low and high Fe and Cu (Fig. 2). The rates of Fe uptake were strongly dependent on the growth conditions of the cells. Rates of uptake by both species were significantly higher in the low- than in the high-Fe cells, as expected with Fe-limited growth. Additionally, when both species were grown under Fe-deficient conditions, the Cu-replete cells had the faster rates of Fe uptake. Iron-replete *T. oceanica* also had significantly higher rates of Fe-uptake when cultivated with high than with low Cu (ANOVA,  $F_{3,32} = 11.2$ ,  $p = 0.001$ , Tukey; and ANOVA;  $F_{3,18} = 392$ ,  $p = 0.001$ , Tukey for *T. weissflogii* and *T. oceanica*, respectively).

The Cu requirement for Fe transport was further documented in Cu enrichment experiments where Cu-deficient *T. oceanica* was briefly exposed to 10 nmol L<sup>-1</sup> Cu prior to measuring Fe uptake. In these experiments, cells were washed and resuspended in Cu-free medium after 1-h exposure to the Cu to avoid Cu carryover in the uptake medium and any aqueous Fe-Cu interactions that could bias the results. As shown in Table 2, the instantaneous rate of Fe transport increased after brief Cu exposure. In both the high- and low-Fe-acclimated cells, the relative increase in Fe transport rates was 1.5, although the results were only statistically significant ( $p < 0.05$ ) in the Fe-limited cells. Collectively, the results demonstrate that Cu was required for efficient Fe transport by both coastal and oceanic diatoms.

Because of the Cu requirement for Fe transport shown here, the low Cu growth data presented in Fig. 1 could be interpreted as a direct effect of Cu on the Fe nutritional

Table 2. Effect of 1-h Cu exposure on the short-term Fe uptake rate,  $\rho^{ST}$ , of Cu-deficient cultures of *Thalassiosira oceanica* (CCMP 1005) grown in Fe-sufficient and -deficient seawater. Acclimated cultures were harvested and resuspended in growth medium with (+ Cu) and without (– Cu) Cu addition (10 nmol L<sup>-1</sup>). Iron uptake rates were measured after the cells were resuspended in seawater medium containing 50 nmol L<sup>-1</sup> <sup>55</sup>Fe equilibrated with 100  $\mu$ mol L<sup>-1</sup> EDTA. Values are reported as single measurements of replicate cultures. Significance of the treatment was calculated using paired *t*-tests.

[Fe] (nmol L <sup>-1</sup> )	[Cu] (nmol L <sup>-1</sup> )	$\rho^{ST}$ (nmol Fe m <sup>-2</sup> h <sup>-1</sup> )		% Increase	Significance
		– Cu	+ Cu		
4.0	1.0	1.36	2.04	151	<i>p</i> = 0.019
		1.64	2.86	175	
		0.80	1.31	163	
		1.90	3.24	170	
1250	1.0	0.39	0.53	135	<i>p</i> = 0.11
		0.80	1.02	126	
		0.83	1.29	155	

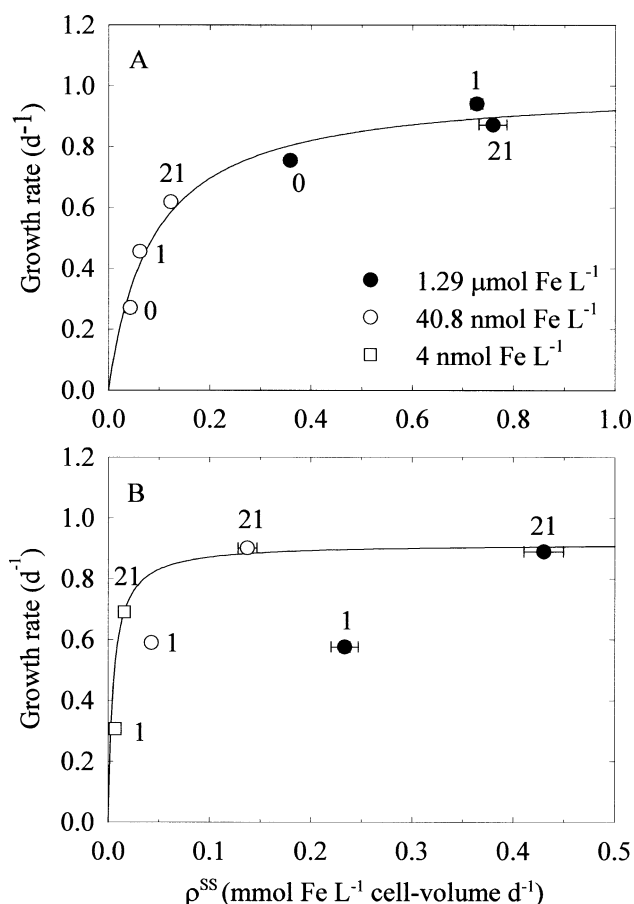


Fig. 3. Growth rate as a function of steady-state Fe uptake rate ( $\rho^{SS}$ ) of a coastal and oceanic diatom, *T. weissflogii* (CCMP 1336) (A) and *T. oceanica* (CCMP 1005) (B). Copper concentrations (nmol L<sup>-1</sup>) are indicated by the numbers beside the individual data points. Plotted lines are hyperbola fit by a least-squares fitting procedure (Sigmaplot 10) to all data (*T. weissflogii*) or to the high-Cu (21.4 nmol L<sup>-1</sup>) data (*T. oceanica*). Data points are means  $\pm$  standard errors (*n* = 3–9).

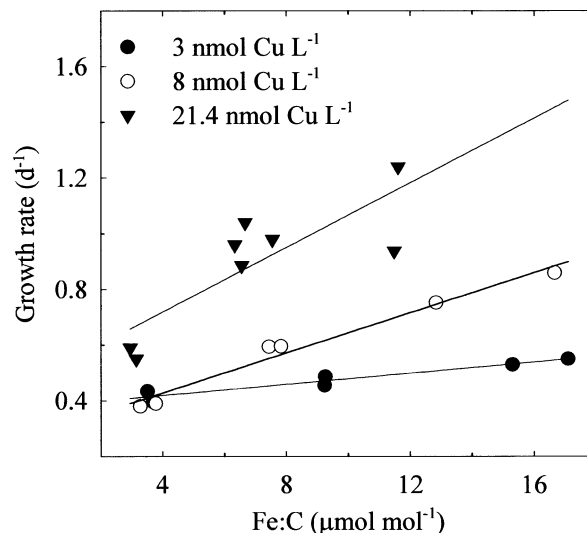


Fig. 4. Growth rates of fully acclimated cultures of *T. oceanica* (CCMP 1005) as a function of Fe:C ratio measured in Fe-deficient cells grown at three different concentrations of Cu. Iron concentrations ranged from 1.2 to 10 nmol L<sup>-1</sup>. Plotted lines represent best-fit regression lines with the following equations: 3 nmol L<sup>-1</sup> Cu,  $y = 0.010x + 0.379$ ,  $r^2 = 0.923$ ; 8 nmol L<sup>-1</sup> Cu,  $y = 0.036x + 0.284$ ,  $r^2 = 0.974$ ; 21.4 nmol L<sup>-1</sup> Cu,  $y = 0.058x + 0.487$ ,  $r^2 = 0.689$ .

status of the phytoplankton. Low Cu could induce Fe limitation, because Cu is required for Fe transport. Indeed, when growth rate of *T. weissflogii* was plotted as a function of the steady-state Fe transport rate, all of the data points fell along a hyperbolic curve as expected if Fe transport were the determinant of growth rate (Fig. 3A). In this species, the effect of low Cu was to reduce the Fe transport rate and thereby reduce growth. Even in the presence of high concentrations of Fe, Cu influenced steady-state Fe transport. The results for *T. oceanica*, however, were dramatically different and suggest that Cu had a more important role in some other cellular process(es) that was independent of Fe uptake. Iron transport rate in the oceanic strain was Cu dependent (as illustrated by the slower rates of Fe transport in the presence of 1 compared to 21.4 nmol L<sup>-1</sup> Cu at each Fe concentration, Fig. 3B), but at low Cu the growth rate was disproportionately reduced. Under Fe-limiting conditions the transport rate of Fe appeared to control growth even at low Cu. These results suggest that the reduced growth rate under low Cu in the coastal diatom was primarily due to its reduced ability to acquire Fe. The oceanic species also required Cu for Fe transport, but had some other major requirement.

The Cu dependence of growth of the oceanic phytoplankton, *T. oceanica*, was examined over a range of Fe-deficient conditions at three different levels of Cu. Each Cu treatment resulted in a different linear relationship between Fe:C and growth rate (ANCOVA,  $F_{2,16} = 28.2$ ,  $p = 0.000$ ) (Fig. 4). A reduction in the Cu concentration decreased growth rate at a constant Fe:C ratio. If Cu were involved in Fe uptake alone, all treatments would be expected to be described by the same relationship between growth and cellular Fe.

The importance of Cu to a Fe-limited phytoplankton com-

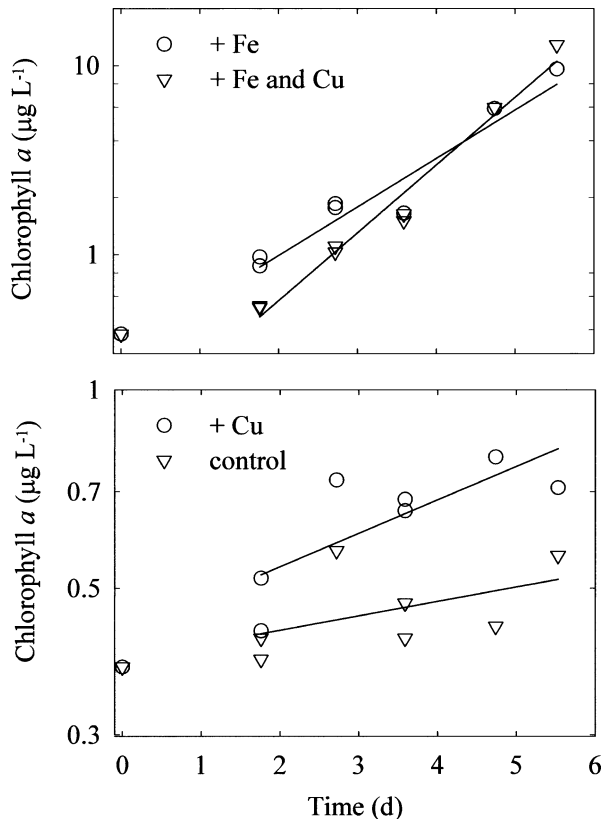


Fig. 5. Net biomass accumulation ( $\mu\text{g L}^{-1}$  Chl *a*) of Bering Sea phytoplankton community following trace metal enrichment. Replicate seawater samples were amended with  $2 \text{ nmol L}^{-1}$  Fe (+Fe),  $2 \text{ nmol L}^{-1}$  Cu (+Cu),  $2 \text{ nmol L}^{-1}$  Fe and  $2 \text{ nmol L}^{-1}$  Cu (+Fe +Cu), or nothing (control) and monitored for 6 d. Each data point for each treatment was obtained from an independent replicate (single bottle). On most days two replicate bottles of each treatment were sampled for Chl *a*. The linear regression lines through the data points (days 2–6) were fit using the least-squares fitting procedure of Systat 10.2. Note difference in scale between graphs.

community in the Bering Sea was evaluated by a metal addition experiment. The experimental design consisted of 8–10 independent replicates per treatment. Iron enrichment increased the initial Chl *a* concentration by 25-fold after 6 d of incubation (Fig. 5). Control samples showed little change (a 1.2-fold increase) over the same period. Net phytoplankton community growth rates (per day) were estimated from linear regression of natural logarithm-transformed concentrations of Chl *a* (shown in Fig. 5). Values from day 2 to day 6 were used in the analyses to avoid the initial lag phase as phytoplankton responded to the metal treatments. Addition of Cu alone significantly increased net community growth rate from  $0.05 \text{ d}^{-1}$  to  $0.12 \text{ d}^{-1}$  (ANCOVA,  $F_{1,18} = 15.8$ ,  $p = 0.002$ ) and additions of both Cu and Fe increased community growth rates to  $0.77 \text{ d}^{-1}$  compared to Fe alone ( $0.59 \text{ d}^{-1}$ ), although this result was only weakly significant (ANCOVA,  $F_{1,13} = 2.8$ ,  $p = 0.11$ ). Inexplicably, the Chl *a* concentrations measured on day 2.5 were abnormally high in the control and Cu-amended treatments. When these data

were omitted from the analyses the difference between the treatments became even more significant.

## Discussion

*Copper requirements for growth*—The habitat-related dependence of essential metal requirements of phytoplankton is well documented. Numerous studies show consistently that species of oceanic provenance are able to grow at much lower concentrations of metals than species from coastal regions (Brand et al. 1983; Maldonado and Price 1996; Tortell and Price 1996). Many of these metals, including Fe, Mn, and Zn, are more concentrated in coastal than in offshore waters (Bruland et al. 1991) so the differences in the biological requirements are thought to reflect phytoplankton adaptations to metal availabilities in the environment. In the case of Fe, these adaptations have reduced the metabolic requirements of the oceanic species to roughly one-tenth of those of the coastal species (Maldonado and Price 1996).

The results reported here on the Cu requirements of six diatoms (Fig. 1) are in stark contrast to all the other bioactive metals. They show that diatoms from oceanic environments require higher concentrations of Cu to grow than coastal isolates. Indeed, the oceanic diatoms we examined were unable to grow at concentrations of Cu that supported maximum growth rates of the coastal species. Total dissolved Cu varies from  $10 \text{ nmol L}^{-1}$  in coastal waters to  $1 \text{ nmol L}^{-1}$  in oceanic waters, similar to the pattern seen for other metals. As pointed out by Mann et al. (2002), however, the free Cu is kept reasonably constant across this gradient by complexation with organic ligands, so its availability may be similar in both environments. Thus, it seems somewhat paradoxical that oceanic species should have evolved higher requirements for Cu, unless they have some unique requirements that are necessary to inhabit these environments.

Copper limitation of phytoplankton growth has thus far only been reported in a few species of phytoplankton (Manahan and Smith 1973; Schenck 1984), so in this regard our results are noteworthy. More important is why have oceanic species evolved a requirement for relatively high concentrations of Cu for growth, whereas coastal species need so little? Two obvious hypotheses come to mind. The first of these is that oceanic diatoms have inefficient Cu transport compared to coastal species and the second is that they require more metabolic Cu to grow. Although Cu uptake was not measured directly in our experiments, we can use published data (Sunda and Huntsman 1995b; Chang and Reinfelder 2000) to test this hypothesis. The relevant data are for *T. oceanica* and *T. weissflogii* from experiments conducted at low concentrations of Cu. In both diatoms, the calculated steady-state Cu uptake rates are  $\sim 25 \mu\text{mol L}^{-1} \text{ d}^{-1}$  at a pCu of 14.42 and 14.79, respectively. Thus, Cu transport per se does not appear to be fundamentally different in these two species. We surmise then that the results presented in Fig. 1 are not a consequence of differences in Cu acquisition between coastal and oceanic isolates, but that they may reflect differences in the amounts of metabolic Cu they require for growth.

Copper quotas of both *T. oceanica* and *T. weissflogii* are

about  $1 \mu\text{mol mol}^{-1} \text{C}$  (Sunda and Huntsman 1995b; Chang and Reinfelder 2000) growing under the conditions described above. In neither case, however, were these quotas determined under Cu limitation and so are likely overestimates of metabolic Cu. Estimating the elemental requirement for growth of phytoplankton requires measurements of quotas under optimal or rate-limiting conditions and must consider the metabolic rate of the organism. So far these estimates do not exist for Cu. Luxury consumption increases the cellular content of many elements when they are abundant and can obscure the true metabolic requirements (Droop 1974). A good example of this phenomenon is cellular Fe in *T. oceanica*, which increases concomitantly with increasing dissolved Fe even though growth rates remain constant and Fe sufficient (Sunda and Huntsman 1995a).

We note that Sunda and Huntsman (1995b) measured a small (5%) decrease in growth rate of *T. oceanica* at the lowest Cu concentrations, suggesting that they may have been on the verge of achieving Cu-limited growth. The small decrease in growth rate observed in their experiments is surprising given the results we obtained. Indeed, Cu concentrations in both experiments were remarkably similar. At  $1 \text{ nmol L}^{-1} \text{ Cu}$ , for example, Sunda and Huntsman (1995b) measured near-maximum growth rates of *T. oceanica* (CCMP 1005), whereas we observed a 50% decline in growth (Fig. 1). Either Cu contamination was a confounding variable or some other differences in the composition of our media are the cause of this discrepancy. As discussed below, our results have established one important role for Cu in Fe transport in both coastal and oceanic diatoms. Measurement of Cu quotas under Cu-deficient conditions will be essential to establish the Cu requirements of these phytoplankton.

*The role of Cu in Fe transport*—High-affinity Fe transport in several model eukaryotic organisms requires Cu. In *Saccharomyces cerevisiae*, for example, Fe is first reduced by a Fe(III) chelate reductase and then reoxidized during uptake (Stearman et al. 1996). The reoxidation step is Cu dependent, and is mediated by an MCO that has also recently been identified in the green alga, *Chlamydomonas reinhardtii* (Herbik et al. 2002; La Fontaine et al. 2002). Several of our physiological observations are consistent with a Cu-dependent step in Fe uptake by marine diatoms and suggest that an MCO may be involved.

Judging from changes in absolute growth rate, the copper requirements of the species we examined were enhanced by Fe deficiency (Fig. 1). Growth rates of the oceanic diatoms under Fe limitation, for example, were even slower than under Fe-replete conditions when Cu was lowered in the medium. Thus, Cu and Fe were interacting essential resources, like Mn and Fe (Peers and Price 2004), and at low concentrations were colimiting to growth. This interaction was less clear-cut in the coastal isolates, possibly because they were much more difficult to limit for Cu in the first place.

Direct evidence for the involvement of Cu in Fe transport of diatoms was provided by measurements of Fe quotas and short-term uptake rates. Cellular Fe declined as Cu was lowered in the medium (Table 1), implying that transport rates of Fe were decreased. Indeed, the instantaneous Fe uptake rate was much slower in cells grown in low than in high Cu

(Fig. 2). This effect was most pronounced in the Fe-limited cells that had the fastest rates of Fe uptake. In Fe-replete cells, the effect of Cu was only statistically significant in the oceanic strain, although the mean rates of uptake by *T. weissflogii* were also greater in the high-Cu-grown cells. Exposure of Cu-depleted *T. oceanica* to  $10 \text{ nmol L}^{-1} \text{ Cu}_T$  (complexed with  $100 \mu\text{mol L}^{-1} \text{ EDTA}$ ) for 1 h increased Fe transport rates by 1.5-fold (Table 2), regardless of the Fe status of the cells. *T. weissflogii* was not examined. Note that the variability among the replicates of these experiments was quite high, likely because the additional steps of filtration and resuspension (see Methods) were detrimental to the cells. Despite this variation all individual experiments showed the same Cu stimulation. We note that treatment with Cu for 1 h was not sufficient to cause the Fe transport rates to increase to the levels observed under Cu-replete conditions.

The results presented in Fig. 3 show that Cu limitation of growth in the coastal diatom can be most simply interpreted as an indirect effect caused by a decrease in the steady-state Fe transport rate. Although a number of other metabolic functions of Cu are undoubtedly important, they may be less sensitive to changes in Cu availability, perhaps because of their overriding importance or small Cu requirement. In Fe-replete cultures of *T. oceanica*, Cu deficiency reduced Fe uptake but also caused a reduction in growth rate that was independent of Fe uptake, suggesting an additional, important role for Cu in this oceanic species (see below).

Although our data are the first to show a link between Cu and Fe acquisition in diatoms, previous studies have provided important evidence for the existence of such a pathway of Fe uptake. Extracellular Fe reduction, the first step of high-affinity Fe uptake, has been shown to occur in Fe-limited diatoms (Maldonado and Price 2000, 2001) and in natural plankton communities (Maldonado and Price 1999; Maldonado et al. 2001; Shaked et al. 2004). Many freshwater algae also possess this ability (Weger 1999). It should be noted that higher plants (Curie and Briat 2003) and yeast (Jones 2003) internalize Fe(II) directly, but in the case of yeast, this pathway has a much lower affinity for Fe compared to the MCO-dependent pathway. In the Fe transport pathways of higher plants there has been no documented role for an MCO.

The genome of the coastal diatom *T. pseudonana* has recently been sequenced and shown to contain a gene homologous with the MCOs of other eukaryotes and to possess a ferric chelate reductase (Armbrust et al. 2004). Although we have no evidence for a Cu requirement in an MCO, we have detected MCO activity in partially purified membrane fractions from these diatoms (Quesnel and Price unpubl. data).

As the majority of dissolved Fe in surface waters of the open ocean is bound to ligands (Rue and Bruland 1995), a Fe(III) chelate reductase system like that described for other eukaryotes may be an important way for phytoplankton to acquire Fe (Maldonado and Price 1999, 2000, 2001). Hutchins et al. (1999), Maldonado and Price (1999), and Maldonado et al. (2001) have all reported that natural populations of plankton are able to obtain Fe bound to a variety of ligands, possibly through a Fe(III) chelate reductase. Such a pathway could be compromised by a lack of Cu so that the

reduced Fe may be free to diffuse away from the cell before it is oxidized and transported.

*Copper requirements in oceanic diatoms*—Possible explanations for the higher Cu requirement of the oceanic species could include a greater need for Cu in Fe transport as discussed above. However, oceanic and coastal diatoms take up Fe at similar, diffusion-limited rates (Sunda and Huntsman 1995a) so one would have to postulate that the oceanic strains have a less Cu-efficient Fe uptake system. If additional Cu were required for Fe uptake it does not appear to confer any apparent advantage to oceanic diatoms and is therefore unlikely.

The results of Fig. 4 illustrate that Cu limitation in the oceanic diatom was independent of Fe. Decreasing the Cu concentration in the medium from 21.4 to 8 nmol L<sup>-1</sup> decreased growth rate at a constant cellular Fe:C ratio. At the extreme, growth rate at 3 nmol L<sup>-1</sup> Cu was completely independent of Fe:C. If the only significant effect of Cu limitation was on Fe acquisition (through inhibition of an MCO), then we would expect a single relationship between Fe:C and growth.

A number of other metabolic uses for Cu are well documented in cellular metabolism. Notable Cu-containing enzymes include those such as Cu/Zn superoxide dismutase, and the Cu-containing electron transport protein plastocyanin (Raven et al. 1999). We have observed that superoxide dismutases in diatoms contain Mn, not Fe (Peers and Price 2004, unpubl. data), so it is unlikely that any Fe-sparing effect would be accomplished by replacing this enzyme with a Cu-containing homologue. The interreplacement of the photosynthetic electron transport proteins plastocyanin and cytochrome *c*<sub>6</sub> (Fe-containing) may also lead to substantial metal savings (Raven et al. 1999). Photosynthetic organisms that possess both proteins produce one or the other depending on cellular metal status (Wood 1978). Diatoms (and other chromophyte algae) are thought not to possess plastocyanin (Inda et al. 1999; Raven et al. 1999), but so far only a few species from coastal regions have been examined. Strzepek and Harrison (2004) were unable to detect cytochrome *c*<sub>6</sub> in *T. oceanica*, suggesting that this species either contains very low cellular concentrations of the protein or that the protein is completely absent. Such an absence may be compensated by a Cu redox protein such as plastocyanin. Indeed, using rapid fluorescence induction techniques we have found that Cu deficiency impairs photosynthetic electron transport in oceanic but not in coastal diatoms and that this effect is independent of Fe nutrition (Peers and Price unpubl. data). We are currently examining the role of Cu in photosynthetic reactions of diatoms.

Finally, no discussion of important Cu-containing enzymes would be complete without mention of cytochrome *c* oxidase, the terminal enzyme of respiratory electron transport. We have not investigated the effects of Cu deficiency on respiration rates but perhaps oceanic diatoms have a higher biomass-specific respiratory demand than coastal species, which would increase their Cu requirement.

*Oceanographic relevance*—Research into the importance of dissolved copper in the ocean has primarily revolved

around the free cupric ion and its potential toxicity to natural populations of plankton. Algal species are known to differ greatly in their sensitivities to Cu and their abundances in natural waters are thought in part to be influenced by variations in environmental Cu concentrations (Brand et al. 1986; Mann et al. 2002). Of particular interest are the cyanobacteria, the most sensitive of the species, which are able to produce organic ligands to complex Cu and render it non-toxic. The structure of the ligands they produce is unknown, but their Cu binding strength is remarkably similar to the natural Cu ligands in the sea (Moffett and Brand 1996). One hypothesis is that the Cu-sensitive phytoplankton may regulate the bioavailability of Cu in the open ocean by secreting organic ligands, thereby modifying the chemistry of their environment to allow survival. Whereas these ligands may be important in reducing the toxicity of Cu to cyanobacteria in the ocean (Mann et al. 2002), they also lower the biological availability of the Cu to all other organisms. Conceivably, reduced bioavailability of Cu could be deleterious to the growth of eukaryotic phytoplankton or to other species with high Cu requirements.

Very little is known about the forms of dissolved Cu that are available to eukaryotic phytoplankton for growth. Most studies show that biological activity is proportional to the free cupric ion concentration (Sunda and Guillard 1976), but some recent observations suggest that organic complexes may also be used. Hudson (1998) calculated that the diffusive supply of dissolved, inorganic Cu species was too slow to supply most diatom cultures with the Cu they require for maximal growth and concluded that some must be acquiring Cu from Cu-EDTA complexes (although the same type of calculations showed that this was not the case for *T. weissflogii*). Diatoms are able to reduce Cu(II) bound to organic ligands (Jones et al. 1987) and may be able to use these forms of Cu for growth just as they do for Fe. Given the importance of Cu in Fe acquisition, we hypothesized that Cu could be a colimiting resource in low Fe regions of the sea.

To test this hypothesis we conducted an enrichment experiment in the basin region of the Bering Sea. Iron was the primary limiting resource, judging from the large increase in chlorophyll after addition of 2 nmol L<sup>-1</sup> Fe (Fig. 5, LeBlanc et al. unpubl. data). Copper stimulated a small but significant increase in net chlorophyll production, an effect that was observed even in Fe-amended samples (albeit this latter effect was weakly significant). There are three possible ways of interpreting these data: addition of Cu stimulated Fe acquisition, addition of Cu stimulated growth independently of Fe, or addition of Cu inhibited grazers, resulting in a net accumulation of Chl *a*. This latter explanation was proposed by Coale (1991) to account for the Cu-induced increase in plankton biomass in the subarctic Pacific Ocean. However, in a separate experiment, grazing rate was unaffected by addition of 2 nmol L<sup>-1</sup> Cu<sub>T</sub> (R. Kudela pers. comm.), so we suggest that, in this case, net Chl *a* accumulation was not due to grazer inhibition. Thus, in this region of the Bering Sea Cu and Fe were colimiting resources. Our grow-out results, however, cannot discern the mechanism by which Cu stimulated the phytoplankton. According to our lab results, at low ambient Cu concentrations, Cu addition would be expected to stimulate phytoplankton growth in the presence

and absence of Fe. At present we do not know how widespread Cu deficiency is in the Bering Sea or whether it is a general feature of other Fe-limited waters. Collectively, our results show that Cu is required for the acquisition of Fe by diatoms and advocate a role for Cu limitation in regulating phytoplankton growth in the sea.

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