

## Extracellular production of superoxide by marine diatoms: Contrasting effects on iron redox chemistry and bioavailability

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### Abstract

We report the extracellular production of superoxide in cultures of the marine diatoms *Thalassiosira weissflogii* and *Thalassiosira pseudonana*. In EDTA-buffered media with  $\sim 45 \mu\text{mol L}^{-1}$  iron (Fe)(III)', over half the Fe(III) reduction is mediated by extracellular  $\text{O}_2^-$  production. Surprisingly, even though we saw a consistent inhibition of Fe reduction by the addition of superoxide dismutase (SOD) and enhancement of Fe reduction due to superoxide production using the xanthine-xanthine oxidase system (X-XO), we observed no effect of SOD or X-XO on Fe uptake in these cultures. We also observed no effect of SOD on the uptake of Fe from either ferrihydrite, an Fe-labeled porphyrin, or as regenerated by metazoan grazers. Our data reveal that Fe(II)' is formed by  $\text{O}_2^-$  via the reduction of Fe(III)' rather than by the reduction and dissociation of FeEDTA complexes in EDTA-buffered media. This bulk-phase reduction of Fe(III)' by  $\text{O}_2^-$  is a "futile" reductive step, as the Fe(II)' formed is rapidly reoxidized to Fe(III)' in pH 8 oxygenated seawater.

One of the major recent discoveries within the field of biological oceanography is that the growth of phytoplankton, particularly of diatoms, is limited by the availability of iron (Fe) in several oceanic regions (Martin et al. 1994; Boyd et al. 2000). But in view of the very low concentrations of dissolved Fe in most surface waters and the fact that most of this dissolved Fe is bound to strong ligands (Rue and Bruland 1997), it is perhaps surprising that Fe limitation is not even more severe and extensive than it is. Some studies have shown that marine diatoms are able to reduce Fe(III) to Fe(II) and that reduction may be essential for Fe acquisition (Anderson and Morel 1980; Allnut and Bonner 1987; Maldonado and Price 2001). In both regards, this indicates a similarity to the yeast (*Saccharomyces cerevisiae*) high-affinity iron uptake system (Eide 1998). The reduction mechanism of diatoms is apparently effective for a number of Fe(III) and copper (Cu)(II) complexes, however (Jones et al. 1987). In view of this lack of specificity of the reduction, we wondered if it may be caused by reaction with superoxide,  $\text{O}_2^-$ , produced in the culture medium.

The superoxide radical is an effective Fe(III) reductant that is known to be produced at high concentrations by toxic marine phytoplankton of the genera *Chattonella* and *Heterosigma* (Kim et al. 2000) (Raphidophyceae); it is also excreted as part of a defense mechanism against a variety of

pathogens by mammalian macrophages (Segal et al. 1992) as well as by certain tissues of vascular plants (Lamb and Dixon 1997). However, the extracellular production and possible roles of  $\text{O}_2^-$  in nontoxic unicellular organisms has not been investigated.

Here we present the results of experiments designed to test the two related hypotheses that  $\text{O}_2^-$  is produced in diatom cultures as an intermediate reductant and that extracellular production of  $\text{O}_2^-$  by diatoms increases Fe availability by reduction of various Fe species.

### Material and methods

**Cultures**—The centric diatoms *Thalassiosira weissflogii* (CCMP 1336) and *Thalassiosira pseudonana* (CCMP 1335) were maintained axenic in f/2 medium at 20°C under continuous light (80–100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ). Prior to experimentation, cells were transferred to microwave-sterilized, trace metal-defined medium Aquil (Price et al. 1988/89) under Fe-limiting conditions (90 and 100 nmol Fe  $\text{L}^{-1}$  for *T. weissflogii* and *T. pseudonana*, respectively, and 100  $\mu\text{mol EDTA L}^{-1}$ ). Cells were grown for at least 15 generations under these conditions before experimentation. Cell counts were determined using a Coulter Counter. Specific growth rates for these species, calculated by the slope of the regression of  $\ln(\text{cell number})$  versus time, were  $\sim 0.70$ – $0.80 \text{ d}^{-1}$  and  $1.25$ – $1.35 \text{ d}^{-1}$ , respectively, under these Fe-limited conditions (between 70% and 80% of  $\mu_{\text{max}}$  obtained under Fe-replete conditions).

To compare our data to that obtained for other species, cell surface areas of  $\sim 460 \mu\text{m}^2$  and  $51 \mu\text{m}^2 \text{ cell}^{-1}$  were determined for our cultures of *T. weissflogii* and *T. pseu-*

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### Acknowledgments

This work was funded by the Center for Environmental Bioorganic Chemistry (NSF grant CHE-0221978) and the Carbon Mitigation Initiative at Princeton University.

Table 1. Summary of Fe reduction and uptake experiments with *T. weissflogii* and *T. pseudonana* (*Tw* and *Tp*, respectively). Rates of uptake and reduction were determined from time series measurements (typically, four observations) from each experiment and are reported as mol Fe cell<sup>-1</sup> h<sup>-1</sup>. Brackets represent the standard error of each slope (expressed in the same power of 10 as the rate). For each experiment, observed rates of various treatments that were significantly different ( $F_s > F_{\alpha^1(\mu, v)}$ , where  $\alpha=0.05$ ) from unamended cells are indicated by asterisks. Cell densities (expressed as 1,000 cells ml<sup>-1</sup>) for experiments A–J were 65, 59, 65, 174, 65, 35, 192, 116, 546, and 330, respectively. nd, parameter not determined.

	Experiment/treatment	Fe reduction	Fe uptake
(A)	<i>Tw</i> control	3.87×10 <sup>-19</sup> (0.59)	nd
	<i>Tw</i> +10× SOD	1.13×10 <sup>-19</sup> (0.15)*	nd
	<i>Tw</i> +0.06 mg BSA L <sup>-1</sup>	3.20×10 <sup>-19</sup> (0.40)	nd
	<i>Tw</i> +0.6 mg BSA L <sup>-1</sup>	3.15×10 <sup>-19</sup> (0.31)	nd
(B)	<i>Tw</i> control	1.54×10 <sup>-18</sup> (0.22)	nd
	<i>Tw</i> 10× SOD	9.37×10 <sup>-19</sup> (1.9)	nd
	<i>Tw</i> 10× SOD + catalase	8.07×10 <sup>-19</sup> (0.35)*	nd
	<i>Tw</i> +6 mg BSA	4.82×10 <sup>-19</sup> (1.3)*	nd
(C)	<i>Tw</i> control	1.53×10 <sup>-18</sup> (0.20)	nd
	<i>Tw</i> +10× SOD	8.84×10 <sup>-19</sup> (1.1)*	nd
(D)	<i>Tw</i> control	9.6×10 <sup>-19</sup> (0.76)	nd
	<i>Tw</i> +10× SOD	3.71×10 <sup>-19</sup> (0.60)*	nd
	<i>Tw</i> +10× denatured SOD	1.06×10 <sup>-18</sup> (0.13)	nd
(E)	<i>Tw</i> control	8.88×10 <sup>-19</sup> (0.85)	nd
	<i>Tw</i> +10× SOD	3.59×10 <sup>-19</sup> (0.33)*	nd
(F)	<i>Tw</i> control	nd	4.86×10 <sup>-18</sup> (0.25)
	<i>Tw</i> +10× SOD	nd	4.59×10 <sup>-18</sup> (0.21)
	<i>Tw</i> +2 mU XO L <sup>-1</sup>	nd	6.24×10 <sup>-18</sup> (1.1)
	<i>Tw</i> +4 mU XO L <sup>-1</sup>	nd	3.51×10 <sup>-18</sup> (0.20)
(G)	<i>Tp</i> control	4.35×10 <sup>-20</sup> (0.79)	4.59×10 <sup>-19</sup> (0.40)
	<i>Tp</i> +10× SOD	6.29×10 <sup>-21</sup> (7.0)*	5.78×10 <sup>-19</sup> (0.38)
	<i>Tp</i> +100× SOD	-2.8×10 <sup>-21</sup> (9.4)*	5.92×10 <sup>-19</sup> (0.39)
(H)	<i>Tp</i> control	1.69×10 <sup>-19</sup> (0.20)	nd
	<i>Tp</i> +10× SOC	3.43×10 <sup>-20</sup> (0.49)*	nd
(I)	<i>Tp</i> control	4.04×10 <sup>-20</sup> (0.04)	nd
	<i>Tp</i> +10× SOD	7.76×10 <sup>-21</sup> (5.4)*	nd
(J)	<i>Tp</i> control	8.27×10 <sup>-20</sup> (0.37)	1.21×10 <sup>-18</sup> (0.02)
	<i>Tp</i> +10× SOD	5.17×10 <sup>-20</sup> (0.70)*	1.23×10 <sup>-18</sup> (0.06)
	<i>Tp</i> +10× MnSOD	5.84×10 <sup>-20</sup> (0.44)*	1.11×10 <sup>-18</sup> (0.08)

*donana* using a Coulter Counter. For comparison, cell surface areas for *Chattonella* strain 85, *Fibrocapsus japonica*, *Heterosigma akashiwo*, and *Olisthodiscus luteus* (four toxic phytoplankters in which O<sub>2</sub> production rates have been measured; Oda et al. 1997) were approximated based on electron micrographs presented in Oda et al. (1997). For these four species, we estimate the surface area to measure ≈16,000, 1,260, 560, and 600 μm<sup>2</sup> cell<sup>-1</sup>, respectively.

**Extracellular production of Fe(II)**—We investigated Fe(II) production by marine diatoms using relatively low Fe(III) concentrations and trapping <sup>59</sup>Fe(II) with the cell-impermeable Fe(II) ligand ferrozine (FZ; Sigma P-9762), as described in Shaked et al. (2004). Such low concentrations are necessary to both probe a high-affinity system and to ensure that reduction rates are unsaturated. During exponential growth, cells were filtered onto a 47-mm, 3- or 8-μm acid-cleaned polycarbonate filter (depending on the species), rinsed twice with 50 ml Aquil medium, resuspended, and

added to experimental medium containing 90 nmol L<sup>-1</sup> Fe radiolabeled with <sup>59</sup>Fe (after pre-equilibration, this yields an [Fe'] of ~45 pmol L<sup>-1</sup> [Sunda and Huntsman 2003]). During this procedure, special care was taken to prevent exposing the cells to air on the filter manifold. *T. weissflogii* and *T. pseudonana* experimental cell densities were typically 65,000 and 100,000–300,000 cells ml<sup>-1</sup>, respectively (Table 1). In this and all other experiments with radiolabeled Fe and EDTA, the specific activity ranged from 1 to 7 × 10<sup>16</sup> Bq mol Fe<sup>-1</sup>. All experiments were conducted in the dark to avoid photoreduction of Fe(III)EDTA, except for brief (<10-min) exposure of sample aliquots to dim laboratory light during column loading. Immediately after adding cells to each treatment bottle, FZ was added at 100 μmol L<sup>-1</sup>. At each of four time points over 2–3 h, 30-ml aliquots from each treatment were loaded onto Sep-Pak C<sub>18</sub> columns, effectively concentrating the <sup>59</sup>Fe(II)FZ<sub>3</sub> complex. Cells were removed immediately upstream of the columns with in-line 13-mm Versapor filters (0.8 μm, acrylic copolymer). After

eluting the  $\text{FeFZ}_3$  complex from the column with methanol, the methanol was evaporated and the sample was counted using liquid scintillation. Rates of  $\text{Fe(II)}$  production were calculated by the linear regression of  $\text{FeFZ}_3$  produced over time. Scintillation counts of  $\text{FeFZ}_3$  were converted to total  $\text{Fe}$  by concurrent counts of 0.1-ml aliquots of experimental media. Blank rates of  $\text{Fe(II)}$  production were periodically determined in Aquil with radiolabeled  $\text{Fe}$  in the absence of cells, and these rates were always <10% of control rates.

**Superoxide dismutase (SOD) effect on  $\text{Fe(II)}$  production by diatoms**—The effect of extracellular  $\text{O}_2^-$  production by diatoms on  $\text{Fe(II)}$  production was measured by utilizing the  $\text{O}_2^-$  dismutase enzymes, bovine Cu zinc (Zn)SOD and *Escherichia coli* manganese (Mn)SOD (Sigma S-2515 and S-5639) (Fridovich 1970). These were prepared in Milli-Q water containing 100  $\mu\text{mol EDTA L}^{-1}$  and were used fresh or frozen at  $-80^\circ\text{C}$  for up to 60 d. By convention, 1 U Cu-ZnSOD in a 3-ml reaction volume will decrease  $\text{O}_2^-$ -mediated ferricytochrome *c* reduction by 50% in pH 7.8 phosphate buffer solution, and we found that 10 U per 3 ml, hereafter referred to as “10 $\times$  SOD,” decreased cytochrome *c* reduction by >95% in Aquil. In the  $\text{Fe(II)}$  production experiments (above), SOD was added at a concentration of 0.6 mg  $\text{L}^{-1}$ , or “10 $\times$ ”, immediately after ferrozine addition and before cell addition, except as noted. Several independent assays of the effect of SOD were conducted for *T. weissflogii* and *T. pseudonana* ( $n = 5$  and 4, respectively). For each species, a paired comparison, one-tailed *t*-test between unamended and SOD-treated rates of  $\text{Fe(II)}$  production was performed ( $\alpha = 0.05$ ). Short-term (6 h)  $^{14}\text{C-HCO}_3^-$  fixation assays indicated SOD did not affect cell growth (data not shown). Various control treatments included reduction assays with *T. weissflogii* and additions of either bovine serum albumin or heat-denatured (boiled for 10 min and cooled) CuZnSOD. In addition, since the superoxide disproportionation reaction produces  $\text{H}_2\text{O}_2$ , a catalase (Sigma C-1345; 1 U  $\text{L}^{-1}$ ) + SOD treatment was conducted to ensure  $\text{H}_2\text{O}_2$  was not responsible for the observed decrease in  $\text{Fe}$  reduction (Table 1, experiment B).

**$\text{Fe(II)}$  production by superoxide**—The cell-free superoxide-generating xanthine-xanthine oxidase (X-XO; xanthine: Sigma X-0626; xanthine oxidase: Fluka 67684) system was used to continuously generate  $\text{O}_2^-$  (Fridovich 1970) in Aquil containing  $^{59}\text{FeEDTA}$  ( $[\text{X}] = 16 \mu\text{mol L}^{-1}$ ,  $[\text{XO}]$  ranged from 0 to 3.3 U  $\text{L}^{-1}$ ) and  $\text{Fe(II)}$  production over time was measured (as described previously); 1 U XO corresponds to the amount of enzyme that oxidizes 1  $\mu\text{mol}$  xanthine per minute at pH 7.5 at  $25^\circ\text{C}$ . The specific rate of  $\text{O}_2^-$  production achieved by XO in Aquil was determined spectrophotometrically with ferricytochrome *c* reduction assays, using 0.6 U XO  $\text{L}^{-1}$ , with special attention to the caveats for  $\text{O}_2^-$  quantification (Fridovich 1970).

**Superoxide quantification**—We used flow injection analysis (FIA) and the  $\text{O}_2^-$ -specific chemiluminescent reagent 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA, a *Cypridina* luciferin analogue; Zheng et al. 2003) to quantify the production of  $\text{O}_2^-$  by *T.*

*weissflogii*. In this system, the sample stream and reagent stream (containing MCLA) were mixed in the flow cell, and chemiluminescence was measured. Calibration of chemiluminescence to  $\text{O}_2^-$  concentration was done by generating  $\text{O}_2^-$  standards by the photolysis of a solution of benzophenone and 2-propanol (McDowell et al. 1983). The  $\text{O}_2^-$  concentration of this stock was determined spectrophotometrically at 240 nm, and dilutions were subsequently introduced to the FIA system. All solutions were amended with 100  $\mu\text{mol DTPA L}^{-1}$  to prevent metal-assisted Fenton chemistry in the FIA system.

Superoxide production by *T. weissflogii* was measured by loading about  $3.5 \times 10^6$  cells (quantified on a Coulter Counter) on a 25-mm-diameter 0.2- $\mu\text{m}$  filter (Durapore) connected just upstream of the spiral flow cell (in the sample stream), and cells were flushed with seawater at 2 ml  $\text{min}^{-1}$ . To demonstrate the specificity of the assay, the sample stream was switched to either a seawater solution containing 50 $\times$  CuZnSOD or 60  $\mu\text{mol L}^{-1}$  of the transmembrane oxidase inhibitor diphenylene iodonium chloride (DPI; Sigma D-2926) (O'Donnell et al. 1993).

**Role of superoxide on  $\text{Fe}$  uptake**—Iron uptake in *T. weissflogii* and *T. pseudonana* was measured in Aquil containing 90 nmol  $\text{L}^{-1}$   $\text{Fe}$  (with 100  $\mu\text{mol L}^{-1}$  EDTA, resulting in  $[\text{Fe}'] = 45 \text{ pmol L}^{-1}$ ) radiolabeled with  $^{59}\text{Fe}$  by passing cells through in-line, 0.8- $\mu\text{m}$ , 13-mm filters (Versapor) using a peristaltic pump (in the absence of ferrozine). At several time points over 2–3 h, 30-ml aliquots from each treatment were loaded, treated with the Ti-citrate-EDTA wash (Hudson and Morel 1989), and counted on a Wallac 1801 gamma counter. Counts of cellular  $^{59}\text{Fe}$  were converted to total  $\text{Fe}$  by concurrent counts of 0.1-ml aliquots of experimental medium with the appropriate correction for differences in counting geometry. Uptake rates were determined in the presence and absence of CuZnSOD (10 $\times$ ) in several experiments. In a second set of experiments,  $\text{Fe}$  uptake rates by *T. weissflogii* were determined in the presence of the X-XO  $\text{O}_2^-$ -generating system (16  $\mu\text{mol X L}^{-1}$ , 0, 2 and 4 mU XO  $\text{L}^{-1}$ ). These values of X-XO were chosen because they effectively augment the expected  $\text{O}_2^-$  production rates by 125–250%.

**Speciation of superoxide-mediated  $\text{Fe(II)}$  production**—To determine whether  $\text{O}_2^-$  reduces  $\text{Fe(III)}$ ',  $\text{Fe(III)EDTA}$ , or a combination of the two, the X-XO system was used to generate  $\text{O}_2^-$  in Aquil containing 20–100  $\mu\text{mol EDTA L}^{-1}$  and 90 nmol  $\text{Fe L}^{-1}$ . The measured rates of  $\text{Fe(II)}$  production were compared to the calculated steady-state  $\text{Fe(III)}$ ' concentrations. The equilibrium concentrations of  $\text{Fe(III)}$ ' at each  $[\text{EDTA}]$  in the presence of  $\text{O}_2^-$  were calculated using the initial  $\text{Fe(III)}$ ' concentrations (Sunda and Huntsman 2003), the measured rates of  $\text{FeFZ}_3$  formation, and the known rates of  $\text{FeEDTA}$  dissociation and complex formation.

**Effect of superoxide on  $\text{Fe}$  uptake from ferrihydrite, porphyrins, and regenerated  $\text{Fe}$** —Colloidal ferrihydrite was synthesized using two simple methods. First, 10  $\mu\text{l}$  of  $^{59}\text{FeCl}_3$  stock ( $[\text{H}^+] = 2.5 \times 10^{-3} \text{ mol L}^{-1}$ ) was added to 75

$\mu\text{l}$  of seawater amended with  $2 \times 10^{-2}$  mol  $\text{NaHCO}_3 \text{ L}^{-1}$ . This preparation is referred to as “fresh” ferrihydrite. Second, 40  $\mu\text{l}$  of fresh ferrihydrite was heated to  $60^\circ\text{C}$  for 5 min (“baked” ferrihydrite). Immediately after synthesis, 40  $\mu\text{l}$  of each preparation was added to 10 ml of *T. weissflogii* cells that had been resuspended in Gulf Stream seawater. To minimize uptake of dissolved Fe, after mixing for 10 min to allow coagulation of cells with the radiolabeled ferrihydrite, the cells were filtered, rinsed, and resuspended in unlabeled Gulf Stream water. Following resuspension, samples for iron uptake (with and without CuZnSOD; 20 $\times$ ) were periodically collected over  $\sim 5.5$  h. For intracellular uptake, cells were washed with Ti-citrate-EDTA (Hudson and Morel 1989) to remove adsorbed Fe. The total colloidal Fe concentrations carried into the unlabeled media were 9.1 and 9.7  $\text{nmol L}^{-1}$  (baked and fresh colloids, respectively).

Deuteroporphyrin dihydrochloride was obtained from Frontier Scientific (catalog No. D5109) and dissolved in pyridine. Batches of porphyrin were labeled with  $^{59}\text{Fe}$  by adapting the hot acetic acid method (Morell et al. 1961). Briefly, we introduced 100  $\mu\text{l}$  of  $1 \times 10^{-3}$  mol  $\text{L}^{-1}$  porphyrin solution and  $5 \times 10^{-7}$  moles of radiolabeled  $\text{FeSO}_4$  solution (a 5:1 Fe:Y ratio) into 5 ml acetic acid (HAc). This was immediately transferred to an  $80^\circ\text{C}$  water bath for 15–20 min. The conversion of apo-porphyrin to Fe-porphyrin was determined fluorometrically (Excitation/emission = 398/621 nm), as the Fe-porphyrin is no longer fluorescent. The excess Fe was removed via organic-aqueous extraction in a 125-ml Teflon separatory funnel. One milliliter of the HAc mixture was added to 30 ml of high performance–liquid chromatography–grade ethyl acetate, followed by three Milli-Q extractions and three 1 mol  $\text{L}^{-1}$  Q-HCl extractions. The remaining ethyl acetate (some volume is lost to the aqueous phase) was refluxed to dryness at  $45^\circ\text{C}$  using a home-made trace metal clean reflux apparatus. After the solution reached dryness, the walls of the reflux vial were rinsed twice with 3 ml ethyl acetate. The dried product was maintained at  $4^\circ\text{C}$  (dessicator) until it was redissolved in  $2 \times 300 \mu\text{l}$  of dimethyl sulfoxide. Aliquots were dispensed in clean Eppendorf vials and frozen. We chose to avoid experiments with protoporphyrin IX, as previous investigators have reported a high tendency for aggregate formation in high-pH, high-ionic strength solutions (Blauer and Zvilichovsky 1968).

Regenerated Fe was obtained from *Artemia* grazing on radiolabeled *T. weissflogii*. Diatoms were grown in Aquil medium with 90  $\text{nmol L}^{-1}$   $^{59}\text{Fe}$  ( $1.5 \times 10^{13}$  Bq  $\text{mol}^{-1}$ ) and 100  $\mu\text{mol EDTA L}^{-1}$  and were harvested by filtration during late exponential growth. The cells grew in this medium for >10 generations, ensuring isotopic equilibrium between dissolved and cellular Fe. The cells were resuspended in Gulf Stream seawater containing 5-d-old *Artemia nauplii* (grown on *T. weissflogii* and *Isochrysis galbana*). Grazing was determined by cell counts, and the grazers and diatoms were filtered (230- $\mu\text{m}$  mesh followed by 0.2- $\mu\text{m}$  filtration) when diatom biomass was <10% of initial cell density. The filtrate was maintained at  $4^\circ\text{C}$  until uptake experiments with Fe-limited *T. weissflogii* were conducted. Two experiments were conducted; both had control and “10 $\times$ ” CuZnSOD treatments, while experiment 1 included a FZ (200  $\mu\text{mol L}^{-1}$ ) treatment and experiment 2 had a “killed cells” control in

which cells were frozen and thawed twice (a modification of the technique of Bidle and Azam [2001]) before filtration and resuspension into experimental medium. In experiments 1 and 2, the dissolved Fe concentrations were radiometrically determined to be 4.4 and 10.0  $\text{nmol L}^{-1}$  (most of the Fe was retained by the grazers and their fecal pellets).

In experiments conducted with EDTA-buffered medium, model ligands, or grazer-regenerated Fe, each observed rate of reduction or uptake was compared to the rate obtained by unamended cells by a one-tailed single classification analysis of variance test for equality of slopes (Sokal and Rohlf 1995; p. 469) with an experiment-wise Type I error rate of  $\alpha = 0.05$  determined by the Dunn Šidák method (Sokal and Rohlf 1995; p. 239).

## Results

*SOD effect on Fe(II) production by diatoms*—The addition of a strong Fe(II) complexing agent such as FZ has been used to demonstrate reduction of Fe(III) in phytoplankton cultures with high Fe concentrations (Anderson and Morel 1980; Allnut and Bonner 1987; Shaked et al. 2002). This phenomenon can also be observed at low Fe' using a technique employing radiotracers and  $\text{C}_{18}$  extraction (Shaked et al. 2004). Under these conditions, the addition of CuZnSOD (10 $\times$  concentration) consistently and significantly decreased the production of Fe(II) by both *T. weissflogii* and *T. pseudonana* (Fig. 1A,B; Table 1, experiments A–E, G–J). Reduction rates in the CuZnSOD treatments averaged  $45\% \pm 13\%$  and  $29\% \pm 22\%$  of the control rates for *T. weissflogii* and *T. pseudonana*, respectively (significantly different,  $p < 0.05$  in both cases), indicating that over half of the reduction of Fe(III) observed in these cultures might be caused by  $\text{O}_2^-$ . But the CuZn metallotype of SOD has been shown to catalyze the oxidation of aminothiols (primarily cysteine and cysteamine) in solution (Winterbourn et al. 2002), and the inhibitory effect of CuZnSOD on Fe(III) reduction could thus result indirectly from the oxidation of extracellular aminothiols rather than  $\text{O}_2^-$  dismutation. We ruled this possibility out by verifying that MnSOD inhibited Fe reduction by *T. pseudonana* to the same extent as CuZnSOD (Table 1, experiment J). We also verified that the effect of SOD was not due to some nonspecific interference resulting from the high protein concentration: in control treatments, rates of Fe(II) production in the presence of denatured CuZnSOD or bovine serum albumin (BSA) at a concentration equivalent to 10 $\times$  CuZnSOD were not significantly different from those obtained without protein additions (Table 1, experiments A,D). However, we found evidence of nonspecific effects at higher concentrations of BSA ( $\sim 6$  mg BSA  $\text{L}^{-1}$ , equivalent to “100 $\times$ ” CuZnSOD; Table 1, experiment B). We note also that the Fe(II) production time series experiments exhibited non-zero intercepts (i.e., Fig. 1A), corresponding, apparently, to a rapid formation of the  $\text{FeFeZ}_3$  complex. We did not investigate further this phenomenon that has been observed by others and ascribed to reaction with cell surface moieties (Jones et al. 1987).

*Fe(II) production by superoxide*—The reduction of Fe(III) to Fe(II) in an EDTA-buffered medium is a bit surprising

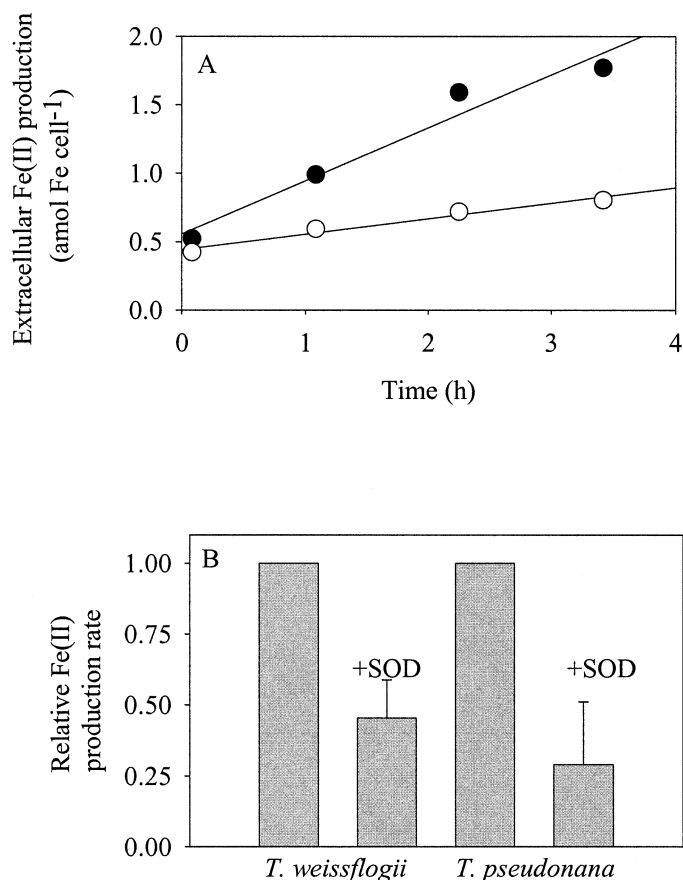


Fig. 1. SOD inhibition of Fe(II) production in diatoms. (A) Typical cellular Fe(II) production by *Thalassiosira weissflogii* (65,000 cells ml<sup>-1</sup>) in the absence (filled circles) and presence (open circles) of Cu,ZnSOD (10×). (B) The relative effects of CuZnSOD on Fe(II) production rates by cultures of *T. weissflogii* and *T. pseudonana*. For each experiment ( $n = 5$  for *T. weissflogii* and  $n = 4$  for *T. pseudonana*), cellular rates were normalized to those obtained in the absence of SOD. *T. weissflogii* exhibited an average Fe(II) production rate of  $1.11 (\pm 0.49) \times 10^{-18}$  mol Fe cell<sup>-1</sup> h<sup>-1</sup>; in the presence of SOD, this rate was 45% ( $\pm 13\%$  SE, shown) of the normalized rate. *T. pseudonana* exhibited an average Fe(II) production rate of  $8.9 (\pm 6.0) \times 10^{-20}$  mol Fe cell<sup>-1</sup> h<sup>-1</sup>; in the presence of SOD, this rate was 29% ( $\pm 22\%$  SE) of the normalized rate. Normalized to surface area, the Fe(II) production rates were  $2.4 \times 10^{-21}$  mol Fe(II)  $\mu\text{m}^{-2}$  h<sup>-1</sup> and  $1.6 \times 10^{-21}$  mol Fe(II)  $\mu\text{m}^{-2}$  h<sup>-1</sup> for *T. weissflogii* and *T. pseudonana*, respectively. Similarly, the SOD-labile Fe(II) production rates averaged  $1.1 \times 10^{-21}$  mol Fe(II)  $\mu\text{m}^{-2}$  h<sup>-1</sup> for both species.

since O<sub>2</sub><sup>-</sup> has been found to result in net oxidation rather than reduction of Fe bound to EDTA (McClune et al. 1977). We thus tested the effect of O<sub>2</sub><sup>-</sup> generated by the X-XO system on Fe(II) production in our EDTA-buffered medium in the absence of cells. We observed Fe(II) production at rates increasing with the concentration of XO and thus with the rate of O<sub>2</sub><sup>-</sup> production (Fig. 2). As determined by cytochrome *c* reduction assays, the rate of O<sub>2</sub><sup>-</sup> production by X-XO in our culture medium (about 570 nmol O<sub>2</sub><sup>-</sup> min<sup>-1</sup> U<sup>-1</sup> XO) falls between rates obtained at pH 7.0 and 10.0 in phosphate buffer solution (Fridovich 1970). The fact that O<sub>2</sub><sup>-</sup> is capable of reducing Fe in our FeEDTA-buffered medium and that

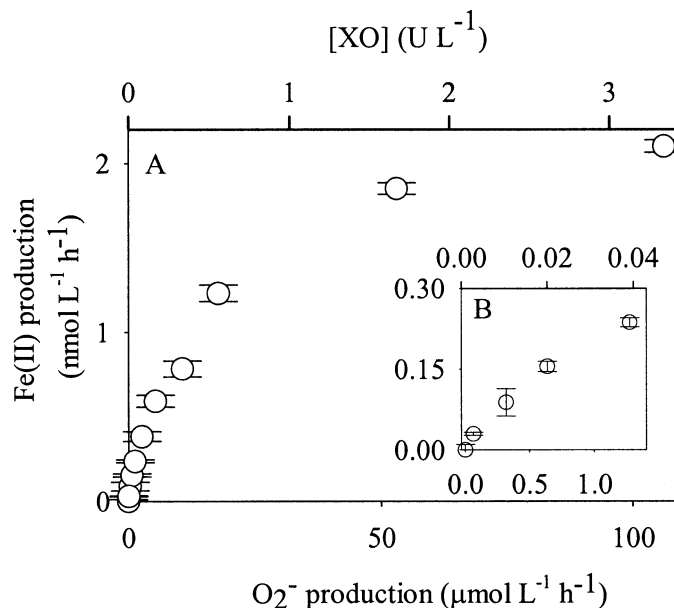


Fig. 2. (A) Superoxide-mediated Fe(II) production in culture medium in the absence of cells. Xanthine-xanthine oxidase (X-XO) was used to generate O<sub>2</sub><sup>-</sup> in solution. Fe(II) production generated from XO concentrations ranging from 0 to 3.3 U L<sup>-1</sup>. (B) Fe(II) production generated at low XO concentrations (0–40 mU L<sup>-1</sup>). The control treatment (where [XO] = 0 mU L<sup>-1</sup>) was conducted in duplicate, while single analyses were conducted for each XO concentration. The error bars represent the standard deviation of the duplicate observations (no XO) and the standard errors of the Fe(II) production rates (all XO additions). The rates of Fe(II) production were corrected for the apparent blank Fe(II) production in the presence of X only (average =  $7.0 \times 10^{-12}$  mol L<sup>-1</sup> h<sup>-1</sup>).

SOD partially inhibits Fe reduction in *T. weissflogii* and *T. pseudonana* cultures strongly indicates that O<sub>2</sub><sup>-</sup> is produced in the medium.

**Superoxide quantification**—To demonstrate and quantify O<sub>2</sub><sup>-</sup> production more directly, we used the superoxide-specific chemiluminescent probe MCLA in a flow injection system (see Materials and methods). When the cells were flushed with seawater in line with the flow injection system, a significant and relatively constant chemiluminescence signal was recorded (Fig. 3). Shortly after introducing Cu-ZnSOD to the flushing seawater, the chemiluminescence signal decreased to background levels. Addition of 60 μmol L<sup>-1</sup> DPI, a known inhibitor of transmembrane oxidoreductases and other NA(D)PH binding enzymes, caused a rapid decrease in O<sub>2</sub><sup>-</sup> production (Fig. 3), indicating that the O<sub>2</sub><sup>-</sup> we measured might be produced enzymatically, possibly by one or more transmembrane NAD(P)H oxidoreductases. The response to DPI addition was not as rapid as with SOD addition, which is consistent with the fact that SOD disproportionates O<sub>2</sub><sup>-</sup> in the medium while DPI must first enter the diatom cell and then prevent the transfer of electrons to O<sub>2</sub> before an effect can be measured. On the basis of the data from Fig. 3 and the calibration with O<sub>2</sub><sup>-</sup> standards (data not shown), we calculated an approximate O<sub>2</sub><sup>-</sup> production rate by *T. weissflogii* of  $8.4 (\pm 1.4) \times 10^{-16}$  mol cell<sup>-1</sup> h<sup>-1</sup> in our

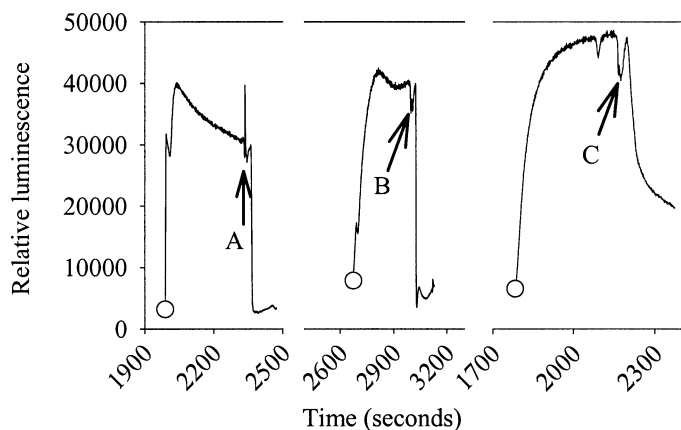


Fig. 3. Superoxide production by *T. weissflogii*. Cell-mediated MCLA chemiluminescence was measured in the presence and absence of SOD and DPI (see Materials and methods). Open circles indicate the point at which the cells (loaded on filters) were introduced to the sample stream. Arrows indicate when 50× CuZnSOD (A and B) or 60 μmol DPI L<sup>-1</sup> (C) were added to the flushing seawater.

flow injection system. For comparison, *Chattonella* strain 85, *Fibrocapsus japonica*, *Heterosigma akashiwo* and *Olithodiscus luteus* (four toxic marine phytoplankton) produce ~0.45–4 × 10<sup>-12</sup> mol O<sub>2</sub><sup>-</sup> cell<sup>-1</sup> h<sup>-1</sup> (Oda et al. 1997). Normalized to cell surface area, the rates of O<sub>2</sub><sup>-</sup> production by *T. weissflogii* and by these toxic species average 1.8 × 10<sup>-18</sup> (±3.0 × 10<sup>-19</sup>) and 4.2 × 10<sup>-16</sup> (±2.2 × 10<sup>-16</sup>) mol O<sub>2</sub><sup>-</sup> μm<sup>-2</sup> h<sup>-1</sup>, respectively.

**Role of superoxide on Fe uptake**—The experiments described above demonstrate both that O<sub>2</sub><sup>-</sup> is produced in the external milieu of the diatoms and that O<sub>2</sub><sup>-</sup> reduces Fe in an EDTA-buffered medium. Since we know that Fe uptake by diatoms is enhanced by photoreduction of Fe(III)EDTA (Anderson and Morel 1982; Sunda and Huntsman 1997), we might expect that O<sub>2</sub><sup>-</sup> reduction of Fe(III) in our culture medium should enhance Fe uptake. Surprisingly, even though we saw a consistent inhibition of Fe reduction by SOD in the medium, we observed no effect of SOD on iron uptake by *T. weissflogii* or *T. pseudonana* (Table 1; experiments F, G, J). We also observed no effect of enhanced O<sub>2</sub><sup>-</sup> production (by the addition of X-XO) on Fe uptake rates (Table 1; experiment F). So, somehow, the reduction of Fe(III) by O<sub>2</sub><sup>-</sup> does not increase the concentration of available Fe in our EDTA-buffered medium. To resolve this apparent paradox, we need to better understand how Fe(III) is reduced by O<sub>2</sub><sup>-</sup> in our medium; in particular, we need to know whether Fe(II)' is formed by reduction of the chelated or unchelated Fe (FeEDTA or Fe(III)').

**Source of Fe(II) production by superoxide**—To determine whether O<sub>2</sub><sup>-</sup> produces Fe(II)' from FeEDTA, Fe(III)', or a combination of both, O<sub>2</sub><sup>-</sup> was generated by the X-XO system, and Fe(II) production was measured at various concentrations of Fe(III)' by varying the EDTA concentration and maintaining a constant [Fe]. We measured decreasing Fe(II) production at increasing EDTA concentrations at rates that

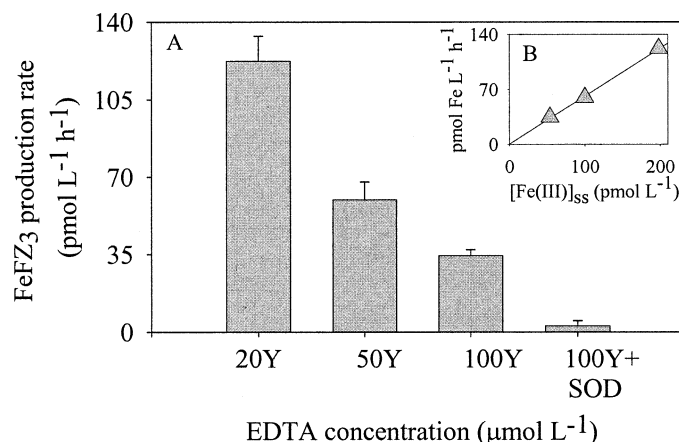


Fig. 4. (A) Superoxide-mediated Fe(II) production at varied concentrations of EDTA. [XO] = 2 mU L<sup>-1</sup>, [X] = 16 μmol L<sup>-1</sup>, and FeEDTA = 90 nmol L<sup>-1</sup>. EDTA was added from the Aquil EDTA-metal stock (minus Fe) to achieve final EDTA concentrations of 20, 50, and 100 μmol L<sup>-1</sup>, with a constant EDTA:metal ratio. (B) Steady-state Fe(II) concentrations were calculated at each EDTA concentration by using the initial Fe(III)' concentrations, the measured rates of FeFZ<sub>3</sub> formation, and the known rates of FeEDTA dissociation and complex formation.

were directly proportional to the calculated [Fe(III)'] (Fig. 4B). Thus, Fe(II)' is formed from reduction of Fe(III)' but not FeEDTA. This finding resolves the apparent discrepancy between our observation of Fe(III) reduction by O<sub>2</sub><sup>-</sup> in our EDTA-buffered medium and in the report by McClune et al. (1977) that O<sub>2</sub><sup>-</sup> does not effectively reduce Fe(III)EDTA. The reduction of Fe(III)', and not FeEDTA, also explains that the rate of Fe(II)' formation should reach a maximum controlled by the rate of dissociation of FeEDTA, as observed in Fig. 2. More importantly, it explains the apparent paradox that formation of Fe(II) by O<sub>2</sub><sup>-</sup> in our culture medium should have no effect on Fe uptake by the diatoms: the rapid reoxidation of Fe(II) to Fe(III)' effectively leaves the speciation of Fe in the culture medium unaffected. Indeed, we can calculate the steady-state Fe(III)' and Fe(II)' concentrations in the medium using the measured SOD-dependent rate of Fe(II) production by *T. weissflogii* (R = 3.6 × 10<sup>-11</sup> mol L<sup>-1</sup> h<sup>-1</sup>; with a cell density of 6.5 × 10<sup>7</sup> cells L<sup>-1</sup>, and average cell-specific rate of 5.5 × 10<sup>-19</sup> mol Fe cell<sup>-1</sup> h<sup>-1</sup>; Table 1), the effective first-order rate constant for oxidation of Fe(II) by O<sub>2</sub> in oxygenated seawater (k<sub>ox</sub><sup>O<sub>2</sub></sup> = 27.2 h<sup>-1</sup>; Lin and Kester 1992, ignoring the additional oxidation by O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>), the dissociation rate of Fe(III)EDTA (k<sub>d</sub>FeY = [3.6 × 10<sup>-3</sup> h<sup>-1</sup>] × [9 × 10<sup>-8</sup> mol L<sup>-1</sup>] = 3.2 × 10<sup>-10</sup> mol L<sup>-1</sup> h<sup>-1</sup>), and the complex formation rate for either Fe(II) or Fe(III) with EDTA after Hudson and Morel (1990) (k<sub>f</sub>Y' = [7.2 × 10<sup>4</sup> mol L<sup>-1</sup> h<sup>-1</sup>] × [1 × 10<sup>-4</sup> mol L<sup>-1</sup>] = 7.2 h<sup>-1</sup>):

$$[\text{Fe(III)'}]_{\text{ss}} = \frac{k_d \text{FeY} + k_{\text{ox}}^{\text{O}_2} \text{Fe(II)'} - R}{k_f Y'} \quad (1)$$

$$[\text{Fe(II)'}]_{\text{ss}} = \frac{R}{k_{\text{ox}}^{\text{O}_2} + k_f Y'} \quad (2)$$

This results in steady-state concentrations of Fe(II)' and

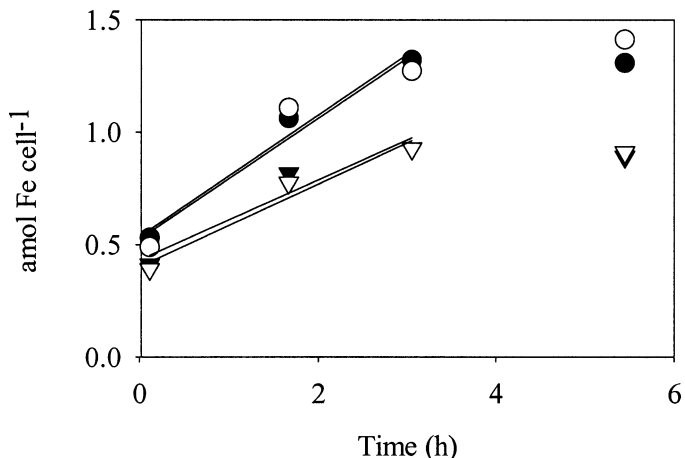


Fig. 5. Effect of SOD on uptake of ferrihydrite-derived Fe by *T. weissflogii*. Radiolabeled ferrihydrite was precipitated onto the surfaces of diatoms as described in the Materials and methods section. Iron uptake was measured in diatoms with “fresh” ferrihydrite with and without SOD (open and closed circles) and baked ferrihydrite with and without SOD (open and closed triangles). At the beginning of the experiment, cell-adsorbed ferrihydrite concentrations were  $9.1 \text{ nmol L}^{-1}$  and  $9.7 \text{ nmol L}^{-1}$  (baked and fresh colloids, respectively), corresponding to  $1.34 \times 10^{-16}$  and  $1.44 \times 10^{-16}$  mol Fe cell $^{-1}$ , respectively. The mean cell density in the four treatments was  $67,700 \text{ cells ml}^{-1}$  (SD = 2,100).

Fe(III)' = 1 and  $44 \text{ pmol L}^{-1}$ , compared to values of 0 and  $45 \text{ pmol L}^{-1}$  in the absence of  $\text{O}_2^-$ -mediated Fe(III) reduction, thus demonstrating that the concentration of unchelated Fe is negligibly affected by the  $\text{O}_2^-$  in the medium.

*Effect of superoxide on Fe uptake from ferrihydrite, porphyrins, and regenerated Fe*—The buffering of Fe(III)' by EDTA in culture media is convenient but, presumably, not representative of the conditions in nature. We thus wondered if  $\text{O}_2^-$  production in the external milieu might play a role in Fe acquisition from other substrates, such as colloidal ferrihydrite, porphyrins, and Fe regenerated from metazoan grazers.

We tested the effect of  $\text{O}_2^-$  on Fe uptake in the presence of colloidal Fe using two preparations of ferrihydrite: one freshly precipitated from  $\text{FeCl}_3$  added to seawater and another heated for 5 min at  $60^\circ\text{C}$  (Wells et al. 1983). As expected, the initial uptake rate was faster in the medium containing the fresh precipitate ( $\sim 2.7 \times 10^{-19}$  vs.  $1.8 \times 10^{-19}$  mol cell $^{-1} \text{ h}^{-1}$ ; Fig. 5) but the removal of  $\text{O}_2^-$  by SOD had no measurable effect in either medium. This result is consistent with the notion that Fe uptake from colloidal Fe depends on the dissolution of the ferrihydrite and is controlled by its kinetics (Rich and Morel 1990). As is the case for the FeEDTA-buffered system,  $\text{O}_2^-$  should have no measurable effect on the Fe concentration and speciation in the medium as long as it reduces only Fe(III)' and not the colloid itself.

Porphyrin-bound Fe compounds display a range of redox potentials that make it potentially suitable for reduction by  $\text{O}_2^-$  (for example,  $E_{1/2}$   $-30 \text{ mV}$  to  $+283 \text{ mV}$  for some Fe-porphyrins at pH 7.8 and  $I = 0.15 \text{ mol L}^{-1}$  [Batinic-Haberle et al. 1999]), given the redox potential for the  $\text{O}_2^-/\text{O}_2$  couple

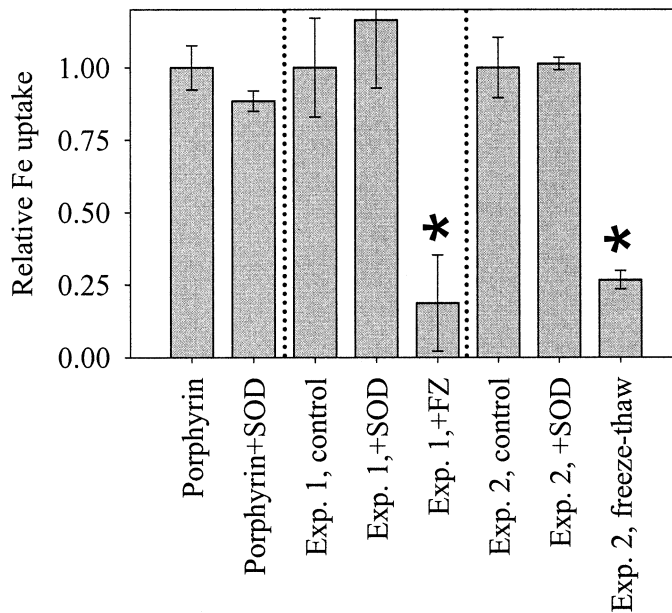


Fig. 6. Fe uptake in *T. weissflogii* of Fe-deuteroporphyrin and regenerated products of grazing from three experiments. Results of each experiment are normalized to control rates (no SOD), and dashed lines separate each experiment. The concentration of Fe-deuteroporphyrin =  $25 \text{ nmol L}^{-1}$ , and the control uptake rate =  $4.8 \times 10^{-18}$  mol cell $^{-1} \text{ h}^{-1}$ . In experiments 1 and 2, the dissolved Fe concentrations (released from previously grazed radiolabeled diatoms) were  $4.4$  and  $10.0 \text{ nmol L}^{-1}$ ; the control uptake rates in each were  $6.9 \times 10^{-20}$  and  $5.2 \times 10^{-19}$  mol cell $^{-1} \text{ h}^{-1}$ . For each experiment, uptake rates that significantly differed from controls (no SOD added) are indicated by asterisks ( $F_s > F_{\alpha', [u, v]}$ , where  $\alpha = 0.05$ ).

(at  $1 \text{ mol L}^{-1} \text{ O}_2$  in aqueous solution,  $E = -160 \text{ mV}$ ). Nonetheless, we observed no measurable effect of SOD on the rate of Fe uptake from deuteroporphyrin (Fig. 6). Uptake of grazer-regenerated Fe, which presumably also contains porphyrins as well as other cellular Fe compounds, (and, perhaps, some extracellular inorganic colloids) was also not affected by SOD (Fig. 6) in two separate experiments. It is interesting to note that addition of  $200 \text{ } \mu\text{mol FZ L}^{-1}$  inhibited uptake by  $\sim 85\%$ , indicating that Fe reduction is necessary for uptake of Fe from the diverse mixture of compounds released by grazers. This is consistent with the empirical observations and model predictions of Shaked et al. (2005).

## Discussion

Our first hypothesis, that extracellular  $\text{O}_2^-$  is produced in diatom cultures, is supported by all our experimental results. Of particular note is the consistency of our data on the rate of  $\text{O}_2^-$  production measured by chemiluminescence and the rate of SOD-dependent Fe(III) reduction in the culture medium. The estimated  $\text{O}_2^-$  production rate in *T. weissflogii* cultures is  $\sim 8.4 \times 10^{-16}$  mol  $\text{O}_2^- \text{ cell}^{-1} \text{ h}^{-1}$ , which should yield an Fe(III) reduction rate of about  $4.4 \times 10^{-19}$  mol Fe cell $^{-1} \text{ h}^{-1}$  (based on interpolation of the data in Fig. 2). This is similar to the average value of SOD-labile  $\text{FeFZ}_3$  production in *T. weissflogii* cultures ( $5.2 \pm 1.4 \times 10^{-19}$  mol Fe

cell<sup>-1</sup> h<sup>-1</sup>), as calculated by the difference between controls and “10×” SOD treatments in Table 1. Thus, O<sub>2</sub><sup>-</sup> production is sufficient to effect a redox cycling of Fe in EDTA-buffered medium.

There are two possible mechanisms of O<sub>2</sub><sup>-</sup> production to explain our results: (i) the diatoms may reduce O<sub>2</sub> directly at the cell surface via reductase enzymes, or (ii) a metabolite released into the medium may reduce O<sub>2</sub> to O<sub>2</sub><sup>-</sup>. In favor of the first (direct) mechanism is the fact that the chemiluminescent detection of O<sub>2</sub><sup>-</sup> decreased upon addition of the transmembrane oxidoreductase inhibitor DPI. In addition, there are at least three genes present in the *T. pseudonana* genome (Armbrust et al. 2004) that encode for the critical motifs present in the NADPH-oxidoreductase protein family (Shatwell et al. 1996). Although both ferric reductases and respiratory burst oxidases belong to this family, we have no direct evidence that the putative proteins in *T. pseudonana* are localized at the plasma membrane (vs. intracellular vacuoles), nor can we identify the specific substrate(s) of these proteins. In support of the second (indirect) mechanism for O<sub>2</sub><sup>-</sup> production are the recent measurements of extracellular glutathione (GSH) release from healthy cells of *T. weissflogii* (Tang et al. 2005). The univalent oxidation of any such released GSH (or similar reductants) by O<sub>2</sub> will produce O<sub>2</sub><sup>-</sup> (Misra 1974), which could then proceed to reduce Fe(III)' in our medium. While the direct enzymatic production of O<sub>2</sub><sup>-</sup> by surface enzymes seems more probable to us at this point, more work is needed to ascertain the mechanism(s) of superoxide production in diatom medium.

We also hypothesized that O<sub>2</sub><sup>-</sup> production may increase the bioavailability of Fe to diatoms by releasing Fe(III) from various complexes and colloids. This second hypothesis is not supported by our results. Several studies with marine diatoms indicate that reduction may be a necessary step for Fe uptake from various chelates (Anderson and Morel 1980; Allnut and Bonner 1987; Maldonado and Price 2001), and we have also found this to be the case when Fe is buffered by EDTA (Shaked et al. 2005). Thus, the reduction of Fe by O<sub>2</sub><sup>-</sup> might have been expected to increase the available Fe by obviating the need for a cell surface reductive process. But, as shown above, in an EDTA-buffered system it is only unchelated Fe, at equilibrium with the chelate, that is reduced by O<sub>2</sub><sup>-</sup>. In oxygenated seawater at pH 8, the reoxidation of Fe(II) by O<sub>2</sub> is so rapid that O<sub>2</sub><sup>-</sup> has a negligible effect on Fe speciation in the medium. In the presence of 100 μmol FZ L<sup>-1</sup>, we calculate that about 95% of Fe(II) is complexed to FZ rather than reoxidized. The formation of the FeFZ<sub>3</sub> complex thus provides an accurate measure of bulk-phase Fe(II) production but says nothing of the availability of Fe(II) for the cell.

We also observed no effect of SOD on Fe uptake by diatoms from ferrihydrite or deuteroporphyrin. The first observation indicates that O<sub>2</sub><sup>-</sup> is not an effective reductant of ferrihydrite, as inferred by earlier studies (Kuma and Matsunaga 1995; Voelker and Sedlak 1995). In a ferrihydrite suspension, as in an EDTA-buffered system, the effect of O<sub>2</sub><sup>-</sup> is then only to promote the reduction of Fe(III)' to Fe(II)', which is rapidly reoxidized back to Fe(III)' by O<sub>2</sub>. In contrast, in the case of deuteroporphyrin, we expect that Fe(III) in the porphyrin should be effectively reduced by

O<sub>2</sub><sup>-</sup>. In this case, the lack of effect of O<sub>2</sub><sup>-</sup> on Fe uptake is most likely explained by the fact that the Fe(II)-porphyrin complex that is produced is not particularly labile and that Fe can be reduced and oxidized within the heme without being released to solution. In all these cases, the effect of O<sub>2</sub><sup>-</sup> on the speciation of Fe in the medium is to promote a futile redox cycling between Fe(III) and Fe(II) without a net increase in the unchelated Fe concentration or in the bioavailability of Fe in the medium. This is in contrast with the effect of light on Fe(III)EDTA, which results in a charge transfer reaction with simultaneous reduction of Fe(III) and oxidation of EDTA. Fe(II) is thus released into the medium, increasing Fe' and the rate of Fe uptake by phytoplankton. In none of our experiments did we find evidence of increased Fe availability as a result of the reaction of Fe(III) species with O<sub>2</sub><sup>-</sup>. We cannot rule out, of course, that such increased bioavailability of Fe may be observed under some particular conditions.

Our results on the fate of Fe(II) produced in the bulk medium, via O<sub>2</sub><sup>-</sup>, have implications for the functioning of the cell surface Fe reductases of diatoms. Since Fe reduction appears to be necessary for uptake of all Fe(III) substrates (Shaked et al. 2005), at least one of the putative Fe reductases (Armbrust et al. 2004) is able to reduce Fe(III) species at the cell surface, but the Fe(II) is apparently not released into the medium (unless a strong Fe(II) complexing agent is added to the medium to compete with the transporter for the Fe(II) at the cell surface). If Fe(II) were to be released into the medium by cell surface ferric reductases, it would be rapidly reoxidized, resulting in a futile redox cycle, as observed when Fe(II) is generated in the bulk medium by O<sub>2</sub><sup>-</sup>. Thus, the cell surface reductases must participate in an effective ligand exchange process whereby the Fe(II) produced by the cell surface reductases is transferred directly to the oxidase/permease complex.

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Received: 22 October 2004

Accepted: 15 February 2005

Amended: 22 March 2005