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Diurnal cycling of glutathione in marine phytoplankton: Field and culture studies

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Abstract

Glutathione is an abundant intracellular thiol that is involved in the detoxification of reactive oxygen species generated in the presence of light. We used short-term continuous cultures of *Emiliania huxleyi* and *Thalassiosira pseudonana* to show that a diurnal light cycle causes intracellular glutathione to undergo at least twofold variations, with maximum concentrations occurring during light periods. Cysteine concentrations also appear to vary in *E. huxleyi* to a similar extent, with the highest concentrations occurring at night. A complementary field study conducted at Salt Pond in Falmouth, Massachusetts, yielded a similar diurnal cycle of glutathione concentrations in particulate samples. This cycling is important to consider when measuring particulate thiols in both culture and field studies and provides additional evidence that glutathione plays an important role as an antioxidant in eukaryotic marine phytoplankton.

Glutathione (γ-glutamylcysteinylglycine) is found in all eukaryotes and some prokaryotes, including cyanobacteria and purple bacteria (Newton et al. 1996). It is often the most abundant small peptide and intracellular thiol. Concentrations are typically in the millimolar range and can account for up to 90% of nonprotein thiols in plants and algae (Rennenberg 1982; Rennenberg and Lamoureux 1990; Ahner et al. 2002). Glutathione has several physiological roles (Fig. 1); therefore, several environmental variables might control glutathione levels. One of the major roles of glutathione is the detoxification of reactive oxygen species (ROS) in the chloroplast, where it acts as an intermediate in the removal of superoxide radicals that can be generated during light saturation of photosynthesis (Polle and Rennenberg 1992). Since this stress is dependent upon light, intracellular concentrations of glutathione might be expected to vary with light intensity and duration in marine phytoplankton.

There are several lines of evidence in the literature on which we formed this hypothesis. Three species of mixotrophic algae, when grown in the presence of light, contained twofold to fivefold more glutathione than when grown heterotrophically in the dark (Fahey et al. 1987). Matrai and Vetter (1988) reported decreases in glutathione concentrations in natural assemblages of coastal phytoplankton following 24 h of dark incubation. Perhaps most importantly, Schupp and Rennenberg (1988) showed that glutathione in pine needles (*Picea abies*) followed a diurnal cycle with high concentrations during midday and low concentrations during the night.

More recently, investigators interested in the potential role of thiols as metal ligands in seawater have measured glutathione and other thiols in the field (Le Gall and van den Berg 1998; Tang et al. 2000; Al-Farawati and van den Berg 2001). Glutathione chelates heavy metals such as Cu, Cd, and Pb through sulfhydryl coordination (Rabenstein 1989) and may form strong Cu (I) complexes in seawater (Leal and van den Berg 1998). Marine phytoplankton are likely to be one of the important sources of low molecular weight thiols such as glutathione in surface seawater, since thiols have been shown to correlate well with Chl *a* levels in coastal seawater (Al-Farawati and van den Berg 2001). Thus, it is important to understand possible controls on the intracellular concentrations of these compounds in marine algae.

In this study, we examined the effects of diurnal light cycles on the intracellular concentrations of the low molecular weight thiols glutathione and cysteine in phytoplankton cultures. We exposed short-term continuous cultures of *Emiliania huxleyi*, a cosmopolitan open ocean coccolithophore, and *Thalassiosira pseudonana*, a coastal diatom, to diurnal light cycles while measuring particulate thiols. Particulate thiols were also monitored over a 24-h period in Salt Pond, a pristine inlet on the south side of Cape Cod in Falmouth, Massachusetts.

Methods

Axenic cultures of *E. huxleyi* (Provasoli-Guillard National Center, CCMP 373) and *T. pseudonana* (CCMP 1336) were grown in Aquil, a synthetic seawater medium developed for trace metal nutrition and toxicity studies (Price et al. 1988/1989), in a plant growth chamber equipped with sodium halide lights to provide a 16 : 8 h light–dark cycle. All containers in contact with the culture media were acid-washed clear polycarbonate, and techniques to minimize trace metal contamination were used throughout. The cultures were grown in a polycarbonate carboy (total liquid volume 10 liters for *E. huxleyi* and 2 liters for *T. pseudonana*) immersed in a fixed temperature water bath (19°C), and agitated by an overhead mixer fitted with a Teflon propeller set at low speed to maintain a well-mixed culture. Growth was monitored by measuring culture fluorescence (Turner Instruments) as a proxy for Chl *a* (Brand et al. 1986). Each species was started

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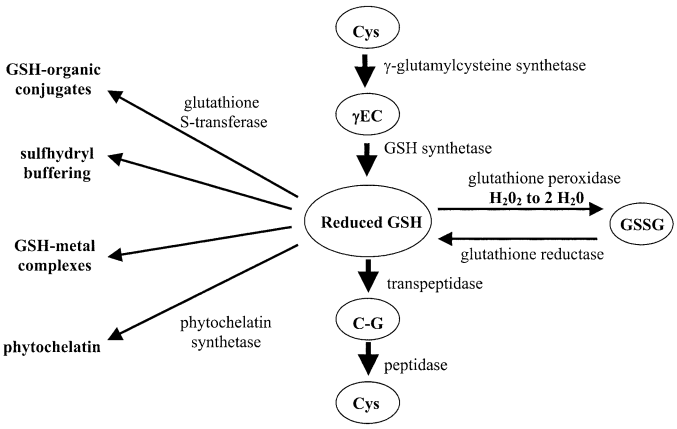

Fig. 1. The multiple physiological roles of glutathione (GSH) and the synthesis and degradation pathways are shown. Conjugation to xenobiotics in secondary metabolism, maintenance of reduced sulfhydryl groups within intracellular proteins, intracellular metal buffering, precursor to the metal binding peptide phytochelatin, and through the action of glutathione peroxidase, detoxification of ROS. GSSG is oxidized glutathione, Cys is cysteine, γEC is γ-glutamate-cysteine, C-G is cysteine–glycine.

as a batch culture, and when midexponential growth was reached a peristaltic pump was used to withdraw medium from the reactor (1.1 and 0.6 ml min^{-1} for *E. huxleyi* and *T. psuedonana* experiments, respectively), while fresh medium was drawn at the same rate into the reactor via hydrostatic force, creating what we have called a short-term continuous culture because our system never reached continuity. Samples for thiol analysis and Chl *a* content were collected from a container receiving the outflow from the culture vessel. Both medium and outflow collecting containers were in the constant temperature bath, and axenic sampling techniques were used to withdraw samples from the outflow container. Because the dilution rate was very low, culture conditions in the outflow container essentially paralleled those in the main carboy.

For the *E. huxleyi* experiment, samples were taken every 3–4 h for 65 h. Light levels were reduced to 110 μmol photons m^{-2} s^{-1} inside the carboy with neutral density screening since cells did not grow well in full light (light was measured in the middle of an empty carboy). On the third day of sampling following 6 h of light, the culture vessel was darkened with black plastic. For the *T. pseudonana* experiment, samples were taken every 4–5 h for 31 h beginning at 0100 h, 4 h into the dark period. Light levels inside the unshaded carboy were 400 μmol photons m^{-2} s^{-1}. Light levels experienced by the algae during the experiments were less than the reported values since light is attenuated by the culture medium.

At each time point three 50-ml and three 5-ml aliquots of culture were gently (<5 psi) filtered onto a Whatman GF/F filters for measurements of particulate thiols and Chl *a*, respectively. Filters were immediately stored in liquid nitrogen until extraction and analysis. Chl *a* was measured using fluorescence (Turner Instruments) following GF/F filter extraction in the dark for 4–6 h at room temperature in a solution of 45% : 45% : 10% dimethyl sulfoxide : acetone : water with 0.1% diethylamine (Shoaf and Lium 1976). The fluorometer was calibrated using a spectrophotometer and a standard dilution curve of Chl *a* extracted from spinach. Duplicate 1-ml samples were also taken at each time point and fixed with Lugol's solution, and cell density was estimated by cell counts made with a Levi haemocytometer (Sournia 1978).

To analyze particulate thiols, sample filters were heated to 70°C in 10 mmol L^{-1} methanosulfonic acid (MSA) for 2 min and then homogenized on ice as described elsewhere (Ahner et al. 1994). The MSA extract was retained for derivatization with the fluorescent tag monobromobimane (Ahner et al. 2002).

Derivatized homogenate was then analyzed on a Beckman high-performance liquid chromatograph (HPLC) equipped with a reversed-phase C-16 amide column (4.6 × 250 mm, Supelco Discovery) and a 100-μl injection loop. Compounds were quantified postcolumn using fluorescence detection (Gilson, excitation 310–410 nm, emission 475–650 nm). The elution gradient and buffers used are described elsewhere (method C; Wei et al. 2003). Stock solutions of cysteine and glutathione (Sigma) were used to develop standard curves for peak area calibration and to verify elution times. For both glutathione and cysteine, the detection limit is about 200 femtomoles per injection.

Salt Pond, Falmouth, Massachusetts, was chosen as a location for a field study because it is closed off from Vineyard Sound, minimizing tidal effects, and there is minimal influence from freshwater sources, thus little salinity gradient. In the summer, it is also a stratified body of water with a stable water column (Wakeham et al. 1987) and is easily sampled on a 24-h basis. Field samples were collected on 4 August 2002 at 1.5 m depth using a 3-m pole sampler and an acid-washed polycarbonate container. The water temperatures varied from 23°C to 27°C during the 24-h sampling period with temperatures peaking at 1600 h. The sky was clear, and the sampling area was in the sun from sunup to sundown (~0600 to 2100 h). Duplicate water samples were immediately filtered (<5 psi) onto Whatman GF/F filters and stored in liquid nitrogen until extraction of Chl *a* and particulate thiols that were then analyzed as previously described.

In order to eliminate temperature as a potential variable, an opaque polycarbonate bottle was partially filled with Salt Pond water at 1100 h, sealed, and left in shallow water for 2 h. At 1300 h, the temperature of the bottle and the surrounding waters was identical and both were sampled.

Results and discussion

For both laboratory experiments, the cultures were prepared as a hybrid of batch and continuous cultures that we have termed short-term continuous. This hybrid technique was used to extend the period of time during which the cultures were in exponential growth and to provide additional volume of culture from which to take the relatively large samples needed for thiol analysis. The dilution rate used during both experiments was lower than the growth rate. In the *E. huxleyi* experiment, a gradual increase in both cell density and extracted Chl *a* was observed over the 65-h sampling period (Fig. 2C). The specific growth rate, μ, was 0.30 d^{-1}

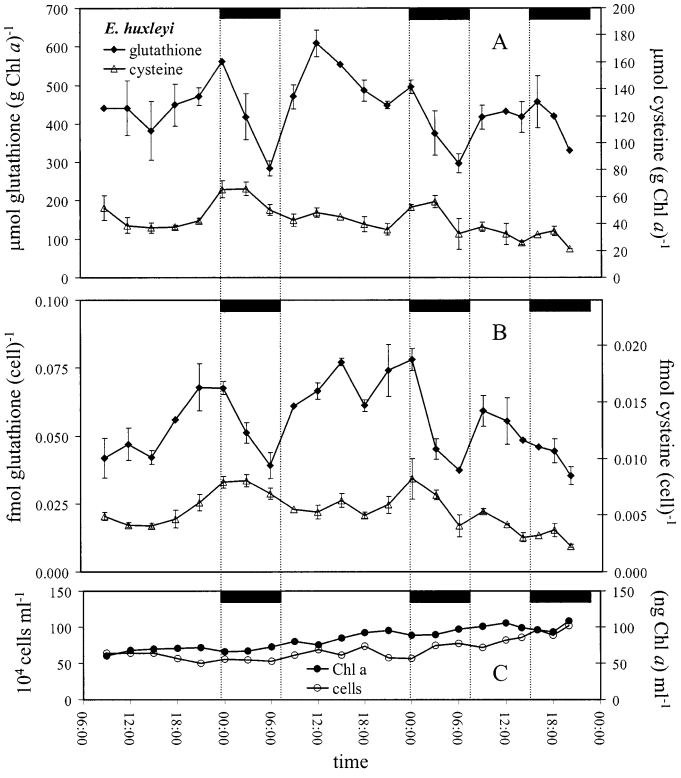

Fig. 2. Time course of intracellular glutathione and cysteine concentrations in a short-term continuous culture of *E. huxleyi* grown with a 16 : 8 h light–dark cycle. Data are normalized to (A) Chl *a* and (B) cell number. (C) Chl *a* concentration and cell numbers ml^{-1}. Solid bars signify dark periods. Error bars are the standard deviations of triplicate samples in panels A and B. The error bars for the cell counts and Chl *a* data are within the symbol size.

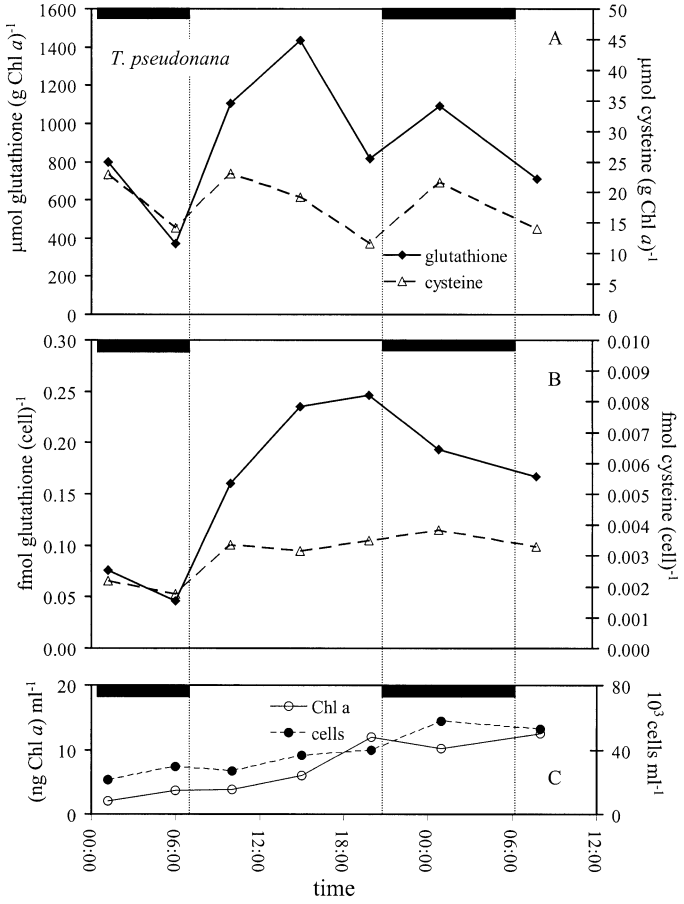

Fig. 3. Time course of intracellular glutathione and cysteine concentrations in a short-term continuous culture of *T. pseudonana* grown with a 16 : 8 h light–dark cycle. Data are normalized to (A) Chl *a* and (B) cell number. (C) Chl *a* concentration and cell numbers ml^{-1}. Solid bars signify dark periods. Duplicate samples were not taken for thiols, cell counts, or Chl *a*.

in the starting batch culture, whereas the dilution rate was 0.16 d^{-1} until $t = 48$ h, when the pump rate was increased to 1.5 ml min^{-1} to better match the dilution rate (0.22 d^{-1}) to the growth rate. The Chl *a* $cell^{-1}$ levels remained fairly constant over the course of the experiment, showing only minor variation (0.10–0.15 pg Chl *a* $cell^{-1}$, calculated from data in Fig. 2C) that did not follow a diurnal pattern. In the *T. pseudonana* experiment, the dilution rate (0.43 d^{-1}) was once again less than the specific growth rate of the algae (1.5 d^{-1}). Cell numbers increased gradually over the course of the experiment (net growth rate of 1.1 d^{-1}), whereas a large jump in Chl *a* occurred at the end of the photoperiod (Fig. 3C), presumably because of a light-induced change in Chl *a* $cell^{-1}$.

Light-mediated changes in intracellular concentrations of glutathione and cysteine were clearly evident in the *E. huxleyi* culture whether the data are normalized to Chl *a* or to cell number (Fig. 2A,B). Over the course of 2 d, the intracellular levels varied by a factor of 2 with a maximum of 600 μmol g^{-1} Chl *a* and a minimum of approximately 300 μmol g^{-1} Chl *a* (Fig. 2A). While the maximum did not occur at the same time both days, concentrations were generally higher during the light period. A sharp decline in glutathione consistently followed light cessation, and minimum levels always occurred at the end of the dark period. When the culture was darkened after 6 h of light on the third day, particulate glutathione levels fell from ~425 μmol g^{-1} Chl *a* to ~325 μmol g^{-1} Chl *a* in 6 h (Fig. 2A).

Cysteine concentrations in *E. huxleyi* exhibited twofold diurnal variations, but maximum concentrations occurred early in the dark period with a gradual decline that persisted until the onset of the next dark period (Fig. 2A,B). An increase was not observed following the truncated light period at the end of the experiment.

In experiments with *T. pseudonana*, even though sampling frequency was lower and we only sampled one light cycle, glutathione also appeared to be modulated by light. Concentrations increased while the lights were on and decreased while the lights were off (Fig. 3A,B). The concentrations varied fourfold when normalized to Chl *a* (Fig. 3A) and fivefold on a per-cell basis (Fig. 3B). The maximum occurred at 2000 h, near the end of the light period, while the minimum was near 0600 h, during both dark periods. No discernable diurnal pattern was evident for cysteine, though concentrations varied twofold throughout the experiment (from 0.002 to nearly 0.004 fmol $cell^{-1}$, Fig. 3B, and 10 to 20 μmol g^{-1} Chl *a*, Fig. 3A). Cysteine concentrations nor-

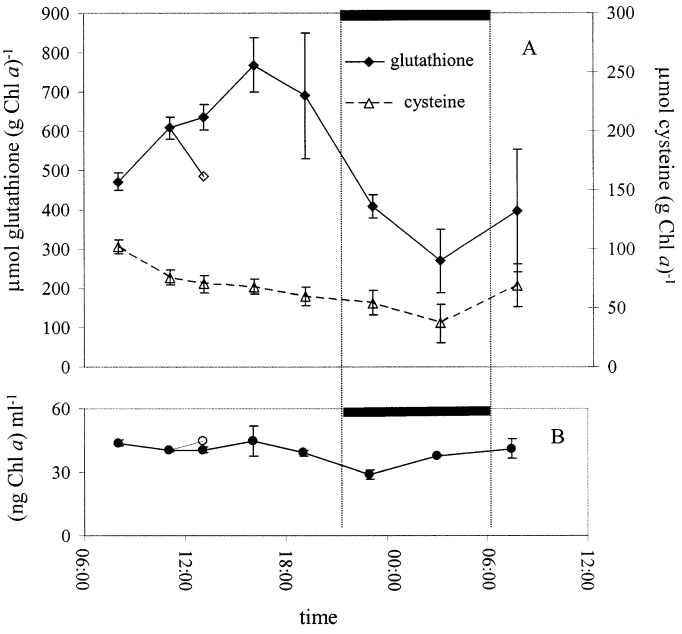

Fig. 4. (A) Time course of intracellular glutathione and cysteine concentrations (μmol g^{-1} Chl *a*) in a natural assemblage from Salt Pond over the course of 24 h. Solid bars show approximate night period (dusk to dawn). Open diamond at 1300 h is from a sample of seawater taken at 1100 h following incubation in an immersed darkened bottle for 2 h. (B) Chl *a* concentration. Open circle is the Chl *a* concentration in the darkened bottle. Where shown, error bars represent the range of duplicate measurements or are within the symbol.

malized to cell numbers were fairly constant following the first dark period (Fig. 3B).

Glutathione concentrations in the *T. pseudonana* cultures were similar in magnitude to those in *E. huxleyi* (Figs. 2A and 3A), but the range of fluctuation was greater perhaps because of the higher light levels in this experiment. Cysteine concentrations on a per-cell basis were slightly greater in *E. huxleyi* despite its smaller size and therefore when normalized to Chl *a* were about twofold to threefold greater compared to *T. pseudonana* (Figs. 2A and 3A). Ahner et al. (2002) found that, of five different algal species examined, *E. huxleyi* contained by far the most cysteine and γ-glu-cys, the intermediary between cysteine and glutathione. The physiological reason for this difference remains unknown, although it may be linked to the very high concentrations of dimethylsulfoniopropionate (DMSP) found in *E. huxleyi* (Sunda et al. 2002).

Remarkably similar results were obtained from the field samples, especially given the significant differences between the experimental conditions in the laboratory and the field (e.g., abrupt vs. gradual light changes, the presence of ultraviolet in sunlight and its absence in the laboratory). Over the course of 24 h, particulate glutathione in Salt Pond displayed a clear diurnal cycle similar to that observed in the culture experiments (Fig. 4A). Levels of glutathione (normalized to Chl *a*) were within the range of those measured in laboratory cultures. Glutathione concentrations gradually increased during the daylight hours (a maximum concentration of 780 μmol g^{-1} Chl *a* was observed at 1600 h) and decreased during the night (a minimum of 270 μmol g^{-1} Chl *a* was measured at 0300 h). Samples taken from a darkened bottle at midday exhibited a significant decline in particulate glutathione concentrations from 600 to 480 μmol g^{-1} Chl *a* in only 2 h, while in the sample taken concurrently from the ambient water the glutathione concentrations continued to increase (Fig. 4A). The cysteine concentration in the darkened bottle was indistinguishable from that in the ambient water. The observed diurnal cycle was not driven by Chl *a* normalization, since the Chl *a* was generally higher during the daylight hours and lower at night (Fig. 4B); normalization to Chl *a* actually serves to dampen the observed variation in total particulate glutathione concentrations if one assumes that algal biomass remains constant.

In the field, cysteine did not appear to cycle with light, but particulate concentrations in Salt Pond (50–100 μmol g^{-1} Chl *a*) were very high, even greater than those reported here for *E. huxleyi.* The high levels of cysteine in Salt Pond cannot be explained by *E. huxleyi,* since this species is generally found in the open ocean. It is interesting to note that Fahey et al. (1987) found that some cyanobacteria (including *Synechococcus lividus*) produce similar or even greater concentrations of cysteine compared to glutathione. It is possible that the elevated cysteine levels observed in Salt Pond were due to the presence of *Synechococcus* sp., which can account for up to 10% of the total Chl *a* in pristine harbors (Moffett et al. 1997). In addition, Wakeham et al. (1987) found significant numbers of purple and green bacteria near the depth from which our samples were taken; however, these organisms have fairly similar ratios of glutathione to cysteine compared to those reported here for marine eukaryotes (Fahey et al. 1987).

Other low molecular weight thiols are also important biochemically and are precursors in glutathione metabolism (Fig. 1). Cysteine (Cys) is a substrate for γ-glutamyl-cysteine synthetase, which forms γ-glutamyl-cysteine (γ-Glu-Cys). γ-Glu-Cys is then adjoined to glycine by glutathione synthetase to produce glutathione. Cysteine–glycine (Cys–Gly) and Cys are also generated during glutathione degradation by transpeptidase and peptidase, respectively (Meister and Anderson 1983). In the *E. huxleyi* experiment, concentrations of γ-Glu–Cys (50–100 μmol g^{-1} Chl *a*) and the newly identified thiols, Arg–Cys (50–120 μmol g^{-1} Chl *a*) and Gln–Cys (150–350 μmol g^{-1} Chl *a*; C.L.D. and B.A.A. unpubl. data) varied by as much as twofold but did not exhibit a consistent diurnal trend. Particulate Cys–Gly (10–20 μmol g^{-1} Chl *a*) and phytochelatin (n = 2; 30–55 μmol g^{-1} Chl *a*) were measured in the field experiments and, although variable, did not follow a diurnal cycle.

As observed in spruce needles (Schupp and Rennenberg 1988), cysteine did not respond to light cycles in *T. pseudonana* or the field. Cysteine did cycle in *E. huxleyi,* although the maximum levels often occurred at night. The peak of cysteine that occurs early in the dark period may be the result of sulfur assimilation in preparation for the synthesis of sulfur-containing proteins, a process that has been shown to occur at night in mixed assemblages in surface seawater (Cuhel et al. 1984). Since we were close to our detection limit for cysteine in the *T. pseudonana* experiment,

it is possible that we missed small yet significant changes in cysteine concentrations.

The variations in glutathione observed in the laboratory and the field are potentially driven by the need to scavenge H_2O_2 generated during photosynthesis saturation at high light levels. Superoxide dismutase (SOD) activity in the red alga *Gracilariopsis tenuifrons* also followed a diurnal cycle in response to visible light, presumably due to photoinduced ROS production (Rossa et al. 2002). The ratio of oxidized to reduced glutathione might also be expected to vary as a function of light (as well as glutathione reductase activity responsible for the cycling between these two pools, Fig. 1), but our analytic technique measures only the total glutathione, which includes both oxidized and reduced pools.

Another possible explanation for the observed diurnal fluctuations is that, to some extent, the cultures become synchronized with respect to cell cycle, a common phenomenon in phytoplankton (Nelson and Brand 1979). Glutathione concentrations in phytoplankton might be linked to this cycle; de novo synthesis of glutathione has been linked to cell cycle progression in mammalian cells (Poot et al. 1995). On the other hand, such diel periodicity in *E. huxleyi* is thought to be tied to a biological clock, as opposed to a light–dark cycle (Brand 1982). Therefore, if the observed diurnal variations were due to diel periodicity or synchronized cell division, then we would not expect the glutathione concentrations to decline when the light was attenuated early in both the *E. huxleyi* and the field experiments. Additionally, a common indicator of a synchronized cell cycle is a diurnal cycle in Chl *a* cell^{-1}, something we did not observe.

Multiple defenses against oxidative stress are common; chronic metal stress in algal chloroplasts leads to heightened SOD and ascorbate peroxidase activity and high glutathione content (Okamoto et al. 2001). The reduced sulfur compounds dimethyl sulfoxide (DMSO) and dimethyl sulfide (DMS) have recently been suggested to act as antioxidants in algae (Sunda et al. 2002), and our results suggest that glutathione adds to this defense in the case of light-induced stress. With increasing evidence that marine phytoplankton influence Cu speciation by exuding thiols such as Cys, Arg–Cys, and Gln–Cys into seawater (C.L.D. and B.A.A. unpubl. data), fieldwork on the presence of specific thiols in seawater should continue. Since the rate at which thiols may be released from the particulate to the dissolved phase through nonspecific means such as viral lysis or grazing is certainly influenced by intracellular concentrations, it is important to understand the various controls on this pool. It is also possible that intracellular pools influence the biologically controlled exudation of specific thiols.

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