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Oxidative stress induced by copper: defense and damage in the marine planktonic diatom *Ditylum brightwellii*, grown in continuous cultures with high and low zinc levels

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Abstract Light-limited cultures of the planktonic diatom *Ditylum brightwellii* (West) Grunow were grown at 14‰ salinity. Cells were subjected to oxidative stress induced by copper, in the presence of zinc. In two continuous cultures with total Zn levels of 40 and 140 nM, respectively, dissolved Cu levels were increased from 3 to 126 nM. This resulted in an increased Cu adsorption capacity of the cell walls, probably due to an increase of surface area and roughness. Sexual reproduction (auxospore formation) was accelerated but was considered as a non-specific stress response. Cu-induced oxidative stress was indicated by a decrease of reduced glutathione (GSH), and a removal of superoxide anions monitored as an increasing activity of superoxide dismutase (SOD). Although Zn has no oxidative potential per se, cell division rates and chlorophyll *c* contents were lower in the culture with high Zn levels. In both cultures, the pro-oxidant Cu caused a decrease of chlorophyll *a*, decreasing photosynthetic O₂ evolution and cell division rates, and a growing number of deformed and broken cells.

Introduction

Oxidative stress in aquatic organisms is a normal phenomenon, caused by a number of natural external factors (e.g. UV-B radiation), but it can be promoted by some pollutants (Stegeman et al. 1992). Many organochemicals (quinones, nitroaromatics, aromatic hydroxylamines) and transition metals (e.g. copper) are pro-oxidants, which catalyze and accelerate the formation of oxyradicals in plants

(Salin 1987). Due to this oxidative potential, copper is a very toxic metal, not just because of its high affinity for thiol-sites (–SH) on proteins, as this is also the case for e.g. cadmium and zinc (Reed and Gadd 1990). An excess of Cu-induced oxyradicals disturbs the pro- vs antioxidant balance and enhances lipid peroxidation (membrane disintegration, failure of osmoregulation), which shortens the lifetime of cells (De Vos and Schat 1991; Lee and Hassan 1985).

At low values, both Cu and Zn stimulate phytoplankton growth (Brand et al. 1983; Verweij et al. 1992), but a slight excess of both metals binds SH sites, while Cu also oxidizes –SH to disulfur (–SS–) bridges in proteinaceous biomolecules, causing a cumulative, inhibitive Cu/Zn effect (Stauber and Florence 1987, 1990; French and Evans 1988; Rijstenbil and Poortvliet 1992). With speciation models it was calculated that for most algae, ion activities of Cu²⁺ limit growth below 10^{–13} M, and become sublethal above 10^{–11} M (Brand et al. 1986; Verweij et al. 1992). *Ditylum brightwellii* is a Cu-sensitive diatom species, blooming in estuaries and coastal waters (Canterford and Canterford 1980; Rijstenbil and Wijnholds 1991; Rijstenbil et al. 1993; Rijstenbil et al. in press). At 10^{–11} M Cu²⁺, its cell division was slightly inhibited, but its growth ceased abruptly between 10^{–10.3} and 10^{–9.3} M Cu²⁺ (Brand et al. 1986; Gerringa et al. in press). It is expected that even at low Cu and Zn levels, *D. brightwellii* shows a poor defense against metal toxicity and suffers from oxidative damage (Rijstenbil et al. in press).

We investigated how *Ditylum brightwellii* was affected by increasing levels of available Cu, which exceeded the equivalent of the ligand in the medium (Rijstenbil and Wijnholds 1991). Although Zn has no oxidative effect, earlier observations showed that it indirectly stimulated superoxide dismutase activity in this diatom (Rijstenbil et al. in press). For this reason, the cumulative effect of Zn was further investigated. In the present paper, continuous cultures were run at two different zinc levels: 40 nM (Zn–) and 140 nM (Zn+). For 121 d dissolved Cu was stepwise, but gradually increased from ~3 to 126 nM in the medium. The data concerning external metal speciation and metal ad-

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sorption to the cell wall have been described elsewhere (Gerringa et al. in press). Here, we have examined whether or not metabolic responses of *D. brightwellii* to Cu were: (1) related to additional Zn in the medium; (2) part of its antioxidant defense; or (3) attributable to oxidative cell damage.

Materials and methods

Media and culture conditions

Artificial brackish medium was prepared as described in Rijstenbil et al. (1989). Salinity (14‰) was measured with a WTW-LF2000-CONX conductivity meter. Metals were removed from the salt solution by CHELEX-100 (Morel et al. 1979; Rijstenbil and Wijnholds 1991). Media were enriched with 200 μM nitrate, 108 μM silicate and 18 μM orthophosphate, and 56.5 nM Na_3EDTA was used as a metal chelator. The pH of the medium was 7.8 (PHM 80 Radiometer Copenhagen). Media were filter-sterilized (0.2 μm). Axenic cells of the marine planktonic diatom *Ditylum brightwellii* (West) Grunow were grown in 6-liter glass vessels (siliconized borosilicate; Techné, Cambridge UK), at 10°C. Cells were irradiated at a mean level of 83 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ measured inside the culture suspension with a QSL-100 4 π -sensor (Biospherical Instruments) in a light (L):dark (D) cycle of 2 h L:2 h D:2 h L:2 h D:2 h L:14 h D. This alternate photoperiodicity was designed to simulate vertical mixing of phytoplankton and to keep the pH-values in the cultures below 8.4. On reaching a density of ~ 8 million cells l^{-1} in batch mode (i.e., medium dilution rate $D=0$), the peristaltic pumps were started to keep the cells in exponential-phase growth. Pumping rates were varied until D balanced cell division rates (μ) that were at maximum. $D = v/V$ in which v = pumping rate (l d^{-1}); V = culture volume (l). $\mu = D + [\ln(N_t/N_{t-\Delta t})/\Delta t]$ in which N = cell number (cells l^{-1}); t = time (d); Δt = time interval of sampling (d). Steady-state ($\mu = D$) light-limited growth thus achieved was monitored by cell counting. When cell numbers showed a fluctuation of more than $\sim 20\%$ the pumping rate (v) was corrected (Rijstenbil and Wijnholds 1991).

Experimental design

In the first culture (Zn-) the medium contained 40 nM; in the second culture (Zn+) the medium contained 140 nM total dissolved Zn. Light-limited cells grew at an initial Cu concentration of ~ 3 nM. The Cu concentration in the continuous cultures was then stepwise increased via dilution, using media with 40, 80, and 120 nM CuSO_4 , respectively (Fig. 1). Using the MINEQL (Mineral Equilibrium) model, Cu speciation in the cultures was calculated in relation to the other trace metals (Fe, Mn, Co, Zn) added to the medium, as well as

to the ligands added (EDTA) or produced by the algae (Gerringa et al. in press).

To compare effects of increasing Cu at two different levels of Zn, samples were taken in the course of the 121-d experiment, at the end of the first photoperiod (10:00 hrs). Metal-induced decreases of growth rates (μ) were monitored by cell counting and balanced by the pump speed to restore the steady state (light-limited growth).

Cell counting and sinking rate, SEM (scanning electron microscopic) preparations

Cell samples (2-ml) were taken from the cultures and preserved in Lugol's iodine. Cells were counted (cells l^{-1}) under an inverted microscope (Olympus CKBIN). Length and diameter (μm) of 100 cell frustules per sample were measured with an Olympus IM VIDS image analyzer. Mean cell volumes (μm^3) were calculated. To normalize cell parameters to units cell volume, we calculated with the biovolume as a biomass standard (Rijstenbil et al. 1989):

$$\text{Biovolume} = (\text{cell volume} \times \text{cell numbers}) \quad (1)$$

Cell reproductive stages were examined, distinguishing vegetative and presexual cells, zygotes and auxospores, according to Waite and Harrison (1992). Sinking rates of *Ditylum brightwellii* were determined at 15°C in a 5-ml tubular plankton settling chamber with a thin bottom glass (Hydrobios, Kiel), placed on the inverted microscope. The cylinder was filled with 4 ml culture medium [column height (h) = 2 cm] with the appropriate Cu concentration. Using a variable pipette, 0.2 ml algal suspension was carefully transferred in a thin layer on top of the medium, at $t=0$ (Bienfang 1980). From $t=0$ to 90 min, at 10-min intervals, the cells (N) settled on the bottom of the chamber were counted (cells cm^{-2}). Final cell numbers (N_∞) on the bottom of the chamber were determined at $t=90$ min. The microscope lamp was on only during counting. Accumulated numbers of settled cells followed a normal S-shaped frequency distribution pattern in time (Bienfang 1980). The (bell-shaped) differential curve with the change in cell numbers at each time (ΔN) was derived. Fractions of settled cells ($F_t = \Delta N/N_\infty$) were given for each interval. The mean ($\approx$ optimum) settling time is

$$t_{\text{AVG}} = (1/N_\infty) \times \sum_{t=0}^{t=90} [\Delta N \times t] = \sum_{t=0}^{t=90} [F_t \times t] \quad (\text{min}). \quad (2)$$

With t_{AVG} and $h=2$ cm, we calculated the mean sinking rates of *D. brightwellii* as

$$v_{\text{AVG}} = h/[t_{\text{AVG}}/(60 \times 24)] = 0.02 \times 60 \times 24/t_{\text{AVG}} = 28.8/t_{\text{AVG}} \quad (\text{m d}^{-1}). \quad (3)$$

for cells growing at 3, 40, 80 and 120 nM total dissolved Cu.

For SEM, 15 ml cell suspension of *Ditylum brightwellii* was fixed (1 h) with 2% (w/v) glutaraldehyde (GA) in HEPES pH 7.8, washed three times in 14‰ S isotonic medium, post-fixed with 2% (w/v) OsO_4 (1 h), and washed again in isotonic medium. Microscope slides were pretreated with poly-L-lysine to make cells stick to the glass. Attached cells were washed in subsequently 30, 50, 70, 90 and (three times) 100% (v/v) ethanol, and transferred into a CO_2 atmosphere in a critical point dryer (Balzers CPD 020). Temperature was increased to 40°C (85 bar), and CO_2 was then slowly evacuated. After drying, samples were gold-coated at 0.1 Torr. Cells were observed with a Jeol JSM-T300 scanning electron microscope.

HPLC (high performance liquid chromatographic) analysis of cellular thiols

For the HPLC-analysis of the free -SH groups in the reduced thiol antioxidants, we applied a post-column derivatization with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), according to Gekeler et al. (1988) with sulfosalicylic acid (SSA) instead of sodium borohydride. Cells were harvested and concentrated by centrifugation (1000 g; 15 min; 0°C); 50 to 100 mg freeze-dried cells (48 h) were ground with a pestle and mortar (0°C). The powder was suspended in 1 ml of buffer with 236 mM SSA and 6.3 mM DTPA (diethylenetriaminepentaacetic acid, pH 2) to keep SH reduced. The suspension was sonicated (0°C; MSE Soniprep 10, amplitude 14 μm) and centrifuged (12 000 g; 20 min; 0°C) to remove cell residues. The supernatants (cytosol extracts) were stored at -70°C and used later for SH-anal-

Fig. 1 *Ditylum brightwellii*. Increase of total dissolved Cu with time, in light-limited continuous cultures grown at 40 nM (Zn-) and 140 nM Zn (Zn+); days of sampling on horizontal axis

ysis (Rijstenbil et al. in press). 50- μ l extracts were injected into an HPLC-system (LKB Pharmacia) consisting of a controller, PMV-7 injection valve, mixing chamber and HPLC pump 2248-107 and UV-VIS detector 2141. Optimum separation of SH peaks was obtained, using a reverse-phase C₂/C₁₈ HPLC column (Pharmacia Superpac PepS) and the solvents A [water (0.05% v/v H₃PO₄, pH 2.5)] and B (100% acetonitril). At a pump flow of 1 ml min⁻¹, the following separation programme was used: 0 to 6 min 100% solvent A (isocratic); 6 to 33 min, 0 to 40% solvent B (linear). Thiols were detected via a post-column SH reaction (0.6 mM DTNB in 50 mM KH₂PO₄ pH 7.6; flow = 1 ml min⁻¹; reaction time = 1.0 min; wavelength = 412 nm). Retention times and SH concentrations were obtained via standards of gammaglutamylcystein (Sigma), glutathione (Merck) and purified *Silene*-phytychelatins (gammaglutamylcysteinyl_n-glycines: n = 2, 3; Free Univ. Amsterdam), in 236 mM SSA. SH-concentrations were normalized to g dry weight (μ mol SH g⁻¹ dry wt).

Superoxide dismutase activity

Duplicate 100-ml samples of cells were filtered on 0.45- μ m Tuffryn membrane discs (Gelman Sciences), and suspended into 2 ml 0.05 M KH₂PO₄ extraction buffer (pH 7.8) containing 0.1 mM EDTA, and sonicated on ice (0 °C) for 3 min (MSE Soniprep 150, amplitude 14 μ m). Cell residues were removed by a 15 min centrifugation at 8000 g (0 °C). The supernatant was used as enzyme solution for measurement of the superoxide dismutase (SOD) activity according to McCord and Fridovich (1969). SOD activities were calculated according to Asada et al. (1974). The principle is that superoxide anion radicals (O₂⁻), generated by the xanthine-xanthine oxidase (XOD) system, reduce cytochrome *c*. Inhibition of cytochrome *c* reduction by SOD was continuously monitored at 550 nm (Kontron Uvikon 940 spectrophotometer). Each assay was performed five times at 25 °C in 1.5 ml reaction mixture with: 0.05 M KH₂PO₄ (pH 7.8), 0.1 mM EDTA, 10 μ M ferricytochrome *c*, 50 μ M xanthine (Sigma) and 5.4 μ g XOD (Sigma). The SOD unit (per mm³ cell volume) is defined as the amount of enzyme required for a 50% inhibition of ferricytochrome *c* reduction.

Photosynthesis

O₂-evolution was measured in 200-ml cell suspensions as described by Rijstenbil et al. (1989) and Rijstenbil and Wijnholds (1991). Photosynthetic rates were measured at irradiance values of 0, 100, 200, 300, 400, 500 and 600 μ mol quanta m⁻² s⁻¹. These rates were used to draw the *P-I* (photosynthesis-irradiance) curves, and initial slopes of this curve rendered the photosynthetic affinity (α). As described in Rijstenbil et al. (1989), the α values were normalized to cell volume [mM O₂ h⁻¹ (μ mol quanta m⁻² s⁻¹)⁻¹].

Analyses of pigments and cellular carbon

100-ml cell suspensions were collected on Whatman GF/C glass-fibre filters. Diatom pigments (chlorophyll *a* and *c*) were HPLC-analyzed (Waters Ass.) using 100% methanol and 2% ammonium acetate as an extraction mixture instead of 90% acetone (Gieskes and Kraay 1983). Cellular carbon was measured on a Carlo Erba NA1500 NC-analyzer (Rijstenbil and Wijnholds 1991).

Metal analyses

Culture samples were collected in glass bottles, previously cleaned with dilute acid and were equilibrated with medium before use. Samples were filtered under a low N₂ pressure through 0.45- μ m cellulose nitrate filters in a polyethylene filtration set. To avoid cell rupture, the collecting bottle was removed before filters became dry. Filters were destroyed in a Plasma Processor 200-G (Technics GmbH) and ashes were dissolved in HCl/HNO₃. Particulate Cu and Zn were measured with GFAAS (Perkin Elmer Zeeman 3030). Cellular metal contents were normalized to cell volume. For total dissolved Cu and Zn, filtrates (<0.45 μ m) were acidified to pH=2. After a 4-h UV/H₂O₂ destruction, metals were analyzed with differential pulse anodic stripping voltammetry (DPASV) using a hanging drop mercury electrode and analyzer (EG & G PAR 303A/384B, Princeton, NJ – USA), collection potential –0.6 V.

Fig. 2 *Ditylum brightwellii*. Valves volume of intact cells grown at 40 nM (Zn-) and 140 nM Zn (Zn+) vs dissolved Cu in the medium

Fig. 3 *Ditylum brightwellii*. Percentage of the volume of broken valves relative to total biovolume; cells grown at 40 nM (Zn-) and 140 nM Zn (Zn+) vs total dissolved Cu [Arrow increase of free Cu²⁺ (MINEQL-calculated)]. Cellular Cu content ~8 μ M

Fig. 4 *Ditylum brightwellii*. Percentage of vegetative and sexual reproductive cell stages at 3, 40, 80 and 120 nM total dissolved Cu. Mean cell sinking rates (m d⁻¹) indicated to the left

Fig. 5 *Ditylum brightwellii*. Scanning electron microphotographs of cells grown at 10 nM (a, c, e) and 80 nM (b, d, f) total dissolved Cu. **a** Intact cell at 10 nM Cu; **b** presexual cell with increased surface roughness at 80 nM Cu; **c** close-up of a smooth surface at 10 nM Cu; **d** wrinkled cell at 80 nM Cu; **e** normal, single labiate processus; **f** double labiate processi, appearing after extended exposure to Cu (80 nM)

Results

Total dissolved Zn was 45 ± 11 nM in the Zn⁻ culture (40 nM added), and 64 ± 36 nM in the Zn⁺ culture (140 nM added). Loss of dissolved Zn in the Zn⁺ culture was a result of co-precipitation with silicate at pH 8.4. The amount of Zn adhering to the glass of the culture vessel was negligible, as the sum of total dissolved and algal Zn (146 nM) gave a full recovery of the amount added to the culture media (140 nM). About 72% of total Zn was incorporated into the Si-coated cell wall matrix of *Ditylum*

brightwellii (Gerringa et al. in press). Algal Zn contents showed wide variations. Normalizing Zn to algal cell carbon gave $110 \pm 30 \mu\text{mol Zn mol}^{-1} \text{C}$ for the Zn- cells, and $172 \pm 53 \mu\text{mol Zn mol}^{-1} \text{C}$ for the Zn+ cells. The same applied to Zn contents normalized to diatom biovolume. Zn- cells contained $179 \pm 54 \mu\text{M Zn}$, Zn+ cells contained $337 \pm 175 \mu\text{M Zn}$. A *t*-test for difference among pairs of means among Zn- and Zn+ cells (Sokal and Rohlf 1981) proved that Zn contents were significantly higher in the Zn+ culture ($p < 0.01$). Moreover, cellular Zn increased significantly with cellular Cu in the Zn+ culture, as $[\text{Zn}] = 15[\text{Cu}] + 90 (\mu\text{M})$ ($n = 11$; $r = 0.54$; $p < 0.05$). The designed increase of total dissolved Cu with time (Day 1 to 121) was the same in cultures Zn- and Zn+ (Fig. 1). In both cultures, total algal Cu contents comprised $\sim 10\%$ of total dissolved Cu throughout the experiment. Cellular Cu, normalized to biovolume (μM) increased with dissolved Cu (Zn-: $n = 12$; $r = 0.76$; $p < 0.005$ vs Zn+: $n = 11$; $r = 0.59$; $p < 0.05$), but Cu levels in Zn+ cells were not significantly higher (*t*-test) than in Zn- cells. As a result of an increase of cell volume in the total dissolved Cu-gradient from 10 to 80 nM (Fig. 2), $\mu\text{mol Cu l}^{-1}$ biovolume did not increase linearly with dissolved Cu. The relative biovolume of broken cells frustules increased when total dissolved Cu exceeded 25 nM (Fig. 3) and calculated ionic Cu increased from 10^{-11} to $10^{-9.7} \text{ M}$ (MINEQL model; Gerringa et al. in press). Due to cell breakage, part of the cellular Cu may have been released into the medium, with the remaining Cu still adsorbed to the cell fragments.

Cells intensified their sexual reproduction at increasing Cu (Fig. 4). Increasing cell volumes were probably caused by a larger proportion of auxospores and post-auxospore, vegetative cells. Sinking rates showed a minor decrease with increasing Cu (Fig. 4). However, due to an overlap of frequency distributions of settled cell fractions vs time, this decrease was not significant. Apart from disintegrated cells and reproductive stages, deformed cells were found in increasing numbers. It was estimated that a 3-fold increase of curved, presexual (Fig. 5b) and "wrinkled" (Fig. 5d) cells occurred at 80 nM Cu, relative to observations at 10 nM Cu (Fig. 5a, c). Cells with more than one labiate process at both ends were more frequently present (Fig. 5f).

At the start of the experiment, steady-state cell division rates were $\sim 0.45 \text{ d}^{-1}$ in the Zn-, and $\sim 0.37 \text{ d}^{-1}$ in the Zn+ culture. Growth rates decreased gradually with increasing Cu, and dilution rates (*D*) were reduced accordingly to maintain a biomass of ~ 12.5 million cells l^{-1} . Division rates ($\mu/\ln 2$) in the Zn+ culture (140 nM Zn) were lower than those in the Zn- culture (140 nM Zn), and indicated a cumulative effect of Zn (Fig. 6). In both cultures division rates decreased significantly with cellular Cu (Zn-: $n = 15$; $r = -0.47$; $p < 0.05$ and Zn+: $n = 14$; $r = -0.67$; $p < 0.005$).

A decrease of reduced glutathione (GSH) was observed at increasing cellular Cu (Fig. 7). GSH was the only thiol detectable via this HPLC-DTNB method. Phytochelatin, if present, were below the limit of detection. SOD activity per cell increased with Cu in Zn- ($n = 10$; $r = 0.54$; $p < 0.05$)

Fig. 6 *Ditylum brightwellii*. Cell division rates vs increasing concentrations of total dissolved Cu in light-limited continuous cultures, grown at 40 nM (Zn-) and 140 nM Zn (Zn+)

Fig. 7 *Ditylum brightwellii*. Reduced glutathione (GSH) in cytosolic fractions of cells grown at 40 nM (Zn-) and 140 nM Zn (Zn+) vs concentrations of cellular Cu. The concentration of free thiol groups in GSH (reduced glutathione) is expressed in $\mu\text{mol SH g}^{-1}$ dry wt

and Zn+ cells ($n = 9$; $r = 0.59$; $p < 0.05$). SOD activity, normalized to cell volume, increased with cellular Cu in both Zn- and Zn+ cultures (Fig. 8).

Chlorophyll *c* contents, normalized to cell volume, were not influenced by increasing cellular Cu. Contents were lower in Zn+ cells than in Zn- cells ($0.09 \pm 0.03 \text{ g l}^{-1}$ and $0.15 \pm 0.04 \text{ g l}^{-1}$, respectively; *t*-test: $p < 0.0005$). In both Zn- and Zn+ cells, chlorophyll *a* decreased considerably with increasing cellular Cu (Fig. 9a). In chlorophyll *a*:*c* ratios, however, clear differences existed between Zn- and Zn+ cells (Fig. 9b). This was the result of an initial chlorophyll *a*:*c* ratio of ~ 5 in Zn+ cells, whereas in Zn- cells initial ratios were ~ 3 . The decrease in chlorophyll *a* resulted in decreased photosynthetic affinities (α), which means that the cells gradually lost their ability to efficiently evolve O_2 . Cu damaged the photosynthetic apparatus equally in Zn- and Zn+ cells (Fig. 9c).

Fig. 8 *Ditylum brightwellii*. Superoxide dismutase activity (SOD) vs cellular Cu in Zn- and Zn+ cultures (combined data)

Fig. 9 *Ditylum brightwellii*. Values of: **a** chlorophyll *a* contents; **b** chlorophyll *a*:*c* ratio; **c** photosynthetic affinities (α) plotted vs Cu contents of cells grown at 40 nM (Zn-) and 140 nM Zn (Zn+)

Discussion

Increasing Cu levels have affected the cell structure and integrity of *Ditylum brightwellii*. An increase of cell volumes (Fig. 2) upon Cu stress has been reported before in diatoms (Fisher et al. 1981; Rijstenbil and Wijnholds 1991). Disturbance of silicon deposition, due to a Cu-induced lipid peroxidation in the Golgi apparatus (Lee and Li 1991), might have caused wall deformation. Increasing volumes and surface roughness (Fig. 5d) may contribute to an increased metal binding capacity. Gerringa et al. (in press) observed an increase of the adsorption capacity from ~ 2.5 to $7.5 \mu\text{equiv Cu m}^{-2}$ cell surface upon a gradual increase from 10 to 126 nM Cu in the medium. Although an increased metal sorption in the wall may diminish internalization of Cu in *D. brightwellii*, it is doubtful that the gradual increase of Cu induced a tolerance, as suggested by Twiss et al. (1993). Such an adaptive response would be in contradiction with the increase of broken cells (Fig. 3). In general, breakage follows membrane leakage due to lipid peroxidation (Fisher et al. 1981). Probably as a result of an increased leakage of organic Cu-complexing ligands (Gerringa et al. in press), the competition between ligands and cells (Hudson and Morel 1993) caused a stabilization of calculated Cu^{2+} at 0.2 nM (MINEQL model) in spite of a further Cu increase. Thus, ligands excreted by *D. brightwellii* passively reduced Cu availability and cannot be regarded as a tolerance (detoxification) mechanism (Foster 1977; Härdstedt-Roméo and Gnassia-Barelli 1980).

The relative increase of sexual stages in *Ditylum brightwellii* was one of the major causes of cell enlargement (Fig. 4). According to Waite and Harrison (1992), these cell types have a higher sinking speed and may use this feature to escape from a toxic environment. However, as sinking rates were hardly affected by Cu, it is possible that the increased wall roughness gave some additional resistance against sinking (Fig. 5d, f). Cu-induced oxyradicals damage DNA, oxidize GSH, and hence, affect genetic composition and mitosis (Lewis 1984; Floyd et al. 1986; Stauber and Florence 1987; Wingate et al. 1988; Milne et al. 1993). Auxospore formation resulted from an accelerated recombination of DNA through male gametes and egg cells. We cannot rule out the possibility that metal-tolerant cells evolve via genetic selection (Cha and Cooksey 1993; Voloudakis et al. 1993), as it is a common feature of diatoms to constantly change their genetic composition (Armbrust and Chisolm 1992). Here, sexual reproduction should be considered as a non-specific and, as regards avoidance of oxidative damage, ineffective stress response (Drebes 1977).

SH-groups in cysteine are effective in detoxification, forming cytosolic metal-complexes (Kosakowska et al. 1988). In many algae, phytochelatins (poly-gammaglutamylcysteinyl-glycines) are induced by heavy metals (Gekeler et al. 1988; Reed and Gadd 1990; Wikfors et al. 1991; De Vos et al. 1992). Phytochelatins were not detectable in *Ditylum brightwellii*, and the rapid decline of the small pool of reduced GSH (Fig. 7) indicated that it might be oxidized

by Cu. Glutathione (GSH: gammaglutamylcysteinyl-glycine) is the major cellular ligand in metal homeostasis and implicitly protects against oxyradicals whose formation is catalyzed by the transition metal Cu (Miller et al. 1990). Due to its low GSH:GSSG redox ratios, *D. brightwellii* might be rather sensitive to Cu-induced oxidative stress (Rijstenbil et al. in press).

SOD is a prominent enzymatic biomarker of defense against oxidative stress (Van Assche and Clijsters 1990; Chongpraditnun et al. 1992). It transfers O_2^- radicals into H_2O_2 . In previous work, SOD activities increased in Zn-exposed *Ditylum brightwellii* (Rijstenbil et al. in press). SOD activities increased with Cu, independent of the Zn contents (present work), which indicates that Cu is the only oxidative stressor (Salin 1987; Van Assche and Clijsters 1990).

A lack of antioxidant defense will cause oxyradical damage in chloroplasts, and inhibition of algal photosynthesis. Chlorophyll *c* contents were low, and chlorophyll *a:c* ratios were high in Zn+ cells (Stauber and Jeffrey 1988). A doubled amount of cellular Zn did not affect photosynthesis (Fig. 9c), but cell division was suppressed in Zn+ cells (Fig. 6) (Stauber and Florence 1990; Rijstenbil and Poortvliet 1992). Chlorophyll *a* proved to be sensitive to Cu, and its decrease coincided with the decrease of photosynthetic activity. As in former experiments, a decrease of photosynthetic affinity (α) was caused by Cu (Rijstenbil and Wijnholds 1991, Rijstenbil et al. in press). A competitive inhibition at metal binding sites in the photosystems, disturbance of photosynthetic electron transport, pigment breakdown and lipid peroxidation in chloroplasts have been described as damaging effects of the oxidative metal Cu (Sandmann and Böger 1980; Baszynski et al. 1988; De Vos et al. 1989; De Vos and Schat 1991; Ahmed and Abdel-Basset 1992; Mishra et al. 1993).

To summarize, the long-term exposure to Cu did not increase tolerance in *Ditylum brightwellii*. Evidence for stress avoidance, as a result of increasing Cu adsorption capacity or accelerated sexual reproduction, could not be provided. Metal detoxification mechanisms were not activated, and GSH pools were soon depleted. Although SOD increasingly eliminated the O_2^- radicals formed, the pro-oxidant Cu gradually caused a loss of photosynthetic performance, a decrease of chlorophyll *a*, enlargement, deformation and breakage of cells.

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