Causes of Shear Sensitivity of the Toxic Dinoflagellate *Protoceratium reticulatum*

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DOI 10.1021/bp.161
Published online April 27, 2009 in Wiley InterScience (www.interscience.wiley.com).

Dinoflagellates have proven extremely difficult to culture because they are inhibited by low-level shear forces. Specific growth rate of the toxic dinoflagellate Protoceratium reticulatum *was greatly decreased compared with static control culture by intermittent exposure to a turbulent hydrodynamic environment with a bulk average shear rate that was as low as 0.3 s^{-1}. Hydrodynamic forces appeared to induce the production of reactive oxygen species (ROS) within the cells and this caused peroxidation of cellular lipids and ultimately cell damage. Exposure to damaging levels of shear rate correlated with the elevated level of lipoperoxides in the cells, but ROS levels measured directly by flow cytometry did not correlate with shear induced cell damage. This was apparently because the measured level of ROS could not distinguish between the ROS that are normally generated by photosynthesis and the additional ROS produced as a consequence of hydrodynamic shear forces. Continuously subjecting the cells to a bulk average shear rate value of about 0.3 s^{-1} for 24-h caused an elevation in the levels of chlorophyll a, peridinin and dinoxanthin, as the cells apparently attempted to counter the damaging effects of shear fields by producing pigments that are potential antioxidants. In static culture, limitation of carbon dioxide produced a small but measureable increase in ROS. The addition of ascorbic acid (0.1 mM) to the culture medium resulted in a significant protective effect on lipid peroxidation, allowing cells to grow under damaging levels of shear rates. This confirmed the use of antioxidant additives as an efficient strategy to counter the damaging effects of turbulence in photobioreactors where shear sensitive dinoflagellates are cultivated.*
 Biotechnol. Prog., 25: 792–800, 2009
Keywords: dinoflagellates, microalgae, lipoperoxide, oxidative stress, Protoceratium reticulatum*, shear sensitivity, turbulence*

Introduction

Dinoflagellates are mostly photosynthetic microalgae which belong to the class Dinophyceae. Many dinoflagellates are toxic at low concentrations, causing fish kills and poisonings of humans, marine birds, and mammals. For example, *Protoceratium reticulatum* produces yessotoxins.[1–6] *P. reticulatum* is widely associated with "red-tides," or red discoloration of water caused by algal blooms.[7] Marine and freshwater dinoflagellates are potentially useful for commercial production of various toxins and other compounds for research.[8]

Although microalgae have been successfully cultured in photobioreactors for producing numerous products,[9–11] dinoflagellates have proven exceptionally difficult to culture[8] because they are extraordinarily sensitive to turbulence-associated shear stresses[8,12–16] that occur in culture devices. An exception is *Crypthecodinium cohnii*, Hu et al.[17] have shown that this obligate heterotrophic dinoflagellate can sustain energy dissipation rates of up to 5.8×10^7 W m^{-3} (5.8×10^5 cm^{-2} s^{-3}) without lysis, although an evidently sublethal loss of flagella was observed at lower energy dissipation levels.

Data published since 1975 on shear sensitivity of dinoflagellates have been comprehensively reviewed.[18] All evidence suggests that dinoflagellates are affected by small-scale turbulence, at least the turbulence levels that are within the realm of what occurs in nature. In 28 out of 39 studies, where the bulk specific energy dissipation rate (ε) was measured or estimated, growth inhibition or cell death were observed in the turbulence regimen such that $0.011 \leq \varepsilon \leq 10$ cm^2 s^{-3}. Only two of these studies were carried out at energy dissipation rate value of above 10 cm^2 s^{-3}. In seven studies, energy dissipation rates in the range of $10^{-4} \leq \varepsilon \leq 1$ cm^2 s^{-3} were found to have no effect. In five studies, energy dissipation rates in the range of $0.05 \leq \varepsilon \leq 1$ cm^2 s^{-3} enhanced growth relative to controls. The 12 studies, which did not report any adverse effect of energy dissipation rate on dinoflagellates, were all carried out at

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$\varepsilon < 1$ cm^2 s^{-3} and, therefore, are not relevant to photobioreactor culture.

Understanding the nature of the shear sensitivity of dinoflagellates is necessary for developing improved photobioreactor culture of these microorganisms.[6] This work demonstrates shear sensitivity of *P. reticulatum* and provides evidence for the hydrodynamic shear stress mediated production of reactive oxygen species and lipid oxidation within cells, as the cause of cell damage.

Photobioreactor culture of microalgae is typically carried out at a high cell density as this maximizes the biomass productivity of the photobioreactor and ensures complete use of the available light. High cell density cultures necessarily require a high level of turbulence. Sufficiently intense turbulence assures that the algal cells do not reside continuously in the dark interior zone of the photobioreactor for longer than a few seconds.[19–21] Cells in the dark zone are limited by light and do not grow. Bulk specific energy-dissipation rates in bubble columns and stirred tanks vary commonly within the range $1{,}000 \leq \varepsilon \leq 40{,}000$ cm^2 s^{-3}.[22,23] Furthermore, local rates several magnitude orders higher than the aforementioned ones can be encountered in bioreactors (e.g., behind a rupturing bubble, in the vicinity of impellers, etc.). Therefore, the utility of ecophysiological studies for developing commercially interesting dinoflagellate culture strategies is quite limited because the turbulence levels studied were much lower than the values that occur in bioreactors.

Effects of hydrodynamic shear forces on microalgae and other sensitive cells have been discussed in the literature.[17,21–28] Animal cells are perhaps the most shear sensitive of cells, but methods have been developed for successfully culturing them in many industrial processes.[22,23] The low shear rates that prove lethal to microorganisms such as dinoflagellates often do not produce any observable external damage to the cellular envelope. One mechanism of cell damage that has been suggested[29,30] is that hydrodynamic shear forces somehow trigger a metabolic cascade that leads to an elevation in the intracellular concentration of reactive oxygen species (ROS), which ultimately damage the cellular organelles.[13]

ROS directly and indirectly damage cellular organelles and essential molecules.[31] For example, ROS cause peroxidation of cellular lipids.[32] Organelles such as chloroplasts and nuclei depend on lipid membranes for their integrity and functioning. Peroxidation of lipids produces lipoperoxides that can be readily detected.[32,33] Reactive oxygen species within cells can be detected directly by reacting with various fluorescent probes.[34]

Oxidative stress in cyanobacteria and microalgae has been shown to be induced by factors such as intense sunlight, ultraviolet light,[35,36] carbon dioxide limitation,[31,37] nutrient limitations,[38] and the presence of specific compounds in the culture medium.[39] Photosynthesis itself produces ROS. ROS in dinoflagellates and other photosynthetic microorganisms have been measured mainly by using the nonspecific fluorescent dyes 2,7-dichlorodihydrofluorescein (DCFH) and dihydrorhodamine 123 (DHR).[13] ROS produced by hydrodynamic stimulation of the cells may interfere with photosynthesis. Antioxidant additives such as ascorbic acid (AA) in the culture medium have been shown to reduce oxidative stress in some cases.[40] Data presented here demonstrate that shear stress provokes oxidative stress, which can be indirectly detected by measuring intracellular lipoperoxides. Evidence for the protective effect of ascorbic acid (AA) is also provided.

Materials and Methods

Culture and growth conditions

Monocultures of the toxic dinoflagellate *Protoceratium reticulatum* (GG1AM) were used. This yessotoxins (YTXs)-producer strain was obtained from the culture collection of the Centro Oceanográfico, Vigo, Spain. Inocula were grown under a 12:12 h light-dark cycle at 18°C ± 1°C. Filter sterilized (0.22-μm Millipore filter; Millipore Corporation, Billerica, MA, USA) L1 medium prepared in natural Mediterranean Sea water was used in all experiments. Growth vessels were inoculated using cultures that were in the exponential growth phase. Cells were acclimated to the illumination level of the assays by maintaining the stock cultures at exponential growth for several dilutions under the irradiance level of the experiments.

Erlenmeyer flasks (1-L) were used as growth vessels. The fill volume was 400 mL. Flasks were held static (control cultures) and on an orbital shaker (3-cm of shaking diameter) at various specified agitation speeds. Headspace of some of the control and test flasks was sparged with air. Headspace aerated flasks had pH electrodes installed and pure carbon dioxide was mixed with the ingoing air to control pH automatically (pH was maintained at 8.7[6]). All experiments were carried out in duplicate.

In addition to static controls, two distinct turbulent regimes were examined as follows: (1) continuous agitation at 60 rpm for only the first 24-h of culture, to constitute a "Lethal Short-Time Stress Treatment (LST)" and (2) a 12-h on 12-h off daily agitation (60 rpm) cycle that constituted a "Inhibiting Long-Time Stress Treatment (IST)." These conditions have been previously tested for this dinoflagellate.[16] The approximate bulk shear rate during agitation in both regimens was 0.3 s^{-1} ($\varepsilon \approx 172.8$ cm^{-2} s^{-3}), but the duration of application was different (first 24-h in LST; during 12-h daily illumination throughout the entire culture period in IST). The time-average shear rate (γ) during each turbulence cycle was calculated as follows[16]:

$$\gamma = \left[\frac{0.0676}{\mu_L}\left(\frac{d_c}{\lambda}\right)^2 (\rho_L \mu_L \varepsilon)^{0.5}\right].\phi \qquad (1)$$

where, d_c is the average diameter of the cells (see Flow cytometric measurements), μ_L is the viscosity of the culture, ρ_L is the density of the culture, λ is the Kolmogoroff microscale of turbulence, ε is the bulk mass-specific energy dissipation rate in the shake flask,[41] and ϕ is a dimensionless time of shaking within one turbulence cycle. The viscosity of algal suspensions was measured using a conventional Cannon-Fenske viscometer.[16] The viscosity of seawater was 1.3×10^{-3} Pas. The bulk density of the algal suspensions was measured using a pyknometer (1,030 kg m^{-3}).[16] Changes in physical properties during culture were not significant. The values of λ, ε, and ϕ were calculated[16,41] using the following equations:

$$\gamma = \left(\frac{\gamma_L}{\rho_L}\right)^{3/4} \varepsilon^{-1/4} \qquad (2)$$

$$\varepsilon = \frac{1.94 n^3 d_s^4}{V_L^{2/3}\left(\frac{\rho_L n d_s^2}{\mu_L}\right)^{0.2}} \qquad (3)$$

$$\phi = \frac{t_t}{t_s + t_t} = \frac{t_t}{t_c} \qquad (4)$$

where, d_s is the diameter of the shake flask, V_L is the volume of the culture in the flask (V_L was maintained constant by replenishing the volume removed for analyses with fresh medium), n is the rotational speed of the flask, t_t is the duration of turbulence within one cycle (t_c), and t_s is the duration of the quiescent period without turbulence ($\phi = 1$ in LST treatment and $\phi = 0.5$ in IST).

Both static and agitated cultures were illuminated identically from the top by four Philips TLD 36W/54 fluorescent lamps at an average irradiance of 50 μmol photons m^{-2} s^{-1}, measured at the surface of the culture. The irradiance was measured by a 4π sensor (QSL-2101; Biospherical Instruments, San Diego, CA, USA).

Growth was measured by cell counts. Thus, 1 mL samples of the culture were collected daily, fixed with Lugol's solution, and the cells were counted on a Sedgewick-Rafter counting slide. Maximum specific growth rates were calculated from the cell concentration (N) as the slope of the regression line of ln N vs. time t. Only portions of the growth curve showing exponential increase were used for calculations.

Flow cytometric measurements

Flow cytometry was used to measure the relative mean cell size and the concentration of reactive oxygen species (ROS) in the cells. All flow cytometric measurements were carried out with a Coulter Epics® XL-MCL (Beckman Coulter) flow cytometer.

A suspension of cells scatters light and this can be related to relative mean size of the scattering particles. Forward scatter of light from the cytometer source laser was used to quantify the relative mean cell size by comparison with the forward scatter produced by suspensions of latex beads of known sizes. A calibration curve was determined with latex beads with diameter values of 5, 10, 15, 20, 25, and 30 μm. Cells in a suspension, which produced the same forward scatter as a suspension of beads of a given size, had an equivalent diameter that was the same as that of the beads.

Reactive oxygen species (ROS) were quantified by measuring the fluorescence level produced after staining with the reactive fluorescent dye 2′,7′-dichlorodihydrofluorecein diacetate (H_2DCFDA, product code WA12360; Sigma-Aldrich, St. Louis, MO, USA). H_2DCFDA is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. The measured fluorescence is then proportional to the amount of oxidative species in the cell.[35,36,40] Culture samples were taken at 10:00 a.m. and mixed with H_2DCFDA at a final reagent concentration of 25 μM. Samples were incubated for 30 min in the dark at room temperature before measurement.

Determination of lipid hydroperoxides in biomass

Lipid hydroperoxides were measured with a PeroxiDetect™ kit (product code PD1; Sigma-Aldrich, St. Louis, MO, USA).[33] For measurement, lipoperoxides were extracted from cells as follows: culture sample was centrifuged (1,000g) and the pellet was resuspended in 2 mL of 98% methanol. This suspension was sonicated in a water bath (40-min; 15–18°C) and centrifuged again as specified above. The supernatant was recovered and held at 4–5°C until analyzed in accordance with the instructions for PeroxiDetect™ kit. The storage period of the samples did not exceed 1 week.

Determination of phosphate and nitrate concentrations

The nitrate and phosphate concentrations in the supernatant were measured daily. These nutrients have been demonstrated to be limiting in the medium[6] and were replenished when their concentration in the medium was less than 40% of the initial concentration of L1. The additions prevented growth limitation so that nutrient limitations could not affect the responses to experimental conditions. These additions were concentrated enough to maintain the culture volume constant (the added volume was 0.05–0.1% of culture volume).

Only the phosphorous species that respond to colorimetric methods were determined and quantified as PO_4^{-3}.[42] Thus, molybdic acid and ammonia–potassium tartarate reacted with orthophosphate in acid solution to produce phosphomolybdic acid, which was reduced by ascorbic acid, to develop a blue color that allowed the spectrophotometric quantitative analysis of phosphate at 885 nm.

Quantitative analysis of nitrate was done by HPLC, using an array-diode detector at 210 nm wavelength.[6] The column used was a Tetra-Pak C18 (4 × 12.5 mm) at a fixed temperature of 25°C. The mobile phase was acidified water (0.01 N H_2SO_4) supplied at a constant flow rate of 1 mL min^{-1}.

Peridinin, dinoxanthin, and chlorophyll a *determination*

For determining the carotenoids peridinin and dinoxanthin and chlorophyll a in the cells, a sample of the culture was suction filtered through a 10 mm Whatman GF/F filter disc (Whatman, Maidstone, UK). The filter disc was then extracted by placing it in a 10 mL tube and adding 3 mL of methanol. The tube was held in the dark in a sonicated water bath for at least 30 min. The resulting suspension was centrifuged for 5 min at 13,000g to obtain a cell-free methanol extract.

Carotenoids and chlorophyll a were quantified by HPLC (Shimadzu AV10; Shimadzu Corporation, Kyoto, Japan) with a fluorescence detector (RF-10AX). The HPLC-fluorometric method is highly sensitive and rapid to determine pigments in comparison with the HPLC-UV method. The HPLC-fluorometric method allows the use of very small culture samples. Excitation and emission wavelengths were fixed at 488 and 525 nm, respectively. Isocratic elution was carried out at a flow rate of 1.0 mL min^{-1}. The mobile phase was 25% solvent A (8:2 v/v methanol: water MiliQ) and 75% solvent B (1:1 v/v acetone:methanol). The column used was a Merck LiChrospher 100 reverse phase C18 column (4.6 mm ID, 125 mm long, 5 μm particle size; Merck KGaA, Darmstadt, Germany). The standards, chlorophyll a, peridinin and dinoxanthin were supplied by Sigma-Aldrich (chlorophyll a) and by DHI Water & Environment, Horsholm, Denmark (peridinin and dinoxanthin). The separation of chlorophyll and carotenoid pigment standards yielded satisfactory results. Calibration curves were prepared based on a series of injections of known concentrations of pigment standards on the chromatographic system. Six to ten different concentrations were injected for each pigment. This method of calibration using external standards allowed the relationships to be established between the mass of the pigment

injected on the chromatographic column and the area under the resulting peak.

Statistical analyses

Comparison of treatments over time was performed using the nonparametric paired sign test (95% confidence level, $\alpha = 0.05$).[18] Tests were conducted for the time courses of the seven parameters analyzed in this study (cell concentration, cell diameter, lipoperoxides content, reactive oxygen species, chlorophyll *a* content, peridinin content, and dinoxanthin content) in STATGRAPHICS Plus v4.1 (StatPoint, Herndon, VA, USA).

Results and Discussion

Cell concentration vs. culture time data for various agitated and static (i.e., control) cultures are shown in Figure 1. No significant differences between the two replicates of each treatment over time were observed (nonparametric paired sign tests). Highest growth rate was achieved under static condition (Figure 1a). Growth rates declined substantially with increasing severity of agitation in relation to static control (paired sign tests, $P_{IST} < 0.05$ and $P_{LST} < 0.05$; Figure 1a). This clearly demonstrated an adverse impact of hydrodynamic forces on growth of *P. reticulatum*. Measureable growth did occur under the most severe continuous LST treatment, but only from day 3 of culture or two days after agitation ceased in the LST culture (Figure 1a). The increase in cell number did not occur immediately after cessation of agitation, but the postagitation maximum specific growth rate (0.240 ± 0.051 day^{-1}) was comparable to that measured in the static control (i.e., $\mu = 0.212 \pm 0.002$ day^{-1}). IST regimen did not support cell growth beyond day 8. After day 8, the cells lysed rapidly as a consequence of a cumulative effect of prolonged exposure to shear rates that were at the damaging threshold. Similar observations were reported by García Camacho et al.[16]

All experiments in Figure 1a were conducted without purging of the flask headspace with CO_2-enriched air; therefore, there was some concern about a possible CO_2-limitation influencing the results although any such influence should have affected equally all the cultures shown in Figure 1a. To discount a possible CO_2-limitation, static cultures with and without air–CO_2 flushing of the headspace were compared (Figure 1b). Clearly, CO_2 did not limit growth until day 9 of culture (paired sign test, $P > 0.05$; Figure 1b); however, in the last 2 days of cultivation, the CO_2 supplemented static culture produced distinctly more biomass than did the nonsupplemented static culture. Consequently, for the most part, the possibility of a CO_2 related stress leading to oxidative stress and subsequent cell damage, as observed by others,[31] could be disregarded in interpreting the results in Figure 1.

In addition to CO_2, the other nutrients that could become limiting and induce physiologically damaging stress are nitrate and phosphate. (None of the micronutrients of the L1 medium become limiting at low cell concentrations[6] as in this work.) Both these were measured daily and replenished as necessary to bring their concentrations to the level found in L1 medium; therefore, possible oxidative stress induced by an insufficiency of these nutrients was ruled out.

Effects of agitation and CO_2-supplementation on the average cell diameter are shown in Figure 2. In static cultures,

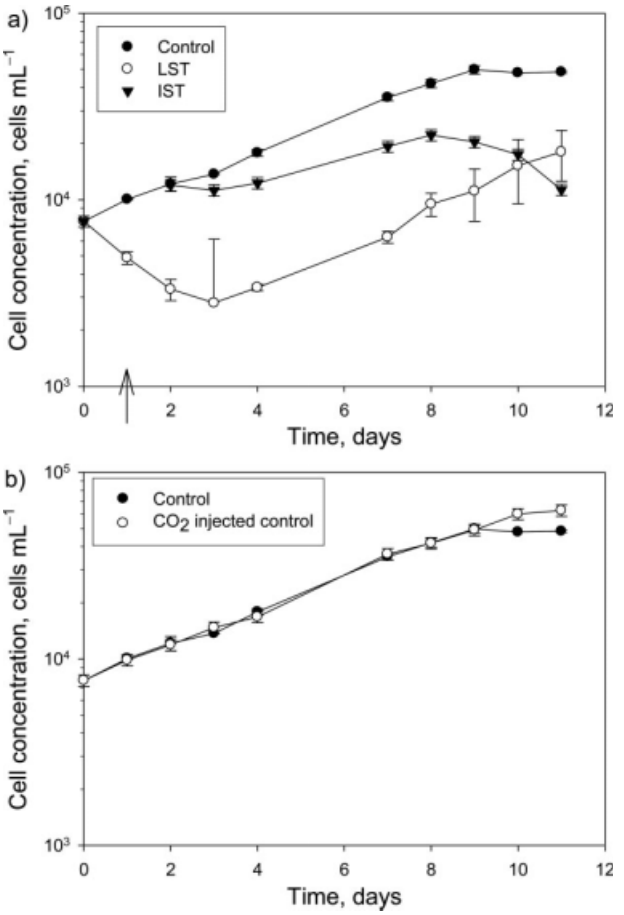

Figure 1. Cell concentration vs. culture time.

(a) Effect of agitation regimens on growth (no pH control; the arrow indicates the instance of agitation being switched off in LST culture); (b) effect of pH control on growth in static cultures. Data points are averages, and vertical bars are standard errors of the means.

irrespective of CO_2 supplementation (Figure 2b), cell diameter at any given stage of growth up to day 9 of culture was quite comparable (paired sign test, $P > 0.05$), demonstrating both an absence of a significant effect of CO_2-supplementation and a high level of reproducibility of these measurements. In both the static cultures (Figure 2b), the average cell diameter increased by about 20% in the first 2 days although the cells were mostly still in the nondividing lag phase (Figure 1b). Subsequently, the cell diameter declined as division became established. The average diameter after the second day was about 20 μm and did not change much during the remainder of the culture period. From day 9 of culture, when CO_2-limitation was evident in the control culture, the cell diameter in flasks supplemented with CO_2 continued to increase (Figure 2b) as cells were still growing (Figure 1b). A similar behavior was seen under the moderate-intensity IST mixing treatment (paired sign test, $P > 0.05$; Figure 2a) up to day 9. From day 9, the cell diameter markedly increased compared with static control (paired sign test, $P < 0.05$), but the cells had aberrant shapes that are typical of programmed cell death (PCD) processes. PCD processes are known to happen in dinoflagellates and are related to high ROS levels.[31,37,43] Under the more severe LST mixing environment, the increase in cell diameter during the first 2 days was substantially greater compared with the static control and the IST agitated culture (paired sign

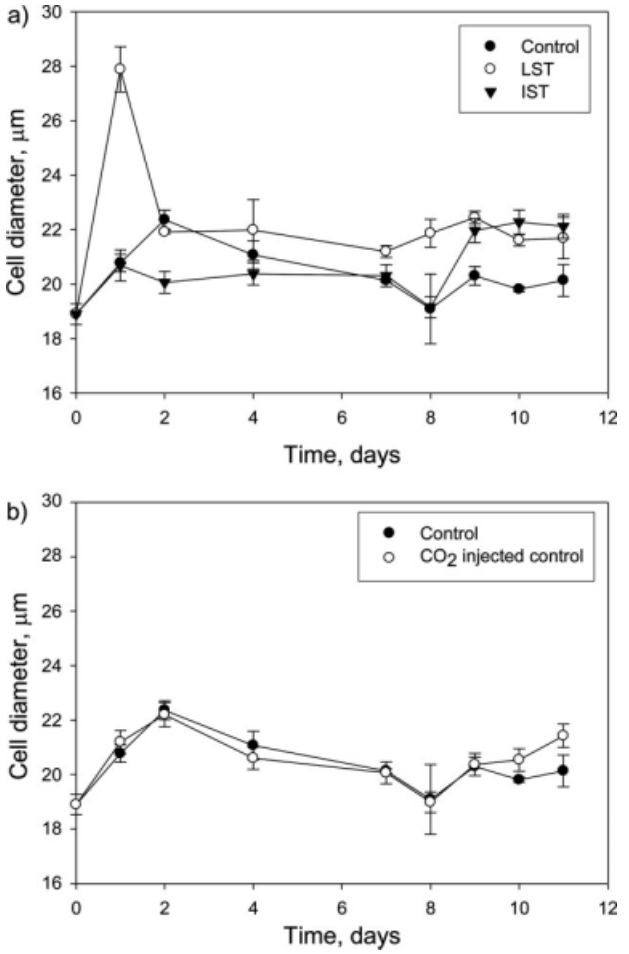

Figure 2. Mean cell diameter vs. culture time.

(a) Effect of agitation regimens (no pH control); (b) effect of pH control in static cultures. Data points are averages, and vertical bars are standard errors of the means.

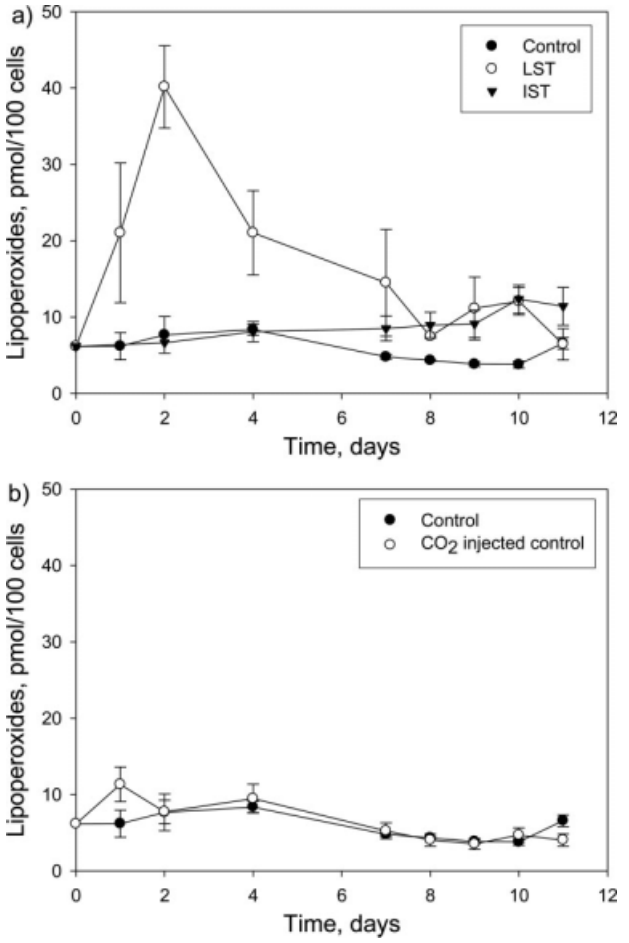

Figure 3. Intracellular lipoperoxides vs. culture time.

(a) Effect of agitation regimens on lipid peroxidation (no pH control) and (b) effect of pH control on lipid peroxidation in static cultures. Data points are averages, and vertical bars are standard errors of the means.

tests, $P < 0.05$; Figure 2a). In LST regimen, even though the agitation was turned off after 24-h, the cell diameter never attained the final value that was seen in the control culture. The increase in cell size of dinoflagellate in response to shear stress has also been previously observed by others (reviewed in Ref. 18). As discussed, the increased cell size during intense agitation closely correlated with increased lipid oxidation products in the intensely agitated cells.

Concentration of the lipid oxidation products or lipoperoxides in the cells during culture is shown in Figure 3. For static cultures, irrespective of CO_2-supplementation, the lipoperoxides remained at a relatively low level, 4–9 pmol per 100 cells, throughout the duration of the culture (Figure 3b). In contrast, in the moderately agitated IST treatment, there was a significant elevation in lipoperoxides to $\geq$10 pmol per 100 cells after the fourth day in comparison with the static control (paired sign test, $P < 0.05$; Figure 3a). In LST treated cells, lipoperoxides rose to a massive 40 pmol per 100 cells within the first 2 days. After the agitation was turned off, the lipoperoxides in LST treated cells declined slowly but only to about 10 pmol per 100 cells. Clearly, exposure to a relatively intense shear stress environment induced quite substantial lipid peroxidation in the cells, possibly through generation of short-lived reactive oxygen species external to the chloroplast.

Concentration of the reactive oxygen species (ROS) within cells, expressed as mean fluorescence intensity per cell, was measured directly by flow cytometry. The relevant data are shown in Figure 4. In static cultures, irrespective of CO_2 supplementation, ROS declined in the first 2 days of the lag phase but subsequently increased with increasing culture time (Figure 4b). Clearly, rapidly growing and photosynthesizing cells were responding strongly to ROS measurements because photosynthesis itself is a major producer of ROS in cells. CO_2 supplemented control culture (Figure 4b) for the most part had a distinctly lower level of ROS species in the cells when compared with the nonsupplemented control (paired sign test, $P < 0.05$), confirming other similar reports.[31] Compared with the static control, much less ROS were detected in the agitated cultures (paired sign test, $P < 0.05$; Figure 4a) after about the fourth day simply because the photosynthetic activity of the agitated cultures was low.

Clearly, measurements of ROS with nonspecific fluorescent dyes do not correlate with shear associated cell damage but measurements of lipoperoxides do. ROS generated by photosynthesis within chloroplasts[36] appear to interfere with detection of ROS, which may be produced as a consequence of hydrodynamic shear stress on cells. The total ROS levels of healthy cells are higher than levels of stressed cells but this did not produce an increase in the lipoperoxides level in

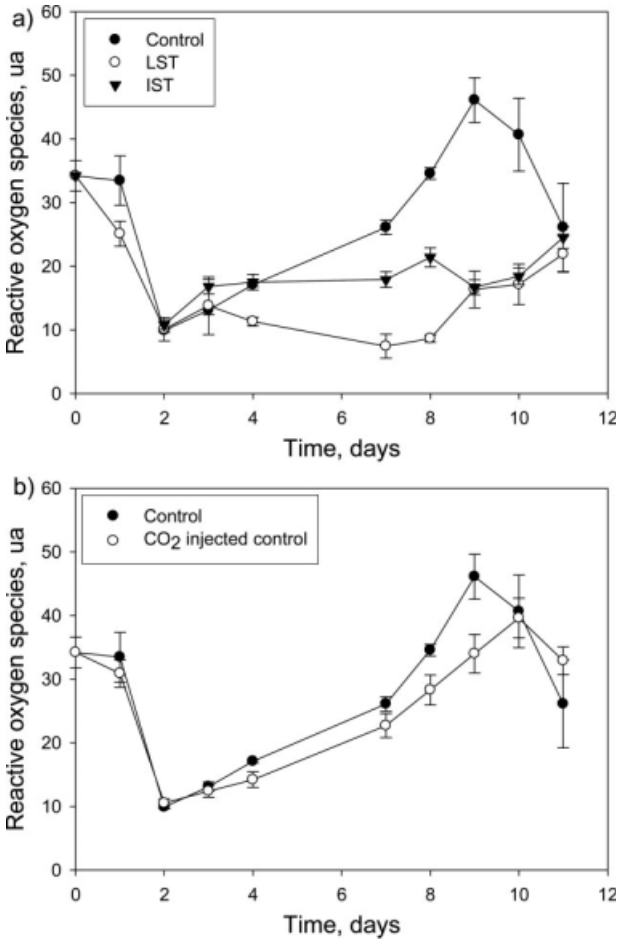

Figure 4. Reactive oxygen species (ROS) concentration in cells, expressed as mean fluorescence intensity per cell, vs. culture time.

(a) Effect of agitation regimens (no pH control) and (b) effect of pH control in static cultures. Data points are averages, and vertical bars are standard errors of the means.

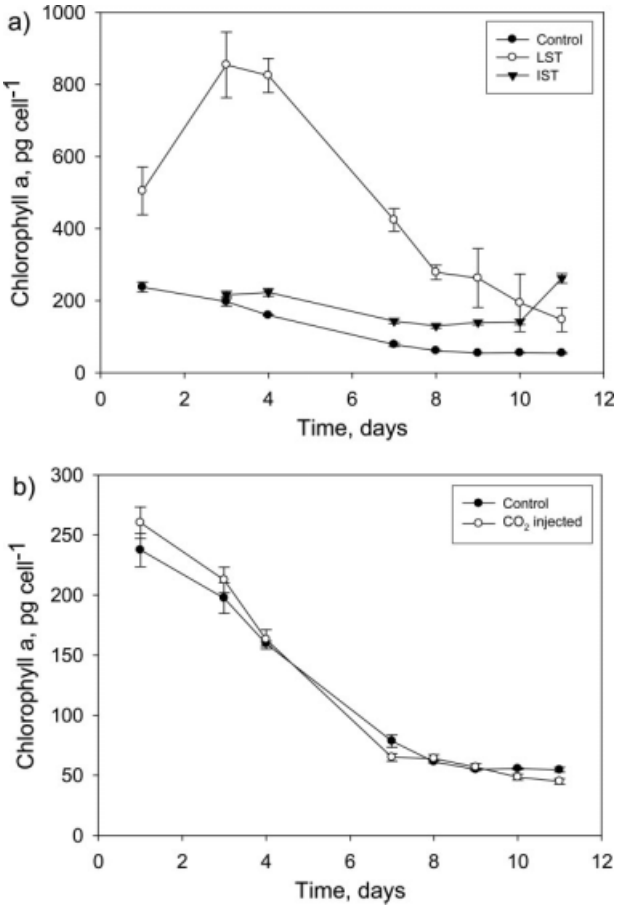

Figure 5. Chlorophyll *a* in cells vs. culture time.

(a) Effect of agitation regimens (no pH control) and (b) effect of pH control in static cultures. Data points are averages, and vertical bars are standard errors of the means.

healthy cells. The photosynthesis-induced ROS are confined to the chloroplasts that are rich in protective pigments. The oxidative species induced by agitation are probably dispersed in the entire cytoplasm and not in organelles such as chloroplast. This is because hydrodynamic shear forces directly affect the cytoplasm through the cell membrane but intracellular organelles are not directly exposed to these forces. Specific fluorescent dyes that only respond to ROS generated by processes other than photosynthesis or react with ROS that are outside the chloroplast are needed to detect any shear-induced elevation in ROS.

The data patterns in Figures 3 and 4 were not altered if ROS and lipoperoxides concentrations were calculated on a cell volume basis instead of a cell number basis (plots not shown). Therefore, the observed effects cannot be ascribed to cell volume variations.

As shown in Figure 5b, the chlorophyll *a* content per cell at any stage of growth in static cultures was virtually identical irrespective of CO_2 supplementation (paired sign test, $P > 0.05$), confirming an absence of a CO_2 limitation for the most part. Chlorophyll *a* was high in the earliest stages of culture, including the lag phase, when the cells were preparing for rapid growth. Rate of increase in chlorophyll *a* in the cells was clearly lower than the rate of cell division during exponential growth and this explains a declining content of chlorophyll *a* per cell (Figure 5b). In the stationary phase, the chlorophyll *a* content per cell was the lowest and stable. In the moderately agitated IST treatment, the chlorophyll *a* content per cell was generally comparable to the static control culture for the first 3 days (paired sign test, $P > 0.05$; Figure 5a). Subsequently, the IST culture had roughly twice the chlorophyll *a* compared with the static control (paired sign test, $P < 0.05$; Figure 5b). Greatly elevated chlorophyll *a* relative to control was observed in the more intensely agitated LST culture (Figure 5a) at all stages of growth. For the most part, chlorophyll *a* increased with increasing agitation intensity (Figure 5a). A comparison of Figures 3 and 5 suggests that chlorophyll *a* content per cell mostly paralleled the lipoperoxides content per cell. The cells may have been attempting to counter the inhibitory effects of lipoperoxides by producing more pigments. The initial build up of chlorophyll *a* in the cells did correlate with an increase in cell diameter (Figure 2) of both agitated and control cultures.

The time profiles of the carotenoid peridinin in the intensely agitated LST culture (Figure 6a) paralleled the profiles for chlorophyll *a*. This was expected as in the cell peridinin is typically complexed with chlorophyll *a* and protein to produce peridinin chlorophyll-*a* protein. In contrast, during the first 3 days, peridinin in the static control cultures and the moderately agitated IST culture increased in the cells (Figure 6a,b) whereas chlorophyll *a* did not (Figure 5a,b). This effect was likely due to an acclimation of cells to an environment that had been freshly replenished with nutrients.

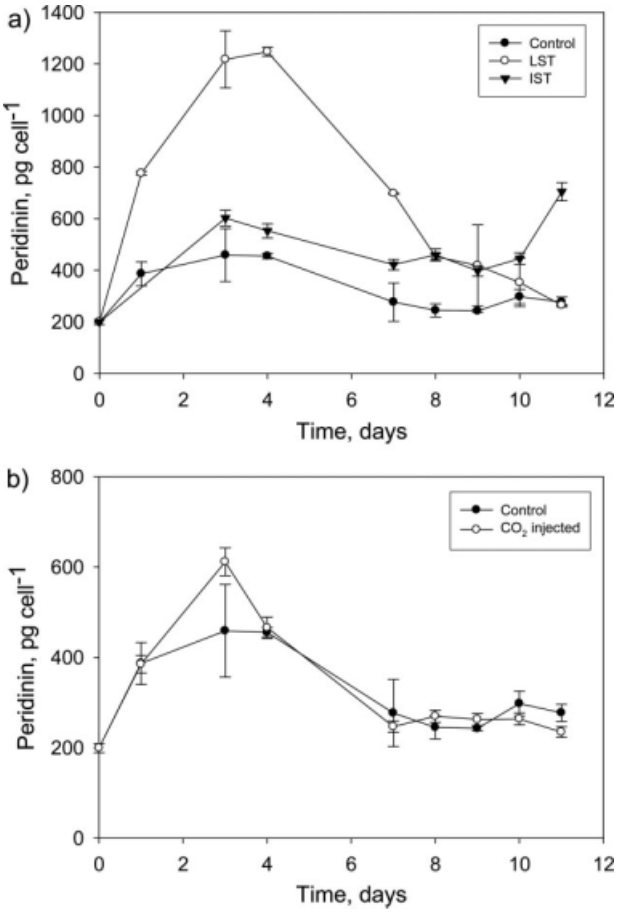

Figure 6. Peridinin in cells vs. culture time.

(a) Effect of agitation regimens (no pH control) and (b) effect of pH control in static cultures. Data points are averages, and vertical bars are standard errors of the means.

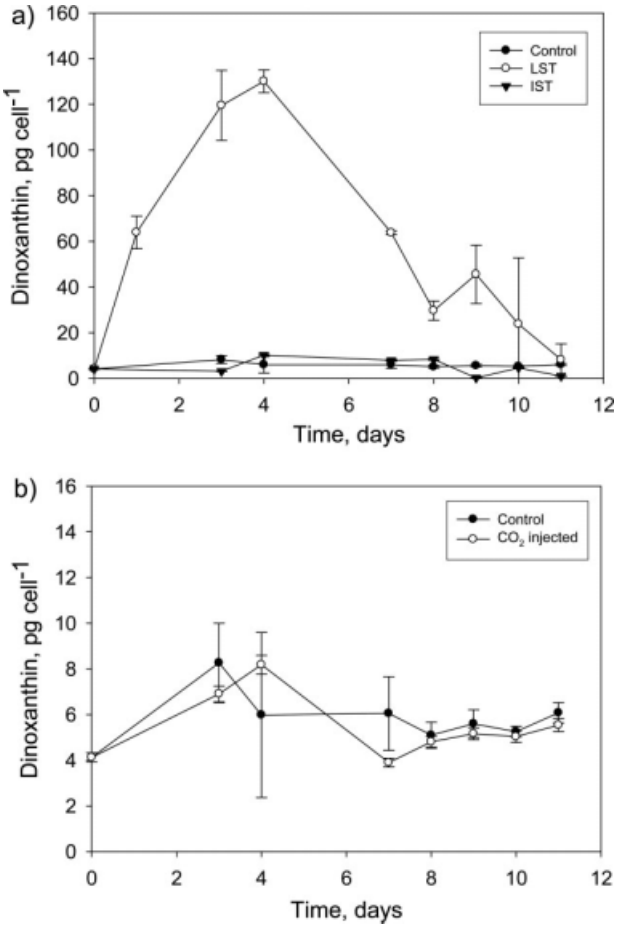

Figure 7. Dinoxanthin in cells vs. culture time.

(a) Effect of agitation regimens (no pH control) and (b) effect of pH control in static cultures. Data points are averages, and vertical bars are standard errors of the means.

Dinoxanthin content per cell in the two static cultures and the moderately agitated IST culture (Figure 7a,b) did not vary much with time and differences among them were not significant in any case (paired sign test, $P > 0.05$). In contrast, in the intensely agitated LST culture (Figure 7a), dinoxanthin level paralleled the initial rapid increase and subsequent slow decline that was seen in the levels of chlorophyll *a* (Figure 5a) and peridinin (Figure 6a). Carotenoids such as dinoxanthin and peridinin are accessory pigments for photosynthesis. These pigments occur in chloroplasts of many phytoplankton species and especially in dinoflagellates. An increase in the level of these pigments within a cell is associated with the induction of the antioxidant defense[44] (Figure 3a). Dinoxanthin and peridinin seem to protect against ROS, for example, during photoinhibition.[45,46] Peridinin is capable of suppressing electronically excited molecules, which have been shown to produce DNA damage and mutagenesis. Peridinin and other antioxidant pigments are well known to be induced and upregulated in response to many kinds of environmental stresses.[44] The data patterns displayed in Figures 5–7 were not altered if pigment contents in the cell were calculated on a cell volume basis instead of the cell number (plots not shown). Therefore, the observed effect cannot be attributed to variations in cell volume.

Changes in intracellular pigment concentration as a consequence of exposure to shear forces have also been previously reported.[47] Unfortunately, these earlier experiments conducted with *Gonyaulax polyedra* were not replicated; the variability was high and seemed to increase with increasing shear, as recognized by the authors. Notwithstanding this, some of the observations reported[47] agree with our results. For example, under inhibitory shear rates pigments per cell increased with turbulence intensity and the time of culture. However, in opposition to what was expected, when the applied turbulence was at a lethal level there was hardly a change in the pigments content of *G. polyedra* in comparison to the control. Except in this study, no one has reported sudden changes in cell carotenoids as a consequence of exposure to lethal shear forces.

In the absence of reactive fluorescent dyes, which can specifically detect ROS that are not derived from photosynthesis, determination of cell lipoperoxides appears to be an easy and effective method for quantifying cell damage caused by oxidative stress produced by excessive shear forces in a fluid.

The results discussed above suggest the use of antioxidants in the medium as a strategy for mitigating the shear-induced oxidative stress. This strategy has been used for reducing the oxidative damage to microalgae caused by excessive exposure to UV-B irradiance,[40,48] but there is no precedent for its use with microalgae for mitigating the damage linked to shear stresses. Ascorbic acid (AA) at 0.05–0.5 mM has inhibited lipid peroxidation in cyanobacteria subjected to UV-B irradiance.[40,48] An experiment tested the protective effect of ascorbic acid. The culture medium was

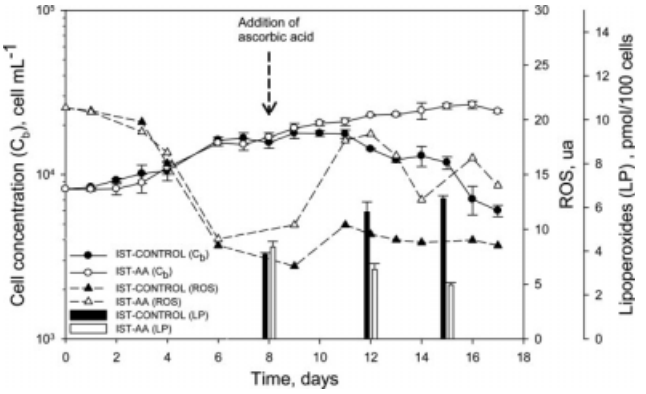

Figure 8. Effect of addition of ascorbic acid (AA) on cell concentration, reactive oxygen species (ROS) level in cells, and intracellular lipoperoxides in cultures under IST regime.

Arrow indicates the instance of AA addition.

supplemented with ascorbic acid at a final concentration of 0.1 mM once the cells had been subjected to the IST regime for 8 days (control culture was always subjected to IST). Figure 8 shows the results obtained. Before addition of AA, the response of cultures was quite similar to that discussed above for the IST cultures. After addition of AA on day 8, the supplemented cultures continued growing, whereas the nonsupplemented cultures began to decline in cell concentration on day 10. As previously observed, cultures under IST regime showed a relatively low ROS level and an elevated concentration of lipoperoxides. On the other hand, the addition of AA inhibited lipid peroxidation significantly, although ROS level was higher than that measured under the IST regime. Nevertheless, supplementation with AA reduced the cellular ROS level in comparison with the static control culture (Figure 4a). These additional data further confirm that the ROS level does not correlate with the shear-induced oxidative stress, as discussed above. In conclusion, addition of AA provided significant protection against the oxidative stress and reduced lipid peroxidation.

Conclusions

Extreme sensitivity to hydrodynamic shear forces is demonstrated for the toxic dinoflagellate *Protoceratium reticulatum*. Intermittent exposure to bulk average shear rate values as low as 0.3 s^{-1} damaged cells. Cell damage correlated strongly with increase in the level of lipoperoxides within cells. Lipoperoxides are oxidation products of lipids that arise through the action of reactive oxygen species (ROS) on lipids. This suggests that hydrodynamic shear forces induced production of ROS that ultimately damaged cells by damaging the lipids, which are essential to the functioning of cell membrane and other organelles. Attempts to directly measure an elevation in cellular ROS by flow cytometry in combination with nonspecific fluorescent dyes that react with ROS could not identify an elevation in ROS apparently because the ROS that were produced naturally by photosynthesis interfered with the measurement. In view of this, use of intracellular concentration of lipoperoxides is concluded to be superior to direct measurement of ROS, for identifying cells under stress. A 24-h continuous exposure to average shear rate values of about 0.3 s^{-1} caused an increase in the cellular chlorophyll *a*, peridinin and dinoxanthin, as the cells apparently attempted to counter the oxidizing effects of ROS by producing these pigments, which are known to have antioxidant properties. Addition of ascorbic acid to the culture medium reduced the turbulence-associated oxidative stress and inhibited the lipid peroxidation. Therefore, to culture shear sensitive dinoflagellates in a necessarily high-shear environment of a photobioreactor, one strategy might be to use powerful antioxidant additives such as ascorbic acid to counter the damaging effects of turbulence. Any antioxidant used must be capable of readily crossing the cell membrane.

Acknowledgments

This research was supported by the Ministerio de Educación y Ciencia (AGL2005-07924-C04-04 and CTQ2008-06754-C04-02/PPQ), Spain.

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Manuscript received July 29, 2008, and revision received Oct. 29, 2008.