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The effects of Cu and Fe availability on the growth and Cu : C ratios of marine diatoms

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Abstract

We investigated the effects of copper (Cu) and iron (Fe) availability on the growth rates, cellular Cu content, and steady-state Cu uptake rates of eight species of centric diatoms (coastal and oceanic strains). Whereas Fe and Cu availability had a significant effect on the growth rates of both costal and oceanic diatoms, an interaction between Fe and Cu availability and growth rates was only observed for the oceanic diatoms. Determination of cellular Cu : carbon (C) quotas using the radiotracers ^{67}Cu and ^{14}C revealed that under Cu-sufficient conditions oceanic diatoms had elevated Cu : C ratios relative to coastal strains, regardless of Fe availability. Two species (one oceanic and one coastal) significantly increased their Cu demands in response to Fe limitation, indicating upregulation of the Cu-dependent high-affinity Fe uptake system in these organisms. The changes in cellular Cu : C ratios were accompanied by variations in steady-state Cu uptake rates. Thus, in some cases Cu uptake rates appear to be regulated by the cell in response to Fe availability. Rates of Cu acquisition also responded significantly to Cu variability. The variation in Cu uptake was more closely correlated with changes in total Cu concentration in the medium than in inorganic, free Cu concentrations, implying that organic Cu complexes may be bioavailable to diatoms. These findings indicate a greater biological role for Cu than was previously thought in open ocean regions.

Iron (Fe) is an essential micronutrient for phytoplankton growth and has been shown to control primary productivity in large oceanic regions (Martin 1990; Martin et al. 1994; Boyd 2004). In these open ocean waters, dissolved Fe is present in extremely low concentrations of ~0.07 nmol L^{-1} (Johnson et al. 1997) and is predominantly complexed by strong organic ligands (Rue and Bruland 1995). Although eukaryotic phytoplankton are not believed to access organically bound Fe directly (Hudson and Morel 1993), lab and field data have shown that these organisms upregulate an inducible high-affinity Fe uptake system in response to Fe limitation that allows them to acquire Fe bound to strong organic complexes, such as the siderophore ferrioxamine B (Maldonado and Price 1999, 2001). The high-affinity Fe uptake system is composed of putative Fe(III) reductases, multi–copper (Cu)-containing Fe(II) oxidases, and Fe(III) permeases (Maldonado and Price 2001; Shaked et al. 2005; Maldonado et al. 2006) and is similar to those previously identified in the yeast *Saccharomyces cerevisiae* (Askwith et al. 1994) and in the green alga *Chlamydomonas reinhardtii* (Herbik et al. 2002; La Fontaine et al. 2002).

The role of Cu in the high-affinity Fe uptake system of Fe-limited *Thalassiosira oceanica* is such that cells grown with low Cu show significantly slower rates of Fe(II) oxidation and Fe acquisition from the siderophore ferrioxamine B (Maldonado et al. 2006). In addition, when *Thalassiosira pseudonana* is subjected to Fe limitation, the transcription levels of the putative gene encoding for the multi–Cu-containing oxidase increase 60-fold (Maldonado et al. 2006). These physiological and genomic data imply that Cu availability may affect the ability of Fe-limited diatoms to acquire organically bound Fe and that the intracellular Cu quotas of Fe-limited diatoms may be significantly higher than those of Fe-sufficient cells.

In addition to the Cu demand of the high-affinity Fe transport system in Fe-limited coastal and oceanic diatoms, oceanic species may have additional Cu requirements associated with the substitution of Fe-containing enzymes by Cu-containing ones, as recently shown for plastocyanin in *T. oceanica* (Peers and Price 2006). The hypothetically higher Cu requirements of the oceanic isolates may help explain how these phytoplankton are able to survive in open ocean waters with intracellular Fe levels that are 70%

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Table 1. Species, strain, size (diameter), source, and oceanic provenance of centric diatoms used in this study.

Species	Strain	Diameter (μm)	Source	Environment
Thalassiosira oceanica	CCMP 1003 Ω 35-81	4.9–7.7	Sargasso Sea	Oceanic
Thalassiosira oceanica	13-1 CCCM 610	4.8–7.0	Sargasso Sea	Oceanic
Skeletonema menzellii	CCMP 787	6.5	Sargasso Sea	Oceanic
Thalassiosira pseudonana	CCMP 1014	2.8–4.4	Sargasso Sea	Oceanic
Thalassiosira pseudonana	3H CCCM 58	3.9–4.9	Moriches Bay, New York	Coastal
Thalassiosira weissflogii	Actin CCMP 1336	9.5–11.4	Long Island, New York	Coastal
Skeletonema costatum	CCCM 782	7.6	Milford Harbour, Connecticut	Coastal
Chaetoceros decipiens	CCCM 537	9×18*	Vancouver, Canada	Coastal

* Dimensions are given for visibly cylindrical cells.

lower than those expected for a typical eukaryotic phytoplankton (based on photosynthetic biochemistry; Raven 1988) or those of their coastal counterparts (Sunda and Huntsman 1995*a*; Maldonado and Price 1996).

To date, direct measurements of Cu quotas have been scarce as a result of the lack of long-lived Cu radiotracers and the expense and difficulty of alternative analytical techniques, such as high-resolution inductively coupled plasma mass spectrometry. Thus, while recent studies (Peers et al. 2005; Wells et al. 2005; Maldonado et al. 2006) have begun to investigate the interaction between Cu and Fe as essential nutrients for phytoplankton, little is known about the Cu requirements of these organisms under various Fe and Cu levels. In the present study, we use the short-lived nuclide ^{67}Cu to examine intracellular Cu levels and steady-state rates of Cu uptake, in addition to growth rates, for eight species of centric diatoms (coastal and oceanic isolates) across a range of Fe and Cu concentrations.

Methods

Study organisms—Eight species of centric diatoms, isolated from both coastal and oceanic environments, were used in this study (Table 1). Cultures were obtained from the Canadian Center for the Culture of Microorganisms (CCCM; University of British Columbia, Vancouver, Canada) and the Center for Culture of Marine Phytoplankton (CCMP; Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine).

Culture media—The chemically well-defined artificial seawater medium AQUIL was used as culture media, prepared according to the method of Price et al. (1988–1989). All culture containers were washed in Extran detergent to remove organic matter and then soaked in 10% HCl for at least 24 h before being washed with Milli-Q water and sterilized. Trace metal additions were buffered with 100 μmol L^{-1} ethylenediaminetetraacetic acid (EDTA) such that manganese (Mn), zinc (Zn), and cobalt free-ion activities were $10^{-8.27}$ mol L^{-1}, $10^{-10.88}$ mol L^{-1}, and $10^{-10.88}$ mol L^{-1}, respectively. Selenium and molybdenum were added at total concentrations of 10^{-8} and 10^{-7} mol L^{-1}, respectively. The trace metal additions required to obtain specific ion activities were calculated using the MINEQL program (Westall et al. 1976). Four culture media were prepared, in combinations of low-Fe ($[Fe_{tot}]$ = 42 nmol L^{-1}), Fe-replete ($[Fe_{tot}]$ = 1.37 μmol L^{-1}), low-Cu ($[Cu_{tot}]$ = 1.96 nmol L^{-1}), and Cu-replete ($[Cu_{tot}]$ = 10.2 nmol L^{-1}) conditions. Free iron concentrations were pFe 20.5 and pFe 19, respectively (where pMetal = negative log of the free metal concentrations). Because some species did not show marked growth rate reduction in pFe 20.5 media, additional media were prepared with very low Fe ($[Fe_{tot}]$ = 4.2 nmol L^{-1}, pFe 21.5), with both high and low Cu.

The free Cu concentration in our Cu-replete media (pCu 14) is approximately that of the standard AQUIL value (pCu = 13.79; Price et al. 1988–1989), and evidence from previous studies (Peers et al. 2005; Maldonado et al. 2006) indicates that Cu may limit growth below this level. Free metal ions are classically considered the bioavailable form of many trace metals, including Cu (Hudson 1998); thus, here we report pCu throughout. Our low-Cu treatment was therefore chosen to provide a 10-fold reduction in free Cu (pCu = 15). Total inorganic Cu for these media was 1.24×10^{-13} and 2.38×10^{-14} mol L^{-1}) for high and low Cu, respectively.

Fe and Cu solutions were precomplexed with EDTA (1 : 1.05, [Me] : [EDTA]) to control free-ion concentrations and prevent precipitation upon addition to the media. All media were allowed to equilibrate chemically overnight before use. Average background Cu contamination in AQUIL has been measured at 1.8 nmol L^{-1} (Wiramanaden 2007).

Growth measurements—Cultures were grown in acid-washed 28-mL polycarbonate tubes and were kept in exponential growth phase in semicontinuous batch cultures (Brand et al. 1981). All cultures were incubated at 19°C ± 1°C under 24-h saturating light level (0.8×10^{16} mol quanta cm^{-2} s^{-1}). Growth of cultures was monitored daily, using fluorescence as a proxy for chlorophyll *a*. In vivo fluorescence was measured by a Turner 10-AU Fluorometer, and $\log_2$ of fluorescence vs. time was used to calculate growth rates, as outlined by Brand et al. (1981). Cultures were considered acclimated to Fe and Cu levels when growth rates in five successive transfers varied by less than 10%. Average growth rates of maintenance cultures were determined based on up to 96 individual growth rate determinations for each species under each treatment level. The standard error was less than 5% in all but four treatments and less than 10% in all cases. Growth rates of cultures in 250-mL bottles (used for the quota experiments) were calculated for each replicate. In all cases the growth

rates of bottle cultures were not significantly different from those of the maintenance cultures.

For experiments, initial cell concentrations and cell size were measured on live samples using a Coulter Z2 Particle Counter and Size Analyzer. Radioactive cell samples fixed with Lugol's preservative were used to determine total final cell concentrations using a Zeiss light microscope and a Palmer–Maloney chamber (0.1 mL). Initial cell size measurements were used for surface area and volume comparisons, assuming spherical shape (except in the case of *Chaetoceros decipiens*, which was assumed to be cylindrical in shape).

Cu quota determination—The gamma-emitting radionuclide ^{67}Cu ($t_{1/2}$ = 62 h; specific activity, $\sim 1 \times 10^9$ MBq mol^{-1}) was produced as a byproduct of ^{67}Ga production via the ^{68}Zn (p,2p) reaction at the MDS Nordion site on the Tri-University Meson Facility campus in Vancouver. For these experiments diatom cultures were grown in 250-mL polycarbonate bottles. Cellular Cu : C quotas were determined during mid-exponential phase by overnight incubation of phytoplankton in media spiked with 80–160 $\times 10^{-3}$ MBq L^{-1} of ^{67}Cu. ^{67}Cu additions were precomplexed with 1 nmol L^{-1} EDTA (minimum 10 : 1 [EDTA] : [Cu]) for ≥30 min to avoid metal precipitation or major variation in free ion concentrations. This ^{67}Cu addition increased by 5%, at most, the total Cu concentration in the growth media. Carbon-14 ($H^{14}CO_3^-$) was also added at a final specific activity of 0.74 MBq L^{-1} (specific activity of stock, 1.665 $\times 10^6$ MBq mol^{-1}; PerkinElmer). All manipulations were performed in a laminar flow hood to minimize metal and bacterial contamination. Cultures were incubated at the same temperature and light levels to which they had been acclimated. After 12–16 h, samples were filtered onto duplicate 2.0-μm pore size, 25-mm AMD Poretics polycarbonate filters. Before running dry, the samples were washed with 1 mmol L^{-1} diethylene triamine pentaacetic acid (dissolved in sterile synthetic ocean water [SOW], pH adjusted to ~8.1; Croot et al. 1999) for 5 min to remove extracellularly bound Cu and were then rinsed with 5 mL of sterile SOW. ^{67}Cu activity was immediately measured in a gamma counter (Minaxiγ Auto-gamma 5000 Series) and corrected for background and decay. Scintillation cocktail was added to samples immediately after analysis of gamma radiation, but measurements of ^{14}C radiation emissions were performed by scintillation counting (Beckman–Coulter LS 6500) only after all the ^{67}Cu had decayed (approximately 3 weeks). Cu : C ratios were then calculated by converting filter activity to total Cu and C per milliliter, using appropriate specific activities. As cultures did not undergo eight cell divisions in radioactive media and thus were not uniformly labeled, cellular concentrations of Cu and C (mol L$^{-1}_{\text{cell volume}}$) were calculated using growth rate and incubation time, according to the method of Morel (1987).

Statistical analyses—Our preliminary statistical analyses consisted of ANOVA Tukey tests for differences between treatment means within each species. However, in order to examine group-wide trends (coastal vs. oceanic, or diatom trends in general) we used generalized mixed-effects model (with both random and fixed effect variables) statistical analyses. Random effects are due to the chosen subjects, consisting of a random sample of all possible subjects, with inferences made about the larger population (Glantz and Slinker 2001). In contrast, fixed effects represent factors that are specifically manipulated for experimental purposes (Glantz and Slinker 2001). Thus, in our mixed-effects model, the continuous dependent variables were growth rate, Cu quota, and steady-state Cu uptake rate. The predictor fixed-effect variables were Cu level (pCu 14 vs. 15), Fe level (pFe 19 vs. 20.5), and species regime (coastal vs. oceanic), as well as the one-way interaction terms for all the fixed effects. Since the diatoms species studied here were chosen randomly among many diatoms cultured in the CCCM and CCMP phytoplankton culture collections, we included a random effect in our model (species). We also assessed assumptions for regression analysis (homoscedasticity, normality, and linearity) by residual diagnostics, including residual plots, outlier detection, and test of normality. We did all statistical analyses with the statistics computer software SYSTAT, version 11.

Results

Effects of Fe and Cu on growth rate—Overall, decreasing Fe availability reduced the growth of coastal (mean reduction, ~23%) and oceanic diatoms (~16%) significantly (Table 2; Fe effect, $p < 0.0001$, Table 3), although species differed in the magnitude of the response (Table 2). Collectively, the growth of coastal and oceanic diatoms was also affected by Cu availability (~14%, and ~8% mean reduction, respectively; Table 2; Cu effect, $p < 0.0001$, Table 3). The mixed-effects statistical model revealed that the growth response of the coastal and oceanic diatom groups to metal availability was significantly different for Fe availability but not for Cu availability (Fe × regime, $p < 0.0001$ vs. Cu × regime, $p = 0.642$, Table 3); the coastal strains exhibited a larger reduction in growth rates than did the oceanic species in response to Fe limitation, but this was not so for Cu. When species were examined individually, all eight showed a significant Fe effect, while the Cu effect on growth rates was only significant for five strains (Table 4). Trace metal limitation was defined here as a statistically significant reduction in mean growth rate ($p < 0.05$, Table 2), in all cases a reduction of at least 14%.

A significant interaction between Fe and Cu availability on the growth rates was only observed for the oceanic group (Fe × Cu effect, $p < 0.007$, Table 3), implying that the Cu effect on growth rates was different between the high- and low-Fe treatments. Under Fe-sufficient conditions the growth of the oceanic group was unaffected by low Cu availability, whereas under Fe-limiting conditions, low Cu availability reduced their growth rates (Table 2).

Effects of metal availability on Cu quotas—Cellular Cu : C ratios varied from 0.32 to 6.3 μmol Cu mol^{-1} C (Table 5; Fig. 1). The highest quotas were observed in the two *T. oceanica* strains, while two coastal strains, *T. weissflogii* and *C. decipiens*, exhibited the lowest quotas.

Table 2. Growth rates of maintenance cultures of all diatom species. Also listed is the percent reduction in growth rate resulting from reduced iron (Fe) availability, relative to Fe-sufficient media with the same concentration of copper (Cu), and vice versa for Cu. Cultures shown in bold were considered limited by either Fe and/or Cu, in which case the growth rate was statistically lower than the metal-replete cultures (p values shown are for two-sample t-test). SE, standard error.

Species (strain)	Free Fe pFe	Free Cu pCu	Growth rate (d^{-1}) (mean±SE [n])	% reduction from Fe sufficient	% reduction from Cu sufficient
Coastal species					
Chaetoceros decipiens	19	14	1.09±0.050(16)		
	19	15	0.835±0.041(15)		**23(p=0.029)**
	20.5	14	1.05±0.050(16)	3(p=0.394)	
	20.5	15	0.659±0.030(17)	**21(p=0.043)**	**37(p=0.001)**
Thalassiosira weissflogii (Actin)	19	14	1.33±0.043(44)		
	19	15	1.01±0.029(30)		**24(p=0.0004)**
	20.5	14	0.908±0.032(27)	**32(p<0.0001)**	
	20.5	15	0.861±0.023(27)	**15(p=0.049)**	5.2(p=0.293)
Thalassiosira pseudonana (3H)	19	14	2.03±0.045(87)		
	19	15	1.97±0.045(96)		3.6(p=0.184)
	20.5	14	1.26±0.040(92)	**38(p<0.0001)**	
	20.5	15	1.26±0.036(91)	**38(p<0.0001)**	−0.22(p=0.485)
Skeletonema costatum	19	14	0.730±0.038(23)		
	19	15	0.707±0.033(20)		3.1(p=0.413)
	20.5	14	0.633±0.059(12)	13(p=0.235)	
	20.5	15	0.498±0.028(22)	**30(p=0.016)**	21(p=0.146)
Oceanic species					
Thalassiosira pseudonana (1014)	19	14	1.59±0.075(23)		
	19	15	1.58±0.059(26)		−0.72(p=0.467)
	20.5	14	1.02±0.043(23)	**35(p<0.0001)**	
	20.5	15	0.936±0.030(24)	**41(p<0.0001)**	7.9(p=0.218)
Thalassiosira oceanica (610)	19	14	1.09±0.051(27)		
	19	15	1.00±0.037(29)		7.5(p=0.228)
	20.5	14	0.954±0.041(23)	12(p=0.124)	
	20.5	15	0.745±0.026(42)	**26(p=0.002)**	**22(p=0.014)**
Thalassiosira oceanica (1003)	19	14	1.16±0.038(43)		
	19	15	1.13±0.040(32)		2.9(p=0.360)
	20.5	14	1.18±0.042(38)	−1.8(p=0.412)	
	20.5	15	0.964±0.031(49)	**14(p=0.034)**	**18(p=0.008)**
	21.5	14	0.719±0.021(16)	**38(p<0.0001)**	
	21.5	15	0.769±0.032(13)	**40(p<0.0001)**	5.5(p=0.342)
Skeletonema menzellii	19	14	0.946±0.038(25)		
	19	15	0.974±0.044(29)		−2.8(p=0.396)
	20.5	14	1.10±0.048(21)	−14.2(p=0.084)	
	20.5	15	1.00±0.042(26)	−2.72(p=0.340)	9.2(p=0.187)
	21.5	14	0.685±0.050(11)	**28(p=0.023)**	

Regardless of Fe availability, the Cu : C ratios of the oceanic diatoms were on average twofold higher than those of the coastal strains under Cu-sufficient conditions (pFe 19: oceanics ~ 3.4 ± 0.62 vs. coastals ~ 1.55 ± 0.81; pFe 20.5: oceanics ~ 4.2 ± 0.8 vs. coastals ~ 1.8 ± 0.9 μmol Cu mol^{-1} C, Table 5). In contrast, in low-Cu conditions there was no significant difference between the Cu quotas of coastal and oceanic diatoms. The mixed-effects model supported these results, showing a significant regime effect on Cu quotas that was dependent on Cu availability (regime effect, $p < 0.004$, and regime × Cu effect, $p < 0.0001$, Table 3).

For all phytoplankton collectively, Fe availability did not have a significant effect on the diatoms' Cu : C ratios (Table 3). In contrast, the Cu quotas of all species, except *T. pseudonana* 3H, were affected by Cu availability (Fig. 1; Tables 4, 5). The oceanic strains showed consistent reductions in Cu : C ratios (mean, ~72% reduction) relative to cells grown in Cu-sufficient media, with the relative reduction of cellular Cu quotas being greatest when Fe was also low (56% reduction at pFe 19; vs. 85% at pFe 20.5). Overall, the coastal diatoms also had lower Cu quotas in low-Cu media (Fig. 1). However, the effect of Cu availability was more pronounced in the low-Fe treatment for some strains (i.e., *C. decipiens*) and in the high-Fe treatment for others (i.e., *T. weissflogii*). Thus, as regime groups, a significant interaction between Fe and Cu availability on Cu : C ratios was only observed for the oceanic diatoms (Fe × Cu effect, $p = 0.022$, Table 3).

In addition to these general trends, some interesting observations were obtained for individual species. While the Cu effect on cellular Cu was widespread (except for *T. pseudonana* 3H), the Fe effect on Cu quotas was only significant for two species, a coastal and an oceanic (*C.*

Table 3. Results from the mixed-effects linear model statistical analyses of the growth rates, copper (Cu) quotas, or steady-state Cu uptake (steady-state Cu uptake rates, $\rho_{ss}Cu$) data presented in Fig. 1 and Tables 4 and 5, respectively. Note that only the data from pFe 19, pCu 14; pFe 19, pCu 15; pFe 20.5, pCu 14; and pFe 20.5, Cu 15 treatments were included in these models. Three separate models were run: the first included all diatoms' data, the second included only the coastal diatoms' data, and the third included only the oceanic diatoms' data. All the significant single fixed factor effects (iron [Fe] or Cu) are positively correlated with the independent variable. Statistically significant results (α=0.05) are given in bold.

Parameter	Effect	Significance level		
		All diatoms	Coastal	Oceanic
Growth rates	Fe	$p<$**0.0001**	$p<$**0.0001**	$p<$**0.0001**
	Cu	$p<$**0.0001**	$p<$**0.0001**	$p<$**0.0001**
	Fe×Cu	p=0.218	p=0.356	$p<$**0.007**
	Regime	$p<$**0.0001**		
	Fe×regime	$p<$**0.0001**		
	Cu×regime	p=0.642		
Cu requirements	Fe	p=0.854	p=0.553	p=0.956
	Cu	$p<$**0.0001**	$p<$**0.005**	$p<$**0.0001**
	Fe×Cu	p=**0.006**	p=0.337	$p<$**0.022**
	Regime	p=**0.004**		
	Fe×regime	p=0.525		
	Cu×regime	$p<$**0.0001**		
$\rho_{ss}Cu$	Fe	p=0.131	p=0.290	p=0.495
	Cu	$p<$**0.0001**	$p<$**0.024**	$p<$**0.0001**
	Fe×Cu	p=**0.045**	p=0.098	p=0.640
	Regime	p=**0.099**		
	Fe×regime	p=0.649		
	Cu×regime	$p<$**0.0001**		

decipiens and *T. oceanica* 1003, respectively). In these two cases, as predicted, the Cu : C quota significantly increased under low-Fe conditions (Fig. 1; Tables 4, 5), but only at pCu 14 (significant Fe effect, Cu effect, and Fe × Cu effect, Table 4).

Effects of metal availability on steady-state Cu uptake—Steady-state Cu uptake rates ($\rho_{ss}Cu$) were calculated for each replicate quota culture based on the equation from Morel (1987) ($\rho_{ss}Cu = Q_{Cu}$ [mol Cu μm^{-2}] × μ [d^{-1}]) and ranged from 0.27 to 8.0 × 10^{-20} mol Cu μm^{-2} d^{-1} (Table 6). As seen for the Cu quotas, Fe availability overall had no effect on the $\rho_{ss}Cu$ for all phytoplankton as a group, while Cu availability had a significant effect (Fe effect, p = 0.131 vs. Cu effect, $p < 0.0001$, Table 3). In nearly all cases, uptake rates were significantly slower (average, ~4.2-fold) in pCu 15 media (Tables 3, 6), and the relative reductions in $\rho_{ss}Cu$ correspond more closely to the reduction in $[Cu]_{total}$ (fivefold) than to that of free Cu^{2+} (10-fold). However, the reduction of $\rho_{ss}Cu$ in response to lower Cu availability was highly species specific, decreasing up to ~10-fold for *T. oceanica* 1003 (Table 6).

In low-Cu media, reduction of Fe availability resulted in a decrease or no change in $\rho_{ss}Cu$ in all cultures (Table 6). However, in Cu-replete conditions, the response varied considerably between species. *T. oceanica* 1003 in pCu 14, pFe 20.5 media had the fastest rate of uptake (7.95 × 10^{-20} mol Cu μm^{-2} d^{-1}), which was a 70% increase over $\rho_{ss}Cu$ in pFe 19 media (Table 6; Fe effect and Fe × Cu effect, Table 4). In contrast, the oceanic strain *T. pseudonana* 1014 exhibited slower rates of $\rho_{ss}Cu$ under Fe limitation regardless of Cu availability (Table 6; Fe effect, Table 4). When considered at a group level (coastal or oceanic), no interaction between Fe and Cu availability was observed in the $\rho_{ss}Cu$ (Table 3).

Cu use efficiency—Metal use efficiency, the rate of C assimilation per unit of cellular metal (mol C μmol^{-1} Cu d^{-1}; Maldonado and Price 1996), was calculated from the specific growth rate and Cu : C ratio of each individual experimental Cu quota culture replicate (Raven 1988). This parameter is usually assessed for metal-limited cultures to ensure that luxury uptake does not affect the calculated efficiency of metal use. We therefore restrict our discussion to *C. decipiens* and *T. oceanica* 610, as these two species allowed comparison between Cu-limited cultures at both Fe treatment levels. In both of these species, Cu use efficiency increased in low-Fe media relative to Fe-sufficient conditions. An increase of ~50% (from 0.993 ± 0.051 to 1.528 ± 0.245 mol C μmol^{-1} Cu d^{-1}) was seen in *C. decipiens*. The increase in Cu use efficiency of the

Table 4. Two-way ANOVA of growth rate, copper (Cu) quota, and steady-state Cu uptake rate ($\rho_{ss}Cu$) data with respect to iron (Fe) and Cu levels. The data are presented in Fig. 1 and Tables 4 and 5, respectively, and include the following treatments: pFe 19, pCu 14; pFe 19, pCu 15; pFe 20.5, pCu 14; and pFe 20.5, pCu 15. In the case of *Thalassiosira oceanica* 1003 only, data for pFe 21.5, pCu 14 and pFe 21.5, pCu 15 were also included. Only significant effects are listed ($p < 0.05$). The single effects are positively correlated with the independent variable, unless otherwise indicated with a (−) symbol.

	Relative growth rates	Q_{Cu}	$\rho_{ss}Cu$
	(μ/μ_{max} × 100)	(μmol Cu mol^{-1} C)	(mol Cu μm^{-2} d^{-1})
Chaetoceros decipiens	Fe,Cu	Fe(−),Cu,Fe×Cu	Fe(−),Cu,Fe×Cu
Thalassiosira weissflogii Actin	Fe,Cu,Fe×Cu	Cu,Fe×Cu	Fe,Cu
Thalassiosira pseudonana 3H	Fe	No effect	No effect
Skeletonema costatum	Fe,Cu	Cu	Cu
Thalassiosira pseudonana 1014	Fe	Cu	Fe,Cu
Thalassiosira oceanica 610	Fe,Cu	Cu,Fe×Cu	Cu, Fe × Cu
Thalassiosira oceanica 1003	Fe,Cu,Fe×Cu	Cu,Fe(−),Fe×Cu	Fe(−),Cu,Fe×Cu
Skeletonema menzellii	Fe	Cu	Fe,Cu,Fe×Cu

Table 5. Cellular concentrations of copper (Cu) and carbon (C) and Cu : C ratios of diatoms in all iron (Fe) and Cu treatments. Growth rates of maintenance cultures are also listed. Statistical analyses of these data are presented in Fig. 1 and Tables 2 and 3. SE, standard error.

	Free Fe	Free Cu		Cellular C (mol C L^{-1})	Cellular Cu (μmol Cu L^{-1})	Cu : C (μmol Cu mol^{-1} C)
Species (strain)	pFe	pCu	*n*	(mean±SE)	(mean±SE)	(mean±SE)
Coastal						
Chaetoceros decipiens	19	14	3	6.33±0.67	3.34±0.50	0.58±0.080
	19	15	3	6.48±0.29	5.84±0.39	0.85±0.060
	20.5	14	4	6.38±0.91	12.7±2.1	1.99±0.33
	20.5	15	6	6.58±0.78	3.31±0.65	0.489±0.10
Thalassiosira weissflogii	19	14	6	10.2±0.32	12.8±3.4	1.28±0.34
(Actin)	19	15	6	10.4±0.42	3.34±0.80	0.33±0.080
	20.5	14	4	11.5±0.43	4.67±0.72	0.406±0.060
	20.5	15	4	10.6±0.51	4.32±0.61	0.407±0.060
Thalassiosira pseudonana	19	14	10	18.4±0.21	35.2±4.5	1.88±0.24
(3H)	19	15	8	18.5±0.90	35.8±2.6	1.96±0.14
	20.5	14	8	12.9±0.16	35.0±9.1	2.67±0.66
	20.5	15	10	12.6±0.19	24.6±10	1.95±0.83
Skeletonema costatum	19	14	4	8.58±0.66	21.1±5.0	2.46±0.58
	19	15	6	8.99±0.79	10.9±1.6	0.97±0.19
	20.5	14	8	10.2±0.74	23.3±1.6	2.28±0.070
	20.5	15	4	10.2±0.76	3.28±0.73	0.321±0.070
Oceanic						
Thalassiosira pseudonana	19	14	6	12.7±0.24	54.1±5.4	4.32±0.41
(1014)	19	15	4	14.1±0.59	36.4±4.0	2.58±0.28
	20.5	14	4	12.7±0.010	56.6±5.1	4.46±0.40
	20.5	15	8	13.9±0.26	21.4±6.5	1.49±0.44
Thalassiosira oceanica	19	14	4	8.97±0.88	28.6±5.0	3.18±0.56
(610)	19	15	4	9.59±0.49	17.6±3.2	1.83±0.34
	20.5	14	2	11.2±0.90	51.8±1.9	4.64±0.35
	20.5	15	4	10.2±1.1	3.43±0.27	0.335±0.030
Thalassiosira oceanica	19	14	4	12.9±0.61	37.8±2.4	2.93±0.19
(1003)	19	15	6	12.6±0.83	11.8±2.6	0.94±0.21
	20.5	14	3	17.0±1.2	79.3±4.8	4.66±0.28
	20.5	15	8	17.8±1.1	11.3±1.1	0.635±0.060
	21.5	14	6	10.8±0.17	68.2±11	6.32±0.98
	21.5	15	6	10.0±0.25	7.43±1.7	0.730±0.17
Skeletonema menzellii	19	14	2	9.38±0.88	30.0±1.0	3.20±0.11
	19	15	6	13.8±0.37	10.1±3.1	0.82±0.29
	20.5	14	4	11.1±0.97	33.8±11	3.03±0.98
	20.5	15	4	10.9±0.72	4.75±0.57	0.437±0.050
	21.5	14	4	14.5±1.8	25.9±6.1	1.84±0.41

oceanic isolate was much greater: nearly fourfold (from 0.591 ± 0.085 to 2.253 ± 0.188 mol C μmol^{-1} Cu d^{-1}) in response to reduced Fe availability, pointing to a greater physiological role for Cu in Fe-limited oceanic cultures.

Discussion

Cu is an essential redox-active trace metal, yet very few studies have focused on Cu as a phytoplankton nutrient. This is the first study to investigate the effects of low Cu and/or Fe on cellular Cu quotas in oceanic and coastal diatoms. The results presented here indicate that oceanic species require more Cu than coastal species and, when Fe-limited, can accumulate cellular Cu concentrations that exceed those of Fe. Reliance on Cu as a redox metal may help explain the ability of oceanic phytoplankton to survive in the low-Fe waters of the open ocean. Contrary to our hypothesis, Fe limitation did not increase the Cu demand of coastal and oceanic diatoms overall.

Evidence of trace metal limitation—Reducing Fe from pFe 19 to pFe 20.5 resulted in an average 16% reduction in growth of oceanic diatoms, with a larger 23% reduction in coastal strains. These results are consistent with those of other studies, which have found oceanic phytoplankton to show little evidence of Fe limitation at Fe concentrations noticeably limiting to their coastal counterparts (Brand 1991; Sunda et al. 1991; Maldonado et al. 2006). These different responses of coastal and oceanic strains to reduced Fe availability can be explained by the well-established higher Fe requirements of coastal phytoplankton (Brand 1991; Sunda et al. 1991; Sunda and Huntsman 1995*a*).

Cu availability also had a modest, but significant, effect on the growth rates of coastal and oceanic diatoms

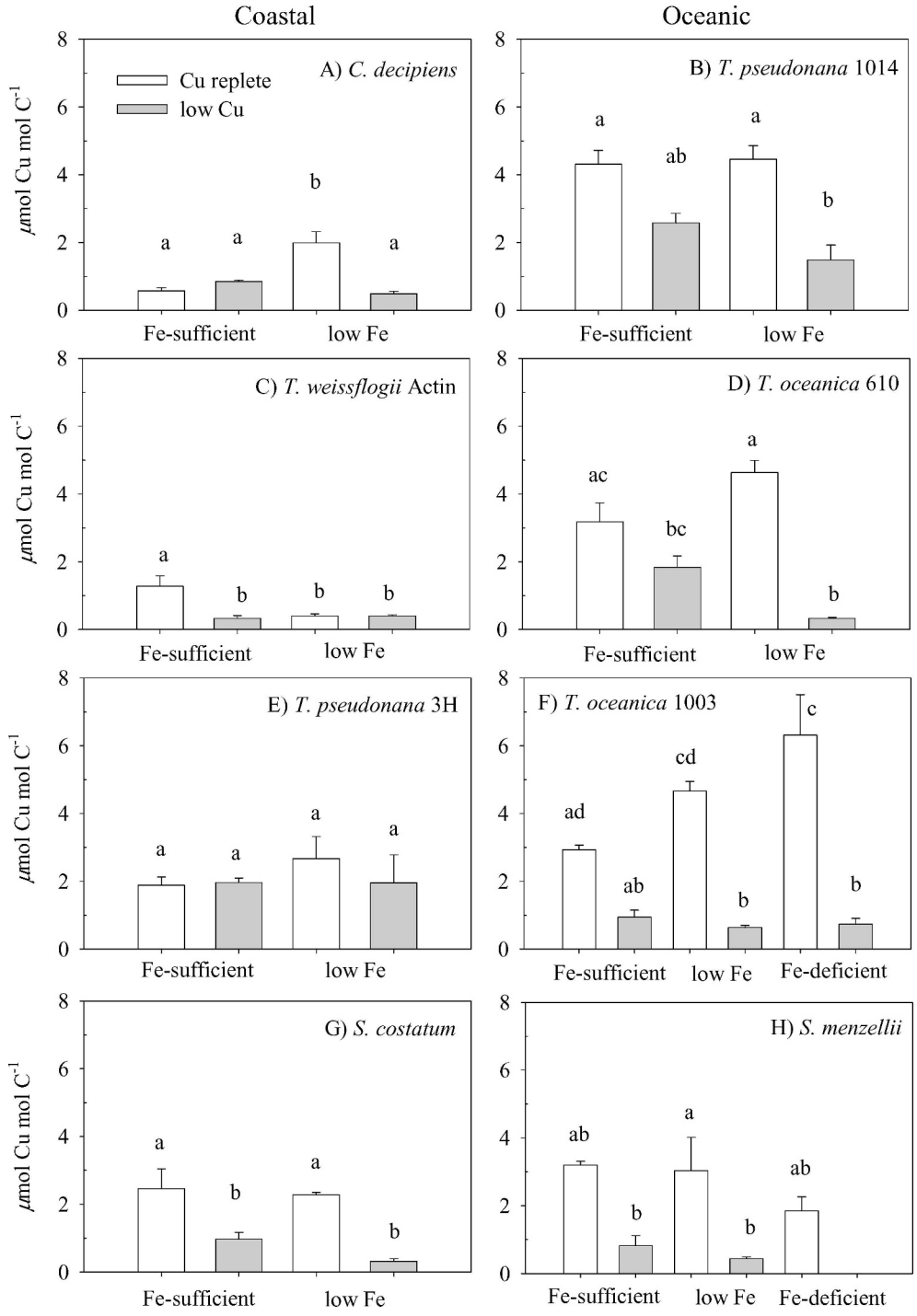

Fig. 1. Copper quotas (μmol Cu mol^{-1} C) of coastal and oceanic diatoms in Fe-sufficient (pFe 19) and low-Fe media (pFe 20.5; pFe 21.5 where specified) evaluated by short-term incubations in media spiked with ^{67}Cu and ^{14}C. Copper treatments were pCu 14 and pCu 15 for Cu replete and low Cu, respectively. Error bars denote standard error. Within each species, all treatments were compared statistically (ANOVA Tukey test); the same letters above bars indicate treatments that were not statistically different ($p > 0.05$). Additional statistical analysis of these data is presented in Tables 2 and 3.

Table 6. Steady-state copper (Cu) uptake rates (ρ_{ss}Cu, $\times 10^{-20}$ mol Cu μm^{-2} d^{-1}) of diatoms in media with sufficient and/or deplete levels of iron (Fe) and Cu. Uptake rates were calculated from cellular Cu and growth rate, according to the method of Morel (1987), and are reported as mean ± standard error (n). Within each species, all treatments were compared statistically (Tukey test); the same letters beside numbers indicate treatments that were not statistically different ($p > 0.05$).

	Free Cu	pFe 19	pFe 20.5	pFe 21.5
	(pCu)	ρ_{ss}Cu ($\times 10^{-20}$ mol Cu μm^{-2} d^{-1})		
Chaetoceros decipiens	14	0.902±0.13(4)[a]	4.05±0.67(4)[b]	
	15	1.79±0.11(4)[b]	0.712±0.14(6)[a]	
Thalassiosira weissflogii Actin	14	1.88±0.35(6)[a]	0.797±0.12(4)[b]	
	15	0.757±0.24(6)[b]	0.747±0.10(4)[b]	
Thalassiosira pseudonana 3H	14	2.92±0.29(10)[a]	2.51±0.73(8)[a]	
	15	3.48±0.52(8)[a]	1.78±0.73(10)[a]	
Skeletonema costatum	14	2.63±0.62(4)[a]	2.52±0.17(2)[a]	
	15	1.49±0.22(4)[ab]	0.533±0.12(4)[b]	
Thalassiosira pseudonana 1014	14	4.26±0.38(6)[a]	2.88±0.26(4)[ac]	
	15	2.13±0.23(4)[cb]	1.19±0.31(8)[b]	
Thalassiosira oceanica 610	14	2.66±0.46(4)[a]	2.81±0.24(2)[a]	
	15	1.94±0.35(4)[a]	0.265±0.021(4)[b]	
Thalassiosira oceanica 1003	14	4.64±0.28(4)[a]	7.98±0.42(3)[c]	6.60±0.72(6)[c]
	15	1.27±0.32(6)[b]	1.26±0.10(8)[b]	0.690±0.15(6)[b]
Skeletonema menzellii	14	5.62±0.19(2)[a]	2.93±0.94(4)[b]	1.29±0.30(4)[bc]
	15	0.575±0.16(8)[c]	0.479±0.058(4)[c]	

(Tables 2–4). Some isolates responded slightly differently to reduced Fe availability when Cu was also low (Table 2). For example, *T. oceanica* 1003 grew significantly slower at pFe 20.5, pCu 15 than at pFe 20.5, pCu 14. Thus, an interaction between Cu and Fe availability on the growth rates of some diatoms was observed (Tables 2, 4). As regime groups, however, this interaction was only significant for the oceanic diatoms (for oceanics, Fe × Cu effect, $p < 0.007$; for coastals, Fe × Cu effect, $p = 0.356$, Table 3). These results agree well with the observations that oceanic diatoms require higher Cu concentrations for growth than do coastal strains (Sunda and Huntsman 1995*b*; Peers et al. 2005; Maldonado et al. 2006), especially when Fe is low (Peers et al. 2005).

Cellular Cu : C ratios—Cellular Cu quotas evaluated in this study using ^{67}Cu range from 0.32 to 6.3 μmol Cu mol^{-1} C. Under optimal growth conditions (pFe 19, pCu 14), cellular Cu quotas varied between 0.58 and 4.3 μmol Cu mol^{-1} C. These Cu : C ratios are in accord with literature values of 1.7 μmol Cu mol^{-1} C for both *T. oceanica* 1003 and *T. pseudonana* 3H at comparable free Cu^{2+} levels (Sunda and Huntsman 1995*b*). Ho et al. (2003) report values of 0.6–2.6 μmol Cu mol^{-1} C for four species of diatoms, with a mean of 1.89 μmol Cu mol^{-1} C (assuming a Redfield C : phosphorus ratio of 106 : 1), very close to the mean of 2.3 μmol Cu mol^{-1} C for diatoms in optimal trace metal conditions presented here.

Several studies (Sunda et al. 1991; Sunda and Huntsman 1995*a*; Maldonado and Price 1996) indicate that the minimal Fe requirement is approximately four times greater in coastal diatoms (i.e., *T. pseudonana* 3H; ~12 μmol Fe mol^{-1} C) than in oceanic diatoms (i.e., *T. oceanica* 1003; ~3.3 μmol Fe mol^{-1} C). In contrast, and regardless of Fe level, Cu-sufficient oceanic isolates in this study had cellular Cu concentrations that were approximately twofold higher than those of coastal strains (Q_{Cu}: regime effect, $p < 0.004$; and Cu × regime effect, $p < 0.0001$, Fig. 1; Table 3). Such high Cu : C ratios in oceanic diatoms may point to the replacement of Fe-containing catalysts with Cu-containing ones. Furthermore, under similar Fe-limiting conditions to those noted in previous studies (pFe 21, pCu 14), *T. oceanica* 1003 had cellular Cu : C ratios (4.66 and 6.32 μmol Cu mol^{-1} C for pFe 20.5 and pFe 21.5, respectively, Table 5) that were similar to or higher than previously reported cellular Fe requirements (i.e., 4.92 ± 0.1 μmol Fe mol^{-1} C for this strain; Maldonado and Price 1996). The similarity of cellular concentrations of Cu and Fe in *T. oceanica* 1003 indicates that Cu nutrition may be particularly important for oceanic diatoms, especially in Fe-limited regions.

Cu is a required redox element for several essential cellular processes. In the respiratory electron transport chain, three Cu atoms are required for cytochrome oxidase, which shuttles electrons from cytochrome *c* to oxygen, reducing it to water. Cu is also required in freshwater green algae and all higher plants for plastocyanin, which carries electrons from the cytochrome b_6f complex of photosystem II to photosystem I (Raven et al. 1999). While diatoms are generally thought to use Fe-containing cytochrome c_6 for this function, a recent study by Peers and Price (2006) indicates that *T. oceanica* synthesizes putative Cu-containing plastocyanin instead of cytochrome c_6 (Strzepek and Harrison 2004). In some phytoplankton, Cu has been shown to be involved in the oxidation of organic nitrogen (Palenik et al. 1988–1989) and the detoxification of superoxide radicals (i.e., Cu-containing superoxide dismutase; Chadd et al. 1996). In addition to these cellular Cu demands, Fe limitation may enhance the Cu requirements of phytoplankton as a result of the involvement of putative multi–Cu-containing oxidases in their high-affinity Fe transport system (Peers et al. 2005; Wells et al. 2005; Maldonado et al. 2006).

Based on the multiple potential roles for Cu in alleviating Fe limitation, we hypothesized that the Cu quotas would increase under low-Fe conditions. However, this hypothesis was not supported by the data (Fe effect, $p = 0.854$, Table 3). Statistical analysis revealed that neither the coastal nor the oceanic isolates showed a group-wide Cu : C response to Fe limitation (Fe effect: coastals, $p = 0.55$, and oceanics, $p = 0.96$, Table 3), although individually, *T. oceanica* 1003 and *C. decipiens* did increase cellular Cu in response to Fe limitation (Fig. 1; Tables 4, 5). The variability in Cu quotas with Fe limitation indicates that the role of Cu in the high-affinity Fe uptake system is not consistent among species, nor within coastal or oceanic diatom groups. It is possible that the Cu demand of the high-affinity Fe transport system is low relative to other cellular Cu pools and may vary between species. The identification and quantification of Cu-containing proteins in these organisms merit further investigation.

Steady-state Cu uptake rates—A notable feature of nearly all isolates analyzed is the decrease in cellular Cu between Cu-sufficient and low-Cu treatments (Cu effect in Q_{Cu} in Tables 3, 4). Reduction in Cu availability resulted in an average 4.1-fold decrease in cellular Cu (Table 5). Similarly, the reduction in steady-state Cu uptake was 4.2-fold (Table 6; Cu effect in $\rho_{ss}Cu$ in Tables 3, 4). While this reduction is certainly a function of reduced Cu availability, free Cu^{2+} in the media was reduced by a factor of 10, whereas $[Cu]_{total}$ in the media was reduced fivefold. The relative reduction in $\rho_{ss}Cu$ closely matches the reduction in $[Cu]_{total}$, indicating that Cu transport is an active process that may involve acquisition not only of free Cu^{2+}, but also of Cu from organically complexed Cu. Similar conclusions were reached in a modeling study investigating the aqueous species controlling rates of Cu uptake in phytoplankton (Hudson 1998) and in a laboratory study investigating Cu uptake kinetics (Quigg et al. 2006).

Uptake of organically bound Fe involves the dissociation of Fe(III) from organic ligands, and it is possible that uptake of Cu may also involve such a process. Analyses by Hudson (for *T. oceanica* and *T. pseudonana*, 1998) and Quigg et al. (for *T. pseudonana* and *T. weissflogii*, 2006) indicate that many diatom species are able to acquire Cu faster than the diffusion-limited rate of Cu^{2+} supply to the cell surface. In addition, Jones et al. (1987) report the ability of several phytoplankton species to reduce Cu^{2+} to Cu^{1+}, which provides support for the bioavailability of Cu from organic complexes, as recently suggested (Quigg et al. 2006).

Such a Cu uptake system has been established in other eukaryotes. For example, in the freshwater green alga *Chlamydomonas reinhardtii*, Cu uptake is associated with a cupric reductase (Hill et al. 1996). In yeast, Cu uptake is well characterized and is facilitated by both low- and high-affinity transporters. The high-affinity Cu uptake involves reduction of Cu^{2+} to Cu^{1+}, but unlike Fe transport, it is the reduced species, Cu^{1+}, that is transported across the membrane (Askwith and Kaplan 1998). Low-affinity Cu transport involves proteins that also transport Fe and Mn.

Examination of $\rho_{ss}Cu$ indicates that in some strains, Cu uptake is upregulated by the cell in response to low Fe or Cu availability. For example, the $\rho_{ss}Cu$ of Fe-sufficient *C. decipiens* was twofold faster in low-Cu than in high-Cu conditions (Table 6). The well-studied algal species *C. reinhardtii* appears to regulate Cu uptake through a homeostatic cellular mechanism that responds to changes in Cu levels. Cultures of *C. reinhardtii* acclimated to Cu deficiency were observed to increase the maximum Cu uptake velocity (V_{max}) approximately 20-fold compared to Cu-replete cells (Hill et al. 1996). Remarkably, even in Cu-replete conditions, intracellular Cu did not exceed metabolic requirements (Hill et al. 1996), indicating that these organisms possess a homeostatic mechanism for Cu metabolism. Interestingly, low Fe availability also enhances the $\rho_{ss}Cu$ of some Cu-sufficient strains (*T. oceanica* 1003 and *C. decipiens* in response to reduced Fe availability [pFe 20.5], Table 6; Fe effect [−], Table 4). These findings are in agreement with those for other eukaryotes, which have shown that the expression of some of the genes encoding for components of the high-affinity Cu transport system or Cu homeostasis are upregulated by Fe deficiency (Peña et al. 1999; La Fontaine et al. 2002; Puig et al. 2007).

Oceanographic implications—The results presented here indicate that Cu plays an important role in oceanic diatoms, which have elevated Cu demands with respect to coastal diatoms. These findings potentially have major implications for understanding the control of primary production, influences of the biota on trace metal availability, and the biogeochemical cycles of Fe and Cu in oceanic surface waters. In the open ocean, Cu, like Fe, is predominantly bound to strong organic ligands; nevertheless, it is present at concentrations up to two orders of magnitude higher than Fe ($[Cu]_{total} < 2$ nmol L^{-1}, Coale and Bruland 1988; $[Fe]_{total} \sim 0.07$ nmol L^{-1}, Johnson et al. 1997), making it far more readily available to phytoplankton. Further studies investigating the roles of Cu in oceanic phytoplankton will be invaluable for our understanding of the unique physiology of these organisms (Strzepek and Harrison 2004; Peers and Price 2006).

In addition, some phytoplankton can significantly increase their Cu demand in response to Fe limitation (an oceanic and a coastal diatom in our study). These findings indicate that in some diatoms, Cu is able to mitigate, in part, some of the stresses of Fe limitation; this ability may be attributed to the role of Cu in the high-affinity Fe uptake system (Maldonado et al. 2006) and, in the case of the oceanic diatom, to the replacement of Fe-containing proteins (Peers and Price 2006). Thus, it is possible that phytoplankton in high-nutrient, low-chlorophyll regions may have higher demand for Cu than those in other oceanic regions. Indeed, analysis of published field data provides some support for this. Calculated Cu : C ratios (Web Appendix 1: www.aslo.org/lo/toc/vol_53/issue_6/2451a1.pdf)—based on linear relationships between dissolved Cu and PO_4^{3-} concentrations in oceanic nutriclines and Redfield ratios—reveal that the elemental composition of phytoplankton (Cu : C ratios) in Fe-deficient waters are significantly higher than those of other oceanic regions (7.2 vs. 4.4 μmol Cu mol^{-1} C, *t*-test, $p < 0.02$). These results indicate that the rates of Cu uptake by indigenous

phytoplankton are significantly elevated in low-Fe waters, a result that is consistent with our data for *C. decipiens* and *T. oceanica* 1003, and, thus, phytoplankton may exert a significant influence over the distribution and speciation of Cu in Fe-limited regions. These findings have important implications for studies of oceanic primary production and CO_2 drawdown, especially in light of the interaction between Fe limitation and Cu demand in an Fe-limited oceanic region (Peers et al. 2005). As Fe limitation was shown here to increase Cu demand in some diatoms, Cu may impart an additional stress to these organisms in Fe-limited regions when Cu may also be low. Elucidating the Cu and Fe physiology of phytoplankton inhabiting Fe-limited waters (i.e., *Phaeocystis* sp.) thus warrants further study.

In this and other studies (Peers et al. 2005; Wells et al. 2005), oceanic diatoms in culture were not Cu limited at free Cu concentrations (pCu 14) representative of open ocean conditions (i.e., subarctic north Pacific: pCu 13.4–13.9, Coale and Bruland 1988; Moffett and Dupont 2007). In apparent contradiction, field experiments have demonstrated stimulated phytoplankton growth following Cu enrichment (Peers et al. 2005; Wells et al. 2005). While Cu^{2+} levels similar to those in the open ocean do not appear to limit oceanic diatoms in culture, total Cu in the surface ocean is much lower than that of artificial seawater at a given pCu (i.e., at pCu 14, $\sim$2 nmol L^{-1} [Moffett and Dupont 2007] vs. 10.2 nmol L^{-1} [Price et al. 1988–1989] in natural and synthetic seawater, respectively). In considering $[Cu_{tot}]$, values from the subarctic North Pacific ($\leq$2 nmol L^{-1}, Coale and Bruland 1988; Moffett and Dupont 2007) are most comparable to our low-Cu media ($[Cu_{tot}]$ = 1.96 nmol L^{-1}), where some species were Cu limited. This study and previous studies (Jones et al. 1987; Hudson 1998; Quigg et al. 2006) indicate that organically complexed Cu is bioavailable. Hence, $[Cu_{tot}]$ may be a better indication of Cu availability to phytoplankton, implying that in some oceanic regions, the reported total Cu concentrations may hinder the growth of these organisms. Further studies focusing on Cu^{2+} vs. Cu_{tot} control of Cu uptake may elucidate whether natural Cu concentrations do indeed represent a stress or limitation to already Fe-limited diatoms, as suggested here.

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