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ORIGINAL PAPER

Carboxymethyl cellulose and Pluronic F68 protect the dinoflagellate *Protoceratium reticulatum* against shear-associated damage

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Abstract The red-tide dinoflagellate *Protoceratium reticulatum* is shown to be protected against turbulence-associated damage by the use of the additives Pluronic F68 (PF68) and carboxymethyl cellulose (CMC) in the culture medium. Relative to agitated controls, these additives had a dose-dependent protective effect at concentrations of up to 0.4 and 0.5 g L^{-1} for CMC and F68, respectively. In static cultures, these additives inhibited growth directly or indirectly at a concentration of >0.5 g L^{-1}. Compared to CMC, PF68 was a better protectant overall. Cell-specific production of yessotoxins was enhanced under elevated shear stress regimens so long as the turbulence intensity was insufficient to damage the cells outright. Shear-induced production of reactive oxygen species and direct effects of turbulence on the cell cycle contributed to the observed shear effects.

Keywords Dinoflagellate · Microalgae · Shear stress · *Protoceratium reticulatum* · Cell cycle · Yessotoxin

List of symbols

CMC	Carboxymethyl cellulose
d_{p}	Diameter of the cells (m)
d_{s}	Diameter of the shake flask (m)
n	Rotational speed of the shake flask (s^{-1})
PF68	Pluronic F68
ROS	Reactive oxygen species
t_{c}	Total cycle time (s)
t_{s}	Duration of the quiescent period within one agitation cycle (s)
t_{t}	Duration of turbulence within one agitation cycle (s)
V_{L}	Volume of the broth in the flask (m^3)
YTX	Yessotoxin

Greek symbols

γ_{ar}	Reference value of average shear rate calculated using $d_{\mathrm{p}} = 20.5$ μm and seawater viscosity (s^{-1})
γ_{av}	Average shear rate (s^{-1})
γ_{t}	Applied shear rate (s^{-1})
μ_{L}	Viscosity of the broth (Pa s)
μ_{r}	Ratio of the maximum specific growth rates of the agitated culture and the corresponding static control
μ_{ra}	Ratio of the maximum specific growth rate in an agitated culture with protective additive and the maximum specific growth rate in an identically agitated culture but without the additive
v	Cycle frequency (= $1/t_{\mathrm{c}}$) (s^{-1})
ρ_{L}	Density of the broth (kg m^{-3})
τ_{ar}	Reference value of average shear stress calculated using $d_{\mathrm{p}} = 20.5$ μm (Pa) and seawater viscosity (s^{-1})
τ_{av}	Average shear stress (Pa)
τ_{t}	Applied shear stress (Pa)
ϕ	Fraction of the time shaken in one agitation cycle

Introduction

Dinoflagellates are a group of motile microalgae. Some dinoflagellates are toxic [1] and are responsible for

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producing harmful algal blooms in the oceans [2–5]. Toxins of different species of dinoflagellates are required as reference standards for monitoring natural algal blooms and the concentrations of toxins in species affected by the blooms. Some of the toxins have biomedical and other applications [1, 2] and others are needed for developing analytical protocols for their monitoring. Only a few of the toxins are commercially available, often at a low purity and high price [2].

In view of the structural complexity of some of the dinoflagellate toxins [2], mass culture appears to be the only viable option for producing these toxins in useful amounts and at an affordable cost. Unfortunately, dinoflagellates can be exceedingly difficult to grow in large-scale culture devices because of their extreme sensitivity to hydrodynamic shear forces [2, 6–10]. Shear rates encountered in some photobioreactors and other downstream bioprocessing equipment can be damaging to dinoflagellates [11].

Dinoflagellates are known to respond to hydrodynamic shear forces in diverse ways. The responses include inhibition of growth [8], disturbance of the cell cycle [6, 10, 12], production of peroxides by the cells [7, 13], changes in the fluidity of the cell membrane [14] and calcium mobilization [12]. The effect of shear forces appears to depend on the duration exposure, the magnitude of the forces, and the stage of the growth cycle that the cells were in during exposure [6, 7, 10]. Turbulence sensitivity of dinoflagellates has been ascribed to various other possible physiological factors [5].

Shear protective additives such as carboxymethyl cellulose (CMC) and Pluronic F68 have been used previously to enhance survival of fragile cells [15–17] including some microalgae [18, 19] in conditions that would be otherwise damaging. Similar methods have been found useful for culturing dinoflagellates [11, 13, 20], although few studies exist. This work reports on the enhanced shear tolerance of the toxic dinoflagellate *Protoceratium reticulatum* in the presence of the protective additives CMC and Pluronic F68 (PF68) in the culture medium. The mechanisms of protection are discussed.

Materials and methods

Cultures and growth conditions

Nonaxenic monocultures of the red-tide dinoflagellate *P. reticulatum* (GG1AM) were used in all experiments. This yessotoxins (YTXs)-producer strain was obtained from the Culture Collection of Harmful Microalgae of IEO, Vigo, Spain. Inocula were grown in filter sterilized L1 medium (0.22-μm Millipore filter) prepared in Mediterranean seawater. Culture temperature was 18 ± 1 °C. A 12:12-h light–dark cycle was used. During the photoperiod, the cultures were illuminated from the top by four Phillips TLD 36 W/54 fluorescent lamps at an average irradiance of 50 μE m^{-2} s^{-1} measured at the surface of the culture. Culture vessels consisted of 1- and 2-L Erlenmeyer flasks with diameter values of 13.1 and 16.7 cm, respectively. The flasks were not sparged with any gas. An exponentially growing inoculum (approximately 20%, v/v) was added to the fresh medium to attain an initial cell concentration of 2,750 ± 150 cells mL^{-1}.

Culture flasks were placed on an orbital shaker with a 3-cm diameter of throw. Control cultures were held under static conditions unless otherwise specified. Agitation of experimental flasks commenced after a 1–3-day acclimation period under static conditions. Depending on the selected turbulence regime, the flasks were shook for 4–6 days.

The shear protectants Pluronic F68 (Sigma-Aldrich, St. Louis, MO, USA) or low-viscosity CMC (Sigma-Aldrich) were added separately to different batches of the culture medium prior to sterilization by filtration. The following concentrations of the protective additives were tested: 0.25, 0.5 and 1 g L^{-1} for Pluronic F68 and 0.1, 0.2, 0.4 and 1 g L^{-1} for CMC. A set of static control experiments were performed with the additives present at the various specified concentrations, to determine if the growth was affected by the additive.

All experiments including the controls were performed in duplicate. Specific growth rates were calculated from the cell counts made on duplicate samples collected daily from each culture. A relative growth inhibition (μ_r) in an agitated culture was calculated as the ratio of the maximum specific growth rates of the agitated culture and the corresponding control (static) culture during a given experimental period. A second measure of growth inhibition due to shear forces (μ_{ra}) was defined as the ratio of the maximum specific growth rate in an agitated culture with protective additive and the maximum specific growth rate in an identically agitated culture, but without any protective additive.

The average shear stress τ_{av} and the average shear rate γ_{av} in the different turbulence regimens used were estimated [11] using the following equations:

$$\tau_{av} = \phi\tau_t \tag{1}$$

$$\gamma_{av} = \phi\gamma_t \tag{2}$$

In the above equations, ϕ is the fraction of the time shaken in one agitation cycle [11], τ_t is the applied shear stress and γ_t is the applied shear rate. Values of ϕ, τ_t and γ_t were calculated as previously specified [11]; thus,

$$\phi = \frac{t_t}{t_s + t_t} = \frac{t_t}{t_c} \tag{3}$$

$$\tau_t = 0.0676\left(\frac{d_p^2 \rho_L^2}{\mu_L}\right) \frac{1.94 n^3 d_s^4}{V_L^{2/3}\left(\frac{\rho_L n d_s^2}{\mu_L}\right)^{0.2}} \tag{4}$$

$$\gamma_t = \frac{\tau_t}{\mu_L} \tag{5}$$

In these equations, t_t is the duration of turbulence within one cycle, t_s the duration of the quiescent period, t_c is the total cycle time ($t_c = 1/v$ where v is the cycle frequency), d_p is the diameter of the cells, d_s is the diameter of the shake flask, ρ_L is the density of the broth, μ_L is the viscosity of the broth, V_L is the volume of the broth in the flask (V_L was kept constant by replenishing the volume removed for analyses, with the fresh medium), and n is the rotational speed of the flask.

CMC increased the initial viscosity of culture broth, in turn decreasing τ_{av} and γ_{av} according to Eqs. 4 and 5. PF68 did not affect the viscosity. For comparison, a reference value of the average shear stress (τ_{ar}) and a reference average shear rate (γ_{ar}) were defined for a broth having a viscosity of 1.2×10^{-3} Pa s and containing dinoflagellate cells of a diameter of 20.5 µm. The various turbulence regimens are summarized in Tables 1 and 2.

Flow cytometry

All flow cytometric measurements were carried out with a Coulter Epics® XL-MCL (Beckman Coulter, Inc., Brea, CA, USA) flow cytometer. 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. The latex beads used had diameter values of 5, 10, 15, 20, 25 and 30 µm. Cells in a suspension that 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.

To establish the cell cycle status, samples were taken during the mid-exponential phase. Cells were then first fixed in 70% ethanol and washed twice with phosphate-buffered saline (0.15 M NaCl, 0.01 M Na_2HPO_4, 0.01 M KH_2PO_4, pH 7.4), and kept at 4 °C until analysis. Cells were treated with 10 µg mL^{-1} RNaseH and held at 37 °C for 30 min prior to staining with propidium iodide. The data were analyzed using the FlowJo software (Tree Star Inc., Ashland, OR, USA).

Lipid hydroperoxides in the biomass

Lipid hydroperoxides were measured with a PeroxiDetect^{TM} Kit (product code PD1; Sigma-Aldrich) [21]. For the measurement, lipoperoxides were extracted from cells as follows: a 15-mL sample of the broth was centrifuged (×1,000*g*, 10 min) and the pellet was resuspended in 2 mL of 98% methanol. This suspension was sonicated for 40 min in a temperature controlled (15–18 °C) sonic bath and centrifuged again as specified above. The supernatant was recovered and held at 4–5 °C until analyzed in accordance with the instructions for the PeroxiDetect^{TM} kit. The storage period of the samples did not exceed 7 days.

Yessotoxins

Yessotoxin production was measured in the culture supernatant at the beginning and the end of each experiment. YTXs were determined by fluorescence HPLC as detailed by Paz et al. [22]. The YTXs were first reacted with DMETAD (4-[2-(6,7-dimethoxy-methyl-3-oxo-3,3dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione) to transform them to a fluorescent molecule [22]. The resulting fluorescent complex was quantified using a Shidmadzu AV10 fluorescence HPLC system (Shimadzu Corporation, Tokyo, Japan) equipped with an autoinjector (SIL-10ADVP), an affinity HPLC column (LiChrospher 100 RP-15, 5 µm, 125 × 4 mm; Agilent Technologies, Santa Clara, CA, USA) and a fluorescence detector (RF-10AX). The excitation and emission wavelengths were 370 and 440 nm, respectively. The mobile phase was 0.1 M ammonium acetate (pH = 5.8): MeOH (3:7) at a flow rate of 0.75 mL min^{-1}.

Results and discussion

Mass culture of microalgae in photobioreactors and other culture devices necessarily requires a certain level of turbulence to keep the cells in suspension and prevent them from residing for extended periods in unproductive

Table 1 Agitation regimens

Regimen	t_c (h)	$v \times 10^5$ (Hz)	ϕ	Nature of turbulence
A	24	1.16	0.5	Agitation during the dark period only
B	Uninterrupted agitation	–	1	Continuous exposure to turbulence
C	2	13.9	0.5	Agitation during the light and the dark periods

Table 2 Summary of agitation treatments

No.	Condition	$V_L \times 10^3$ (m^3)	CMC (g L^{-1})	PF68 (g L^{-1})	τ_{ar} (mN m^{-2})	γ_{ar} (s^{-1})	d_p (μm)	τ_t (mN m^{-2})	γ_t (s^{-1})	μ_r
1	C	0.25[a]	0	0	0.60	0.50	22.7 ± 0.3	1.46	1.21	0.44 ± 0.01
2	C	0.25[a]	0.1	0	0.60	0.50	18.1 ± 0.7	0.75	0.47	0.55 ± 0.02
3	C	0.25[a]	0.2	0	0.60	0.50	18.8 ± 0.3	0.71	0.39	0.62 ± 0.03
4	C	0.25[a]	0.4	0	0.60	0.50	18.2 ± 0.1	0.56	0.24	0.66 ± 0.03
5	C	0.25[a]	0	0.25	0.60	0.50	23.0 ± 0.5	1.50	1.25	0.95 ± 0.02
6	C	0.25[a]	0	0.5	0.60	0.50	22.0 ± 0.5	1.37	1.14	1.07 ± 0.05
7	A	0.25[a]	0.4	0	0.60	0.50	21.0 ± 0.4	0.74	0.32	−1.02 ± 0.06
8	A	0.25[a]	0	0.5	0.60	0.50	22.0 ± 0.4	1.36	1.13	0.34 ± 0.03
9	A	0.25[a]	0	0	0.60	0.50	19.6 ± 3.0	1.08	0.9	−0.81 ± 0.04
10	B	0.25[a]	0.4	0	0.54	0.45	23.0 ± 0.6	0.40	0.17	0.31 ± 0.00
11	B	0.25[a]	0	0.5	0.54	0.45	22.2 ± 0.6	0.62	0.52	0.76 ± 0.02
12	B	0.25[a]	0	0	0.54	0.45	22.16 ± 2.4	0.62	0.52	−0.5 ± 0.09
13	C	0.25[a]	0.4	0	0.60	0.50	18.5 ± 0.3	0.58	0.50	0.61 ± 0.03
14	C	0.25[a]	0	0.5	0.60	0.50	22.8 ± 0.2	1.47	1.23	1.07 ± 0.03
15	B	0.2[a]	0	0	6.57	5.48	21.6 ± 0.4	7.32	6.10	−0.60 ± 0.02
16	B	0.2[a]	0	0.5	6.57	5.48	22.4 ± 0.3	7.82	6.52	−1.15 ± 0.07
17	B	0.2[a]	0	0	18.09	15.08	21.6 ± 0.4	19.95	16.62	−2.83 ± 0.14
18	B	0.2[a]	0	0.5	18.09	15.08	21.7 ± 0.8	20.00	16.67	−0.98 ± 0.05
19	B	0.2[b]	0	0	43.36	36.13	19.8 ± 0.4	39.97	33.31	−2.68 ± 0.24
20	B	0.2[b]	0	0.5	43.36	36.13	19.7 ± 0.4	39.49	32.91	−1.31 ± 0.13

V_L culture volume, d_s flask diameter, τ_{ar} and γ_{ar} reference average shear stress and shear rate, respectively (calculated with $d_p = 20.5$ μm and a seawater viscosity of 1.2×10^{-3} Pa s), d_p average cell diameter ± standard deviation, μ_r relative specific growth rate (static control) ± standard deviation, τ_t applied shear stress, γ_t applied shear rate

[a] Shake flask diameter was 0.131 m

[b] Shake flask diameter was 0.167 m

optically dark zones of the culture device [23]. Unfortunately, even low-level turbulence is detrimental to dinoflagellates [2, 6–9, 11, 24]. One approach to improving survival of dinoflagellates in a relatively turbulent environment may be to use protective additives such as carboxymethyl cellulose (CMC) or Pluronic F68 (PF68) that have been shown to reduce or prevent shear-associated damage to fragile animal cells [15–17] and certain nondinoflagellate shear-sensitive microalgae [18, 19]. This was the rationale for assessing the usefulness of CMC and PF68 in culturing *P. reticulatum*. Different turbulence regimens, including static conditions, were tested in shake flasks. The specific culture conditions of the various experiments are summarized in Table 2.

Static cultures

Additives such as CMC and PF68 have the potential to be toxic to a microorganism either directly or indirectly. Therefore, the effect of CMC and PF68 on growth of *P. reticulatum* was initially assessed in static flasks at various concentrations of the additives. The control flasks were also held static but did not have any CMC or PF68 present. The maximum specific growth rate relative to the control culture (μ_r) was calculated for each flask. The results are shown in Fig. 1. A negative value of μ_r implies cell death in the presence of the additive. Both CMC and PF68 either directly or indirectly inhibited cell growth at all concentrations (Fig. 1). The additives actually killed cells once the concentration of the additive was >0.5 g L^{-1} for CMC and >1 g L^{-1} for PF68 (Fig. 1). In view of Fig. 1, in all future experiments the maximum concentration of an additive was limited to 0.5 g L^{-1} which had reduced the growth rate in static cultures relative to controls, but had not caused direct death of cells. The specific causes of the growth inhibitory effect of CMC and PF68 in static cultures are not relevant here, as both additives were shown to enhance survival of the cells in a turbulent environment as discussed in the following section.

Agitated cultures

A set of experimental runs (control runs) were carried out such that the shear stress level was inhibitory in the

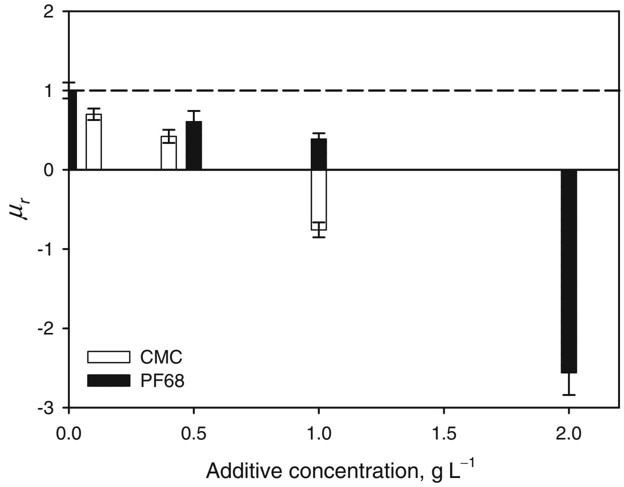

Fig. 1 Effect of the concentration of the protectant on relative protection μ_r. All data are for static conditions

absence of protective additives. A parallel sets of identical runs but with various concentrations of the additives were used to determine the effective concentration for protection, if any. The agitation regimen was fixed at C in Table 1 ($\phi = 0.5$, $t_c = 2$ h) and $\gamma_{ar} = 0.5$ s^{-1} (runs 1–6 in Table 2). This value of γ_{ar} was threefold greater than the damaging threshold value reported [11] for the exact same strain of *P. reticulatum* as used in the present work.

As shown in Fig. 2, the μ_{ra} values for the cultures supplemented with either of the additives were greater than for the agitated controls ($\mu_{ra} = 1$). Clearly, therefore, both the additives had a protective effect and this effect was dose-dependent (Fig. 2). The protection afforded by PF68 was slightly superior to that produced by CMC at similar concentrations in the culture medium (Fig. 2). The protective effect of CMC was due to a decrease in the real average shear rate as a result of increased initial viscosity of the culture medium by the presence of CMC (γ_{ar} ranged from 0.30 to 0.15 s^{-1} for 0.1 and 0.4 g L^{-1} of CMC, respectively). Highest levels of protection were achieved at concentrations of 0.4 and 0.5 g L^{-1} for CMC and PF68, respectively. These concentrations were therefore used in all subsequent work.

Influence of strategy of agitation

For the same intensity of the reference average shear stress, three different conditions of agitation (regimens A–C, Table 1) were tested using the previously determined optimal concentrations of the two additives. As shown in Fig. 3, for all the agitation regimens, PF68 afforded greater protection than did CMC. PF68-supplemented cultures had a positive value of μ_r for all agitation regimens (Fig. 3) and, for intermittent agitation regimen C, the specific growth rate exceeded that of the static control. Thus, relative to static conditions, enhanced mixing actually improved growth once the adverse effect of mixing was relieved by the additive.

The proportion of the relative cell population in various phases of the cell cycle for cells exposed to turbulence regimens A–C is shown in Fig. 4. Figure 5 shows the relative volume for cells exposed to the same regimens and Fig. 6 shows the corresponding relative viability of the cells. Data in Figs. 4–6 are relative to static controls.

In regimen A, the cells were agitated only during the dark. Damage was most severe in this regimen and the cells had a small relative volume compared to the other regimens (Fig. 5). Supplementation with either PF68 or CMC barely had any effect on the relative cell volume (Fig. 5). Relative cell viability was low compared to cells in

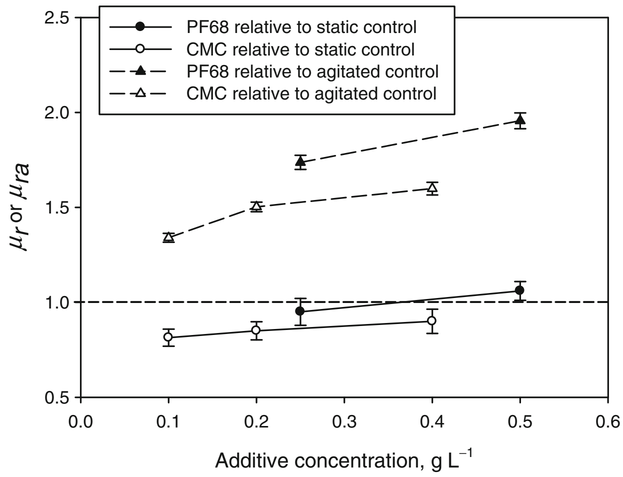

Fig. 2 Influence of the concentration of the protectant on μ_r and μ_{ra}. Data are for the agitation regimen C at $\tau_{pr} = 0.6$ mN m^{-2} (runs 1–6, Table 2). Control cultures for μ_r were static. Control cultures for μ_{ra} were agitated under the conditions specified for runs 1–6, but without the additives

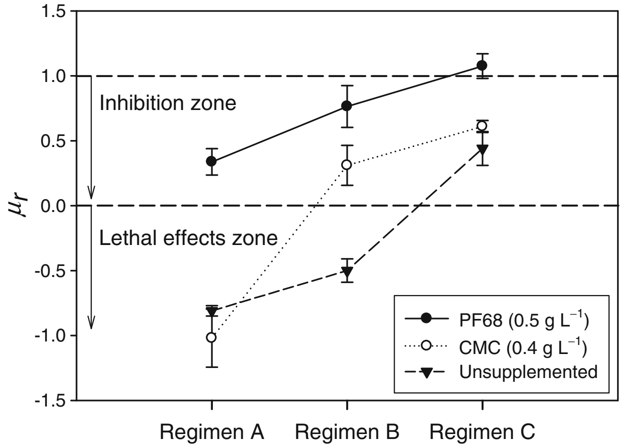

Fig. 3 Relative cell damage (μ_r) under turbulence regimens A–C with and without the protective additives (runs 1 and 7–14, Table 2)

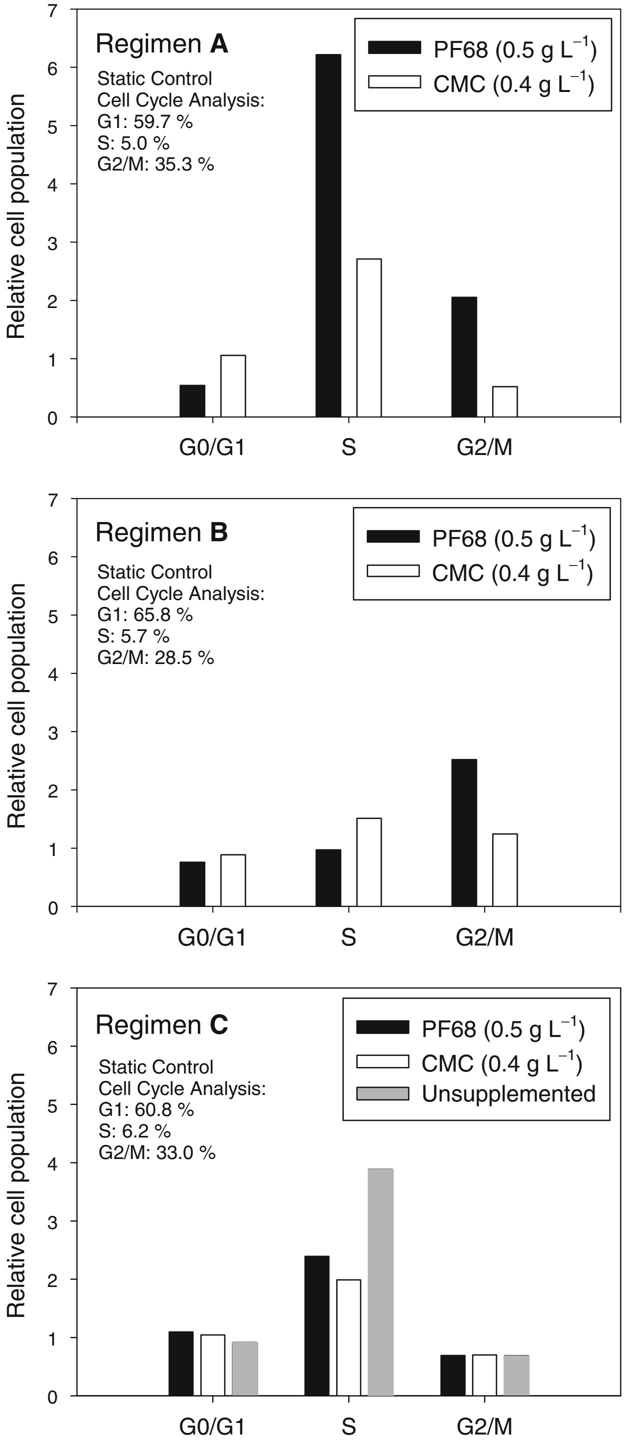

Fig. 4 Cell population relative to static control in various phases of the cell cycle in regimens A–C. Data are for samples taken at the second hour of the light period after 3 days of agitation treatment (runs 1, 7, 8, 10, 11, 13 and 14 of Table 2)

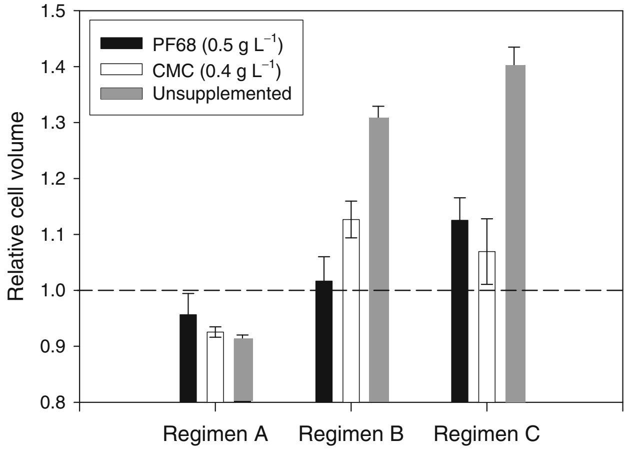

Fig. 5 Cell volume relative to static controls in regimens A–C (runs 1 and 7–14, Table 2)

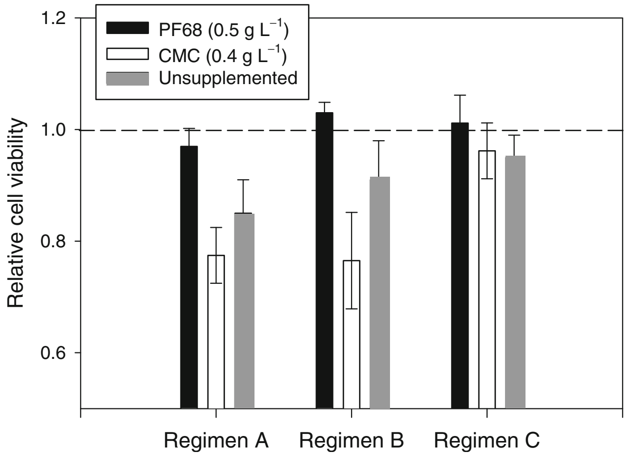

Fig. 6 Cell viability relative to static controls in regimens A–C (runs 1 and 7–14, Table 2)

regimen C (Fig. 6), but supplementation with PF68 did enhance viability compared to either unsupplemented cells or cells that were supplemented with CMC (Fig. 6). A greater number of cells in regimen A relative to the static control was in the S-phase of the cell cycle irrespective of the protective supplement used, but a significant fraction of the PF68-supplemented cells were in the G2/M phase (Fig. 4).

In a light–dark cycle, dinoflagellates divide mainly at the end of the dark phase [5]. Regimen A did not use agitation during the photoperiod, i.e. the period during which cells would normally divide. Most studies of shear-induced inhibition of growth in dinoflagellates suggest that the cells are most sensitive to turbulence when they are close to cell division and have increased in size [8, 9, 11]. Our observations confirm this, although a reduced cell size during dark agitation had not been previously reported. During the dark in quiescent conditions, the cells normally reduce in size because of the consumption of carbohydrates but turbulence appears to accelerate this. Consumption of other nutrients was shown to be enhanced by inhibitory and lethal turbulence in dinoflagellates [11]. This suggests an enhanced consumption of stored cellular material as a consequence of sublethal turbulence. Therefore, energy appears to be required to counter the effects of turbulence. Part of the cellular energy loss in a turbulent environment may be due to turbulence-associated fluorescence that is well documented for dinoflagellates [2]. Furthermore, a reduced cell size may actually better adapt the cells to withstand turbulent forces and may be a survival response. Notwithstanding, other explanations could be implicated in the loss of cell volume. It seems likely that when negative growth is produced it is caused by the greater percentage of dividing and pre-dividing lysed cells. As these cells have a bigger size the disruption of them results in a smaller average size. For diverse microbial species in a given turbulence regimen, cells that are larger are generally more susceptible to disruption compared to smaller cells [16, 17].

In regimen B, the cultures were continuously agitated irrespective of whether they were in the dark or the light period. In this regimen, the cells in the unsupplemented culture had the highest relative volume (Fig. 5). The CMC-supplemented cells had a higher relative volume than the PF68-supplemented cells (Fig. 5). Relative viability of the PF68-supplemented cells was substantially greater than the CMC-supplemented cells (Fig. 6). The latter had a lower relative viability than the unsupplemented cells (Fig. 6). The PF68-supplemented cells were mainly arrested in the G2/M phase of the cell cycle (Fig. 4), but this did not translate into a high growth rate. In contrast, the CMC-supplemented cells were mostly arrested in the S-phase, but there was a good proportion of the cells arrested in the G2/M phase (Fig. 4). An arrest of the population in G2/M phase is a postulated consequence of turbulence linked damage [10] but has not been actually observed.

In regimen C, the relative cell viability was barely affected by the use of protective supplements. The relative cell volume was high compared to the other regimens (Fig. 5), but the supplemented cells had a significantly lower relative volume than the unsupplemented cells (Fig. 5). A greater number of cells in this regimen relative to the static control were in the S-phase (Fig. 4), but the unsupplemented culture had nearly twice as many cells in this phase than the cultures supplemented with the protective additives (Fig. 4). An arrest of dinoflagellate cells in the G1 phase as a consequence of agitation has been previously reported [10], but was not observed in the present work.

Data presented here confirm that hydrodynamic shear forces affect the cell cycle of dinoflagellates. Under turbulent conditions, the cells were mainly arrested in the S-phase and to a lesser extent in the G2/M phase, but introduction of a quiescent period of 1 h between periods of turbulence overcame the adverse effect of turbulence on the cell cycle (Condition C; Table 1). A short quiescent period apparently enabled the cells to repair the damage of the previous turbulence period and continue the cell cycle. This occurred only if the power input in the previous turbulence period was such as to inhibit growth and not cause outright cell death.

Compared to the other eukaryotes, dinoflagellates are cytologically significantly different [5]. Some of the unusual features of the dinoflagellate cells have been speculated to contribute to their characteristic shear sensitivity [5], but mechanisms responsible for this have not been conclusively demonstrated. At certain levels, turbulence appears to be able to reversibly arrest the progression of cell cycle and hence cell division, without causing cell death. At lower levels, turbulence may merely slow the progression of the cell cycle rather than arrest it. Moreover, as was shown in Figs. 3 and 4, the observed cell cycle arrest was not directly related to cell death or either cell growth inhibition (at least it is not the main cause of it) since PF-68 supplemented cells were arrested in a greater extension than CMC-supplemented cells were. This would indicate that PF-68 reduced the number of irreversibly damaged cells in the culture.

Dinoflagellates have enormous genomes packed in relatively small volumes. This increases the likelihood of entanglement of the DNA strands during replication [5]. To prevent this, much of the DNA in a dinoflagellate cell appears to be in a liquid crystal state [5]. During mitosis (dinomitosis) the nuclear envelope expands but remains intact possibly in attempts to control changes in the phase state of the DNA. Mechanical stress associated with turbulence might be transmitted via the cytoskeleton to an expanding nuclear envelope [5] to provoke changes in the phase state of the DNA and consequent sensitivity to turbulence. This potentially explains the cell's increased susceptibility to shear forces at certain stages of the cell cycle.

Changes in the fluidity of the cell membrane are another factor that is known to affect the susceptibility of certain cells to hydrodynamic forces [15, 25]. PF68 reduces fluidity of the membrane of animal cells [25] to make them more robust and may have a similar action on dinoflagellates. In the absence of protective additives, turbulence has been claimed to increase the fluidity of the dinoflagellate membrane [6, 14] and, therefore, further increase their sensitivity to turbulence. PF68 may prevent this, as has been demonstrated for certain nondinoflagellates [16, 25].

PF68 protective effect under high shear rates

In view of efficacy of PF68 in protecting *P. reticulatum* shaken cultures, this protectant was further evaluated under conditions that subjected the cells to continuously applied high reference average shear rate levels of up to ~35 s^{-1}. The results are shown in Fig. 7 relative to static controls.

The agitated cultures generally displayed negative values of μ_r (Fig. 7 inset), but at the highest shear rate values PF68 still had a protective effect as unsupplemented cultures had lower values of μ_r. Up to a reference shear rate value of about 5 s^{-1} loss in viability and changes in cell size were minimal, but at higher shear rates both viability and cell size were significantly reduced relative to controls (Fig. 7). Clearly, a high shear rate arrested metabolic processes that cause an increase in cell size and eventual division. Additionally, at high shear rate levels there was a greater percentage of dividing and pre-dividing lysed cells, as previously discussed.

Shear-induced oxidative stress

Animal cells are well known to undergo programmed death, or apoptosis. Evidence suggests that similar death occurs in phytoplanktons and is linked to oxidative stress. For example, carbon dioxide limitation is known to cause the generation of reactive oxygen species (ROS) in dinoflagellate cells to a level that induces apoptosis [26]. Hydrodynamic shear forces appear to be able to induce the production of ROS in dinoflagellates and this may be one of the mechanisms for shear-related cell damage [7].

ROS are generally short lived and difficult to measure directly [24], but the products of their reactions with the components of the cell can be measured. Lipoperoxides are among such products. Lipoperoxides result from the action of ROS on unsaturated lipids. In *P. reticulatum*, shear-damage has been shown to strongly correlate with an increased level of intracellular lipoperoxides [24]. Damage could be suppressed by supplementing the culture medium with ascorbic acid (vitamin C), a powerful scavenger of ROS [24].

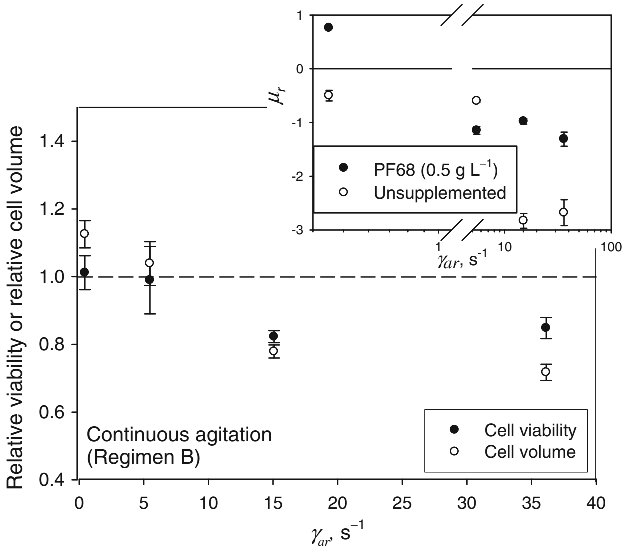

Fig. 7 Influence of average shear rate on relative cell viability, relative cell volume and relative growth rate (inset) in PF68 supplemented continuously agitated cultures (runs 11, 12, 15–20, Table 2). All data are relative to static controls

In the present study, the concentration of intracellular lipoperoxides was measured to assess the oxidative history of the cells that had been subjected to various shear treatments. The cellular lipid hydroperoxides contents relative to static controls are shown in Fig. 8 for treatment regimens A–C. Control cultures were either unsupplemented or contained the shear protectants at the concentrations specified in Fig. 8. Samples were taken at the onset of the stationary phase in which the concentrations of the lipid hydroperoxides were determined to be the highest [24]. In cultures supplemented with the protectants, the lipid hydroperoxides had the highest concentration in regimen A (i.e. agitation during the dark, in the absence of photosynthesis).

The elevated relative lipoperoxides concentration measured in the unsupplemented cultures was nearly prevented from occurring by the addition of CMC and PF68 (Fig. 8). It seems likely, therefore, that the extent of peroxidation in the cell population correlates directly to the proportion of the population undergoing apoptosis. This explains the more severe damage suffered by cells when agitation was provided during the dark (regimen A). Note that for regimen C in the PF68-supplemented culture (Fig. 8), the relative lipoperoxides content is <1. This agitated culture attained a higher growth rate and cell concentration than did the static control. Agitation may have prevented a limitation of carbon dioxide and this may have delayed the appearance of the aforementioned oxidative stress relative to static control. Oxidative stress and apoptosis have been

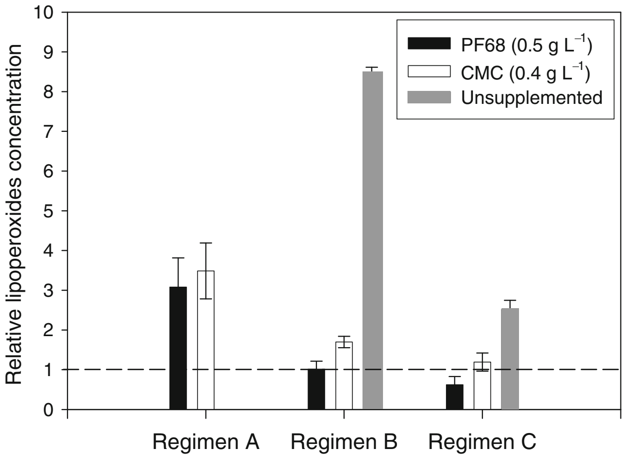

Fig. 8 Relative concentration of lipoperoxides in the cells in mixing regimens A–C (runs 1, 7, 8, 10-14, Table 2). Data are relative to static controls

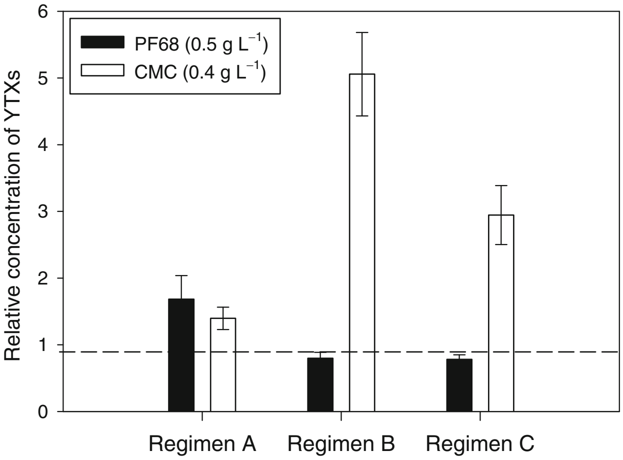

Fig. 9 Relative yessotoxins (YTXs) production per cell in mixing regimens A–C (runs 7, 8, 10, 11, 13, 14, Table 2). Data are relative to static controls

shown to be mediated by carbon dioxide limitation in at least one dinoflagellate [26].

YTXs production in agitated cultures

In a pioneering study, Juhl et al. [27] showed that shear forces affected production of toxins by the dinoflagellate *Alexandrium fundyense*. By lengthening the duration of the daily shear period, toxin production per cell could be increased by nearly threefold compared to controls [27]. Higher toxin production coincided with periods of low growth and cell death [27]. Our results for *P. reticulatum* (Fig. 9) are consistent with these observations for *A. fundyense*. In Fig. 9 relative YTXs production data are given on a per cell basis.

Relative to controls, toxin contents per cell were generally lower in PF68-supplemented culture than in CMC-supplemented cultures (Fig. 9) because of a higher protective effect of PF68. The mechanisms linking toxin production to shear stress are unknown, but production appears to be inversely related to growth. Therefore, any phenomenon that suppresses growth without killing the cells outright has the potential for enhancing toxin production. Shear induced toxin production may also be a response that is triggered in undamaged cell by chemical signals released by cells as a consequence of exposure to shear forces.

Conclusions

Pluronic F68 (PF68) and carboxymethyl cellulose (CMC) added to the culture medium at a concentration of ≤ 0.5 g L^{-1} protected the *P. reticulatum* against shear-related damage. This protective effect of the additives was dose-dependent. At higher concentrations in static cultures, both additives proved to inhibit cell growth. Compared to CMC, PF68 was a superior protectant. Elevated shear regimens enhanced the cell specific production of the YTXs as long as the intensity of the shear stress was insufficient to kill the cells outright. Shear-induced production of ROS and direct effects of turbulence on the cell cycle contributed to the observed shear effects.

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