

Effect of growth conditions on flow-induced inhibition of population growth of a red-tide dinoflagellate

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Abstract

The population growth of some dinoflagellates is known to be reduced by exposure to fluid flow. The red-tide dinoflagellate *Lingulodinium polyedrum* was used to examine the effect of growth conditions on flow-induced inhibition of population growth. Three factors were tested: time of exposure relative to the light:dark (LD) cycle, illumination level, and culture growth phase (early vs. late exponential phase). Cultures maintained on a 12:12 h LD cycle were exposed to one of two flow conditions: quantified laminar shear produced by Couette flow or unquantified flow generated in shaken flasks. The duration of exposure to flow was 1 h d⁻¹ for 5–8 d in all experiments; the shear stress in Couette shear experiments was 0.004 N m⁻². There were many qualitative similarities in the pattern of response to flow in the two hydrodynamic conditions. In both cases, exposure to flow in the last hour of the dark phase resulted in greater reduction of net growth than exposure during the light phase. Cultures grown under lower illumination had proportionally greater reductions in net growth than cultures under higher light. Finally, late exponential phase cultures exhibited much greater reductions in net growth following a given flow exposure than early exponential phase cultures. The higher sensitivity of late exponential phase cultures did not appear to be linked to nutrient limitation or changes in pH of the medium; it may be partially attributed to exudates from late exponential phase cells. These results suggest that the response of red-tide dinoflagellate population growth to in situ turbulence may depend on both environmental conditions and the physiological state of the cells.

Population growth is reduced in laboratory cultures of some dinoflagellates due to agitation by shaking, aeration, or stirring (e.g., White 1976; Pollinger and Zemel 1981; Berdalet 1992). Such observations have led to the hypothesis that in situ dinoflagellate growth can be inhibited by turbulence (Pollinger and Zemel 1981; Thomas and Gibson 1990a; Smayda 1997). This hypothesis is supported by field data showing a negative correlation between dinoflagellate abundance and high winds or waves. For example, Allen (1946) noted that red tides commonly occur during periods of calm weather. Quantitative data showing a similar trend were described by Pollinger and Zemel (1981), Tynan (1993), Berman and Shteinman (1998), and Rapoport and Latz (1998), although the larger scale dissipative or advective properties of turbulence could also explain the correlations found.

At small spatial scales, individual dinoflagellate cells experience the motion of turbulent eddies as laminar shear because the cells are smaller than the dissipation scale of the turbulence (Lazier and Mann 1989). Therefore, the effects of turbulence on small organisms can be simulated through experimental use of laminar shear. Thomas and Gibson (1990a, 1992) measured the inhibition of net population growth in several dinoflagellate species as a function of quantified hydrodynamic parameters such as shear stress and energy dissipation in experiments which subjected the cells to laminar shear in experimental vessels. Use of a fully quantified flow field permitted the comparison between experi-

mental and in situ hydrodynamic parameters. Population growth of the red tide species *Lingulodinium polyedrum* (= *Gonyaulax polyedra*) was inhibited by shear stress values >0.002 N m⁻², plausible for a stratified upper ocean during moderate winds (Thomas and Gibson 1990a). While in situ flows can indirectly influence growth rates, e.g., through vertical mixing of cells (Denman and Gargett 1995), Thomas and Gibson (1990a, 1992) demonstrated that simple laminar shear flow can have a direct effect on dinoflagellate population growth. The mechanisms by which shear influences population growth are not currently known, although Pollinger and Zemel (1981) and Berdalet (1992) both suggested that fluid motion interferes with the process of cell division.

The effect of laminar shear on dinoflagellate population growth has been studied in *L. polyedrum* (Thomas and Gibson 1990a; Gibson and Thomas 1995), *Gymnodinium sanguineum* (Thomas and Gibson 1992; Tynan 1993), *Prorocentrum micans* (Tynan 1993), *Alexandrium minutum* (Chen et al. 1998), and *Ceratocorys horrida* (Zirbel et al. 2000). Growth inhibition in response to unquantified flow has also been described for *Gymnodinium nelsonii* (Berdalet 1992), *Amphidinium carterae* (Galleron 1976), *Peridinium gatunense* (Pollinger and Zemel 1981), and the toxic dinoflagellate *Alexandrium tamarense* (= *Gonyaulax excavata*) (White 1976). Although only the studies using laminar shear were able to fully quantify the flow conditions, the available evidence, as well as anecdotal observations, suggests that the population growth of dinoflagellates is more sensitive to fluid shear than are other phytoplankton groups (Thomas and Gibson 1990b; Berdalet and Estrada 1993). Not only are dinoflagellates one of the most shear-sensitive organisms in the marine environment, they appear to be more shear-sensitive than any other cell type, with growth inhibition occurring at shear stress levels one to two orders of magnitude

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lower than those which affect animal and plant cells (Namdev and Dunlop 1995; Joshi et al. 1996). If shear does interfere with the process of dinoflagellate cell division, their unusual flow sensitivity may be related to the unique characteristics of dinoflagellate mitosis (Karentz 1989; Berdalet 1992).

The studies of Thomas and Gibson (1990a, 1992) describe the response of dinoflagellate growth rates to increasing levels of shear, demonstrating a shear threshold above which growth inhibition occurs. However, their experiments included only a single set of growth conditions for each species. Pollinger and Zemel (1981) had already shown, using an unquantified flow, that the response of *P. gatunense* growth varies depending on the time of day when the flow treatment is applied. Their results suggest that the dinoflagellate response to shear varies depending on environmental conditions. Characterizing the response of dinoflagellate growth to shear under different growth conditions may help to predict both when in situ flows are most likely to inhibit dinoflagellate growth and also the level of the resultant growth inhibition. The first set of experiments in this study tested whether the results of Pollinger and Zemel (1981) apply to a red-tide dinoflagellate species. Flow induced growth inhibition of *L. polyedrum* cultures grown on a light:dark (LD) cycle was measured following flow treatments at different times of their day. Next, because light level is one of the major determinants of growth rate for in situ, as well as cultured dinoflagellates, the second set of experiments tested whether flow-induced growth inhibition of *L. polyedrum* cultures varied with illumination level. Finally, because the phases of batch culture growth are in some ways similar to the phases of in situ blooms (Jensen and Sakshaug 1973; Sakshaug and Myklestad 1973; Bates 1981), the last set of experiments tested whether the shear threshold and magnitude of the response to a given shear treatment varied depending on culture growth phase. All experiments indicated that the shear response varied with growth conditions. This variability may provide insight into the physiological processes underlying the dinoflagellate response to shear. Extrapolation of the results to field conditions may further an understanding of how in situ turbulence influences the formation or demise of red tides.

Methods

Cultures—Nonaxenic monocultures of the red-tide dinoflagellate, *L. polyedrum* (Stein) Dodge (= *G. polyedra*) were used in all experiments. *L. polyedrum* is a thecate, autotrophic, and roughly spherical cell approximately 35 μm in diameter. The culture was initially isolated from water samples collected near Scripps pier in La Jolla, CA, USA. All stock and experimental cultures were grown on a 12:12 h LD cycle at 20–23°C. Except where specified, f/4 medium minus silicate (i.e., 1/4 the added nutrients of full-strength f medium; Guillard and Ryther 1962) was used. For several experiments, f, f/2, or f/40 media minus silicate were used.

Flow fields—Parallel experiments were conducted for two different flow conditions: Couette flow and shaken flasks. Both flows have advantages and disadvantages; however, no

small scale laboratory flow can simulate all aspects of in situ turbulence (Peters and Redondo 1997). Two flow fields were used with the hope that if different flow conditions elicited similar responses, the experimental results could be extrapolated with greater confidence to additional flow fields, including in situ turbulence.

Couette flow generates a constant shear level and has been previously used for testing the effect of quantified shear on dinoflagellates (e.g., Thomas and Gibson 1990a, 1992; Tynan 1993; Latz et al. 1994). Couette flow has also been used to study the shear sensitivity of many other cell types (Midler and Finn 1966; Mead and Denny 1995; Joshi et al. 1996).

Simple Couette flow, in which the outer cylinder rotates while the inner cylinder remains at rest, is extremely stable and Taylor instabilities do not occur (Coles 1965). Shear stresses corresponding to the entire range of typical oceanic values can be generated in the gap by changing the rotation speed of the outer cylinder. The flow field generated is characterized by a nearly linear velocity gradient within the gap between the cylinders. The flow can be described by the Reynolds number within the gap (Re_{gap}):

$$\text{Re}_{\text{gap}} = \frac{\omega \rho r_o (r_o - r_i)}{\mu}$$

where ω is the angular velocity ($\omega = 2\pi N/60$, where N is the rotational speed in rpm), ρ is the fluid density, μ is the fluid dynamic viscosity, and r_o and r_i are the outer and inner cylinder radii, respectively. On the basis of the Navier-Stokes equations, the fluid velocity (u) profile within the gap is a function of rotation rate:

$$u(r) = \frac{\omega_o r_o^2 (r^2 - r_i^2)}{(r_o^2 - r_i^2)r}$$

where r is the radial position within the gap (Schlichting 1979). The mean shear rate γ ($\gamma \equiv \partial u / \partial r$) within the gap can be calculated as:

$$\gamma = \left[\frac{2\omega_o r_o r_i}{(r_o^2 - r_i^2)} \right],$$

while the mean shear stress (τ) is the product of γ and the dynamic viscosity (μ).

The above calculations describe the fluid shear within the gap space of the chamber, a parameter which can be compared to fluid shear in other flows. However, the true stress on the cell is a function not only of the calculated values for the fluid, but also the degree to which the cell is deformed by the flow. Additionally, at any point on the cell surface, shear stress is predicted to vary in an oscillatory manner as the cell rotates in the flow (Cherry and Kwon 1990). Rather than try to account for these factors, further mention of shear will refer to the fluid shear only.

The Couette flow chambers used in this study consisted of two concentric glass cylinders enclosing a seawater-filled gap (Fig. 1). The endcaps and the axle were constructed of nontoxic plastics. The ratio of annular gap to outer cylinder radius was <0.2 , which is within the range used by Taylor (1936); a narrow gap configuration is preferred due to more stable flow (van Duuren 1968). In an improvement over the design of Thomas and Gibson (1990a, 1992), the bottoms

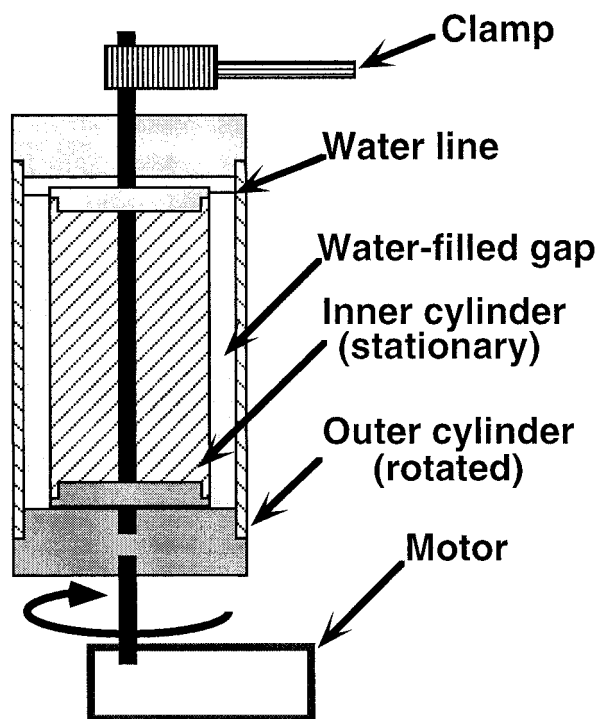


Fig. 1. Schematic cutaway view of a Couette flow chamber. Not drawn to scale. Both cylinders had glass walls. The inner cylinder axle and upper endcaps of both cylinders were transparent acrylic while the lower endcaps were white polyethylene. The inner cylinder had an outer radius of 42.5 mm, while the outer cylinder had an inner radius of 47.5 mm, leaving a 5 mm gap. The inner cylinder height was 110 mm providing a volume within the gap of 165 ml. The outer cylinder was mounted on the shaft of a small AC motor which rotated at 4.5 rpm in all experiments. The inner cylinder was clamped in place by its axle.

of the two cylinders met with no dead space between them. Thus the entire fluid volume (165 ml) was within the gap. Couette flow is experimentally convenient because all relevant hydrodynamic aspects of the flow within the gap can be calculated a priori and are constant throughout the fluid volume for the duration of the treatment. Unfortunately, these convenient properties make Couette flow quite different from in situ flows which are highly variable in time and space. The only deviation from uniformity in the flow field is expected near the bottom of the rotating cylinder where shear will be elevated, especially very close to the inner cylinder. However, the volume of this region with elevated shear is estimated to be less than 4% of the total fluid volume.

In addition to Couette flow, parallel experiments were run in shaken flasks. All experiments used 500 ml glass Erlenmeyer flasks initially filled with 260 ml of medium. Water column height within the flasks was approximately 4.75 cm. The shaker table was of the reciprocating type, producing a linear, back and forth motion. This was preferable to an orbital shaker because a retention zone did not form in the center of the flasks. The table oscillated with a frequency of 50 cycles per minute (cpm) but the intensity of the fluid motion was varied by changing the stroke length, with lon-

ger stroke lengths producing more intense shaking. Only stroke lengths of 8.8 and 13.8 cm were used in this study. The 8.8 cm stroke length setting did not cause growth inhibition of early exponential phase *L. polyedrum* cultures while the 13.8 cm setting produced partial growth inhibition. During shaking, the water surface rocked up and down inside the flasks in a seiche-like manner. The amplitude of this wave varied with stroke length, ranging from approximately ± 0.7 cm from the mean water level for the 8.8 cm stroke length to ± 1.3 cm for the 13.8 cm stroke length. Observations of dye tracer suggested that the flow in shaken flasks was dissipative with eddy-like structures. The majority of the shear was probably produced near the flask wall and bottom (Zirbel et al. 2000). Thus, cells were exposed to variable shear as they were advected from one region of the flask to another. The disadvantage of shaken flasks was that the fluid motion was unquantified, making direct comparison to in situ turbulence impossible. However, any culture shaken using the same stroke length and shaking duration experienced the same physical forcing. Thus, shaken flasks could be used to address questions involving changes in response to a given flow exposure. Moreover, shaken flasks were convenient to use and many replicates could be run within a single experiment. Much of the published data on dinoflagellate sensitivity to flow derives from studies using shaken flasks (e.g., White 1976; Pollinger and Zemel 1981; Berdalet 1992).

In both the Couette flow and shaken flask experiments, a 1 h d^{-1} exposure to flow was selected as the standard treatment so that exposure during different times of the photoperiod could be compared. Gibson and Thomas (1995) demonstrated that a 1 h d^{-1} shear exposure caused measurable growth inhibition in *L. polyedrum*. Once selected, the 1 h exposure was maintained throughout the study to facilitate intercomparison of different treatment combinations.

Growth experiments—*L. polyedrum* cells were inoculated into the experimental containers (Couette flow chambers or flasks) at an approximate concentration of $500 \text{ cells ml}^{-1}$. Except for the conditioned medium and low nutrient experiments described below, cells were inoculated into fresh f/4 medium minus silicate. Each experiment consisted of initial and treatment periods. During the initial period of 3–6 d, daily monitoring ensured that all cultures were healthy and growing similarly. Poorly growing cultures were discarded before the treatment period. During the treatment period, half the chambers or flasks were sheared or shaken, while the other half remained still as controls. The exponential population growth rate was calculated from cell counts made every 1–2 d. Triplicate 0.5 ml samples were removed from each container after gentle swirling to mix the culture. Before sampling, the inner cylinder was removed from Couette flow chambers. Samples were always collected during the middle of the light phase (i.e., the time when growth of *L. polyedrum* is least sensitive to fluid motion, see Results). From each 0.5 ml sample, two subsamples were taken for counting. The subsample volumes were adjusted so that each subsample generally contained 20–30 cells. Because *L. polyedrum* is motile, the subsamples were briefly chilled to minimize swimming during counting, avoiding the need for

preservatives. A minimum of five consecutive cell counts were used to calculate the average daily population growth rate within each experimental container. Counts, expressed as cells ml⁻¹, were ln-transformed and fitted with a linear regression of ln (cell concentration) vs. time (d). The regression slope was taken as the best estimate of mean daily population growth rate. For each experiment, treatment and control growth rates were calculated from data collected over the same period.

The volume removed over the course of an experiment for counting had a negligible effect on flow in the Couette chambers because the water column height was still large relative to the gap width. In the flasks, removal of volume likely resulted in a slight increase in the intensity of the fluid motion over time. In order to minimize changes in the flow field due to decreasing fluid volume, the duration of the treatment period was kept as short as was possible while providing sufficient data to calculate a valid growth rate.

Effect of exposure to flow at different times of the LD cycle—The effect of exposure to flow was tested at three different times during the LD cycle: mid-day (6–7 h into the light phase), early-night (2–3 h into the dark phase), and predawn (the last hour of the dark phase). Early exponential phase cultures were used in all experiments. Three pairs of control and sheared Couette flow chambers were used to test each exposure time. Treated Couette flow chambers were rotated at 4.5 rpm producing a shear stress of 0.004 N m⁻² ($\gamma = 4 \text{ s}^{-1}$, $Re_{\text{gap}} \approx 110$) for 1 h d⁻¹ during the treatment period. Illumination level was approximately 55 $\mu\text{E m}^{-2} \text{ s}^{-1}$ incident radiation outside the chambers as measured using a photosynthetically available radiation (400–700 nm) scalar irradiance meter (Biospherical Instruments QSL-100). The same exposure times were tested using shaken flasks. Three to four flasks each of control and shaken flasks were used for each exposure time. Treated flasks were shaken at 50 cpm during the appropriate hour using a stroke length of 13.8 cm. Illumination level was approximately 65 $\mu\text{E m}^{-2} \text{ s}^{-1}$.

Effect of exposure to flow under different illumination levels—Experiments were conducted at illumination levels ranging from 45–100 $\mu\text{E m}^{-2} \text{ s}^{-1}$. All cultures were in early exponential phase. Flow conditions were the same for all illumination levels. In the Couette flow experiments, the shear stress was 0.004 N m⁻² and exposure occurred during the predawn hour. In the shaken flask experiments, flasks were shaken during the predawn hour as well, at 50 cpm using a 13.8 cm stroke length. One to four pairs of treated and control flasks were used for each illumination level.

Effect of exposure to flow during early vs. late exponential phase—The level of growth inhibition in response to shear exposure was compared in early exponential and late exponential phase cultures. In the Couette flow experiment, cells from a healthy stock culture were inoculated into 1.5 liters of f/4 medium minus silicate. Two Couette flow chambers were filled the next day using the freshly inoculated culture. Following the initial still period, the treated culture was exposed to shear stress of 0.004 N m⁻² for 1 h d⁻¹ during the

predawn hour each day of the treatment period. Illumination level was 55 $\mu\text{E m}^{-2} \text{ s}^{-1}$. After the growth rate of the original source culture had decreased, indicating the onset of late exponential phase (24 d after inoculation), the Couette flow chambers were filled again from the original culture. The shear treatment was then repeated.

A similar experiment was conducted using shaken flasks. Twelve flasks were inoculated from a single stock culture. Four control flasks were not shaken during the experiment (still controls). Four flasks were shaken beginning four days after inoculation (early exponential phase). The remaining four were shaken during late exponential phase. Illumination level was 65 $\mu\text{E m}^{-2} \text{ s}^{-1}$. A shorter stroke length of 8.8 cm, was selected for these experiments. Using this shorter stroke length, shaking of the early exponential phase cultures did not result in detectable growth inhibition. Any inhibition of growth from shaking during late exponential phase therefore implied that a threshold shift in sensitivity to shaking had occurred as growth conditions changed.

Several follow-up experiments were designed to investigate the increased growth inhibition that was found during late exponential phase. In all these experiments, flasks were shaken 1 h d⁻¹ during the predawn hour at 50 cpm with an 8.8 cm stroke length. Illumination level was always 65 $\mu\text{E m}^{-2} \text{ s}^{-1}$. The experiments tested the effect on shear sensitivity of three chemical factors known to change during batch culture growth: nutrient concentration, pH, and exudate concentration.

Two experiments tested the role of decreased nutrients in late exponential phase. In the low nutrient experiment, the comparison of early and late exponential phase cultures was repeated with 1/10 of the initially added nutrients (f/40 medium minus silicate). Stock cultures grown in f/40 medium were inoculated into 12 flasks containing f/40 medium. As before, four flasks remained as still controls, four were shaken soon after inoculation and four were shaken once they and the controls reached late exponential phase. The low amount of added nutrients caused the culture in f/40 medium to reach late exponential phase when factors other than nutrient concentration (cell concentration, pH, and exudate concentration) could be assumed to be similar to the early exponential phase of cultures in f/4 medium.

In the nutrient supplementation experiment, nutrients were added to late exponential phase cultures to remove potential nutrient limitation. Eight flasks with cultures in f/4 medium minus silicate were grown to late exponential phase and then supplemented with nutrient stock solutions, raising the nutrients-added levels up to full strength f medium concentrations. After one day of acclimation, four of the flasks were shaken while the others remained as still controls.

Because pH can increase to 8.5 during batch culture of *L. polyedrum*, the pH of early exponential phase cultures was increased to determine if elevated pH changed their sensitivity to shaking. Eight flasks were inoculated with an early exponential phase culture in f/4 medium minus silicate. The pH of all flasks was raised with addition of NaOH to 8.2–8.5. Four of the flasks were shaken while the remaining four were still controls. Daily monitoring and addition of NaOH maintained the elevated pH.

The exudate experiment was designed to test whether the

exudates of a late exponential phase culture would change the sensitivity to shaking of early exponential phase cells. A large volume culture was grown to late exponential phase. The culture was sterile-filtered first through autoclaved GF/F filters (Whatman) and then through autoclaved 0.22 μm pore diameter polycarbonate membrane filters (Nuclepore) to remove all cells and debris, including bacteria. Nutrients were added, bringing the nutrient concentrations up to f/2 medium levels. This enriched medium was poured into flasks and inoculated with an early exponential phase culture that was first concentrated according to the method of Dodson and Thomas (1964) to reduce dilution of the medium. After four days, half of the flasks were shaken while the others remained as still controls. The cultures thus had the cell concentration, nutrient concentration, and pH of early exponential phase cultures but included the exudates of late exponential phase cultures. The exudate experiment was conducted twice, the first trial used 4 controls and 3 shaken flasks while the second trial included 3 controls and 3 shaken flasks.

Data presentation and statistical analyses—Experimental results were described in terms of absolute and relative growth inhibition. Absolute growth inhibition was calculated as the difference between control and treated growth rates, while relative growth inhibition was that value normalized by the control growth rate ($\times 100\%$). All mean values presented in the text are given with the standard error and the number of replicates. Comparisons between means were analyzed using analysis of variance (ANOVA) or *t*-tests using the assumption of equal variances. Significance of regression slopes were tested using ANOVA; comparison between slopes used *t*-tests. All *t*-tests were two-tailed. Test results were presented with the *F* or *t*-value, degrees of freedom and the corresponding *P*-value. As recommended by Carner and Walker (1982), Fisher's PLSD was used for ANOVA post-hoc comparisons.

Results

Effect of exposure to flow at different times of the LD cycle—Data from a typical experiment testing the effect of predawn Couette shear on population growth is shown in Fig. 2. Two Couette chambers were inoculated with cells from a single stock culture on day 0. The initial still phase lasted through day 4 during which time cell counts from both chambers were similar. Cell abundance in the two chambers began to diverge following initiation of the treatment period on day 5. Net population growth rates were calculated using mean cell counts from days 4–12. The sheared growth rate (0.067 d^{-1}) was lower than the control growth rate (0.134 d^{-1}). Absolute growth inhibition of the sheared culture was thus 0.067 d^{-1} while relative growth inhibition was 50%. Further analyses will use mean values of such parameters averaged over several replicate experiments.

In the Couette shear experiments, the highest mean growth rate occurred in cultures sheared at mid-day, while predawn shearing resulted in the lowest mean growth rate (Fig. 3A). Control growth rates were similar for all exposure times allowing statistical comparison based on the mean absolute

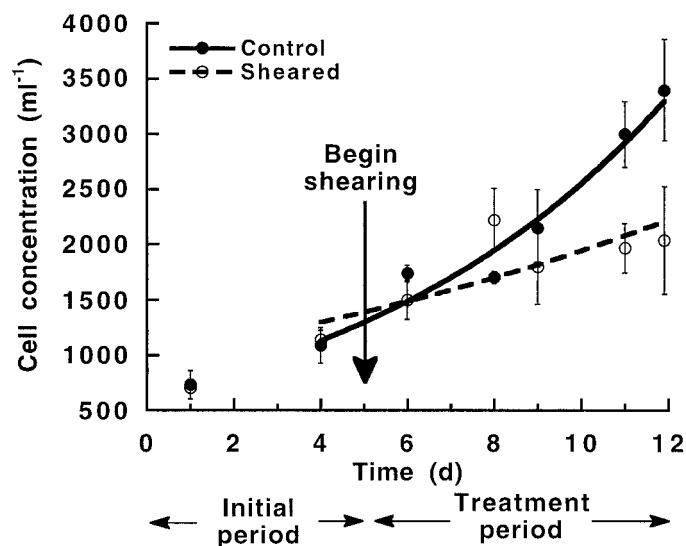


Fig. 2. Representative experiment showing the effect of Couette shear on net population growth of *L. polyedrum*. Each data point is the mean cell concentration (± 1 SE) determined from triplicate subsamples. During the initial period both chambers were still. The treatment period began on the morning of day 5. The sheared Couette flow chamber was rotated 1 h d^{-1} at 4.5 rpm ($\tau = 0.004\text{ N m}^{-2}$) during the predawn hour. The control chamber was still throughout the experiment. Curves represent the exponential regression of \ln (cell concentration) vs. time, where the slope equals the net population growth rate. From day 4 to day 12, the growth rate in the sheared Couette (0.067 d^{-1}) was lower than in the still control (0.134 d^{-1}).

growth inhibition. There was a significant difference in mean absolute growth inhibition for the three exposure times ($F_{2,6} = 32.87$, $P = 0.006$). Each of the three means was significantly different from the other two (Fisher's PLSD, $P < 0.009$ in each case). For the predawn shear treatment, mean relative growth inhibition was 65%.

Shaken cultures showed a similar pattern of growth rates (Fig. 3B). Of the shaken cultures, the highest mean growth rate occurred in cultures shaken at mid-day, while the lowest mean growth rate was measured in the predawn-shaken cultures. Mean absolute growth inhibition of shaken cultures differed between the three exposure times ($F_{2,7} = 6.40$, $P = 0.026$). However, only the mid-day and predawn means were significantly different (Fisher's PLSD, $P = 0.01$; other means comparisons $P > 0.05$). For the predawn shaking treatment, mean relative growth inhibition was 72%.

Effect of exposure to flow under different illumination levels—Control and treated growth rates increased with illumination in both Couette sheared and shaken cultures (Fig. 4). The relationships between illumination and growth rates were significant for both control and sheared Couette cultures (control slope = 0.55 , $r^2 = 0.94$, $F_{1,6} = 89.9$, $P < 0.0001$; sheared slope = 0.66 , $r^2 = 0.92$, $F_{1,6} = 65.1$, $P = 0.0002$) but were not significantly different from each other ($t_{12} = 1.10$, $P = 0.29$), although the curves were offset. Illumination was also significantly related to growth rate in the flask experiments (control slope = 0.48 , $r^2 = 0.94$, $F_{1,7}$

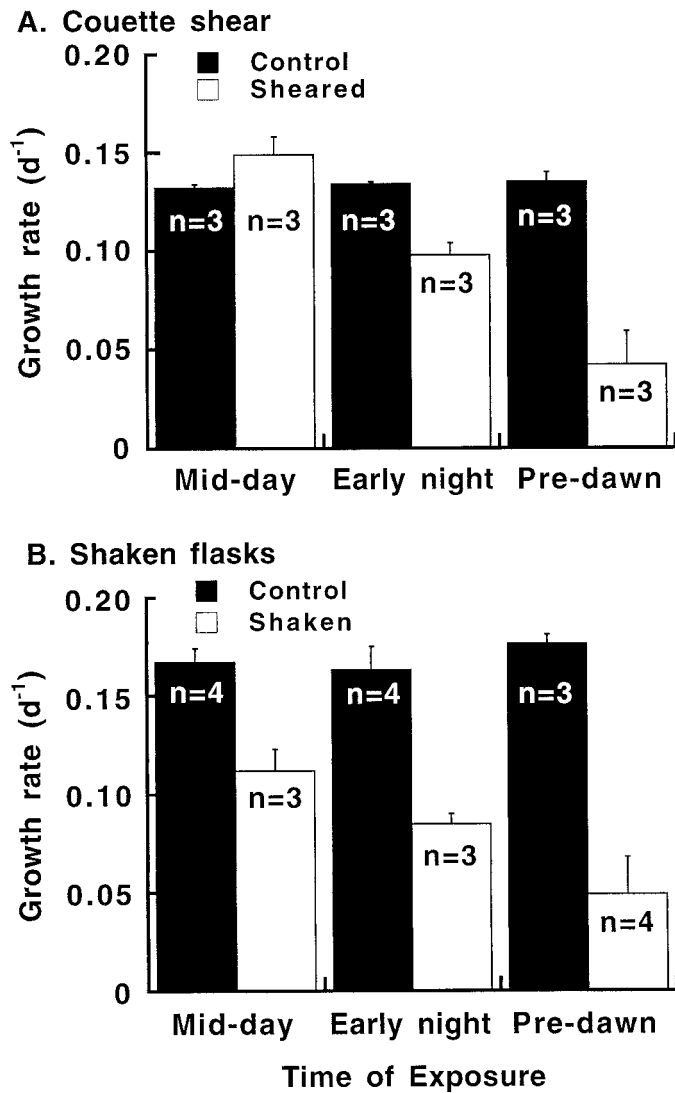


Fig. 3. Effect of exposure to fluid flow at different times of the day. Exposure duration was 1 h d⁻¹ for 5–8 d at the specified times. Bars represent mean net population growth rates (± 1 SE). A. Effect of Couette shear ($\tau = 0.004$ N m⁻²). B. Effect of shaking flasks (50 cpm with a 13.8 cm stroke length). In both cases the difference between treatment and control depended on the timing of exposure to fluid flow. The greatest effect of flow on growth occurred during the predawn exposure.

= 103.4, $P < 0.0001$; shaken slope = 0.65, $r^2 = 0.87$, $F_{1,8} = 53.5$, $P < 0.0001$). As with the Couette flow results, there was no significant difference between the slopes of the control and shaken growth rate data ($t_{12} = 1.67$, $P = 0.12$) although the curves were offset. The similar slopes of the control and treated data imply that the offset between the curves, or the absolute growth inhibition, was consistent regardless of illumination. However, relative growth inhibition decreased as illumination increased. For example, in the Couette flow experiments at an illumination level of 55 $\mu\text{E m}^{-2} \text{s}^{-1}$, there was 69% mean relative growth inhibition. The same treatment at an illumination level of 75 $\mu\text{E m}^{-2} \text{s}^{-1}$ produced only 22% mean relative growth inhibition.

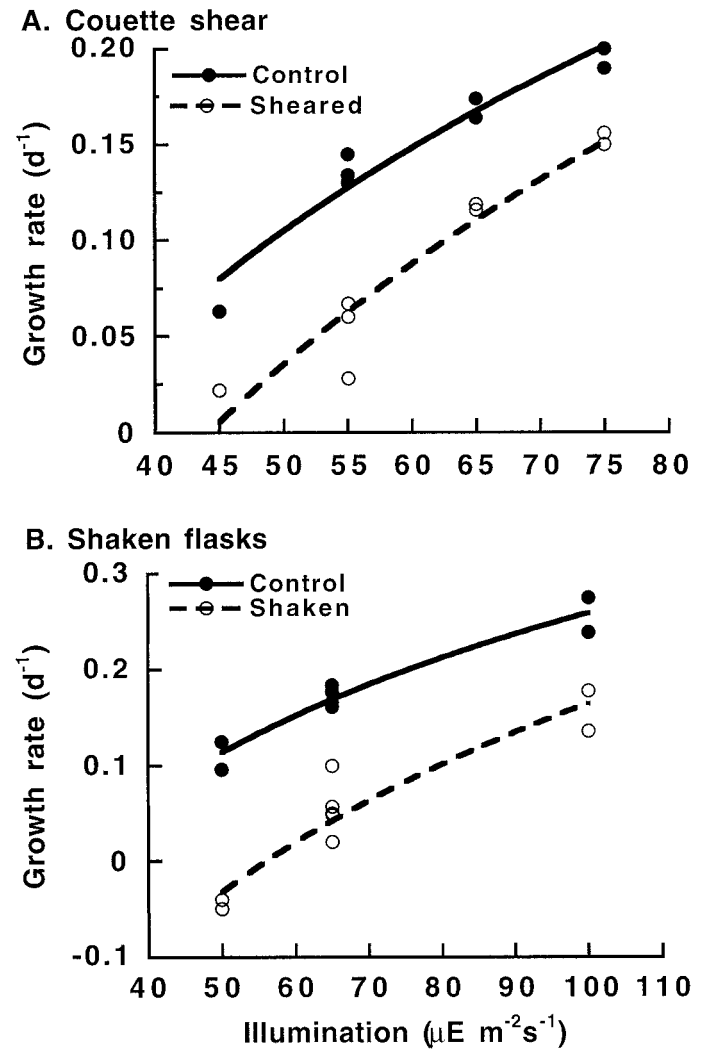


Fig. 4. Effect of illumination on the response to flow. Exposure duration was 1 h d⁻¹ for 5–8 d during the predawn hour. Each point represents the net population growth rate in a single flask or Couette chamber. Curves are the least-squares regression of growth rate on log (illumination). A. Control and sheared ($\tau = 0.004$ N m⁻²) Couette flow chambers. B. Control and shaken (50 cpm with a 13.8 cm stroke length) flasks. For both flow fields, treated and control growth rates increased with increasing illumination. The relative effect of flow on net population growth diminished as illumination increased.

Effect of exposure to flow during early vs. late exponential phase—In both control and treated cultures, growth rates during late exponential phase were lower than during early exponential phase (Fig. 5). Couette shearing during early exponential phase resulted in 71% relative growth inhibition, although the sheared culture still had a net positive growth rate (Fig. 5A). While the absolute growth inhibition caused by Couette shear during late exponential phase was less than that during early exponential phase, net population growth was negative and relative growth inhibition in late exponential phase was greater (194%). In the shaken flask experiment (Fig. 5B), there was no significant difference between mean control and mean shaken growth rates during early

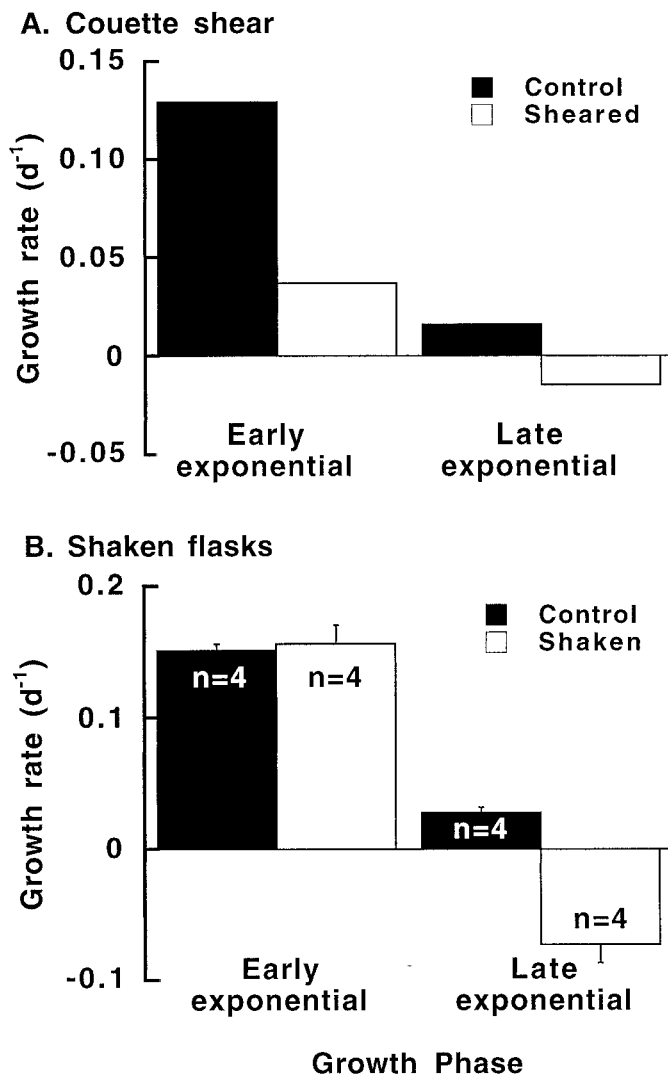


Fig. 5. Effect of culture growth phase on the response to flow. Exposure duration was 1 h d^{-1} for 5–8 d during the predawn hour. Both control and treatment growth rates refer to net population growth only during either the early or late exponential phase treatment periods. A. Effect of Couette shear ($\tau = 0.004 \text{ N m}^{-2}$). Growth rates were measured for both early and late exponential phase cultures under control and sheared conditions. The experiment was not replicated; $n = 1$ for each bar. B. Effect of shaking flasks (50 cpm with an 8.8 cm stroke length). Bars represent mean net population growth rates (± 1 SE). Shaken flasks were shaken either in early or late exponential phases. In both Couette shear and shaken conditions, the effect of motion was greater when the treatment occurred during late exponential phase.

exponential phase ($t_6 = 0.339$, $P = 0.75$). However, in late exponential phase the mean shaken growth rate was again negative and significantly lower than the mean control growth rate ($t_6 = 6.9$, $P = 0.0005$).

When cultures were grown in $f/40$ medium (Fig. 6), shaking during early exponential phase did not affect the mean growth rate relative to the control ($t_5 = 1.33$, $P = 0.24$). Late exponential phase was reached at a lower cell concentration in the $f/40$ than in $f/4$ medium, approximately 1,500 cells ml^{-1} vs. 7,000 cells ml^{-1} , respectively. However, unlike

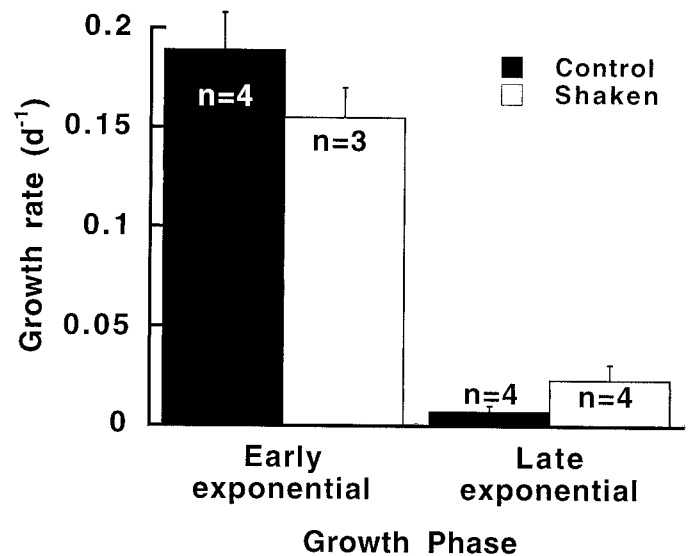


Fig. 6. Effect of low initial nutrients on sensitivity to shaking during early and late exponential phase. Bars represent mean net population growth rates (± 1 SE). Repeat of experiment shown in Fig. 5B except that $f/40$ instead of $f/4$ medium was used. As a result, late exponential phase was reached at lower cell concentration than when $f/4$ medium was used. Shaking during late exponential phase did not decrease net population growth rate, unlike the experiments in $f/4$ medium.

the experiment in $f/4$ medium, there was no significant difference in mean growth rate between shaken and control flasks ($t_6 = 1.82$, $P = 0.12$).

In the nutrient supplementation experiment, the mean control growth rate was $0.027 \pm 0.005 \text{ d}^{-1}$ ($n = 4$), comparable to the growth rate of other cultures in late exponential phase (e.g., Fig. 5). The shaken growth rate was $-0.177 \pm 0.040 \text{ d}^{-1}$ ($n = 4$), significantly different from the mean control growth rate ($t_6 = 5.03$, $P = 0.0024$).

Following elevation of pH of the medium, mean control growth rate was $0.150 \pm 0.003 \text{ d}^{-1}$ ($n = 4$), while that of the shaken flasks was lower at $0.136 \pm 0.012 \text{ d}^{-1}$ ($n = 4$) although the difference was not significant ($t_6 = 2.17$, $P = 0.073$).

In the first exudate experiment, the mean control growth rate was $0.199 \pm 0.010 \text{ d}^{-1}$ ($n = 4$). The mean growth rate in shaken flasks, $0.107 \pm 0.020 \text{ d}^{-1}$ ($n = 3$), was significantly different from the mean control growth rate ($t_5 = 6.13$, $P = 0.002$). Similar results were found when the experiment was repeated. The mean control growth rate in the second trial was $0.201 \pm 0.020 \text{ d}^{-1}$ ($n = 3$). The mean growth rate in shaken flasks, $0.143 \pm 0.002 \text{ d}^{-1}$ ($n = 3$), was again significantly different from the mean control growth rate ($t_5 = 2.95$, $P = 0.042$). However, in both experiments the shaken growth rates were still positive and the absolute growth inhibition in shaken flasks was less than that previously described for late exponential phase cultures.

Discussion

The present results demonstrate that the flow-induced reduction in net population growth of *L. polyedrum* is affected

by environmental and physiological factors. While Thomas and Gibson (1990a) described a threshold for growth inhibition of *L. polyedrum* of $\tau = 0.001\text{--}0.002\text{ N m}^{-2}$, this study suggests that a fixed threshold is inadequate. However, the threshold may vary in predictable ways.

Because the results from Couette shear flow and shaken flask experiments were qualitatively similar, it is likely that growth inhibition is caused by a common property of fluid motion as opposed to any artifacts of the particular flows used (such as potential effects on nutrient uptake, e.g., Karp-Boss et al. 1996). The relevant flow parameter is probably related to shear because Thomas and Gibson (1990a) showed that growth inhibition in *L. polyedrum* increased with shear level. Several facts suggest that flow-induced growth inhibition may be dependent on shear stress, the mechanical force acting on the cell due to fluid viscosity. Shear stress has been implicated in flow-induced changes in growth rate, morphology, and mortality of other cell types (Midler and Finn 1966; Namdev and Dunlop 1995; Joshi et al. 1996). Furthermore, bioluminescence, another response of dinoflagellates to flow, appears to be shear stress dependent (Nauen 1998). Further discussion will therefore assume that shear stress is the causal variable in these experiments.

For this study, the goal was not to simulate in situ conditions of shear stress, but rather to determine whether the response to shear stress exposure was variable under controlled experimental conditions. Nevertheless, the physiological relevance of the shear stress levels used can be demonstrated in two ways. First, neither Couette shear nor shaking stimulated bioluminescence. The shear stress threshold of *L. polyedrum* bioluminescence (Latz et al. 1994; Latz and Rohr 1999) is several orders of magnitude higher than the shear stresses in this study. Additionally, the energy dissipation of the Couette flow experiments can be estimated as $\epsilon = \nu(\gamma)^2$ (Thomas and Gibson 1990a), where ϵ is the rate of viscous energy dissipation and ν is the kinematic viscosity. Thus the standard Couette shear treatment of $\tau = 0.004\text{ N m}^{-2}$ ($\gamma = 4\text{ s}^{-1}$) generates ϵ of approximately $2 \times 10^{-5}\text{ m}^2\text{ s}^{-3}$. This is plausible for the upper 5–10 m of the ocean during a moderate wind with breaking waves (Thomas and Gibson 1990a; Brainerd and Gregg 1993; Anis and Moum 1995). In Lake Kinneret (Israel), the only year of a 32 year record which lacked a spring dinoflagellate bloom was 1996, also the first recorded year with energy dissipation rates as high as $10^{-5}\text{ m}^2\text{ s}^{-3}$ (Berman and Shteinman 1998).

Greater growth inhibition was found when cells were exposed to shear during the dark phase, especially during the predawn hour, than for light-phase exposure. Because similar results have been described for *P. gatunense* (Pollinger and Zemel 1981), this may be a general property of dinoflagellates. Unfortunately, because many cellular processes are correlated with the LD cycle in dinoflagellates (Sweeney 1984), it is difficult to determine what factors contribute to greater growth inhibition at night. Both Pollinger and Zemel (1981) and Berdalet (1992) have suggested that exposure to flow disrupts the cellular division process in dinoflagellates. In fact, the cell cycle of *L. polyedrum* is synchronized to the LD cycle, with most cells dividing near dawn (Sweeney and Hastings 1958; Vicker et al. 1988; Cetta and Anderson 1990). It is therefore plausible that cell cycle

processes explain the increase in sensitivity near dawn; however, other possibilities cannot be excluded. Experiments on species that divide at other times in the LD cycle might reveal what processes ultimately cause diel changes in sensitivity.

If in situ dinoflagellate populations have a similar diel cycle in shear sensitivity, the effect of turbulence on their population growth is predicted to be greatest at night. Diel cycles of turbulence intensity can be generated by winds, tides, or night-time convection (e.g., Moum et al. 1989). Assessment of the effect of turbulence on formation or persistence of red-tide events should take into account the timing of turbulence intensity, not just mean values.

Illumination level had a large effect on shear-induced growth inhibition, with proportionally less growth inhibition at higher illumination levels. All the illumination levels used in this study were below growth-saturating conditions. On a 12:12 LD cycle, the maximum growth rate for this culture of *L. polyedrum* is approximately $0.3\text{--}0.35\text{ d}^{-1}$ (Juhl unpubl. data). Even the highest light level used in this study resulted in only 2/3 of the maximum growth rate. At higher growth rates, experiments were impractical because the early exponential phase was too short in duration. Furthermore, by extrapolating the curves in Fig. 4 to higher levels of illumination, it would be difficult to detect an effect of the shear exposures used in this study (although longer duration or higher shear intensities might still cause significant growth inhibition). It seems likely that the effect of illumination on sensitivity to shear is not due to an intrinsic property of the light but, rather, is mediated by the higher amount of energy available to the cell through photosynthesis. In this sense, other factors that affect cellular energy utilization (such as temperature) might also affect sensitivity to shear.

In both sheared and shaken cultures, greater growth inhibition was observed in the late compared to early exponential phase. In the shaken flask experiments, growth inhibition was not detected when flasks were shaken during the early exponential phase in both f/4 and f/40 media. The dramatic inhibition of growth when shaking occurred in the late exponential phase of cultures in f/4 medium implied a threshold shift in the response to shear stress. Similar shifts in shear sensitivity have been reported in the later phases of batch cultures of mammalian cells (Petersen et al. 1988) and for the ciliate *Tetrahymena* (Midler and Finn 1966). In mammalian cells, increased shear sensitivity is induced in early exponential phase cultures by lowering the pH of the medium and by growing cells in elevated ammonia (Petersen et al. 1988), conditions characteristic of dense heterotrophic cell cultures. The follow-up experiments to the growth phase comparisons were designed to test if factors related to dense dinoflagellate cultures could explain the increase in shear sensitivity of late exponential phase cultures of *L. polyedrum*.

During growth of nonaxenic batch cultures there is an increase in cell concentration, bacterial biomass, pH, and exudates with a corresponding decrease in nutrients. Eventually these changes lead to lowered growth rate (Fogg and Thake 1987). The potential role of nutrient limitation in increasing sensitivity to shear exposure was addressed by supplementing a late exponential phase culture with additional

nutrients and by growing cultures to late exponential phase in low nutrient medium. The results of these experiments should be considered together with the results from the original experiment on shaking of late exponential phase cultures in f/4 medium. The interpretation of the three experiments was somewhat confounded by conflicting observations. On the one hand, low cell yield at late exponential phase of the cultures in f/40 medium suggested that nutrients were limiting to growth. On the other hand, adding nutrients during the late exponential phase of cultures in f/4 medium did not increase growth rate of the controls, suggesting that nutrient limitation did not induce late exponential phase. It may be that late exponential phase was induced by different factors in the different media. Nevertheless, nutrients were probably in low concentration in the late exponential phase cultures in both f/4 and f/40 media, while nutrients were certainly high in the nutrient supplementation experiment. However, there does not seem to be a correlation between growth inhibition due to shaking and nutrient concentration. Results from the cultures in f/40 medium suggest that the increase in shear sensitivity was not an intrinsic property of late exponential phase cultures but rather was due to factors that were correlated to higher cell concentration. Although dense dinoflagellate cultures have elevated pH, the pH experiment demonstrated that high pH by itself does not increase shear sensitivity.

The only treatment which significantly increased the shear sensitivity of early exponential phase cultures was their growth in medium with exudates from late exponential phase cells. Because conditions were similar to early exponential phase cultures in terms of cell concentration, nutrient concentrations, and pH, the increase in shear sensitivity must have been due to a dissolved factor which was exuded into the medium by the previously-resident, late exponential phase cells. Presumably the same factor also increased the shear sensitivity of the other cultures in f/4 medium when they reached late exponential phase. However, the level of growth inhibition in the exudate experiments was less than that measured when late exponential phase cultures were shaken. The causal agent may be labile. Alternatively, a combination of factors may lead to the high shear sensitivity of late exponential phase cultures. For example, cellular changes such as increased vacuolization occur as batch cultures approach stationary phase (Prezelin 1982; Costas et al. 1987). While exudates may contain some sensitizing factors, the full increase in shear sensitivity may only be apparent in conjunction with other changes. However, such changes by themselves do not lead to higher shear sensitivity, as evidenced by the lack of increased shear sensitivity in the late exponential phase of cultures in f/40 medium. The identity of the sensitizing factors is purely speculative. Dinoflagellate exudates include allelopathic compounds whose activity increases as cultures age (Kayser 1979; Arzul et al. 1999). Such allelopathic substances might play a role in shear sensitivity.

The results of the illumination and growth phase experiments suggest that *L. polyedrum* cells are most sensitive to the effect of turbulence when growing under suboptimal conditions. If so, and if the result applies to other species, the effect of shear on field populations of dinoflagellates may

be greatest when growth rates are already low. If red tides are most likely to form when growth rates are high, turbulence may be more effective at terminating blooms rather than influencing bloom initiation.

Reductions in net population growth rate can be driven by a decrease in cellular division rate, increased mortality, or both. Cell death or lysis has typically been used to explain flow-induced reductions in growth of other cell types (e.g., Midler and Finn 1966; Petersen et al. 1988; Lakhotia et al. 1992). However, in this study, net growth rates of treated cultures remained positive in most experiments, so either mechanism may contribute to the growth rate reduction. In experiments where the treated growth rates were negative, some mortality must have occurred. Mortality may have been more important in shaken flask than in Couette shear treatments. Empty, broken thecae often accumulated in shaken flasks by the end of experiments. This was generally not the case in control flask or sheared Couette cultures. Empty thecae may indicate mortality, although thecae could also have been cast off during the formation of temporary cysts (Schmitter 1979). Shaken flasks have regions of high shear (near the walls of the vessel) through which the cells are advected (Zirbel et al. 2000). Some cells could thus briefly experience greater shear forces than the highest levels in the Couette flow chambers. The higher levels or time-varying nature of shear forces in the flasks may cause mortality during shaking. The relative contributions of reduced division and mortality to growth inhibition remain to be determined for different flow conditions.

The results of this study support the hypothesis that turbulent conditions reduce dinoflagellate growth. However, they also suggest that the response to turbulence may depend on environmental and physiological factors. Future laboratory studies with dinoflagellates should use carefully defined conditions of flow, time of day, illumination, and culture age in their experimental design. While the results are consistent with the hypothesis that the growth inhibition caused by flow is related to interference with cell-cycle processes, other processes could also be involved. Mortality clearly occurs in some flow conditions but may not entirely explain the observed reductions of net growth in all conditions. Extrapolating the described changes in shear sensitivity to in situ dinoflagellate populations may aid in understanding how environmental conditions affect the initiation and persistence of red tides.

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