A reduction-dependent copper uptake pathway in an oceanic diatom

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Copper(II) is reduced to Cu(I) extracellularly by marine and freshwater phytoplankton, but its biological significance is not firmly established. We studied the relationship between Cu(II) reduction and uptake in *Thalassiosira oceanica*, a diatom that was recently shown to possess functional copper uptake transporters (CTRs) that take up Cu(I). Inorganic and organic complexes of Cu(II) were reduced directly by reactions at the cell surface in proportion to Cu(II)-ligand reduction potential. The rates of reduction were enhanced twofold in Cu-limited cells, suggesting reduction was regulated by Cu nutritional state. Suppressing Cu(II) reduction caused a decrease in Cu uptake rate by 97% and addition of a Cu(II) complexing agent completely inhibited cell division and reduced Cu quota when Cu concentration was growth limiting. Thus, Cu(II) reduction was an obligatory first step in Cu uptake. Cu(II) reduction rate and growth rate of *T. oceanica* were proportional to Cu-ethylenediaminetetraacetic acid concentration and independent of inorganic Cu concentration in bulk solution. The results suggest that Cu(II) bound to organic ligands was reduced by extracellular cupric reductases and subsequently internalized. This reduction-dependent uptake pathway may enable diatoms to use naturally occurring Cu(II) organic complexes in the sea.

The importance of chemical speciation in determining metal bioavailability to aquatic microorganisms is well established (Campbell 1995; Hudson 1998; Sunda 2012). Uptake of metals by phytoplankton depends on the concentration of hydrated metal ions or kinetically labile species, maintained by complexation reactions with inorganic and organic ligands (the free-ion model: Morel and Hering 1993). Some of the earliest research in this field examined the toxic effects of copper (Cu) on marine phytoplankton (Sunda and Guillard 1976; Anderson and Morel 1978; Jackson and Morgan 1978). These studies showed the response of phytoplankton to high concentrations of Cu varied with the concentration of the cupric ion but not the total Cu or complexed Cu concentration in solution. Experiments at low, growth-limiting concentrations of Cu yielded similar results (Manahan and Smith 1973). Subsequent research confirmed that the general principles of the free-ion activity model were applicable to many metals (e.g., Cd^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}) and organisms (Anderson et al. 1978; Sanders et al. 1983; Stoecker et al. 1986; Sunda et al. 1987): the biological response was directly related to the concentration of free metal ion in solution.

Field and lab experiments recently reveal an unexpected role for organically complexed Cu in phytoplankton nutrition at environmentally relevant concentrations. Cu uptake rates are much faster than the diffusion rates of inorganic Cu to the phytoplankton cell surface (Sunda and Huntsman 1995; Hudson 1998; Quigg et al. 2006; Annett et al. 2008; Semeniuk et al. 2009, 2015; Kim and Price 2017), suggesting that Cu(II) complexes may be utilized directly. The organic complexes are not membrane permeable, and some are photochemically inactive, so other mechanisms may play a role in making the complexed Cu bioavailable. Hudson (1998) speculated that in ethylenediaminetetraacetic acid (EDTA)-buffered media reduction of Cu(II)-EDTA to Cu(I)-EDTA could increase inorganic Cu(I) concentration and allow for faster rates of uptake. Indeed, reducing sites and enzymatically mediated reactions on the surfaces of phytoplankton reduce Cu(II) extracellularly. The cupric reductases reduce Cu(II) bound to a variety of ligands (Jones et al. 1987; Jones and Morel 1988) and their activity is enhanced when phytoplankton are Cu deficient (Hill et al. 1996; Walsh et al. 2015). Secretion of reduced metabolites, like cysteine, also contributes to Cu(II) reduction, increasing the concentration of Cu(I) and the rate of Cu uptake (Walsh et al. 2015). Accordingly, Cu reduction may affect Cu chemical speciation and play a role in making Cu available for phytoplankton.

Experiments in yeast first described how Cu(II) reduction was required for Cu uptake (Hassett and Kosman 1995; Georgatsou et al. 1997). The yeast uptake system consists of two components: a cell surface reductase that reduces Cu(II) to Cu(I) and a copper uptake transporter (CTR) that takes up Cu(I). We recently discovered functional CTRs in *Thalassiosira oceanica* (To) and found homologous genes in many other diatoms (Kong and Price 2019). Two of the...
ToCTRs complemented growth of a CTR-deficient yeast mutant in Cu-depleted medium and transported Cu(I) into the cells. The possession of functional CTRs in the diatom implied that reduction of Cu(II) to Cu(I) was an obligatory step in Cu uptake, because Cu(II) is the predominant redox state of Cu in the surface ocean (Moffett and Zika 1983). In *T. pseudonana*, a related coastal species, putative reductase genes were down-regulated as Cu concentration increased (Guo et al. 2015), suggesting they may be involved in Cu homeostasis.

Extracellular Cu(II) reduction is observed in diatoms, green algae, and in a coccolithophorid (Jones et al. 1987; Jones and Morel 1988; Hill et al. 1996; Weger 1999; Weger et al. 2007; Walsh et al. 2015), but its role in Cu uptake is not established. Here we report some of our findings on the regulation, activity, and significance of Cu(II) reductases in *T. oceanica*. The results show that Cu(II) reduction is required for Cu uptake at low, environmentally relevant Cu concentrations and have important implications for Cu nutrition of diatoms in the open sea.

**Methods**

**Culture and growth condition**

*T. oceanica* CCMP1005 was obtained from the National Center for Marine Algae and Microbiota, and grown in artificial seawater medium, Aquil. The medium, prepared according to Price et al. (1989), contained a modified trace metal nutrient enrichment, consisting of Fe (1290 nmol L⁻¹), Mn (125 nmol L⁻¹), Zn (79.3 nmol L⁻¹), Mo (100 nmol L⁻¹), Co (50 nmol L⁻¹) and Se (10 nmol L⁻¹), and 100 μmol L⁻¹ EDTA. Cu was added separately as a Cu-EDTA complex in a 1 : 1.05 molar ratio at a concentration of 1 or 21.4 nmol L⁻¹. The media were designated as Cu-deplete (1 nmol L⁻¹) or Cu-replete (21.4 nmol L⁻¹) according to the growth phenotype of acclimated cultures. Semiconinuous cultures were grown in 28 mL polycarbonate tubes at 20°C under a continuous illumination of 200 μmol photons m⁻² s⁻¹ supplied by cool white fluorescent bulbs. In vivo chlorophyll fluorescence was measured using a Turner Designs model 10-AU fluorometer (CA) and specific growth rate (d⁻¹) calculated from linear regression of ln fluorescence vs. elapsed time. All phytoplankton cultures were fully acclimated to growth conditions before they were used for experiments.

**Cu reduction assay**

Cu(II) reduction was measured by monitoring the production of Cu(I) in cell suspensions using bathocuproinedisulfonate (BCDS), a membrane-impermeable, colorimetric reagent that forms a strong, stable Cu(I)(BCDS)₂ complex (logₐ K_{Cu(I)(BCDS)₂} = 20.8) (Blair and Diehl 1961; Bagchi et al. 2013). The reduction assays were conducted in O₂-free and in air-equilibrated seawater. In the O₂-free assays, cells were harvested from mid-exponential phase cultures by filtration onto acid-washed 25 mm, 3-μm pore size polycarbonate membrane filters and then resuspended in N₂-bubbled, O₂-free seawater. Reduction assays were conducted in acid-washed 28-mL polycarbonate tubes (Nalgene). The reaction tubes, containing the resuspended cells, were bubbled with N₂ for 10 min and then Cu(II) substrates were added to initiate the experiment. Cu(II) was added as a CuSO₄ salt or complexed with nitrilotriacetic acid (NTA) or EDTA at a total concentration (Cu(II)) of 50 μmol L⁻¹. Nitrogen gas was continuously bubbled into the samples during the assay. Subsamples taken from each replicate at 10 and 70 min were immediately mixed with 500 μmol L⁻¹ BCDS. The concentration of Cu(I) (BCDS)₂ complex was determined spectrophotometrically at 484 nm using a CARY 1E UV/Vis spectrophotometer (Agilent Technologies, ON, Canada) using an extinction coefficient of 12,250 M⁻¹ cm⁻¹ (Blair and Diehl 1961). Samples were filtered to remove cells before measuring absorbance. Sampling time (15–20 s) was kept constant for each measurement to minimize technical errors among samples. Reduction rate was calculated from the difference between Cu(I) concentrations in the two subsamples divided by elapsed time.

In the air-equilibrated assays, BCDS was added during the reduction experiment to trap any Cu(I) produced by Cu(II) reduction. Three milliliters of phytoplankton culture or culture filtrate were added to acid-washed 4.5-mL polystyrene cuvettes (BrandTech Scientific) containing 500 μmol L⁻¹ BCDS. Cellular rates of Cu(II) reduction were determined by subtracting rates of Cu(II) reduction by dissolved substances in the cultures (secreted by the cells during growth) and by BCDS (Jones et al. 1987). Cell-free filtrate was generated by gently removing the cells from the culture by filtration through an acid-washed 1-μm polycarbonate membrane filter. In some experiments, algal cells were first harvested by filtration and then resuspended in fresh, metal-free medium. Following addition of the Cu(II) substrate, the concentration of Cu(I)(BCDS)₂ was determined after correcting for light scattering caused by the cell suspension (Jones et al. 1987). Absorbance was measured every 10–15 min over a period of 1–1.5 h. Cu(I)(BCDS)₂ concentration was plotted against incubation time and the formation rate of Cu(I)(BCDS)₂ calculated from the slope of the linear portion of the curve. Reduction assays were conducted in the dark. All sample processing and assays were conducted at 20°C. Rates were normalized to cell density or cell surface area if cell size varied among treatments.

**Cu uptake assay**

Cells harvested in exponential phase onto a 3-μm polycarbonate membrane filter were rinsed two times with metal-free culture medium, and then suspended in 100 mL Cu uptake assay buffer (metal-free Aquil medium enriched with different amounts of CuSO₄ and 100 μmol L⁻¹ EDTA, equilibrated for 48 h before use). Two time-point samples were collected from each replicate: the first immediately after resuspension and the second after 60 min of incubation. Uptake assays were conducted in the dark. Cells were harvested using trace metal clean techniques, digested in nitric acid and analyzed for Cu content by graphite furnace atomic absorption spectrometry.
Total concentrations of Cu (CuT) and ligand (LT) are reported. Inorganic Cu (CuWestall et al. 1976). Reduction rates were measured in oxygen-free seawater. Values reported are means ± 1 standard deviation of three biological replicates (**).

Reduction of inorganic and organic Cu(II) complexes

Results

Reduction of inorganic and organic Cu(II) complexes

Initial experiments in O2-free seawater provided unambiguous proof of Cu(II) reduction by Thalassiosira oceanica. In the absence of O2, Cu(II) produced by the cells remained stable in solution and did not reoxidize (data not shown; Moffett and Zika 1983). Reduction rates varied from 94–356 amol cell−1 h−1, depending on Cu substrate and concentration: Cu(II) complexed to inorganic and organic ligands was reduced (Table 1). Rates of Cu(II) production were slowest with Cu–EDTA and varied in direct proportion to Cu concentration. Subsequent experiments in air-equilibrated seawater, in which BCDS was added during the assay to trap the Cu(I) produced by the cells, yielded rates that were about fourfold faster than those in the O2-free assays, possibly because BCDS complexation of Cu(I) provided a driving force for Cu(II) reduction (Thorstensen and Aisen 1990; Mies et al. 2006). Rates of Cu(I) production in the presence of BCDS were similar with and without O2, suggesting reactive oxygen species, like O2·−, were not involved in Cu(II) reduction by the cells (data not shown).

### Table 1. Rates of Cu(II) reduction of inorganic (CuSO4) and organic Cu complexes (Cu–NTA and Cu–EDTA) by Thalassiosira oceanica.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CuT (μmol L−1)</th>
<th>LT (μmol L−1)</th>
<th>Cu′ (μmol L−1)</th>
<th>Reduction rate (amol cell−1 h−1)</th>
<th>Log K′</th>
<th>Reduction potential (V)</th>
</tr>
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<tbody>
<tr>
<td>CuSO4</td>
<td>50</td>
<td>–</td>
<td>50</td>
<td>356 ± 64●</td>
<td>–</td>
<td>0.16‖</td>
</tr>
<tr>
<td>Cu–NTA</td>
<td>50</td>
<td>100</td>
<td>2.7 × 10−2</td>
<td>345 ± 82*</td>
<td>8.4‡</td>
<td>−0.29‖</td>
</tr>
<tr>
<td>Cu–EDTA</td>
<td>50</td>
<td>100</td>
<td>1.1 × 10−3</td>
<td>94 ± 16*</td>
<td>10.1†</td>
<td>−0.46‖</td>
</tr>
<tr>
<td>Cu–EDTA</td>
<td>100</td>
<td>300</td>
<td>5.2 × 10−4</td>
<td>193 ± 10**</td>
<td>10.1†</td>
<td>−0.46‖</td>
</tr>
</tbody>
</table>

EDTA, ethylenediaminetetraacetic acid; NTA, nitrilotriacetic acid.

●Reduction potential of Cu′ (Jones et al. 1987).

‡Conditional stability constants (log K′) of Cu–NTA and Cu–EDTA (Sunda et al. 2005).

†Half wave reduction potentials of Cu–NTA and Cu–EDTA (Croot et al. 1999).

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**Chemical equilibrium modeling**

Cu speciation was calculated using the chemical equilibrium modeling software, MINEQL+ v4.6 (Westall et al. 1976). In Agul medium and assay buffer, Cu(II) is bound in a number of EDTA complexes. The thermodynamic stability constant of Cu(II)(BCDS)_2, logK_{Cu(II)(BCDS)_2} was 12.4 (Bagchi et al. 2013). We do not consider potential side reactions of BCDS with Ca2+, Mg2+, and H+, so estimates of the equilibrium concentration of Cu(II) (BCDS)_2 represent an upper limit. Inorganic Cu (Cu′) concentrations were calculated from [Cu^{2+}] using a side reaction coefficient, a_{Cu} of 16.1 (Sunda et al. 2005). Cu(II)(BCDS)_2 concentration in the Cu–EDTA reduction and uptake assays was measured by reducing the Cu(II) complex to Cu(I)(BCDS)_2 using 200μmol L−1 ascorbic acid and quantifying the amount of the Cu(I) complex formed after subtracting background values. Under standard assay conditions (500μmol L−1 BCDS, 50μmol L−1 Cu, and 160μmol L−1 EDTA), we measured 0.87μmol L−1 Cu(II)(BCDS)_2 in the assays, in good agreement with the equilibrium model calculations (Supporting Information Table S1).

**Results**

**Reduction of inorganic and organic Cu(II) complexes**

Initial experiments in O2-free seawater provided unambiguous proof of Cu(II) reduction by Th. oceanica. In the absence of O2, Cu(II) produced by the cells remained stable in solution and did not reoxidize (data not shown; Moffett and Zika 1983). Reduction rates varied from 94–356 amol cell−1 h−1, depending on Cu substrate and concentration: Cu(II) complexed to inorganic and organic ligands was reduced (Table 1). Rates of Cu(I) production were slowest with Cu–EDTA and varied in direct proportion to Cu concentration. Subsequent experiments in air-equilibrated seawater, in which BCDS was added during the assay to trap the Cu(I) produced by the cells, yielded rates that were about fourfold faster than those in the O2-free assays, possibly because BCDS complexation of Cu(I) provided a driving force for Cu(II) reduction (Thorstensen and Aisen 1990; Mies et al. 2006). Rates of Cu(I) production in the presence of BCDS were similar with and without O2, suggesting reactive oxygen species, like O2·−, were not involved in Cu(II) reduction by the cells (data not shown).

**Fig. 1.** Cu(II) reduction rate (●) of Cu-replete Thalassiosira oceanica with (a) 50 μmol L−1 Cu–NTA or (b) 50 μmol L−1 Cu–EDTA as a function of BCDS concentration. Concentrations of Cu(II) (●), Cu–NTA (○) or Cu–EDTA (△), and Cu(I)(BCDS)_2 (□) were calculated using MINEQL+ v4.6. Rates were measured in air-equilibrated seawater. Error bars represent ± 1 standard deviation of three biological replicates.
Cu(II) reduction in the presence of BCDS increased nonlinearly with BCDS concentration using Cu-NTA as substrate (Fig. 1). The increased rate occurred as the concentrations of inorganic Cu (Cu') and Cu-NTA declined, but coincided with an increase in Cu(II)(BCDS)$_2$ concentration, suggesting Cu(II)(BCDS)$_2$ itself was a substrate for the reaction. Increasing BCDS concentration, however, had no effect on Cu(II) reduction rate with Cu-EDTA as substrate ($t = -1.27, p = 0.25, df = 1, n = 12$, regression analysis; Fig. 1). This result was consistent with the results of chemical equilibrium modeling that showed only a minor effect of BCDS on Cu(II) speciation in EDTA-buffered seawater (Table S1). Addition of BCDS affected Cu speciation in seawater (Table S1) to a minor extent. BCDS did not affect Cu speciation in seawater (Table S1) to a minor extent. BCDS affected Cu speciation in seawater (Table S1) to a minor extent. BCDS affected Cu speciation in seawater (Table S1) to a minor extent.

The substrate for the reductase in the Cu–EDTA experiments was assessed by independently manipulating Cu' and Cu-EDTA in the assay solutions. Cu(II) reduction rate was constant over a 20-fold change in Cu' ($t = 0.55, p = 0.60, df = 1, n = 9$, regression analysis), but positively correlated with Cu-EDTA concentration ($t = 39.9, p < 0.001, df = 1, n = 12$) (Fig. 2). Thus, in EDTA-buffered seawater at high CuT concentrations, Cu(II) reduction rate was proportional to Cu–EDTA concentration and not Cu', which was only present at low nmol L$^{-1}$ levels.

Reduction rate was undersaturated at the Cu concentrations used in our experiments and increased linearly with Cu–EDTA (Table 1, Fig. 2), so the reaction could be described with first-order kinetics: viz. Cu(II) reduction rate (mol cell$^{-1}$ h$^{-1}$) = $k_{\text{red}}$ Cu-Y × [Cu-Y], where $k_{\text{red}}$ Cu-Y is the reduction rate constant (L cell$^{-1}$ h$^{-1}$) and [Cu-Y] the concentration of the Cu(II) substrate (mol L$^{-1}$). A plot of $k_{\text{red}}$ Cu-Y as a function of reduction potential of Cu(II)–Y showed a positive exponential relationship (Fig. 3), as observed for other electron transfer reactions (Meyer et al. 1983).

**Cu reduction rate is upregulated in response to Cu limitation**

Physiological state of *T. oceanica* depended on the Cu concentration in the growth medium and influenced the rates of Cu(II) reduction. When CuT was 1 nmol L$^{-1}$, growth was strongly Cu-limited (0.78 ± 0.10 d$^{-1}$) and equal to roughly one-half of the maximum rate ($\mu_{\text{max}}$) achieved under Cu-replete conditions (21.4 nmol L$^{-1}$; 1.60 ± 0.07 d$^{-1}$). Cu limitation increased the cell surface area-normalized Cu(II) reduction rate by approximately twofold (Fig. 4) compared to Cu-sufficient cells, implying reduction rate was under negative feedback regulation by cellular Cu status. Elevated rates of Cu(II) reduction were detected with both Cu-NTA and Cu-EDTA as substrates ($t = 15.1, p < 0.01, df = 4$ and $t = 2.43, p = 0.036, df = 4$, t-test, respectively) (Fig. 4).

**Inhibition of Cu(II) reduction or Cu(I) supply inhibits Cu uptake**

*T. oceanica* possesses functional CTR Cu(I) transporters that are highly expressed under Cu-limiting conditions and
downregulated at Cu concentrations greater than 10 pmol L$^{-1}$ ([Cu$^{2+}$] = 820 nmol L$^{-1}$; [EDTA] = 100 μmol L$^{-1}$) (Kong and Price 2019). If Cu(II) reduction is required to provide Cu(I) to the transporter, then blocking Cu(I) production or supply should impede Cu uptake, particularly at low Cu. Potassium hexachloroplatinate (K$_2$PtCl$_6$; Sigma-Aldrich), a known inhibitor of Fe and Cu reductases in yeast (Eide et al. 1992; Hassett and Kosman 1995), decreased Cu reduction in *T. oceanica* by 80% and almost completely inhibited Cu uptake (Fig. 5).

Addition of BCDS during the Cu uptake assay also reduced Cu uptake rate by 75% (Fig. 5), suggesting that Cu reduction preceded Cu uptake.

Because of analytical limitations, the uptake and reduction assays reported in Fig. 5 were conducted at high Cu concentrations (0.89 and 50 μmol L$^{-1}$ Cu, respectively) that do not reflect natural levels of Cu encountered by diatoms in the sea. More realistic values are in the low nmol L$^{-1}$ range, so we assessed how disrupting supply of Cu(I) affected *T. oceanica* at 1 nmol L$^{-1}$ Cu (Cu$^{2+}$ = 12.3 fmol L$^{-1}$, 100 μmol L$^{-1}$ EDTA). At this Cu concentration, Cu quota is low and limiting (Kim and Price 2017), so that any change in Cu uptake by the cells is reflected by a corresponding change in growth rate. Addition of BCDS to Cu-limited *T. oceanica* completely inhibited cell division within 48 h (Fig. 6). Calculations showed that BCDS had no effect on Cu(II) speciation (Table S1) and the amount of Cu(I) complexed by BCDS by the end of the experiment was roughly 50 pmol L$^{-1}$, less than 5% of the total Cu added to the medium. This latter result showed that BCDS did not significantly alter the total amount of Cu(II) in the medium. In the presence of high Cu concentration, growth rate and biomass yield of *T. oceanica* were unaffected by BCDS, confirming that BCDS by itself was not inhibitory. Thus, trapping Cu(I) prevented growth of *T. oceanica* under Cu-limiting conditions, as expected if Cu(II) reduction was required for uptake.

To confirm that BCDS reduced Cu uptake by *T. oceanica* in the previous experiment, cells grown with 1 nmol L$^{-1}$ Cu were briefly exposed to BCDS, harvested by filtration, and then resuspended in Cu-free medium. In the absence of dissolved Cu, cell division was entirely dependent on the amount of intracellular Cu, because carryover of Cu in the medium was negligible (<1 pmol L$^{-1}$), and continued until intracellular Cu was reduced to a minimum. The maximum cell density of BCDS-treated cells was approximately fourfold lower than

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**Fig. 4.** Cu(II) reduction rate of Cu-limited (■) and Cu-replete (□) *Thalassiosira oceanica* with 50 μmol L$^{-1}$ Cu-NTA or Cu-EDTA. Rates were measured in cell suspensions in the presence of BCDS in air-equilibrated seawater. Error bars represent ±1 standard deviation of three biological replicates. Asterisks indicate a significant difference between Cu-limited and Cu-replete cells (*t = 2.43, df = 4, p = 0.036; **t = 15.1, df = 4, p < 0.01).**

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**Fig. 5.** (a) Cu(II) reduction rate of Cu-limited *Thalassiosira oceanica* in the absence (control) and presence of 200 μmol L$^{-1}$ K$_2$PtCl$_6$ (+Pt). The reduction assay contained 50 μmol L$^{-1}$ Cu-EDTA as substrate. (b) Short-term Cu uptake rate of Cu-limited *T. oceanica* in the absence (control) and presence of 200 μmol L$^{-1}$ K$_2$PtCl$_6$ (+Pt) or 500 μmol L$^{-1}$ BCDS (+BCDS). The uptake medium contained 0.89 μmol L$^{-1}$ Cu complexed with 100 μmol L$^{-1}$ EDTA. K$_2$PtCl$_6$ and BCDS were added at the beginning of the assays in air-equilibrated seawater. Error bars represent ±1 standard deviation of three biological replicates. Asterisks indicate a significant difference between reduction rates of the control and Pt-treated cells (*t = 6.51, df = 4, p < 0.01) and between uptake rates of the control and Pt (*t = 13.9, df = 4, p < 0.01) or BCDS-treated cells (*t = 10.7, df = 4, p < 0.01).
the experiment. Inorganic Cu concentration (Cu

with different concentrations of Cu

T. oceanica

Cu(II) species that was reduced. To test this hypothesis, should be related to the concentration of the dominant was reduction dependent, so growth at low Cu concentrations

was growth and increased final biomass of BCDS-
treated cells by ~16-fold (Fig. 7).

Growth of Cu-limited T. oceanica is a function of the Cu-ligand concentration

The preceding results demonstrated Cu uptake by T. oceanica was reduction dependent, so growth at low Cu concentrations should be related to the concentration of the dominant Cu(II) species that was reduced. To test this hypothesis, T. oceanica was grown in Cu-free Aquil medium supplemented with different concentrations of Cu-EDTA, at a constant Cu’ concentration of 23.5 fmol L⁻¹. Over the range of concentrations tested, growth rate was Cu-limited and increased from 1 to 1.45 d⁻¹ (0.6–0.9 μmax) as Cu-EDTA concentration increased (Fig. 8). Addition of 20 nmol L⁻¹ Cu to 1 nmol L⁻¹ Cu-EDTA cultures increased growth rate to 1.38 ± 0.06 d⁻¹ within 1 d, demonstrating that the cells were Cu limited.

Slow rates of Cu(II) reduction measured using Cu-cyclam as substrate (Fig. 3) implied that Cu-cyclam could also be a source of Cu for the Cu(I) uptake pathway. Cyclam (1,4,8,11-tetraazacyclotetradecane) has an exceptionally high conditional stability constant for Cu(II) binding (log K’ = 15.3, Semeniuk et al. 2015) compared with EDTA (log K’ = 10.12, Sunda et al. 2005) and can maintain [Cu’] at subfemtomolar levels. Growth of T. oceanica in Aquil medium enriched with 10 μmol L⁻¹ cyclam increased with increasing Cu concentration (Fig. 9). At the two highest Cu-cyclam concentrations, [Cu’] was 300 to 3000 times lower than in the EDTA-buffered medium (Fig. 8), yet growth rates were almost identical (1–1.4 d⁻¹ in the EDTA-only medium and 0.8–1.3 d⁻¹ in the cyclam-enriched medium). Thus, at these low Cu concentrations, the organic Cu complexes appeared to be substrates for growth.

Discussion

The results reported here show Cu(II) uptake by T. oceanica proceeds through a two-step reaction in which Cu(II) is initially reduced to Cu(I) and then internalized. The pathway operates at low, environmentally relevant Cu concentrations and is essential to sustain Cu-limited growth. Disrupting the supply of Cu(I) decreased Cu cell quota and completely suppressed cell division, confirming that Cu uptake was impaired. That Cu(I) reduction rate and growth were proportional to Cu-EDTA concentration, and not Cu’, implies that organically complexed Cu(II) was substrate, although other mechanisms may be involved in making the Cu(II)’ available for reduction. As discussed below, these findings are relevant to understanding how chemical speciation affects metal
uptake by phytoplankton and the means of Cu acquisition by diatoms in the sea.

**Substrates for cell surface Cu reduction: Free and chelated Cu**

Previous work established that phytoplankton reduce a variety of Cu(II) complexes and that reduction rates were independent of Cu**2+** concentration (Jones et al. 1987). Reduction consisted of two parts: a nonenzymatic, cell-wall-associated reduction that was short lived and an enzymatically mediated reduction that exhibited saturation kinetics. The Cu(II) reduction rates reported here are for the linear, enzymatic phase that does not include Cu(II) reduction by reducing sites on the cell surface or secreted metabolites. Normalized to surface area, the maximum rate of Cu(I) production by *T. oceanica* was similar to *Thalassiosira weissflogii* (Cu(II)(BCDS)_{2}: 5.9 vs. 4.5 x 10^{-17} mol μm^{-2} h^{-1}) and increased by about twofold when Cu-limited growth (Fig. 4). Substrates for reduction included inorganic Cu species (Cu'), Cu–NTA, Cu–EDTA, and Cu–cyclam, spanning a range of reduction potentials from 0.16 to −0.86 V. Measurements by Jones et al. (1987) were unable to detect reduction of Cu–EDTA by *T. weissflogii*, possibly because of the low concentration of Cu–EDTA used in their experiments. Indeed, following the experimental protocol described here, we detected Cu(II):EDTA reduction by *T. weissflogii*, but the rate was 1/8 of the rate of *T. oceanica*. The results in O2-free assays show conclusively that Cu–EDTA is reduced by *T. oceanica* and that the rates are independent of [Cu(II)] in bulk solution (Table 1). Furthermore, experiments that manipulated the equilibrium Cu chemical speciation showed reduction rate increased in direct proportion to Cu–EDTA, not Cu' (Fig. 2), suggesting that Cu–EDTA was directly reduced. Thermal dissociation of Cu–EDTA in the diffusive boundary layer is likely too slow to supply Cu' for the reaction (Hudson 1998), but we cannot rule out the possibility that other reactions could enhance the breakdown of organic Cu(II) complexes at the cell surface and thereby increase Cu', which is subsequently reduced. Such a mechanism could potentially be involved in reduction of Cu(II) bound to all of the organic ligands: in which case Cu(II) reduction rate would be proportional to the concentration of the Cu complex and independent of Cu' in bulk solution.

**Cu(II) reduction is a biologically mediated process**

Cu reduction occurs via biologically mediated enzymatic reactions on the cell surface and abiotically mediated photochemical reactions. Although photochemical reduction of Cu–chelates is well known, Cu–EDTA complexes, the dominant Cu species in Aquil medium, are photochemically inert (Natarajan and Endicott 1973; Semeniuk et al. 2009). Photolysis of Cu–EDTA would thus not contribute Cu(I) production. Moreover, the reduction and uptake assays reported here were conducted in the dark, precluding photochemistry. Superoxide radical (O_2^-) is an effective metal reductant and produced in natural seawater and culture medium by cell secretion (Cooper et al. 1989; Voelker and Sedlak 1995; Zafiriou et al. 1998; Rose and Waite 2006). It reduces inorganic Cu and Cu complexed with weak organic ligands and is responsible for maintaining a portion (25% of total inorganic Cu) of reduced Cu(I) in the sea. Cu complexed to strong organic ligands are not very sensitive to O_2^- (Zafiriou et al. 1998; Voelker et al. 2000). In addition, some thiol containing ligands, such as glutathione and other cysteine-derived ligands, are potential Cu-reducing agents produced by algal cells (Dupont et al. 2004; Tang et al. 2005). However, their rates of secretion, for example, glutathione production rate in *T. weissflogii* is ∼4 amol cell^{-1} h^{-1} at pCu = 13.8 (Tang et al. 2005), are too slow to sustain the rates of Cu reduction observed here.

Plasma membrane-associated Cu reduction is mediated by reductases homologous to NADPH oxidases (Jones and Morél 1988; Shatwell et al. 1996; Georgatsou et al. 1997). NADPH oxidases (homologous proteins named respiratory burst oxidase in plants) reduce dissolved oxygen to O_2^- (Chanock et al. 1994; Sagi and Fluhr 2006) and O_2 reduces a broad range of Fe and Cu complexes (Voelker and Sedlak 1995; Zafiriou et al. 1998; Kustka et al. 2005; Rose and Waite 2005, 2006). Thus, metal reduction could occur indirectly through a reactive oxygen intermediate. Although this represents a plausible pathway for Cu(I) production, we observed no differences in Cu(II) reduction in O2-free and air-equilibrated solutions, suggesting it is not a significant pathway of Cu(II) reduction. Collectively, the results suggest that Cu(II) reduction in *T. oceanica* is mediated directly by a cupric reductase.

**A Cu(I)-dependent uptake system is present in *T. oceanica***

Inhibition of Cu(II) (and Fe) reduction and uptake by oxidized platinum (Pt) was attributed to its inhibitory effect on reductase activity (Eide et al. 1992; Hassett and Kosman 1995). However, Pt(II) and Pt(IV) have higher reduction potentials than Cu(II) and could oxidize Cu(I) produced by the reductase. This would make it appear like Pt inhibited Cu(II) reductase activity, when in fact, Pt may have oxidized Cu(I) before it reacted with BCDS. Indeed, we observed that Cu(I) rapidly oxidized in Aquil medium after the addition of Pt(IV) (data not shown), but that Cu(I)(BCDS)_{2} was stable. These results may explain why Pt(IV) inhibited Cu reduction by 80% and almost completely (97%) inhibited Cu uptake (Fig. 5). The high concentration of BCDS in the reduction assays may have stabilized a small amount of the Cu(I) produced (ca. 20%), but in the uptake assays, which lacked BCDS, all the Cu(I) produced was likely reoxidized.

Another possible effect of Pt was to competitively interact with Cu(I) during uptake by the high-affinity Cu transporter, CTR1 (Ishida et al. 2002). Hassett and Kosman (1995) observed Pt(II) had no inhibitory effect on Cu(I) uptake by *Saccharomyces cerevisiae*, but in those experiments, Cu(I) was prepared by adding a high concentration of ascorbic acid to the assay solution. We performed a similar experiment with
Indeed, Cu uptake kinetics of transporter, may thus be the primary route of Cu acquisition. The addition of 500 μmol L⁻¹ (uptake assay) and 100 or 20 μmol L⁻¹ (growth assay) BCDS had no effect on Cu(II) chemical speciation (Table S1) so the effect of BCDS was related to its complexation of Cu(I). Reduced growth and Cu uptake were thus related to a decrease in Cu(I) availability, as predicted if Cu uptake occurred by a Cu(I)-dependent uptake pathway.

Cu uptake is controlled by internalization at high Cu and reduction at low Cu concentrations

Previous work on the impact of Cu on marine phytoplankton demonstrated that the inhibitory effects were related to cupric ion (Cu²⁺) activity (Sunda and Guillard 1976; Anderson and Morel 1978; Brand et al. 1986). Our results appear to contradict these studies and thus the predictions of the free ion model in that growth and Cu(II) reduction rate of T. oceanica were functions of the Cu-EDTA concentration and not Cu⁺. One explanation may be related to the Cu nutritional state of the phytoplankton and the use of high- and low-affinity Cu transport systems depending on the environmental Cu concentration (Fig. 10).

Maximum growth rate of T. oceanica is maintained over a greater than three order of magnitude range in Cu concentration, from 263 fmol L⁻¹ to 640 nmol L⁻¹ Cu⁺ (Kong and Price 2019). At [Cu⁺] > 10 pmol L⁻¹ (820 nmol L⁻¹ CuT, 100 μmol L⁻¹ EDTA), CTR Cu(I) transporters are downregulated and diffusion of Cu(II) to the cell surface fast enough to supply Cu needed for growth (Kong and Price 2019). Low-affinity Cu transport, likely through a nonspecific divalent metal ion transporter, may thus be the primary route of Cu acquisition. Indeed, Cu uptake kinetics of T. oceanica shows that at 10 pmol L⁻¹ Cu⁺ uptake of Cu occurs through a separate transport system with a much higher Kₘ than observed at low Cu concentrations (Guo et al. 2010), although the transport proteins have not yet been identified. A reduction step is unnecessary for Cu acquisition at these Cu levels, judging from the lack of effect of BCDS on growth (Fig. 6). Although Cu(I) is expected to be continuously produced by cellular reduction, even under Cu-sufficient conditions (Fig. 4), a decrease in diatom CTR Cu(I) transporter abundance may suppress Cu(I) uptake (Kong and Price 2019). Our data suggest that the nonreduction pathway of Cu uptake predominates at high Cu. Uptake of Cu through these transporters likely involves Cu(II) binding to the transport ligand followed by a slower internalization step, as predicted by the free ion model. At even higher Cu concentrations, Cu(I) may also competitively interact with Mn(II) transport sites and gain entry to the cells (Sunda and Huntsman 1998). Under these conditions, growth and Cu uptake should be related to Cu⁺ concentration.

At low Cu concentrations, uptake of Cu exceeds the rate of diffusion of Cu⁺ to the cell surface and CTR Cu(I) transporters are maximally induced (Kong and Price 2019). Dissociation of the Cu-EDTA complexes within the cell boundary layer is

T. oceanica and observed that Pt(IV) had no effect on Cu(I) uptake (data not shown), but we note that addition of a strong reducing agent like ascorbic acid may have reduced Pt(IV) or Pt(II) to Pt(0), which would not be expected to be biologically active. Although we are uncertain of the precise mechanism of action of Pt on Cu uptake, all of the potential pathways point to a role for Cu(I) as an intermediate ion in Cu transport.

Additional evidence for a Cu(I)-dependent uptake system in T. oceanica was provided by BCDS experiments in which BCDS was used to trap Cu(I) produced by the reductase. A similar phenomenon was observed in Fe uptake experiments with T. weissflogii, using bathophenanthroline disulfonate to trap Fe(II) in culture medium produced by cellular reduction (Anderson and Morel 1980). The addition of 500 μmol L⁻¹ (uptake assay) and 100 or 20 μmol L⁻¹ (growth assay) BCDS had no effect on Cu(II) chemical speciation (Table S1) so the effect of BCDS was related to its complexation of Cu(I). Reduced growth and Cu uptake were thus related to a decrease in Cu(I) availability, as predicted if Cu uptake occurred by a Cu(I)-dependent uptake pathway.

![Copper reduction and uptake pathways of Thalassiosira oceanica](image)

**Fig. 10.** Copper reduction and uptake pathways of Thalassiosira oceanica in low and high Cu environments. (1) Organically complexed Cu (Cu-L) dominates the total dissolved Cu pool; inorganic Cu(II) accounts for only a minor portion (<0.01%). (2) Inorganic Cu(II) diffuses to the cell surface and is reduced to Cu(I) by cell surface Cu reductases. (3) Cu(II)-L is reduced to Cu(I)-L, which dissociates to inorganic Cu(I) and free ligand L. (4) Cu(I) is transported across the cell membrane by CTR Cu(I) transporters. (5) Inorganic Cu(I) diffuses to the cell surface and is transported into cells by a low-affinity divalent metal transporter(s) of unknown identity. Steps (2)–(5) represent biologically mediated Cu reduction and uptake pathways: The dominant pathway at low or high [CuT] is identified by the thick lines. Dashed arrows indicate the pathway is not functional or contributes negligibly to Cu uptake.
thought to be too slow to supply the additional Cu for uptake (and reduction) (Hudson 1998). Cupric reductases are upregulated coincidentally and reduce Cu(II) organic chelates, like Cu–EDTA, supplying Cu(I) to the transporter. The concentration of Cu(II) is too low and the rate constant of the reaction is so small that only a negligible amount of Cu(I) should be produced directly from inorganic Cu(II)’ reduction. The diffusion rates of the Cu(II) chelates to the cell surface are not limiting because of their much higher concentrations compared to free Cu’. According to this model, Cu(II) reduction is an obligatory step in Cu uptake and is supported by the inhibitor and BCDS trapping experiments reported here (Figs. 6 and 7). Although we provide no short-term measurement of Cu uptake at the low Cu concentrations, the decrease in Cu quota observed following BCDS exposure confirms that trapping Cu(I) suppresses Cu transport. At low Cu concentration, uptake is mainly dependent on Cu(I) availability mediated by a Cu(II) reduction step (Fig. 10).

The requirement of a Cu(II) reduction step opens up the possibility that Cu acquisition by T. oceanica could be limited by the rate of Cu(I) production by the reductase. Indeed, the expression of CTR Cu(I) transporters in T. oceanica remains relatively constant as Cu concentration declines from 21.4 to 1 nmol L^{-1}, yet the maximum rate of uptake of the cells increases twofold (Kong and Price 2019). Over the same concentration range, Cu(II) reductase activity doubles as Cu declines (Fig. 4), suggesting that the rate of Cu(II) reduction may indeed control the rate of Cu(I) uptake. In the ocean, photochemical reactions may also supplement the supply of Cu(I) for diatoms so that irradiance could greatly affect Cu availability.

Implications of a Cu reduction-dependent uptake pathway

Plankton communities in the subarctic Pacific Ocean take up Cu bound to synthetic and natural organic ligands (Semeniuk et al. 2015), but the mechanisms involved are not known. As described here, T. oceanica utilizes extracellular reduction to make the organically complexed Cu(II) available for transport. Other diatoms, which contain homologs of the high-affinity Cu transport system of T. oceanica, may do likewise. Ocean regions typically contain 0.5–3 nmol L^{-1} of dissolved Cu and the majority (> 99.9%) is bound to organic ligands (free Cu^{2+} = 10^{-16} to 10^{-13} mol L^{-1}) (Coale and Bruland 1990; Moffett and Dupont 2007; Bundy et al. 2013; Whitby et al. 2018). The nature of these Cu-ligand complexes is unknown, so it is difficult to assess whether they are reducible by the diatom reductases. One important factor influencing their reactivity is their reduction potential. Cu–EDTA was reduced by T. oceanica and has a half-wave potential (E’_{1/2}) of -0.46 V (Croet et al. 1999), so the reduction potential of the cell surface reductase is expected to be lower. The results also show that Cu(II) bound to cyclam, a Cu complex with an E’_{1/2} of -0.82 V (Croet et al. 1999), is reduced by T. oceanica, but at a slower rate (Fig. 3). Measured E’_{1/2} of Cu organic complexes in natural seawater samples range from -1.25 to -0.33 V (Croet et al. 1999), so they may be available substrates for cell surface Cu(II) reduction. If Cu reduction is an important first step in Cu uptake by phytoplankton, then the concentration and properties of the Cu-binding ligands (electrostatic and steric factors, and reduction potentials) should be important determinants of Cu availability in the sea.

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Conflict of Interest

None declared.

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