

Response of nonprotein thiols to copper stress and extracellular release of glutathione in the diatom *Thalassiosira weissflogii*

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Abstract

We studied the dynamic changes of cellular thiols and the extracellular release of glutathione (GSH) during growth of the marine diatom *Thalassiosira weissflogii* under varying levels of copper (Cu) addition in both metal-buffered (with EDTA) and unbuffered (without EDTA) media. The cell quotas of both thiols and pigments decreased with growth time; however, pigment-normalized cellular thiol concentrations at a given Cu exposure level were more or less conservative. In both media, specific growth rates of greater than 1 per day were obtained at total inorganic Cu concentrations of less than 80 nmol L⁻¹; however, at higher Cu levels, cell growth was significantly suppressed. A dose–response relationship was observed between the phytochelatin-2 : GSH ratio and the Cu exposure level during the exponential growth period. GSH was released from the phytoplankton cells at similar concentrations in both media; therefore, the presence of a synthetic metal chelator does not affect thiol release, substantiating the premise that algae respond to inorganic species of Cu. GSH release was closely related to Cu-induced cell membrane damage. The extracellular GSH release rate was higher in normally grown cultures than in growth-limited cultures but lower than in growth-suppressed cultures. The excretion of GSH apparently reflects physiological conditions during algal growth rather than an enzymatic response of the algae to control trace metal speciation in the media. Therefore, the role of GSH in metal complexation in ambient waters is probably an inadvertent by-product of this process, although it could contribute a significant portion of Cu-complexing ligands in open ocean waters.

Many phytoplankton species have been documented to excrete extracellular copper (Cu)-binding compounds (McKnight and Morel 1979; Gerringa et al. 1995; Gonzalez-Davila et al. 1995), including low-molecular-weight acids (Croot et al. 2000), high-molecular-weight polysaccharides (Myklestad 1995; Pistocchi et al. 2000), and sulfhydryl compounds (Leal et al. 1999), in response to Cu stress. Compounds of different natures in Cu complexation were released at different stages during cell growth (Zhou and Wangersky 1989; Rijstenbil and Gerringa 2002). The release of metal-complexing compounds was further hypothesized to be a mechanism for phytoplankton to modify trace metal speciation in natural seawater and to thereby mediate bio-availability (Moffett and Brand 1996). However, the Cu-induced exudates from one species can either enhance or inhibit growth of another species, dependent on the nature of exudates (Vasconcelos et al. 2002), indicating that excretion of organic compounds may not necessarily be mutually beneficial among algal species. The important issue of whether

the release of metal-complexing compounds by phytoplankton results from an active effort by the alga to extracellularly mediate metal speciation or whether it simply reflects normal metabolic excretion of organic matter (constrained by photosynthesis [Baines and Pace 1991]) from actively growing cells through passive diffusion (Bjornsen 1988) remains unclear.

The induction of intracellular phytochelatins is a known response of eukaryotic microorganisms to aqueous metal exposure (Grill et al. 1988; Ahner and Morel 1995; Rijstenbil and Wijnholds 1996). In addition to serving as the principal precursor in phytochelatin synthesis, glutathione (GSH) has a much wider role as an antioxidant, helping maintain intracellular redox conditions and sensing environmental stress (May et al. 1998; Noctor et al. 1998). The extracellular release of GSH observed in some algal species could, in some cases, contribute a significant fraction of the uncharacterized metal-complexing ligands in their growth environment (Leal et al. 1999; Vasconcelos et al. 2002). However, the lack of information about intracellular GSH quotas and their variation limits our ability to clarify the linkages between physiological change and thiolic compound excretion. A quantitative mass-balance analysis of the release of thiols from algae cells into growth media is needed in order to address the importance of thiols as a source of strong metal-complexing ligands in natural waters (Moffett and Brand 1996; Al-Farawati and Van Den Berg 1999; Tang et al. 2000) as well as its potential role, parallel to that of cellular dimethylsulphoniopropionate and derived dimethylsulfide, in the global sulfur biogeochemical cycle (Sunda et al. 2002).

In most studies of physiological changes in phytoplankton in response to metal exposure, metal-chelated or buffered growth media have been used (Price et al. 1988/1989; Sunda and Huntsman 1995). However, unbuffered growth media

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Table 1. Specific growth rate of *T. weissflogii* at different free Cu concentrations in EDTA-unbuffered and EDTA-buffered media and extracellular glutathione release in the mid-exponential phase.*

	pCu [†]	$\mu_{\ddagger}^{\ddagger}$ (d ⁻¹)	G§ (nmol L ⁻¹ d ⁻¹)	V (nmol L ⁻¹ d ⁻¹)	R¶ (fmol cell ⁻¹ d ⁻¹)	Rs# (d ⁻¹)
-EDTA	10.2	1.18 (0.02)	21.3 (1.9)	0.58 (0.2)	0.084 (0.029)	0.029 (0.012)
	8.8	1.35 (0.06)	23.2 (2.1)	1.28 (0.16)	0.099 (0.016)	0.050 (0.012)
	8.3	1.13 (0.08)	16.6 (1.3)	2.01 (0.17)	0.360 (0.042)	0.12 (0.026)
	8.0	0.31 (0.13)	0.76 (0.27)	2.15 (0.47)	1.35 (0.64)	0.31 (0.12)
	7.8	0.15 (0.05)	-0.68 (0.31)	2.13 (0.22)	1.45 (0.56)	0.37 (0.15)
	13.8	1.00 (0.01)	10.6 (0.7)	0.56 (0.12)	0.091 (0.020)	0.035 (0.013)
+EDTA	10.9	0.29 (0.05)	1.4 (0.7)	0.31 (0.09)	0.11 (0.036)	0.026 (0.011)
	9.5	1.23 (0.07)	17.6 (0.9)	1.92 (0.22)	0.19 (0.026)	0.11 (0.021)
	8.2	1.05 (0.07)	12.7 (0.7)	2.59 (0.21)	0.31 (0.040)	0.14 (0.038)
	7.6	0.26 (0.13)	-0.57 (0.23)	2.35 (0.19)	1.47 (0.76)	0.53 (0.18)

* The italicized data in parentheses represent standard error (from regression and its propagation from calculation), and data in bold represent conditions with normal GSH excretion rates.

† The negative logarithmic concentration of free Cu in nmol L⁻¹, calculated using Mineql+ (Schecher and Mcavoy 1992).

‡ *T. weissflogii* specific growth rate.

§ Flask volume-based cellular GSH accumulation rate.

|| Flask volume-based extracellular GSH release rate after 3 d growth.

¶ The average cell number-based GSH release rate ($R = V/N_{av}$, where average cell density, N_{av} , is obtained according to Eq. 1).

The specific extracellular release rate of GSH, equal to R divided by cell number-averaged GSH quota.

(without a synthetic ligand such as EDTA) have also been effectively applied in several studies of release of metal-complexing ligands from algae after careful application of trace metal clean techniques (Moffett and Brand 1996; Leal et al. 1999). The pros and cons of each medium were discussed in detail by Gerringa et al. (2000). In the metal-buffered media, "free" or ionic metal concentrations are regulated by the excess amount of synthetic ligand present, but the metal association with organic ligands released from algae during growth can be masked by the synthetic ligands (e.g., EDTA). The maintenance of a specific ionic metal level is more challenging when an unbuffered medium is used; however, the additional complexity of ligand-induced metal reduction and uptake is eliminated in these systems (Hudson et al. 1992; Hudson 1998). Consequently, direct comparison of algal chemical and biochemical responses to metal exposure in these two types of growth media are needed, especially in studies in which the role of synthetic ligands on metal-complexing ligand excretion is involved.

In this article we will present results from bioassay experiments using a coastal diatom, *Thalassiosira weissflogii*, results that document (1) the physiological response of the diatom to copper exposure in terms of photosynthetic pigments and intracellular thiols, (2) the induction and release of essential thiols from the diatom cells and the implications of these processes, and (3) comparability of cell growth and excretion of metal-complexing ligands in the metal-buffered and metal-unbuffered media.

Materials and methods

Strict trace metal clean protocols were followed in all steps of the bioassay experiments. The diatom, *T. weissflogii* (CCMP 1336), was maintained in normal Aquil medium (Price et al. 1988/1989). Prior to the bioassay experiments, cells were transferred to a modified Aquil medium that lacked Cu, zinc (Zn), and EDTA, and cells were grown for 6 d, through the exponential phase. Cells were then inoculated at a rate of 1.5×10^6 cells L⁻¹ into a series of trace metal clean polycarbonate flasks containing fresh modified Aquil media and different levels of Cu (as the ⁶⁵Cu stable isotope) in the presence or absence of EDTA. The flasks had been pre-equilibrated with Aquil medium and the appropriate level of Cu. In flasks without added EDTA, Cu was added at the total Cu concentration levels of 0, 26, 76, 141, and 221 nmol L⁻¹, and the resulting free-Cu concentrations are listed in Table 1. The residual EDTA concentration was less than 0.5 nmol L⁻¹ after the two transfers. The EDTA-buffered culture of pCu = 13.8 received 5 μ mol L⁻¹ of EDTA and no additional Cu was added; for other cultures in this series, the total Cu concentration was maintained at 5 μ mol L⁻¹, and the EDTA concentrations decreased in the order of 40.0, 6.5, 5.0, and 4.5 μ mol L⁻¹, resulting in declining pCu values. The free-Cu concentration is about 94% of the total inorganically complexed (Cu'), resulting in pCu' = pCu - 1.2 in this experiment. Cells were grown under continuous illumination (150 μ mol quanta m⁻² s⁻¹) at 20 \pm 1°C in a

HEPA-filtered, polycarbonate culturing facility. The cells were allowed to grow for 9 d, covering both the exponential phase (samples from earlier than day 6) and the stationary phase (samples from days 6 and 9), and were harvested at designated times under gentle vacuum filtration. Samples for cellular pigments and thiols were collected on precombusted GF/F filters, and those for trace metals were collected on acid-cleaned 25-mm, 2- μm -porosity Teflon filters (Savillex). The major pigments were quantified using high-performance liquid chromatography (HPLC) after acetone extraction (Hurley and Garrison 1993). Cellular thiols were quantified using HPLC after sonication extraction and monobromobimane derivatization (Rijstenbil and Wijnholds 1996). The dissolved thiols in the filtrates (growth media) were analyzed using the HPLC monobromobimane fluorescence detection technique (Tang et al. 2003). Instead of the lengthy solid-phase extraction step, direct injection of a large volume (1.5 ml) of extract was employed to shorten the analysis time. Overall precision of the thiol measurements are in the range of 5–6% for GSH concentrations between 1 and 20 nmol L⁻¹ and in the range of 10–15% for PC-2 concentrations between 0.1 and 5 nmol L⁻¹ (Tang et al. 2003). Cells were enumerated using a Beckman Coulter EPICS XL flow cytometer against standardized count beads. A SYTOX Green assay was used to quantify the dead/live cell ratios (Franklin et al. 2001).

Results and discussion

Cell growth—A normal, optimal growth rate of *T. weissflogii* was achieved in both media when pCu was greater than 8.2 (pCu' > 7.0; i.e., approximately 80 nmol L⁻¹ of total inorganic Cu)(Table 1). At similar pCu, *T. weissflogii* growth rates in the two different media were comparable. However, at higher Cu additions, cell growth was suppressed, indicating a toxic effect of Cu on cell growth. Significantly lower growth was also observed at pCu = 10.9, where a much higher level of EDTA (40 $\mu\text{mol L}^{-1}$) than of Cu (5 $\mu\text{mol L}^{-1}$) was used to lower the “free”-Cu concentration. It is likely that cell growth was trace-metal limited (especially Zn) at this high EDTA level in the Zn-deficient media. Analysis of nutrient trace element concentrations in the algal cells by high-resolution inductively-coupled plasma mass spectrometry (HR-ICP-MS) supports our hypothesis that Zn limitation was likely the primary reason for the anomalous growth behavior. Cellular levels of Zn in the EDTA-unbuffered treatment averaged $24.4 \pm 5.7 \text{ fg cell}^{-1}$ across the various Cu levels at 30 h. In the EDTA-buffered treatments of 6.5 and 5.0 $\mu\text{mol L}^{-1}$ EDTA (pCu = 9.5 and 8.2, respectively), the Zn content of the cells averaged $5.2 \pm 1.2 \text{ fg cell}^{-1}$. However, at pCu = 10.9 (where an EDTA level of 40 $\mu\text{mol L}^{-1}$ was used), the Zn concentration in the cells was only 0.7 fg cell⁻¹ (0.011 fmol cell⁻¹), a level very likely to be limiting. Molar cellular iron (Fe):Zn ratios in the EDTA-buffered treatments exhibiting normal growth averaged 2.4, a ratio nearly identical to the 2.3 ratio reported by Ho et al. (2003) for *T. weissflogii* grown under similar conditions. In the anomalous treatment, however, the measured Fe:Zn ratio was 78, again indicating Zn limitation.

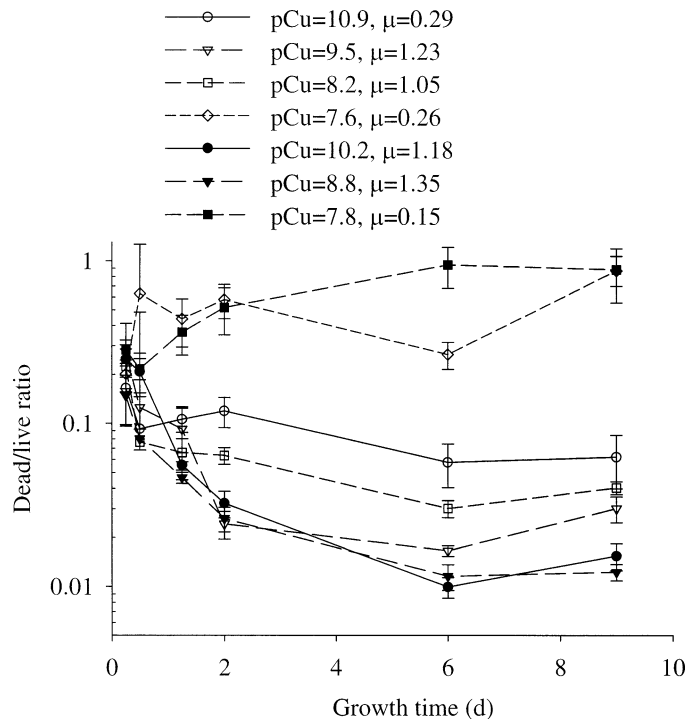


Fig. 1. The dead:live cell ratios during cell growth. Filled symbols represent EDTA-unbuffered cultures and unfilled symbols represent EDTA-buffered cultures. Error bars are calculated from standard deviation of three measurements.

Cell viability—A flow-cytometric assay was used to probe the cell viability (Franklin et al. 2001). In this method, SYTOX Green penetrates damaged plasma membranes of dead cells, producing green fluorescent staining of nucleic acids; however, live cells with intact membranes are unstained. The measured dead:live cell ratios are plotted in Fig. 1 for the different Cu levels during the growth of *T. weissflogii*. Note that in this figure, only data from selected Cu levels are plotted in order to compare the viability of *T. weissflogii* growing in EDTA-buffered and unbuffered media. In general, the dead:live cell ratio in the experimental flasks decreased with time, reflecting the continuous adaptation of cells to the growth media. At the end of exponential growth, less than 10% dead cell contribution was observed in cultures at lower Cu additions (at pCu \geq 8.2); however, notably higher ratios were evident at the highest Cu additions in both media, indicating extensive cell death at much lower growth rate (Table 1). The typically higher dead:live ratios observed early in the experiment likely reflect transfer and inoculation stresses, and the decreasing trend with time principally implies growth dilution by healthy cells over several generations. The cells grown in the EDTA-buffered medium (unfilled symbols) showed slightly higher dead:live ratios than were observed in the EDTA-unbuffered medium (filled symbols in Fig. 1) at similar levels of inorganic Cu. Differences in cell viability between EDTA-buffered and EDTA-unbuffered media indicate that EDTA-complexed Cu species can induce additional membrane damage and/or other toxic effects, even though total inorganic Cu concentrations are well within the range for normal cell growth. The EDTA-com-

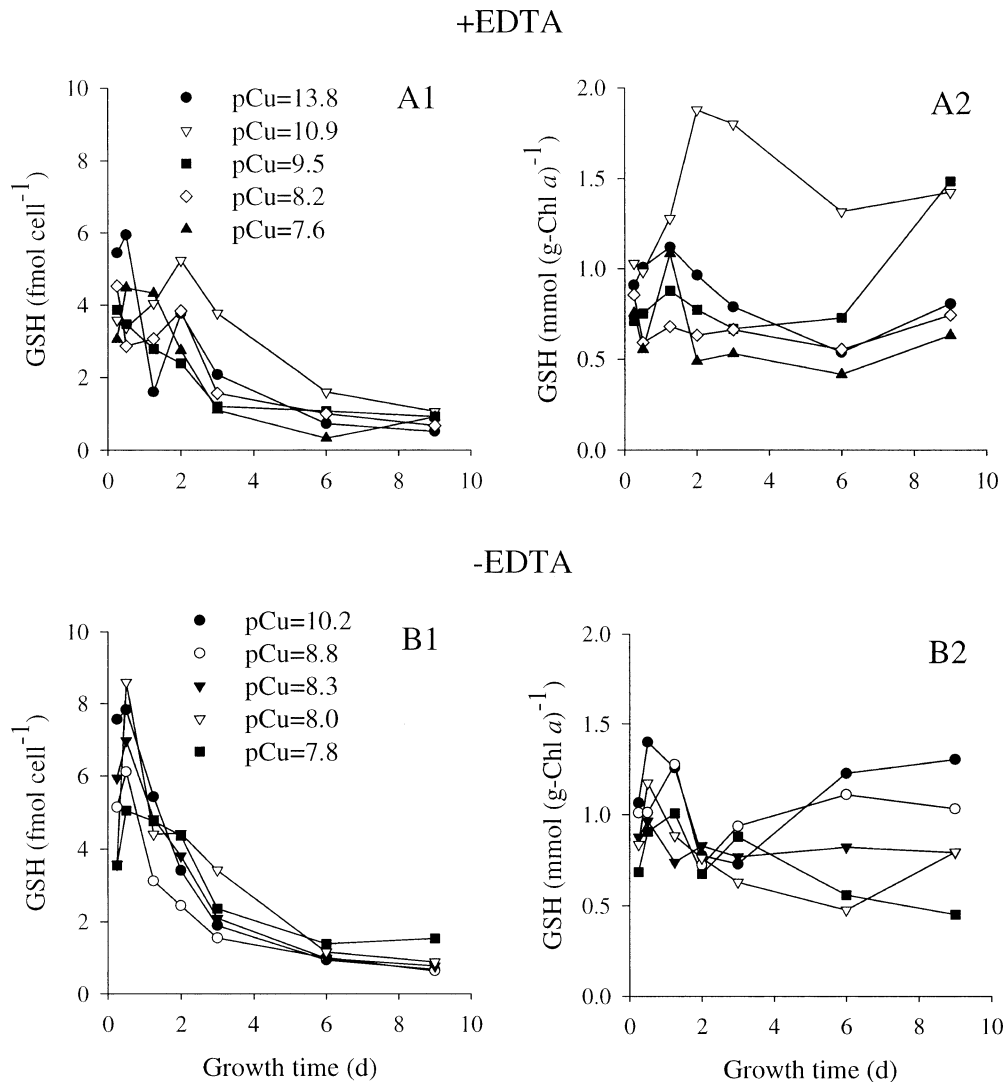


Fig. 2. Time course of glutathione quotas in both media. (A) EDTA-buffered; (B) EDTA-unbuffered; 1 = cell normalized; 2 = chlorophyll normalized.

plexed Cu(II) species has been shown by Jones et al. (1985) to be reduced by plasmalemma redox enzymes and surface redox active sites. The total Cu uptake appears to be enhanced by the EDTA-mediated uptake, in comparison with that of other trace metals (Hudson 1998). The reduction of Cu(II) and ensuing formation of oxidative species could then lead to harmful effects including lipid oxidation, protein fragmentation, and DNA damage (Kohen and Nyska 2002).

Interestingly, in the presumptive metal-limited culture (trace levels of Zn; EDTA-buffered) at pCu = 10.9, a specific growth rate of 0.29 d⁻¹ was observed, similar to that noted in the culture with extensive death (0.26 d⁻¹ at pCu = 7.6). The dead:live cell ratio, however, fell within the range observed for healthy cells (less than 0.1, but slightly higher than that of the average healthy cell). This confirms that these cells are stressed, but not necessarily dead, and that the slower growth rate resulted from a fundamentally different mechanism than that operating in the dying cultures at high Cu exposures.

Cellular thiols—The cell number-normalized quota of GSH (Fig. 2) decreased with growth time in both media, although slightly higher quotas were found in EDTA-unbuffered medium (Fig. 2B1) than in EDTA-buffered medium (Fig. 2A1). The average GSH quotas decreased from about 4 and 6 fmol cell⁻¹ in the EDTA-buffered and unbuffered medium, respectively, in the early exponential phase to about 1 fmol cell⁻¹ at the stationary phase in both cases. The cell number-normalized concentrations of different major pigments (including chlorophyll *a* [Chl *a*], fucoxanthin, and diadinoxanthin) also decreased with growth time (data not shown). When the GSH cell quotas are normalized to Chl *a*, the growth time dependence largely disappears, with normalized values in the range of 0.5 and 1.5 mmol GSH (g-Chl *a*)⁻¹, similar to those reported by Ahner et al. (2002). With few exceptions, GSH quotas (chlorophyll normalized) in cultures with suppressed growth rate (at pCu < 8.2) were slightly lower in both media than in the normally growing cultures (at pCu ≥ 8.2), reflecting an inhibition of the pho-

tosynthetic activity in cells with a reduced viability relative to that of healthy cells (Veldhuis et al. 2001). For normally growing phytoplankton species, studies have shown that Chl *a*-normalized GSH levels are constrained in a narrow range when cells are harvested near the end of the exponential growth period (Ahner et al. 2002). However, much greater variance is observed if GSH levels are normalized to biovolume (Rijstenbil and Wijnholds 1996). The constrained Chl *a*-normalized glutathione levels we measured during growth are consistent with the essential roles of GSH in cell homeostasis.

An exception to the general trend in GSH levels was observed in the EDTA-buffered culture at pCu = 10.9. Significantly higher GSH:Chl *a* ratios were found (Fig. 2A2) in comparison with values obtained from other EDTA-buffered treatments. We suggested that these slow-growing but viable cells (Fig. 1) were Zn limited (pZn' = 12.1). The lack of essential metals can cause oxidative stress, as is the case with Fe deficiency, which limits the electron transfer efficiency during photosynthesis (Sunda et al. 2002). We hypothesize a similar phenomenon resulting from low Zn availability, in turn resulting in an accumulation of intracellular GSH in response to oxidative stress. Cellular Cu concentrations, however, were not high enough to promote hydroxyl radical formation and subsequent cell death, as was the case for normally growing cells (at pCu \geq 8.2) in both media (Fig. 1).

Concentrations of other nonprotein thiols were also observed to change during cell growth. For cysteine (Cys), we found a gradual increase in the ratio of Cys to GSH, up to a value of \sim 0.1 (with time) in both media. Significantly higher Cys:GSH ratios were found for dying cultures in all phases of growth (data not shown). This indicates that the toxic Cu exposure induced more cysteine synthesis and/or impaired other metabolic processes that require cysteine as a substrate. When cysteine homeostasis is disrupted, the accumulation of cellular cysteine may act as a reductant, which in turn promotes oxidative DNA damage (Park and Imlay 2003). The ratio of γ -glutamyl-cysteine (γ EC) to GSH in both EDTA-buffered and unbuffered cultures varied only slightly at different Cu exposure (data not shown).

Phytochelatin-2 (PC2) was generally present at less than 2.5% of GSH in exponentially growing cultures, with a slightly higher ratio observed in the dying cultures (Fig. 3A,B) at high Cu additions, which is indicative of considerable cytoplasmic exposure and cellular toxicity. These results are in agreement with published data for low Cu exposures (less than 2% calculated for *T. weissflogii*, from Ahner et al. 2002). At a given level of Cu, our measured PC2:GSH ratios typically showed a relatively small progressive increase with growth time in EDTA-unbuffered medium (Fig. 3B); however, the ratios were considerably more variable in EDTA-buffered medium (Fig. 3A). For example, at pCu = 8.2 and 9.5, PC2:GSH values were even higher than those observed in dying cultures and were much higher in the late exponential phase than in the early exponential phase. The somewhat higher dead:live cell ratios in EDTA-buffered growth medium (Fig. 1) are consistent with higher Cu stress, indicated here by elevated PC2:GSH values; however, these higher PC2:GSH ratios only occurred after 3 d

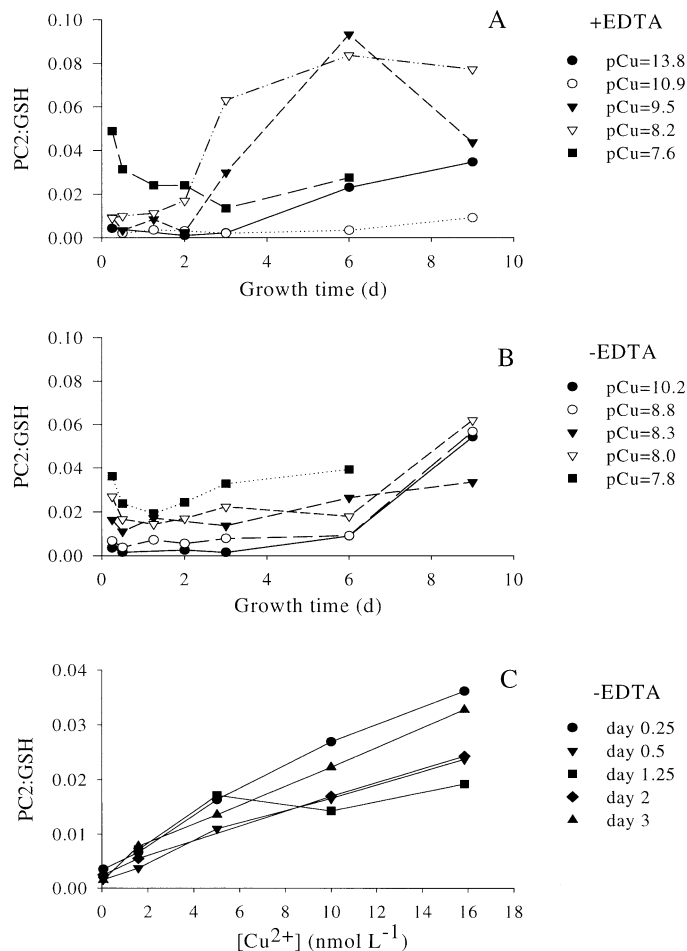


Fig. 3. Changes of phytochelatin-2:glutathione ratios during growth in (A) EDTA-buffered and (B) EDTA-unbuffered media, and (C) the dose-response of phytochelatin-2:glutathione ratios versus [Cu²⁺] during growth in EDTA-unbuffered medium.

during the exponential growth (Fig. 3A). Because phytochelatin production in response to Cu exposure is very fast for *T. weissflogii* (see below), the increase in PC2 production in EDTA-buffered medium in the late exponential growth remains to be explained.

A clear dose-response pattern of PC2 to Cu exposure is evident for the exponentially growing cultures. In EDTA-unbuffered medium, PC2:GSH ratios on a given harvesting day increased approximately linearly with free-Cu concentrations (Fig. 3C). The induction of PC2 was fast, with the highest PC2:GSH ratios observed after 6 h of exposure. This is consistent with the fast activation of enzymatic synthesis of phytochelatin by different trace metals (Grill et al. 1989; Cobbett 2000). The consistent response pattern of phytochelatin induction, such as that evident in Fig. 3C, is important if phytochelatin concentration levels are to be used as a proxy for "toxicity" in Cu bioavailability tests. The PC2:GSH ratios from EDTA-buffered experiments fall along the trend from EDTA-unbuffered medium, except for data points at pCu = 8.2 and 9.5 at days 3 and 6. This demonstrates a general comparability of dose-response patterns in both media, although cells growing in EDTA-buff-

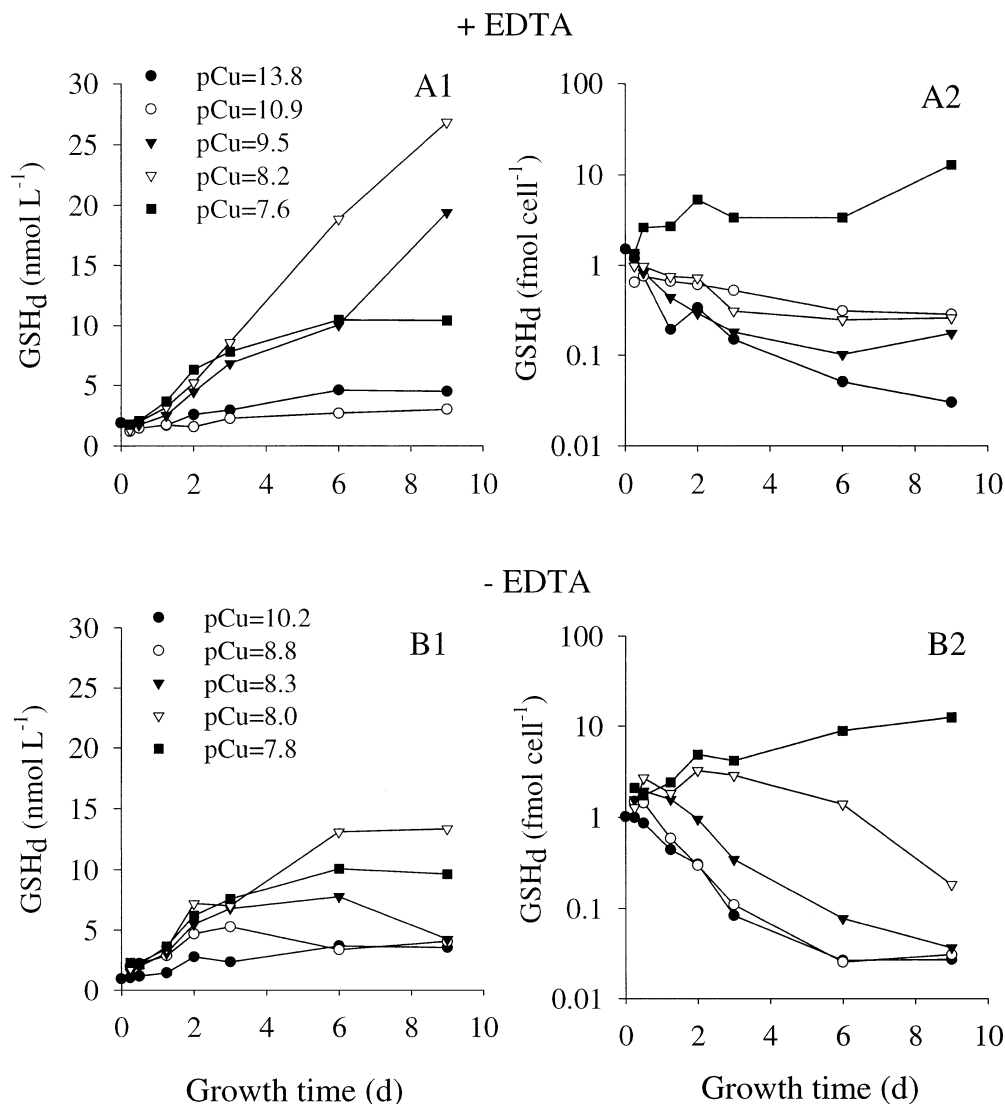


Fig. 4. Extracellular release of dissolved glutathione from *T. weissflogii* during growth. (A) EDTA-buffered; (B) EDTA-unbuffered. 1 = volume-based accumulation in flask (nmol L⁻¹) and 2 = cell number-normalized accumulation (fmol cell⁻¹).

ered medium might have accessed higher metal concentrations at similar free metal concentrations as a result of enzymatic interaction between EDTA-complexed species with cell surface enzymes or abiotic interaction with surface active sites (Jones et al. 1985, 1987). Further study is required to confirm these results and to elucidate those mechanisms leading to possible enhanced availability and cytotoxic effects of metals complexed by EDTA or other synthetic chelators. Nonetheless, these results point to the suitability of using PC2:GSH ratios in short-term toxicity tests in unbuffered medium.

Extracellular release of glutathione—Dissolved glutathione (expressed on a volume-based concentration, GSH_d, with a unit of nmole per liter of media), was detected in both EDTA-buffered and EDTA-unbuffered growth media; however, the dissolved phytochelators were not. In general, GSH_d increased with growth time until the late exponential phase

and leveled off in the stationary phase (Fig. 4A1,B1). GSH_d release profiles were similar in both media except for pCu = 9.5 and 8.2 in EDTA-buffered cultures, in which GSH_d levels continuously increased. It is important to note that at background levels of Cu (~1 nmol L⁻¹), GSH_d was also released from *T. weissflogii* and accumulated in both growth media at concentrations up to 4–5 nmol L⁻¹ at the end of the exponential growth phase. This result was not unexpected given that phytoplankton excrete low levels of organic carbon under normal growth conditions (Bjornsen 1988; Baines and Pace 1991). The release of thiol compounds at background Cu levels has also been reported for *Emiliania huxleyi*; furthermore, the excretion of thiols under Cu stress increased with concentrations of Cu, representing a significant fraction of detected Cu-complexing ligands (Leal et al. 1999; Vasconcelos et al. 2002). Our results are consistent with those of these studies, with GSH_d levels generally increasing with higher Cu concentrations. Dissolved

GSH concentrations were typically lower than total inorganic Cu at high Cu exposures but were greater than Cu at background levels. In addition, the release of GSH depends on neither the presence nor absence of EDTA. The extracellular GSH release was decoupled from both the extent of Cu exposure and the presence/absence of synthetic ligands, indicating that the excretion of Cu-complexing compounds results from indirect responses of algae to Cu exposure.

The cell number-normalized extracellular GSH_d concentrations decreased with growth time in healthy cultures, and the opposite trend was found in dying cultures (Fig. 4A2,B2). The pattern of GSH_d release was similar in both media, although on a per-cell basis, it appears that slightly higher excretion occurred in the EDTA-buffered media than in the unbuffered media. However, the physical meaning of the cell number-normalized GSH_d levels in Fig. 4 is complicated by the fact that the dissolved GSH value represents an accrual of algal excretion from generations before harvesting, but cell density is a discrete property of the culture at the time of harvest. A GSH_d sample at generation n reflects the summation of excretion from $n-1$ generations (assuming conservative thiol behavior). The ratio between cell density at generation n and the generations prior ($\sum n-1$) is 2 at the beginning and approaches 1 later in exponential growth. Therefore, the ratio between GSH_d and cell density at a given time overestimates the excretion on a per-cell basis by a factor of between 1 and 2. This analysis has the effect of enhancing the resolution of the dying cultures, in which cell density change did not follow the aforementioned trend for exponentially growing cells, so that the release of GSH_d due to cell death is clearly distinguished in these figures.

Another way to frame the GSH excretion data is to present the extracellular release and cellular quotas of GSH in the context of the behavior of average cells. Although cell number-normalized GSH quotas decreased during growth (Fig. 2), the total cellular GSH increased as a result of the exponential increase of cell density. Cellular GSH increase rate (G) was thus obtained from the linear regression slope of a plot of total cellular GSH versus growth time, which increased with the specific growth rate (Table 1). From Fig. 4A1 and B1, the volume-based extracellular GSH release rate (V) can also be directly derived at each Cu level. Data shown in Table 1 were based on culture samples collected in the first 3 d of exponential growth, because cell densities after day 6 deviated slightly from the exponential line. It can be seen from Table 1 that the cellular GSH production rates in the cell cultures under both growth-limited and growth-suppressed conditions were much smaller than the rates obtained at normal growth conditions, where moderate extracellular GSH release rates were found. There is, however, no simple relationship between the specific growth rate (μ) and the culture-wise extracellular GSH release rate (V), because the latter is an average property of the culture, related to cell density, GSH quota, and cell physiological conditions. The average cell density (N_a) at any day T during growth can be obtained from Eq. 1:

$$N_a = \frac{1}{T} \int_0^T N(0)e^{\mu t} dt = \frac{N(T)(1 - e^{-\mu T})}{(\mu T)} \quad (1)$$

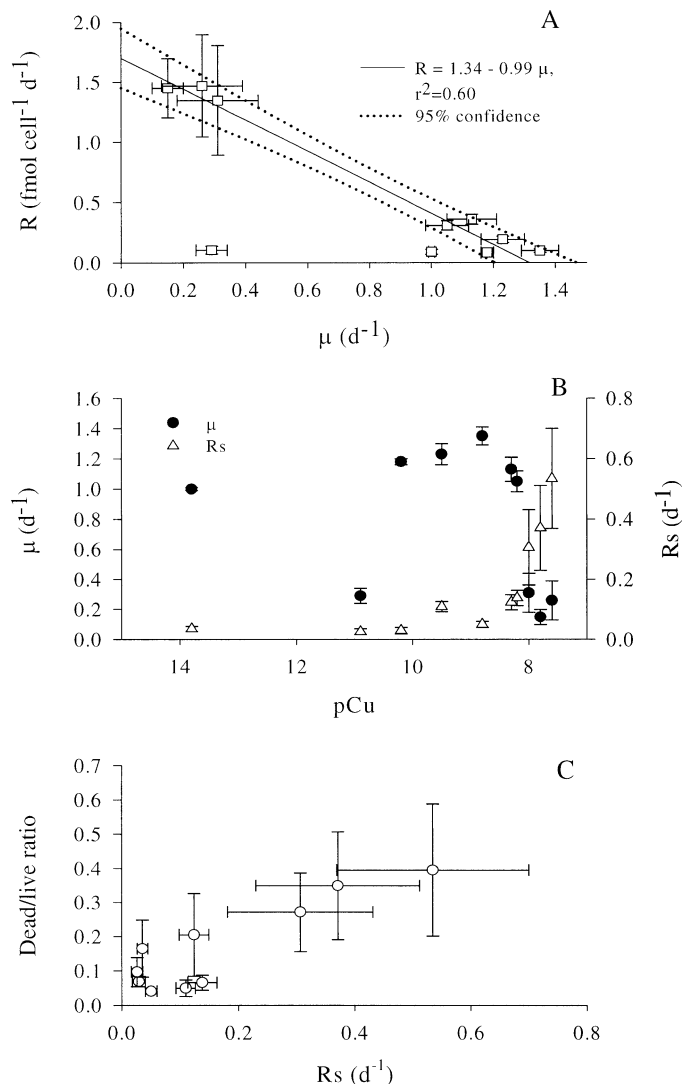


Fig. 5. Relationships between GSH-specific excretion (R_s , d^{-1}) or excretion rate (R , $fmol\ cell^{-1}\ d^{-1}$) and (A) cell growth rate (μ , d^{-1}); (B) Cu levels (pCu); and (C) dead:live cell ratio.

where $N(0)$ and $N(T)$ are the discrete cell densities at sampling time 0 and T day, respectively. The cell number-based release rate (R) is equal to $V:N_a$ with a unit of femto-mole GSH per cell per day. The specific release rate (R_s), with a unit of d^{-1} , is then given by the ratio between R and the cell number-averaged GSH quota. These descriptors are plotted in Fig. 5.

The GSH release rate on a per-cell basis (R) is shown to be negatively related to growth rate (Fig. 5A), with one exception for the growth-limited culture at pCu = 10.9 in EDTA-buffered medium, which was excluded from the linear regression. This implies that fast-growing cells excrete less GSH, which is consistent with data presented in Table 1, in which extracellular excretion in normally growing cultures was shown to be less than in growth-suppressed cultures. Although at Cu concentrations of pCu = 8.2 or lower, normal growth rates are obtained ($> 1\ d^{-1}$), there is already a large pool of extracellularly released GSH (R) resulting

from Cu exposures at $pCu \leq 8.8$ to $pCu = 8.3$, with an average value of 0.099 to 0.36 $\text{fmol cell}^{-1} \text{d}^{-1}$, respectively. Low values in both the cellular GSH accumulation rate (G) and the volume-based extracellular GSH release rate (V) are found in the culture at $pCu = 10.9$ in EDTA-buffered medium; however, the cell-based release rates of GSH (R or R_s) are similar to those observed in normally growing cells. This finding is consistent with the viability results (Fig. 1).

The average GSH quotas decrease from about 4 and 6 fmol cell^{-1} in the EDTA-buffered and unbuffered medium, respectively, in early exponential growth, to about 1 fmol cell^{-1} at the late exponential growth and the stationary phases (Fig. 2). Using the average release rate of 0.11 $\text{fmol cell}^{-1} \text{d}^{-1}$ for normally growing diatom cells with a specific growth rate of 1.2 d^{-1} , about 1.5–2.2% of cellular GSH is calculated to be excreted in the early exponential growth phase and 9.2% released in the late exponential phase. This demonstrates that not only can different compounds be released from algae (Zhou and Wangersky 1989; Rijstenbil and Geringa 2002) at different stages of growth, the same compound can be excreted at different rates as well. The GSH release rates estimated here fall within the generalized calculations of extracellular organic carbon release, with an average of 13% of total fixation (Baines and Pace 1991).

The negative relationship between GSH release and cell growth was closely related to Cu levels. The specific release rate (R_s) of GSH increased from about 0.03 d^{-1} at background Cu level to about 0.12 d^{-1} at Cu levels of $pCu \geq 8.3$. At higher Cu concentrations ($pCu < 8.2$), R_s abruptly increased, and, concomitantly, growth rate decreased dramatically (Fig. 5B). The higher extracellular release rate is closely related to the higher dead:live cell ratios in the cultures, reflecting the induced cell membrane damage from higher Cu levels in the medium (Fig. 5C). The permeability, indicated by Bjornsen (1988), of GSH across the cell membranes was thus enhanced at progressively higher Cu exposures. Although dissolved GSH is likely present as its disulfide and/or Cu-complexed species in synthetic culture media (Tang et al. 2003), the form of GSH excreted from algal cells is still uncertain. If cell membrane damage is the principal cause of increasing permeability, it is reasonable to assume that the dominant intracellular form of GSH (the free GSH) will be the major species excreted.

To refine our understanding of mechanisms responsible for the observed GSH release, we conducted an additional short-term exposure experiment (30 h) with *T. weissflogii* in the EDTA-unbuffered medium. We examined two sources of cellular GSH loss: diffusive (permeation) loss and that due to cell lysis, as a function of Cu exposure level. Culture flasks were inoculated to a cell density of $4 \times 10^6 \text{ cells L}^{-1}$, and Cu additions covered the range of 0 to 400 nmol L^{-1} . As shown in Fig. 6A, cells grew optimally, and the dead cell percentages were less than 1% of total at Cu levels lower than 100 nmol L^{-1} . At higher Cu levels, cell growth was slower, and significantly more dead cells were found. Cellular (particulate) GSH concentrations remained relatively constant with Cu level, with an average value of 1.89 fmol cell^{-1} (Fig. 6B). However, dissolved (extracellular) GSH increased dramatically at Cu concentrations higher than 75 nmol L^{-1} (Fig. 6B). These results are consistent with those

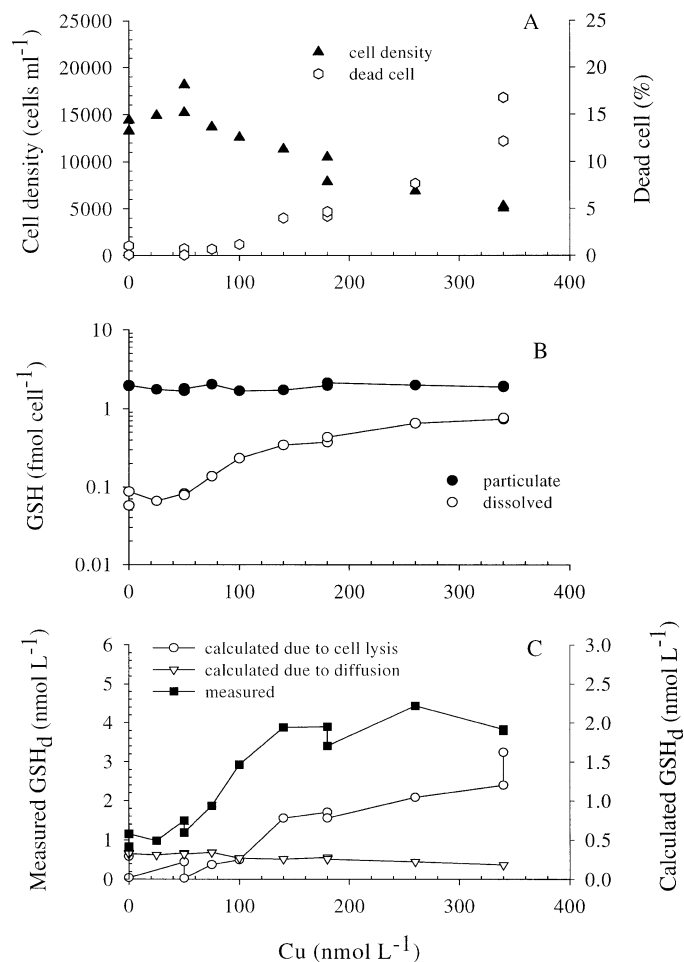


Fig. 6. Changes with the total Cu levels in EDTA-unbuffered medium for (A) cell density and percentage of dead cells, (B) particulate (cellular) production and dissolved (extracellular) release of GSH, and (C) the measured dissolved GSH and the estimated contribution from cell lysis and diffusion, in a 30-h exposure experiment of *T. weissflogii* (replicate analyses are shown at certain Cu levels).

previously discussed from the long-term cell growth experiments in both media (summarized in Table 1).

We now explore two possible extreme modes of GSH excretion, (i) that solely due to diffusion of GSH through the cell membranes into the growth medium and (ii) that solely due to cell lysis upon Cu exposure. In the first scenario, we assume that (a) the centric diatom (with a radius, r , of 6 μm) is growing exponentially with constant GSH quotas (Q) during the 30-h exposure, (b) intracellular cycling of GSH is sufficient to maintain similar concentrations of GSH at the cell surface and inside the cell, and (c) GSH diffuses into the medium with a diffusion coefficient (D) of $10^{-5} \text{ cm}^2 \text{ s}^{-1}$. The cumulative amount of GSH released from time 0 to time T can then be described by Eq. 2, which combines Eq. 1 for an average cell density with GSH surface concentration (C) and the steady-state Fick's radial diffusive flux ($=4\pi rDC$):

$$[\text{GSH}] = \int_0^T 4\pi rDCN(t) dt = 4\pi rDCN(0) \frac{(e^{\mu T} - 1)}{\mu} \quad (2)$$

In the second scenario, we assume that only dead cells (probed by the SYTOX Green assay) release their cellular GSH into the growth medium and that these cells are not growing at all. The calculated release of GSH into the growth media as a result of diffusion/permeation and cell lysis are shown as triangles and circles, respectively, in Fig. 6C (scaled by right-side y-axis). The estimated GSH release due to diffusion fails to describe the observed trend of GSH levels as a function of [Cu] (filled squares in Fig. 6C), likely because the GSH permeability through the cell membrane was implicitly assumed to be constant. However, diffusion of GSH does appear to be important at lower Cu exposure levels, and the predicted release could match that measured if D were increased by a factor of 3 to 5, which is not unreasonable. The estimated GSH release from cell lysis follows the overall measured trend, albeit at lower concentrations. To explain the measured GSH concentrations, it is obvious that GSH must be released from actively growing cells. What is less obvious is that Cu-induced changes in algal membrane permeabilities for GSH and SYTOX Green are apparently different. It appears that membrane damage enhanced the permeation of GSH and that the SYTOX Green assay captured the trend but not the absolute level of GSH permeation. Thus, cell viability as probed by the SYTOX Green assay can only be treated as a semiquantitative or relative indication of permeability changes for GSH. In yeast and plants, glutathione is not normally a passive permeable molecule; its exchange among different intracellular compartments and uptake from external media require specific plasma membrane transport systems (Foyer et al. 2001; Nocctor et al. 2002). Further studies are needed to show if some of these proteins are expressed or activated in algae and are in part responsible for the excretion of GSH and its conjugates. Data reported here support our contention that release of the essential compound GSH is actually decoupled from Cu exposure, in that at lower Cu levels, passive leakage from cells is a significant, if not primary, mechanism of dissolved GSH release, while at higher Cu exposure, Cu-induced membrane damage enhances the excretion.

In summary, we found that, with certain exceptions, cell growth, intracellular thiol induction, and extracellular GSH release were comparable at similar Cu exposure levels in both the EDTA-buffered and unbuffered medium. These findings substantiate the premise that algae respond nearly exclusively to inorganic species of Cu. The cell quotas of thiols and Chl *a* both decreased with growth time, so that Chl *a*-normalized cellular thiol concentrations were more or less conservative, with normalized values in the range of 0.5 and 1.5 mmol GSH (g-Chl *a*)⁻¹. A clear dose-response relationship was observed between PC2:GSH ratio and inorganic Cu levels in EDTA-unbuffered medium, regardless of growth time; however, a more complicated pattern was observed in EDTA-buffered medium. GSH was released from the phytoplankton cells at generally similar concentrations into both the EDTA-buffered and unbuffered growth media—so it does not appear that the presence of a synthetic metal chelator substantially affects thiol release. There is, however, an indication that EDTA possibly enhanced the release of GSH, as indicated by the higher dead:live cell ratios under EDTA-replete conditions.

We also demonstrated that the release of GSH from the diatom and Cu exposure are in some sense decoupled. For normally growing cells, about 1.5–2.2% of cellular GSH is released in the early exponential growth phase and 9.2% released in the late exponential phase, with the difference likely reflecting physiological changes during growth (mainly the decrease of cellular GSH quota). At background Cu conditions (~1 nmol L⁻¹), glutathione was excreted at an average rate of 0.087 fmol cell⁻¹ d⁻¹ in both media, but release increased with increasing inorganic Cu concentrations. At the elevated Cu exposures (total inorganic [Cu] > 100 nmol L⁻¹), substantially greater amounts of GSH were released, corresponding to the changes of the cell membrane integrity. Thus, the Cu-enhanced release of glutathione into ambient waters is probably an inadvertent by-product of cell membrane damage by Cu rather than a feedback mechanism to control the speciation (and toxicity) of aqueous Cu. However, at background levels of Cu, glutathione released from algae could contribute a significant portion of the Cu-complexing ligands in oceanic waters.

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