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Localization and Role of Manganese Superoxide Dismutase in a Marine Diatom[1]

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ABSTRACT

Superoxide dismutase (SOD) catalyzes the transformation of superoxide to molecular oxygen and hydrogen peroxide. Of the four known SOD isoforms, distinguished by their metal cofactor (Fe, Mn, Cu\Zn, Ni), MnSOD is the dominant form in the diatom *Thalassiosira pseudonana*. We cloned the MnSOD gene, *sod*A, using the expression vector pBAD, overexpressed the product in *Escherichia coli* and purified the mature protein (TpMnSOD). This recombinant enzyme was used to generate a polyclonal antibody in rabbit that recognizes MnSOD in *T. pseudonana*. Based on quantitative immunoblots, we calculate that *in vivo* concentrations of TpMnSOD are approximately 0.9 amols per cell using the recombinant protein as a standard. Immunogold staining indicates that TpMnSOD is localized in the chloroplasts, which is in contrast to most other eukaryotic algae (including chlorophytes and embryophytes) where MnSOD is localized exclusively in mitochondria. Based on the photosynthetic Mn complex in photosystem II, cellular Mn budgets cannot account for 50-80% of measured Mn within diatom cells. Our results reveal that chloroplastic MnSOD accounts for 10-20% of cellular Mn, depending on incident light intensity and cellular growth rate. Indeed, our analysis indicates that TpMnSOD accounts for 1.4 (±0.2)% of the total protein in the cell. The TpMnSOD has a rapid turnover rate with an apparent half-life of 6-8 h when grown under continuous light. TpMnSOD concentrations increase relative to chlorophyll, with an increase in incident light intensity in order to minimize photosynthetic oxidative stress. The employment of a Mn-based SOD, linked to photosynthetic stress in *T. pseudonana*, may contribute to the continued success of diatoms in the low-Fe regions of the modern ocean.

INTRODUCTION

All aerobic organisms produce intracellular and extracellular reactive oxygen species (ROS) as metabolic byproducts (Haliwell 1982, Asada 1999, Apel and Hirt 2005). Photoautotrophs also produce ROS through photosynthesis (Falkowski and Raven, 1997; Anderson et al., 1999; Wolfe-Simon et al., 2005). The ROS byproducts include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$) (Haliwell 1982). In addition to being highly reactive, $O_2^{\cdot -}$ is particularly destructive because it cannot diffuse across cell membranes, and therefore, must be destroyed at the site of production. Superoxide dismutases (SODs) are a polyphyletic family of enzymes that protect cells from $O_2^{\cdot -}$. SODs come in four isoforms, recognized by their metal center cofactors (Fe, Mn, Cu\Zn, and Ni), and catalyze the destruction of $O_2^{\cdot -}$ to H_2O_2 and O_2. This key antioxidant has been well studied in many eukaryotic systems, including metazoa and plants (Bowler et al., 1992; Scandalios, 1993; Fridovich, 1995; Raychaudhuri and Deng, 2000; Zelko et al., 2002). However, few studies on the intracellular regulation of SOD in diatoms are available, which is unfortunate as these algae dominate the flux of carbon in the contemporary ocean (Falkowski et al., 2004b).

Diatoms appear to rely primarily on the manganese form of SOD (MnSOD) (Peers and Price, 2004); therefore, understanding the regulation of MnSOD in diatoms is important, as this enzyme must be critical to the cells' ability to cope with oxidative stress. The regulation and subcellular localization of MnSOD varies significantly among algal taxa (Wolfe-Simon et al., 2005). In cyanobacteria, MnSOD is found in the periplasm and is associated with the thylakoid membranes (Herbert et al., 1992; Chen et al., 2001; Li et al., 2002). In contrast, MnSOD is found in the mitochondria of embryophytes, chlorophytes, and dinoflagellates (Kliebenstein et al., 1998; Kitayama et al., 1999; Wu et al., 1999; Okamoto et al., 2001; Okomoto et al., 2001; Fink and Scandalios, 2002). Although some information has been shown regarding SOD in diatoms (Huang et al., 2005; Ken et al., 2005), no information on the subcellular localization of MnSOD and the associated kinetics in diatoms is available.

Given our lack of understanding of MnSOD in diatoms, despite their global significance, we examined the expression and preliminary regulation of MnSOD in the bloom-forming diatom *Thalassiosira pseudonana* CCMP1335 (Ziemann et al., 1991;

Levasseur et al., 1992; Zigone et al., 1995; Cabecadas et al., 1999). Our results indicate that MnSOD is localized in the chloroplast and has a rapid turnover rate mediated by incident light levels which is closely coupled to photosynthetic activity.

RESULTS

Native Molecular Mass and Western Analyses

Western blots from denaturing polyacrylamide gel electrophoresis (PAGE) of crude cell extracts (Fig. 1) probed with anti-TPMnSOD reveal a major band of approximately 23 kDa which corresponds well with the predicted subunit molecular mass of 22.8 kDa for MnSOD based on sequence analysis. The anti-TpMnSOD cross reacted with other diatom species, and weakly recognized MnSOD in several dinoflagellates (Table I). Interestingly, there was no anti-TpMnSOD cross-reactivity with two other heterokonts: *Nannochloropsis oculata* and *Heterosigma akashiwo*. No reactivity was observed in the chlorophytes, cyanobacteria, prymnesiophytes, cryptophytes, and rhodophytes.

MnSOD and the Cellular Manganese Budget in Diatoms

Based on quantitative immuno-analyses of MnSOD in nutrient replete, exponentially growing cultures of *T. pseudonana*, this marine diatom maintains 0.91 amol MnSOD per cell when grown at moderate light levels. This quantity of MnSOD accounts for 1.4 (±0.2)% of the total cellular protein (Table II). This pool turns over rapidly; TpMnSOD is virtually undetectable after 16 h under continuous light when protein synthesis is blocked (Fig. 2), corresponding to a 5-8 h half-life. The turnover was mediated by light as the protein was detectable even after 27 h when cells were kept in darkness, regardless of whether protein synthesis was inhibited.

The total Mn associated with TpMnSOD ranges between 10-20% of the total cellular Mn (Fig. 3, see legend for calculation details). Raven (1990) estimated that between 2 to 4 μmol Mn mol C^{-1} are needed to support the Mn requirement of the PSII Mn-complex in *T. pseudonana* which would account for approximately 60% of total Mn within a cell (Sunda and Huntsman, 1986; Raven, 1990; Sunda and Huntsman, 1998). Based on immuno-quantitative analyses, MnSOD accounts for another 18% of the Mn budget. Thus, ~80% of the total Mn budget is associated with MnSOD and PSII.

Immunolocalization of MnSOD in Plastids

Immunogold labeling measurements suggest that MnSOD is mainly confined to the chloroplast (Fig. 4). The immunogold label is predominantly associated with thylakoid membranes and the pyrenoid. It is not associated with the cytosol or the mitochondria. Since the chloroplast localized MnSOD is regulated by the nuclear-encoded *sod*A gene, plastid/endoplasmic reticulum transit peptides must be present, but they have not yet been identified.

Impact of light on TpMnSOD expression

When acclimated to a range of irradiance levels (25, 50, 120, 350, 800 µmol m^{-2} s^{-1}), *T. pseudonana* cells show a 73% increase in growth rate (Fig. 5). Total chlorophyll *a* $cell^{-1}$ is constant at low light levels (25 to 50 µmol m^{-2} s^{-1}), but decreases by 63% as the incident light intensity increases from 50 to 800 µmol m^{-2} s^{-1}. (Note: Cells were kept optically thin in semi-continuous batch cultures to avoid self shading.) Over this range of irradiances, the amount of TpMnSOD per unit chlorophyll increased by 60% (Fig. 5) reflecting changes in the chlorophyll concentration not MnSOD. Although the chlorophyll *a*-normalized MnSOD content of *T. pseudonana* increased with increasing light, the amount of MnSOD per total cellular protein was constant (data not shown). Thus the demand for MnSOD per cell in these cells appears to be constant over these light levels despite declining chlorophyll. A similar relationship between light intensity, SOD, and reduced cellular chlorophyll was also seen for the chloroplastic CuZnSOD in bean and other higher plants (Gonzalez et al., 1998 and references therein).

To further examine the relationship between light and MnSOD, the time course of TpMnSOD expression was followed over 30 h in cells acclimated to a 12/12 h photoperiod. TpMnSOD expression did not vary significantly over the photoperiod when grown at 120 µmol m^{-2} s^{-1} incident light (control), but increased by 40% within 24 h and after one dark period when transferred to high light (>800 µmol m^{-2} s^{-1}) (Fig. 6A-B). The maximum photosynthetic quantum yield (Fv/Fm, Kolber et al., 1998) initially decreased by 50% under high light, but recovered and exceeded the control within 30 h (Fig. 6A-B). After a period of recovery (the dark cycle) to reorganize their metabolic profile, the cells then effectively cope with the high light stress with increased MnSOD expression. The increase in MnSOD expression per unit protein in the high light treatment (12:12 L:D) is significant ($p= 0.0007$) and is not exhibited by cells exposed to continuous high light. MnSOD expression, normalized to cell protein doubled after transfer from culture grown in continuous light to a 12:12 light:dark cycle (data not shown). Thus, continuous light apparently results in greater oxidative stress in diatoms than does a diel light cycle.

DISCUSSION

Our results clearly indicate that in *Thalassiosira pseudonana* MnSOD is localized in the chloroplasts. This subcellular location is in contrast with all other cellular MnSOD distributions in eukaryotic photoautotrophs, where MnSOD is found exclusively in the mitochondria (Grace, 1990; Moller, 2001; del Rio et al., 2003). The presence of MnSOD in the chloroplast results in cells having a high cellular Mn requirement, given the substantial need for Mn of the photosynthetic machinery. The localization of this nuclear encoded gene in a secondary symbiont presumably facilitates the rapid destruction of SOD, which is inevitably photochemically generated from the reaction centers in both photosystems. For example, the D1 protein (PsbA) has a turnover rate of approximately 30 minutes, one of the fastest turnover protein rates on Earth (Kim et al., 1993; Sundby et al., 1993; Andersson and Aro, 1997; Neidhardt et al., 1998). This high turnover is due, in large part, to the production of radical oxygen on the donor side of photosystem II (PSII) (Mattoo et al., 1984). Chloroplast specific SODs influence the D1 protein turnover due to their role in catalyzing the destruction of ROS in the chloroplast (Barber and Andersson, 1992; Aro et al., 1993; Andersson and Aro, 1997). If diatoms use MnSOD to suppress oxidative stress associated with photosynthesis in the chloroplast, we would expect diatoms to have higher Mn requirements than other classes of phytoplankton. Indeed, the measured cellular Mn quota of diatoms is significantly higher than that reported for other eukaryotic algae (Raven, 1990; Raven et al., 1999; Ho et al., 2003; Quigg et al., 2003).

Given the potentially large Mn requirement associated with photosynthesis, a great deal of effort has been focused on determining the cellular Mn budget in marine phytoplankton. Current cellular Mn budget estimates for diatoms have been based solely on the Mn associated with PSII (Raven, 1990). These budgets significantly underestimate measured cellular Mn concentrations (Sunda and Huntsman, 1986; Sunda and Huntsman, 1998; Peers and Price, 2004); however, including MnSOD (10-20% of total cellular Mn), up to 80% of the total cellular Mn can be accounted for, all of which is in the chloroplasts (Sunda and Huntsman, 1986; Raven, 1990; Sunda and Huntsman, 1998; Peers and Price, 2004).

Manganese does not appear to be bio-limiting in the oceans. Concentration profiles from numerous ocean basins show that Mn is often at biologically accessible concentrations while Fe is typically undetectable in surface waters (Li, 1991; Shiller, 1997; Nozaki et al., 1998; Whitfield, 2001). Measured values for Mn in the Atlantic basin range from about 25 $\mathrm{nmol\,kg^{-1}}$ in coastal regions to between 2 to 5 $\mathrm{nmol\,kg^{-1}}$ at open ocean stations (Shiller, 1997). Using average values of chlorophyll as a proxy for biomass: 5 $\mu\mathrm{g\,kg^{-1}}$ and 1 $\mu\mathrm{g\,kg^{-1}}$ for coastal and oceanic regions, respectively (Falkowski and Raven, 1997), and an average of 77 μg MnSOD mg chl a^{-1} (based on our measurements, see fig. 5), we calculated that oceanic Mn concentrations support more than 1000 turnovers of Mn in diatoms assuming growth rate between 1 to 2 $\mathrm{d^{-1}}$. Mn could thus serve as a possible metal replacement for iron and other bio-limiting metals in marine algae (Whitfield, 2001; Peers and Price, 2004).

The high Mn requirement of diatoms is significant to the ecology of these eukaryotic algae, as the role of trace metals has been shown to structure oceanic phytoplankton productivity and community composition in many regions (Saito et al., 2003; Coale et al., 2004). A major focus of research has been on Fe, which limits productivity and is only present in sub nM concentrations in most of the world's oceans (Boyd et al., 2000; Coale et al., 2004). Therefore, it is not surprising that some photoautotrophs have evolved mechanisms to compensate for low Fe availability. For example, diatoms can use flavodoxin, instead of the Fe-requiring ferredoxin, under low Fe conditions to support electron transport in PSI (LaRoche et al., 1993; McKay et al., 1999). Similarly, some cyanobacteria and chlorophytes substitute the Cu-containing plastocyanin for the Fe-heme cytochrome c_6 in PSI to transfer electrons between the cytochrome b_6f complex and P700$^+$ (Quinn and Merchant, 1999). This strategy appears to have been selected on substituting a limiting metal in a pathway with a more available and accessible metal in the same biochemical role.

This strategic biochemical substitution suggests that phytoplankton living in chronically Fe-limited waters may gain a competitive advantage if they can use alternative metals. Cyanobacteria from oligotrophic areas contain either NiSOD alone or both Ni and MnSOD instead of FeSOD found in freshwater species (Partensky et al., 1999; Palenik et al., 2003; Wolfe-Simon et al., 2005). Thus, two of the most successful groups of marine phytoplankton (diatoms and cyanobacteria, Falkowski et al., 2004a; 2004b) use non-Fe SODs to cope with oxidative stress in the Fe-poor regions of the modern ocean. Modern chlorophytes, including embryophytes, do not use Fe enzyme replacements. Therefore, it is not surprising that these taxa are not dominant in the oceans and are found primarily in terrestrial, freshwater, and estuarine systems that have abundant Fe concentrations (Sterner et al., 2004, and others). Thus, utilization of MnSODs may be one more mechanism underlying the dominance of red alga taxa over the last 275 Ma (Falkowski et al., 2004a; 2004b).

The ancient aquatic ecosystem is thought to have been chemically reduced, which would have made iron abundantly available to evolving organisms (Canfield, 1998; Brocks et al., 1999; Anbar and Knoll, 2002). As oxygen increased and oxidized most of the Fe to an insoluble oxide form, organisms were forced to evolve alternative options for biochemical pathways. Fe is still biochemically utilized for many electron transfer reactions.   However, at protein active sites where Mn could substitute for Fe with few genetic mutations Mn was often appropriated. This is especially true for electron transfer reactions involving O_2.

Chlorophytes (as well as some embryophytes) typically utilize FeSOD isoforms in the chloroplast (Sakurai et al., 1993; Chen et al., 1996; Kitayama et al., 1999). Consequently, the use of MnSOD in the chloroplast should lower a cell's iron demand because there is less FeSOD in use. These proteomic differences are reflected in the metal quotas of various marine phytoplankton taxa as diatoms have significantly lower Fe requirements than chlorophytes (Ho et al., 2003; Quigg et al., 2003). Furthermore, this biochemical difference may reflect the environments under which the diverse photosynthetic taxa evolved (Williams, 1981). Thus, the nutritional difference of Mn between chlorophytes and diatoms may contribute to the success of the diatoms in the low Fe modern marine environment (Falkowski et al., 2004a).

MATERIALS AND METHODS

Organisms, culture conditions, and standard protocols

Axenic cultures of *Thalassiosira pseudonana* CCMP1335 cells were used for all manipulations, including nucleic acid isolation and physiological studies. Cells were maintained in F/2+Si medium (Guillard and Ryther, 1962; Guillard, 1975) at a salinity of 35 practical salinity units (psu) and 20ºC with aeration under fluorescent cool white lamps with an incident light intensity of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ unless otherwise stated. Chlorophyll *a* was analyzed using standard 90% acetone extractions from glass fiber filtered culture (Jeffrey and Humphrey, 1975) measured on an spectrophotometer (Agilent 8453E, Agilent Technologies Inc.). Variable fluorescence (Fv/Fm) was acquired using a fast repetition rate flourometer (Kolber et al., 1998). Total carbon (inorganic and organic, subset of samples acidified to remove inorganic carbon) and nitrogen content of cultures were measured with a Carlo Erba NA-1500 elemental analyzer.

Cloning and Purification of Recombinant TpMnSOD

Total nucleic acids from mid-log growth *Thalassiosira pseudonana* cells were extracted and treated with DNA-free (Ambion, Inc. Austin, TX, catalog no. 1906) to remove DNA. First strand cDNA was synthesized with total RNA using M-MLV Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA, catalog no. 28025-013). The cDNA was then used as a template for PCR to amplify *sod*A with the specific primers 5’ATGAAAATCCATCATGATAAGCAT3’ and 5’TCCTCGCACGGGGACTCCTG3’. The full copy of the gene was then cloned into the pBAD vector (Invitrogen Corp., Carlsbad, CA, catalog no. K4300-40) and transformed into *Escherichia coli* for overexpression. The expression of the protein was controlled by varying the concentration of arabinose to achieve ideal expressed product. The vector contains a poly his-tag as well as a V5 epitope region. Thus, the recombinant protein was purified using Ni-NTA resin (Qiagen, Valencia, CA, catalog nos. 30230 and 30410) using both gravity chromatography and FPLC.

Antibody Production Against TpMnSOD

For the initial immunization, equal volumes of recombinant TpMnSOD protein in a 2mg/ml concentration and Freunds Complete adjuvant were emulsified using a micro-emulsifying needle. 0.8 ml of the emulsified protein and adjuvant were injected subcutaneously into two New Zealand White rabbits in four sites (max 0.2 ml per site). A subsequent injection was given 30 days later and was prepared using equal volumes of the provided antigen in a 1mg/ml concentration and Freund's incomplete adjuvant. The emulsified protein and adjuvant were injected subcutaneously, with a maximum of 0.2 ml/site. Subsequent injections were given at 30-35 day intervals. Blood draws from the central ear artery were performed between 10-20 days after each subsequent injection. The maximum blood withdrawn did not exceed the standard recommendation of blood amount withdrawn of 15% of total blood volume, or 1% of body weight.

Immunoblot Analyses

Protein was extracted from cell pellets in 2% SDS, 0.05M sodium carbonate, 7.5% glycerol, 0.025% bromothymol blue, 5mM PMSF, and 0.1M DTT. Sample protein concentration was quantified using either the bicinchoninic acid method (Pierce Biotechnology, Inc. Rockford, IL, cat no. 23227) or a fluorescent method (Invitrogen Corp., Eugene, OR, cat. no. R33200). Samples were then run on either 12, 15, or 18% (w/v) polyacrylamide gels and then blotted onto polyvinylidene fluoride (PVDF) membrane (Towbin et al., 1979). The blots were then probed with anti-TpMnSOD, the antibody raised against the recombinant MnSOD in *Thalassiosira pseudonana*. An HRP conjugated secondary antibody (BioRad Laboratories, Inc. Hercules, CA, catalog no. 172-1019) was used according to instructions and the blots were visualized with a chemiluminescent substrate system on film (Pierce Biotechnology, Inc. Rockford, IL catalog no. 34080). For quantitative immunoblot, known concentrations of both cells (total number) and protein (total ug) were run on 3 separate gels and then compared to unknown samples using densitometry.

Immunogold Staining

After fixation for 3 h in a modified EM Fixative (3% sodium chloride, 0.1M sodium cacodylate, 2.5% Glutaraldehyde, pH 7.4), cell pellets were rinsed three times in

Eppendorf tubes (2 x 15 min and 1x overnight) in 3% sodium chloride, 0.1M sodium cacodylate, pH 7.4 (Cells for TEM imaging only were also post-fixed for 2 h in 1% buffered osmium tetroxide). After the washes the cells were then dehydrated through a graded series of Ethanol washes, starting with 50% ethanol to 100% ethanol. The pellets were then embedded in Dr. Spurr's Low Viscosity Embedding Media within the Eppendorf tubes.

Sections were cut using a LKB 2088 ultramicrotome (LKB-Produkter, S-161 25 Bromma, Sweden), collected on 300-mesh gold grids and immunostained. Briefly, each grid was incubated for 1 h. in Tris-buffered saline-tween + 0.5% BSA (TBS-t: 0.02M Tris, 0.15M NaCl, 0.1% Tween-20), pH 7.6. The grids were then transferred to primary antibody diluted in TBS-t (50µl drops). The grids were then incubated overnight in a humidified chamber at 4˚C. The next morning, the grids and solutions were left to come to room temperature and then the grids were washed 10 times, 1 min each time. Then the grids were transferred to the appropriate gold-labeled secondary antibody (1:20 or 1:15, Sigma-Aldrich Co., St. Louis, Mo, catalog no. G7402) diluted in TBS-t. They were incubated in the secondary antibody for 1 h at room temperature. Control grids were stained only with the secondary gold-labeled antibody. The grids were washed 10 times, 1 minute each in TBS-t, then the same amount of times in ultra pure water. The grids were then counter-stained with uranyl acetate and lead citrate and photographed in a JEM-100CXII Electron Microscope (JEOL LTD., Tokyo, Japan) at 80 kV.

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Figure Legends

Figure 1. Immunoblot of Selected Diatom Species.
Immunoblots showing the anti-TpMnSOD antibody we produced cross-reacted with multiple diatom species. The phylum specificity of this antibody suggests that all diatoms have an MnSOD with similar structure. All lanes loaded with 30µg total protein (except control loaded with 10 ng pure recombinant TpMnSOD): lane 1, *Ditylum brightwellii* CCMP358; lane 2, *Navicula incerta* CCMP542; lane 3, *Nitzchia brevirostris* CCMP551; lane 4, *Stephanopyxis turris* CCMP815; lane 5, *Thalassiosira pseudonana* CCMP1010; lane 6, *Skeletonema costatum* CCMP1332; con, control protein overexpressed and purified recombinant TpMnSOD. Note: *T. pseudonana* strain used in this immunoblot is CCMP1010 and different than CCMP1335, which is the one used for cloning and over-expression of *sod*A. Marker indicates molecular weight standards in kDa.

Figure 2. Immunoblot of *Thalassiosira pseudonana* CCMP1335 cells and cells treated with 10mg/ml cycloheximide (L = light, D = dark, Lc = light + cycloheximide, Dc = dark + cycloheximide) to inhibit protein synthesis. After 27 h the protein is below detection in the cells grown under light with protein synthesis inhibited. This suggests that the turnover of TpMnSOD is related to processes that occur when cells are exposed to light. Conversely, cells exposed to continuous darkness show evidence of TpMnSOD through out the experiment. Each lane is loaded with 8 µg of total protein extracts. Antibody was specifically raised against recombinant protein in control lane.

Figure 3. Intracellular Distribution of Manganese for *Thalassiosira pseudonana* CCMP1335. Total cellular Mn (Mn_{tot}) was estimated from the C_{org}-specific Mn quotas (µmol Mn mol C^{-1}) of Sunda and Huntsman (1998, Fig. 7 and Table 2) and the C_{org} content of mid-log exponentially growing cells (0.89 pmols C_{org} cell^{-1}). Mn in photosystem II (Mn_{PSII}) is that modeled by Raven (1990). Mn in SOD was estimated as the average measured MnSOD concentration using the quantitative immuno-technique (mols of MnSOD cell^{-1}, Table II). Note that as C_{org} decreases in cells as light increases

while moles of MnSOD cell^{-1} stays constant across light levels, the percent of Mn in MnSOD may increase with irradiance (see also Fig. 5). Error indicated is standard error (values are means, *n*= 2 ±SE).

Figure 4. Immunogold localization of MnSOD in *Thalassiosira pseudonana* CCMP1335. A, Osmium tetroxide stained electron micrograph of whole cell. B, A second different view of osmium tetroxide stained cell. C, Immunogold labeling of the chloroplast with the anti-TpMnSOD antibody. D, Magnified view of delineated area in B. E, Immunogold labeling of the chloroplast in another cell of *T. pseudonana*. F, Magnified view of delineated area in E. Note the absence of labeling of mitochondrial and cytosolic regions. Key: c, chloroplast; p, pyrenoid; m, mitochondrion; n, nucleus; nc, nucleolus; v, vacuole. Arrows indicate black, electron dense gold label corresponding to TpMnSOD. Note, faint and less electron dense granules also apparent in the pyrenoid are crystalline formations of almost pure RuBISCO (Falkowski and Raven, 2007). Scale bars = length as indicated.

Figure 5. Comparison of growth rate, total cellular chlorophyll, and MnSOD per unit chlorophyll of *Thalassiosira pseudonana* CCMP1335 cells grown at different continuous light intensities. Immunoblot images above the graph are of protein samples loaded according to equal chlorophyll concentrations. Growth rate (solid black circles and solid line) increases by two-fold over these light levels. Concurrently, cellular chlorophyll (solid black squares, dotted line) decreases. Although MnSOD is constant per unit protein (data not shown), MnSOD per unit chlorophyll (solid black triangles, dashed and dotted line; and western blot above image) increases. This supports the strong association of the relative contribution of MnSOD in the chloroplast to protecting the photosynthetic machinery; especially as the light harvesting pigments decrease. Values are means, *n*= 2 ±SD.

Figure 6. Diel expression of MnSOD in *Thalassiosira pseudonana* CCMP1335. This figure demonstrates the quantum yield (Fv/Fm), A, of cells exposed to 12:12 L:D cycle under high light (800μmol m^{-2}s^{-1}, solid black triangles and dashed line) and control light

(120 µmol $m^{-2}s^{-1}$, solid black circles solid line) over time (*x*-axis). Fv/Fm decreases in the high light over the first 12 h when compared to the control and then recovered during and after the dark period. The dark period is represented by the shaded area. Immunoblot densitometric analysis, B, shows significant recovery after the dark period of TpMnSOD in the high light (hatched bars) treatment as compared to the expression of TpMnSOD in the control light (solid bars) cultures (values are means, *n*= 2 ±SD). Asterisks denote significant differences between treatments ($p = 0.0105$).

TABLES

Table I. Antibody Cross Reactivity. We reverse transcribed, amplified and cloned the gene for MnSOD from freshly extracted *Thalassiosira pseudonana* mRNA. We then raised an antibody in rabbits to the recombinant protein and tested the antibody against a wide range of whole cell protein extractions from cyanobacteria, primary green, primary red and secondary red algae. Here we present data showing the specificity of this antibody. It primarily recognizes only diatoms and only weakly some dinoflagellates. It did not cross react with any other phylum or class of algae. All algae were grown in pure culture at optimal conditions as recommended by the Culture Collection of Marine Phytoplankton* (www.bigelow.org).

TAXA IDENTFICATION	**CCMP***	**RECOGNITION**
BACILLARYOPHYTA		
Ditylum brightwellii	358	+
Navicula incerta	542	+
Nitzchia brevirostris	551	+
Stephanopyxis turris	815	+
Thalassiosira pseudonana	1010	+
Skeletonema costatum	1332	+
DINOPHYCEAE		
Karlodinium micrum	415	~
Heterocapsa triquetra	449	~
Amphidinium carterae	1314	~
Prorocentrum minimum	1329	~
EUSTIGMATOPHYCEAE		
Nannochloropsis oculata	525	-
RAPHIDOPHYCEAE		
Heterosigma akashiwo	1680	-
CHLOROPHYTA		
Dunaliella tertiolecta	1320	-
Pyramimonas parkeae	724	-
Nannochloris atomus	509	-
Pycnococcus provasolii	1203	-
Tetraselmis marina	898	-
PRYMNESIOPHYTA		
Isochrysis galbana	1323	-
Emiliania huxleyi	373	-
CRYPTOPHYCEAE		
Rhodomonas salina	1319	-
RHODOPHYTA		
Porphyridium sp.	X	-
CYANOBACTERIA		
Trichodesmium sp. IMS101	X	-
Synechocystis sp.	PCC6803	-

Table II. Statistics for MnSOD in *Thalassiosira pseudonana* CCMP1335. These data show the cell specific MnSOD budget based on quantitative immuno-analyses (see methods section). Although MnSOD accounts for a small percentage of the total protein, it is an important pool of manganese in the cell. Cells grown under continuous light, 120 μmol m^{-2}s^{-1}, 20ºC.

Protein mass per cell	19.8 ±3 fg MnSOD cell^{-1}
Molecules per cell	5.5 ±0.9 x 10^{5} MnSOD cell^{-1} [1]
Molecules per cell	2.7 ±0.5 x 10^{5} HOLO- MnSOD cell^{-1} [2]
Moles per cell	0.9 ±0.2 amol cell^{-1}
Cell volume	19.6 ±0.8 fL [3]
Percent of total protein	1.4 ±0.2 %

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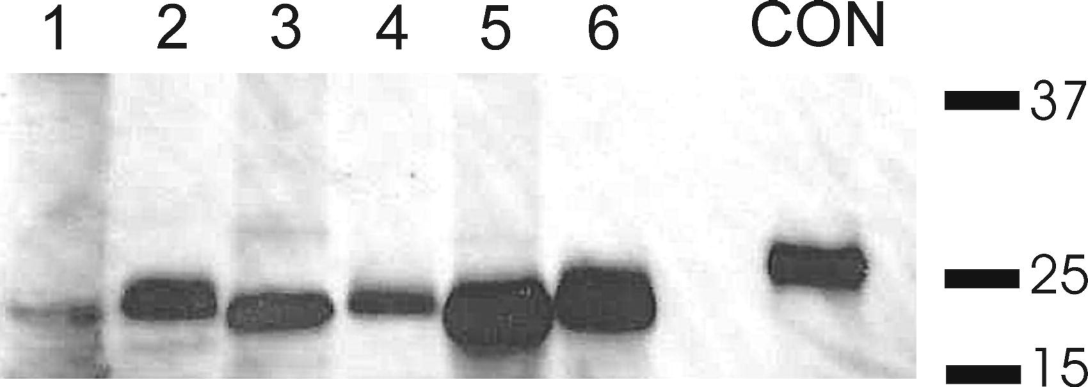
[1] based on M.W. of 21.798 kDa

[2] Based on hypothetical homodimer with a M.W. of 43.696 kDa

[3] Based on scanning electron micrography (SEM)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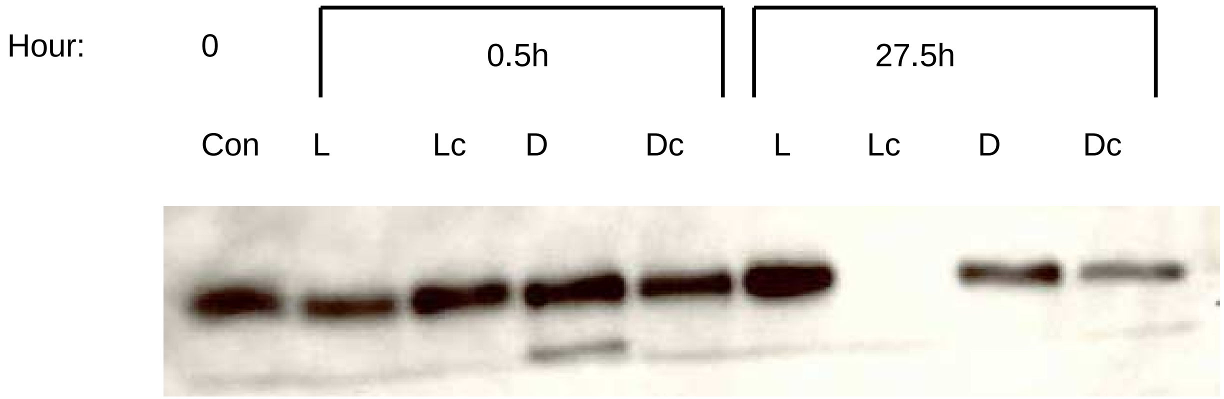
Hour:
0
0.5h
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Con
L
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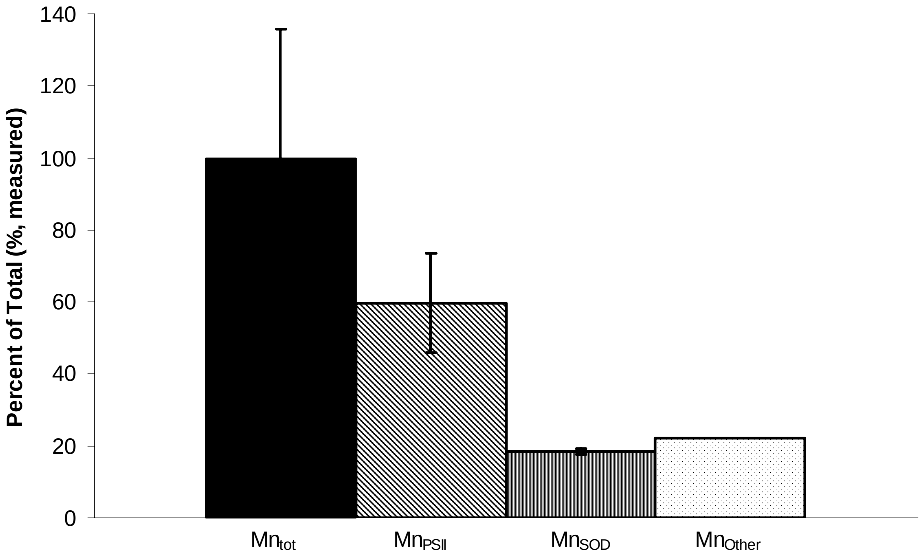
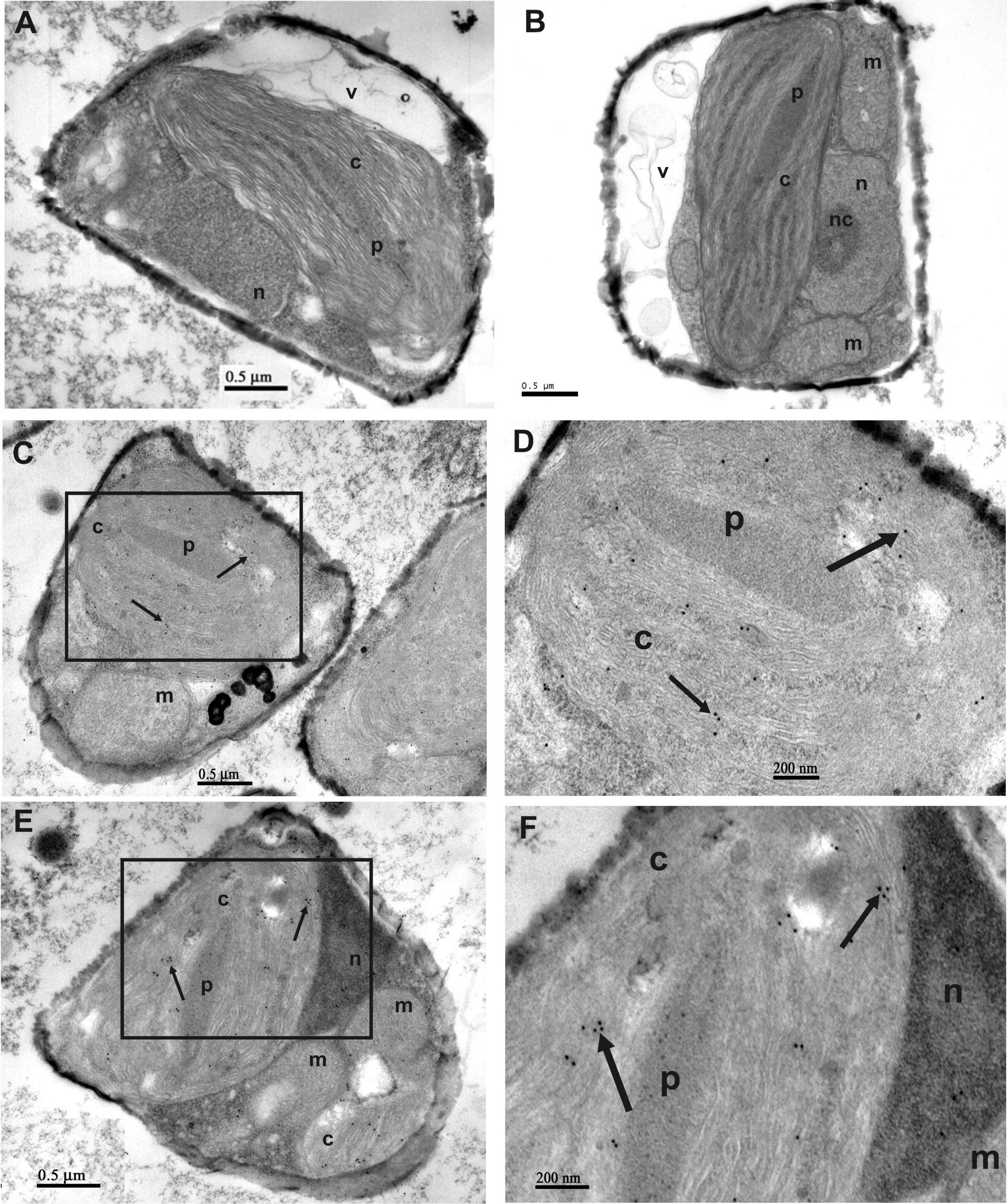

Percent of Total (%, measured)
140
120
100
80
60
40
20
0
Mn$_{tot}$
Mn$_{PSII}$
Mn$_{SOD}$
Mn$_{Other}$


A
v
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m
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0.5 µm
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200 nm
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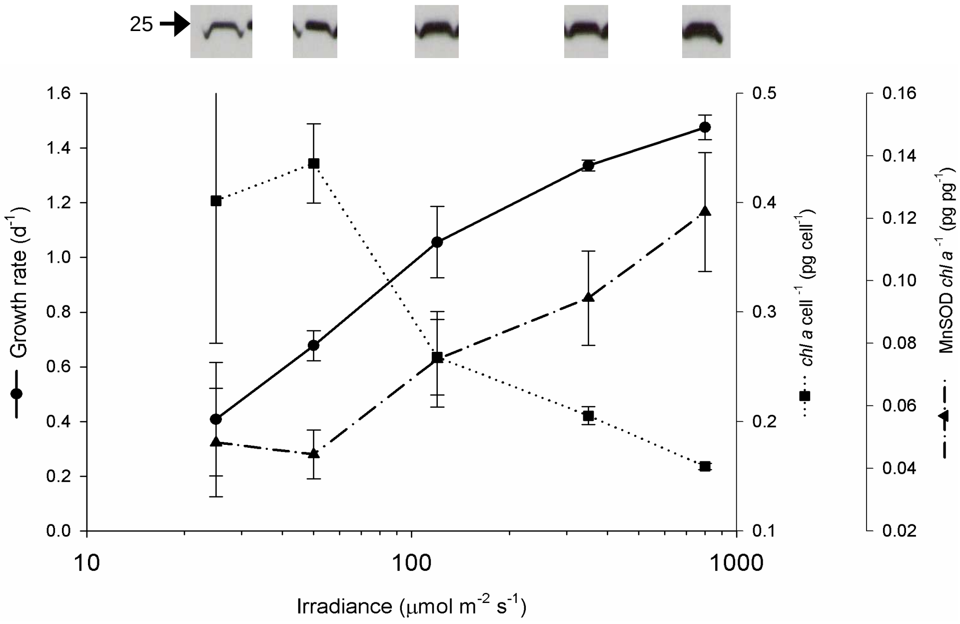
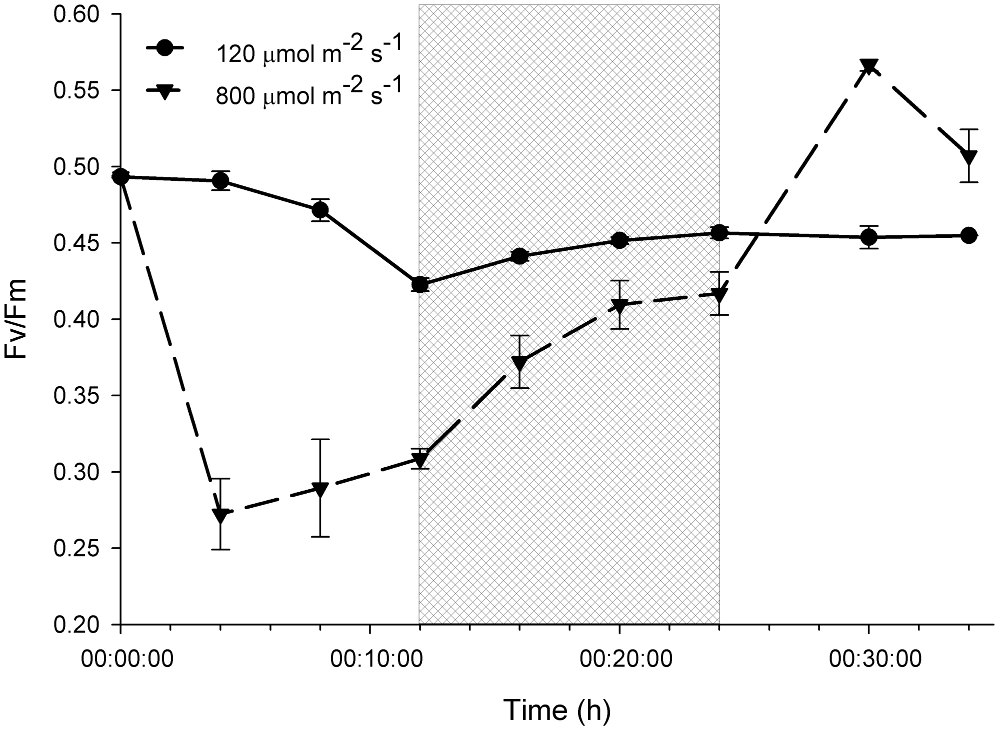
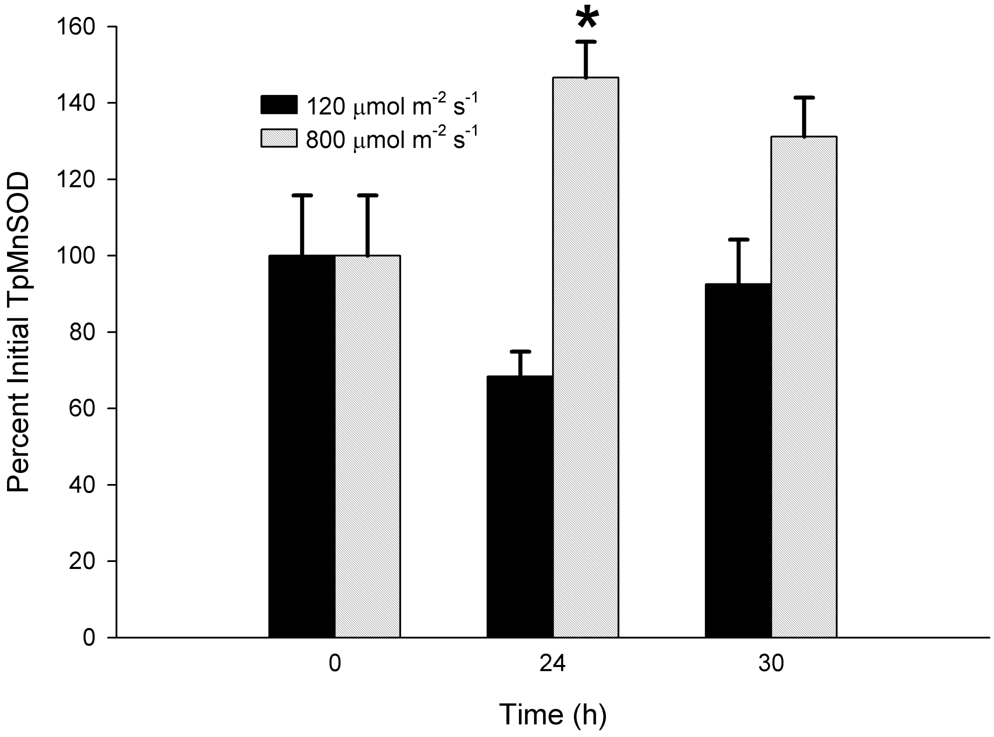

25
Growth rate (d^{-1})
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
chl a cell^{-1} (pg cell^{-1})
0.5
0.4
0.3
0.2
0.1
MnSOD *chl a*$^{-1}$ (pg pg^{-1})
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
10
100
1000
Irradiance (µmol m^{-2} s^{-1})

A


0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
Fv/Fm
● 120 μmol m^{-2} s^{-1}
▼ 800 μmol m^{-2} s^{-1}
00:00:00
00:10:00
00:20:00
00:30:00
Time (h)

B


160
140
120
100
80
60
40
20
0
Percent Initial TpMnSOD
■ 120 μmol m^{-2} s^{-1}
▨ 800 μmol m^{-2} s^{-1}
*
0
24
30
Time (h)