

Shear effects on suspended marine sponge cells

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Abstract

Fractions of viable cells, apoptotic and irreversibly damaged cells, dead whole cells and cell fragments were measured by flow cytometry during the production of freely suspended primary cells from explants of the marine sponge *Axinella damicornis*. The explants were disintegrated using the well-known Müller protocol [W.E.G. Müller, M. Wiens, R. Batel, R. Steffen, R. Borojevic, M.R. Custodio, Establishment of a primary cell culture from a sponge: primmorphs from *Suberites domuncula*, Mar. Ecol. Progr. Ser. 178 (1999) 205–219]. Supplementation of the standard Ca²⁺- and Mg²⁺-free artificial seawater of the Müller protocol, with the shear protectant Pluronic F68 (0.1%, w/v) greatly reduced the cell damage and enhanced the recovery of viable cells at each of the four stages of the protocol. Agitation of cells on an orbital shaker at 75 rpm essentially killed all the viable cells within 2.5 h, but no loss of viability occurred at a higher agitation speed of 100 rpm for up to 6 h when the cells were supplemented with Pluronic F68. This time-dependent loss in viability could be significantly reduced by processing at 3 °C instead of the normal 17 °C. A four-step mechanistic model was shown to describe the kinetics of cell death and fragmentation within ±10% of the measured values. The damage to cells was modeled as a web of first-order processes that did not depend on cell–cell interactions. The forces in the agitated fluid killed the viable cells by impact, which was not accompanied by cell rupture (i.e. the cell was left dead, but intact).

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1. Introduction

Marine sponges are increasingly being examined as possible sources of unusual bioactive compounds [1,2]. Sponge biomass for recovery of metabolites is generally obtained from explants grown in the sea and aquariums. Sponges cultivated by these methods are invariably contaminated with various microbial populations. In view of this, interest is emerging in producing metabolites using pure cultures of freely suspended sponge cells and cell aggregates (primmorphs). Protocols have been established for producing primary cell suspensions from sponge tissue [3], although no cell lines capable of indefinite growth have been obtained [2]. In many cases, the primary cells have been cultured and multiplied as primmorphs, or morphologically well-defined

cell aggregates that form spontaneously from suspended cells [2,4,5].

In suspension culture, cells are exposed to hydrodynamic and mechanical forces that they do not normally encounter in the sponge matrix. Like most types of animal cells [6,7], freely suspended sponge cells are susceptible to damage by these forces, but no data exist on such effects. Here we show that the most commonly used method of generating primary cell suspensions from sponge tissue [3], causes damage to cells and provide an improved variant of the method to reduce the damage. The improved method combines the effect of temperature and the use of shear protective additive Pluronic® F68 for enhancing cell survival. A mathematical model of cell damage is used to show that in media free of gas bubbles, damage to cells is a first-order process that does not depend on cell–cell interactions. This is consistent with other published observations [6,7]. The results are useful for possible future long-term suspension culture of sponge cells in bioreactors.

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Nomenclature

A	percentage of apoptotic or irreversibly damaged cells
A_0	initial percentage of irreversibly damaged or apoptotic cells
CMFSW	calcium and magnesium free artificial seawater
D	percentage of cell debris
D_0	initial percentage of cell debris
K_{1-4}	first-order rate constants for the various events in Fig. 1
M	percentage of dead cells
M_0	initial percentage of dead cells
N	agitation speed
N_A	number of events associated with irreversibly damaged cells
N_D	number of events associated with cell debris or fragments
N_M	number of dead cell events
N_T	total number of events measured by the flow cytometer
N_V	number of viable cell events
t	time
V	percentage of viable cells
V_0	initial percentage of viable cells

Greek letters

α	parameter defined in Eq. (13)
β	parameter defined in Eq. (14)
γ	parameter defined in Eq. (15)

2. Materials and methods

Specimens of the marine sponge *Axinella damicornis* were collected by SCUBA divers in the Mediterranean coastal waters (Almería, Spain), immediately transported to the laboratory and maintained in an aquarium as described previously [8]. The sponge was kindly identified at Centro de Estudios Avanzados de Blanes (Girona, Spain).

Suspensions of the sponge cells were generated using the well-known method of Müller et al. [3]. Briefly, $\approx 0.5 \text{ cm}^3$ explants of the mother sponge were cut under water, using a sterile razor-sharp scalpel. The explants were placed in 40 ml of Ca^{2+} and Mg^{2+} -free artificial seawater (CMFSW) containing EDTA. The explant-containing vial was gently agitated on an orbital shaker (20 min) and the supernatant was discarded. A further portion (40 ml) of CMFSW was added and agitation continued for a further 40 min. The supernatant was discarded and this cycle was repeated 2–3 times. The cell suspension generated was held in the same vial with or without added Pluronic® F68 (0.1%, w/v; Sigma, St. Louis, MO, USA) for specified periods at various agitation intensities (orbital shaker; 0, 70 and 100 rpm). The CMFSW was pre-

pared by dissolving the following components in deionized water (1 l): 31.6 g NaCl, 2.42 g Tris-HCl, 0.994 g Na_2SO_4 , 0.746 g KCl and 0.0168 g NaHCO_3 . The pH was adjusted to 8.2 prior to filter sterilization with a $0.45 \mu\text{m}$ membrane filter. The CMFSW was always supplemented with antibiotics (100 IU ml^{-1} penicillin and $100 \mu\text{g ml}^{-1}$ streptomycin). All experiments were carried out at 17°C , the typical habitat temperature of the sponge.

A flow cytometer (Coulter Epics XL-MCL) was used to quantify the total cell and debris count. The fraction of viable and nonviable cells were distinguished by staining with propidium iodide and fluorescein diacetate [9]. Measurements were made for up to 96 h from the generation of cell suspension. All results are averages of triplicate measurements.

3. A model of shear damage

The total number of events (N_T) measured by the flow cytometer in a sample are made up of the following: (1) the number of viable cell events (N_V); (2) the dead cell events (N_M); (3) events associated with irreversibly damaged cells (N_A); (4) events associated with cell debris or fragments (N_D). Thus, a total event balance can be written as follows:

$$N_T = N_V + N_M + N_D + N_A \quad (1)$$

Dividing Eq. (1) by N_T and multiplying by 100, we obtain the event balance in percentage terms:

$$100 = \frac{N_V}{N_T} 100 + \frac{N_M}{N_T} 100 + \frac{N_D}{N_T} 100 + \frac{N_A}{N_T} 100 \quad (2)$$

$$100 = V + M + D + A \quad (3)$$

where V , M , A and D are the percentages of viable cells, dead cells, apoptotic or irreversibly damaged cells and debris, respectively, as measured by flow cytometry.

In an agitated cell suspension the cells are transformed from the initially viable state to some of the other possible states through a dynamic process, as schematized in Fig. 1a. Steps 5 and 6 represent direct cellular disintegration from whole cells to debris when the bioreactor is intensely agitated (i.e. internal impeller and/or bubbling). When suspensions of animal cells are gently agitated like in this work (i.e. orbital shaker) constants $K_{5,6}$ logically are close to zero, and the scheme of Fig. 1a is simplified to Fig. 1b. The four steps in Fig. 1b are obviously irreversible. In keeping with prior evidence for various animal cell suspensions [6,7], we assume that each step is a first-order process, i.e. the damage is not due to collisions or other interactions among cells, but hydrodynamic shear forces that are independent of the cell concentration. Thus, for cells that do not grow, following balances can be written for the fractions of viable, dead and apoptotic and/or irreversibly damaged cells, respectively:

$$\frac{dV}{dt} = -(K_1 + K_3)V \quad (4)$$

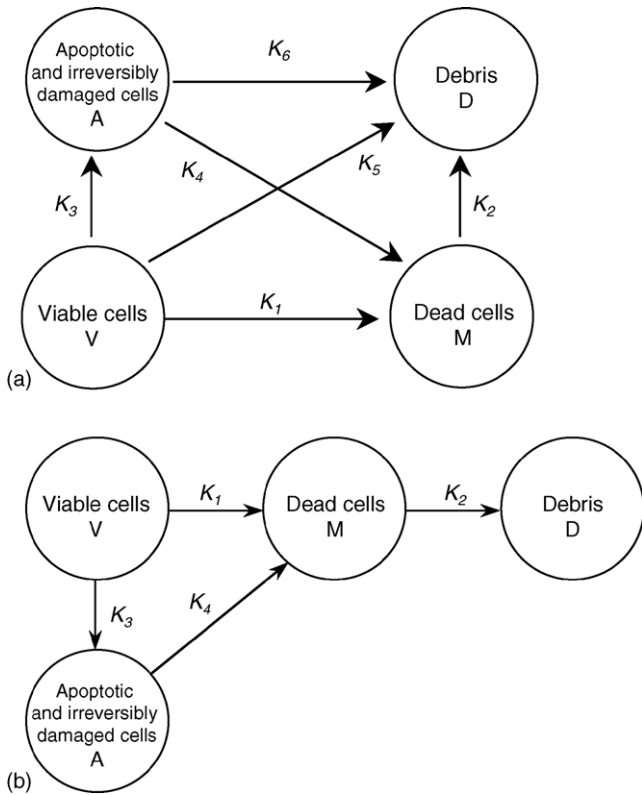


Fig. 1. A model for the emergence of apoptotic cells, irreversibly damaged cells, dead cells and cell debris from viable cells. The first-order rate constants for the various steps are K_{1-6} . (a) Whole cells may be directly disintegrated ($K_{5,6} \neq 0$); (b) whole cells are not directly disintegrated ($K_{5,6} = 0$).

$$\frac{dM}{dt} = -K_2M + K_1V + K_4A \quad (5)$$

$$\frac{dA}{dt} = -K_4A + K_3V \quad (6)$$

where K_{1-4} are the first-order rate constants for the steps shown in Fig. 1b.

Simultaneously solving Eqs. (4)–(6), leads to the following equations:

$$V = V_0 \exp[-(K_1 + K_3)t] \quad (7)$$

$$M = \frac{\alpha + \beta}{K_2 - K_1 - K_3} [e^{-(K_1+K_3)t} - e^{-K_2t}] + \frac{\gamma - \beta}{K_2 - K_4} [e^{-K_4t} - e^{-K_2t}] + M_0 e^{-K_2t} \quad (8)$$

$$A = \frac{K_3 V_0}{K_4 - K_1 - K_3} [e^{-(K_1+K_3)t} - e^{-K_4t}] + A_0 e^{-K_4t} \quad (9)$$

$$D = 100 - V - M - A \quad (10)$$

Assuming, logically, that the apoptotic or irreversibly damaged cells die slowly, the time scale for going from A to M (Fig. 1b) will be much larger than the time scales for

the other steps in Fig. 1b, i.e. $K_4 \ll K_{1-3}$ (K_4 is close to zero). Thus, Eqs. (8) and (9) can be simplified to the following equations:

$$M = \frac{\alpha}{K_2 - K_1 - K_3} [e^{-(K_1+K_3)t} - e^{-K_2t}] + M_0 e^{-K_2t} \quad (11)$$

$$A = A_0 - \frac{K_3 V_0}{K_1 + K_3} [e^{-(K_1+K_3)t} - 1] \quad (12)$$

where

$$\alpha = K_1 V_0 \quad (13)$$

$$\beta = \frac{K_4 K_3 V_0}{K_4 - K_1 - K_3} \quad (14)$$

$$\gamma = K_4 A_0 \quad (15)$$

In the above equations, V_0 is the initial percentage of viable cells, M_0 is the initial percentage of dead cells, A_0 is the initial percentage of apoptotic or irreversibly damaged cells and D_0 is the initial percentage of cell debris. The constants K_i s are likely to depend on factors such as temperature, agitation speed and the viscosity of the broth.

4. Results and discussion

The time profiles of the variables A , D , M and V are shown in Fig. 2 at the agitation intensities of 0 rpm (i.e. controls) and 75 rpm. Note that the x -axes of the two plots (Fig. 2) have different scales. In both cases, the fraction of the apoptotic or irreversibly damaged cells (i.e. A) is always low, but the viable fraction of the population (i.e. V) is lost much more rapidly in the agitated culture. The standard deviation and the average values of the measured variables show a high level of reproducibility of the measurements (Fig. 2).

To facilitate comparison, the data on viable cell fraction (Fig. 2) at the two agitation intensities are replotted in Fig. 3. Clearly, in the absence of agitation viable cells can be detected at up to 96 h, but fewer than half the original live cells have survived by 2 h in the agitated environment (Fig. 3). This is clear evidence of an exceptionally high sensitivity of the marine sponge cells to hydrodynamic forces.

The model-generated profiles and the experimental data for the variables V , A , D and M are shown in Fig. 4 at two values of the agitation speed. The figure demonstrates an excellent ability of the model to reproduce the measured values. Eqs. (7)–(10) are used to generate the various modeled profiles shown in Fig. 4. The viable fraction of the cells declined extremely rapidly at 100 rpm. A more gradual decline occurred under more gentle agitation of 75 rpm (Fig. 4). Interestingly, the fraction of the apoptotic or irreversible damaged cells was always low throughout the experimental period and irrespective of the agitation intensity (Fig. 4). This suggests that the progression to apoptosis or irreversibly damage from the viable state did not depend on hydrodynamic conditions and the cells that attained this state remained in it a long time.

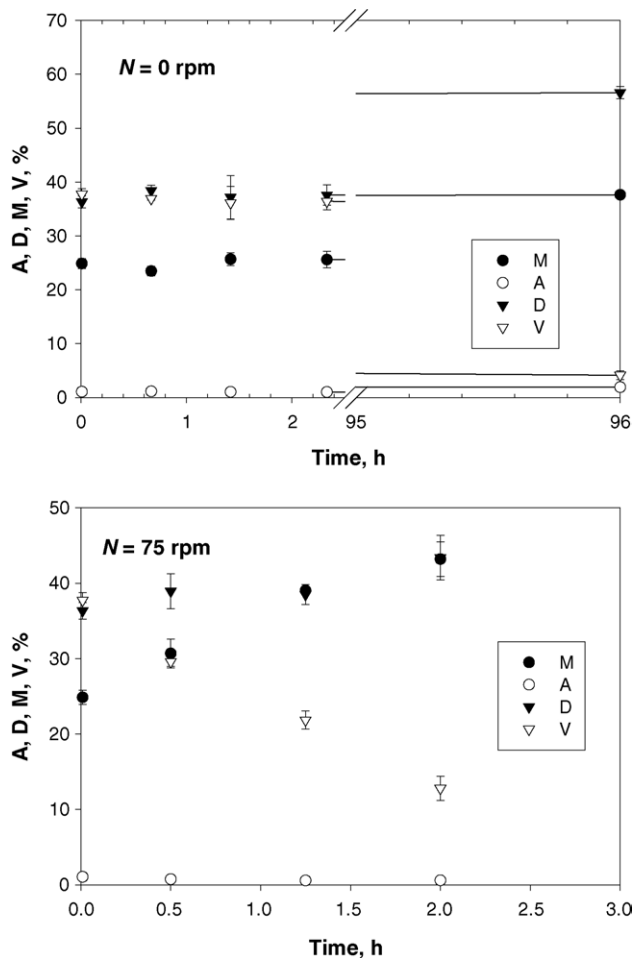


Fig. 2. The variables A , D , M and V vs. time at two values of agitation speed. No Pluronic® F68.

Unlike the fraction of the apoptotic or irreversibly damaged cells, the cell debris fraction (D) rose with time. This and the profiles of M (i.e. dead whole cells) in Fig. 4 suggest that the viable cells were lost in two consecutive ways (steps 1 and

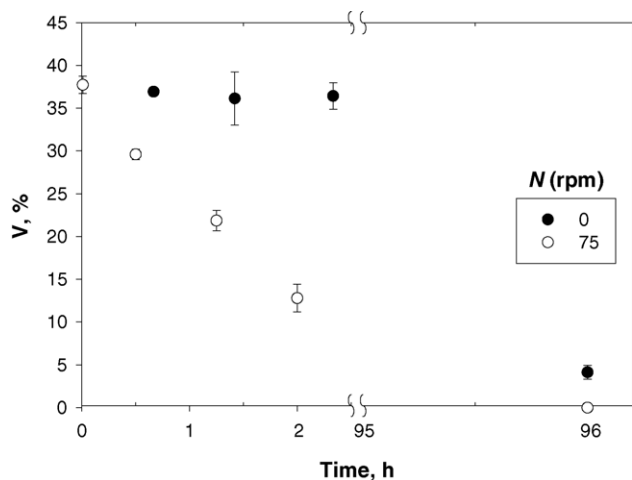


Fig. 3. Effect of agitation speed on viable fraction V of cells. No Pluronic® F68.

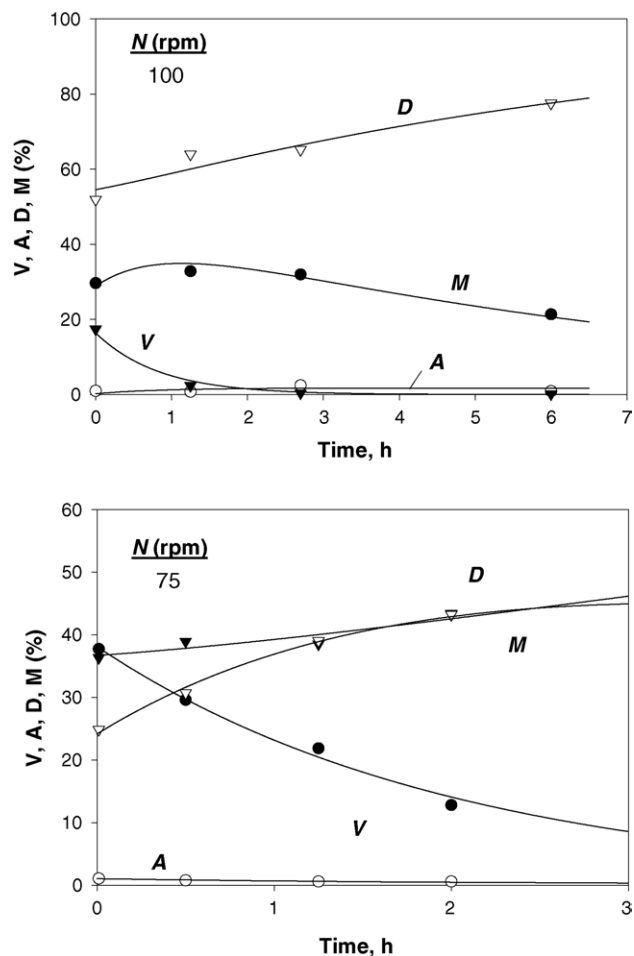


Fig. 4. Fit of the model (solid lines) to the measured data (V , A , D and M) at 100 and 75 rpm.

2 in Fig. 1b): (1) by being killed while remaining physically intact, by the damaging forces and (2) by being subsequently disintegrated into debris by physical breakage. The shape of the M and D profiles suggest that the second process was slower than the one that initially had caused the kill (i.e. $K_1 > K_2$).

The values of K_{1-3} that produced the best fit of the modeled profiles to the measured values of V , A , D and M , are shown in Table 1 for the various agitation speeds. A negative value of K_3 in one case may suggest that the viable-to-apoptotic or irreversible damage transition may not be completely irreversible, or simply that both reversible and irreversible damage to a cell are possible scenarios. The negative value cannot be ascribed to experimental error, as the values of A used in the fit were all within experimental error. The K_{1-3} values

Table 1
Values of the rate constants K_{1-3} for various agitation speeds N

N (rpm)	K_1 (h^{-1})	K_2 (h^{-1})	K_3 (h^{-1})
0	0.0225	0.0055	0.0006
75	0.5096	0.0819	-0.0118
100	1.0810	0.1318	0.1095

(Table 1) confirm a nonlinear dependence of these rate constants on the hydrodynamic regimen (i.e. N -value).

For all experiments, the model predicted fraction of the viable cells consistently agreed with the measurements within $\pm 10\%$ for the full range of agitation intensities (including the nonagitated controls), as shown by the parity plots in Fig. 5.

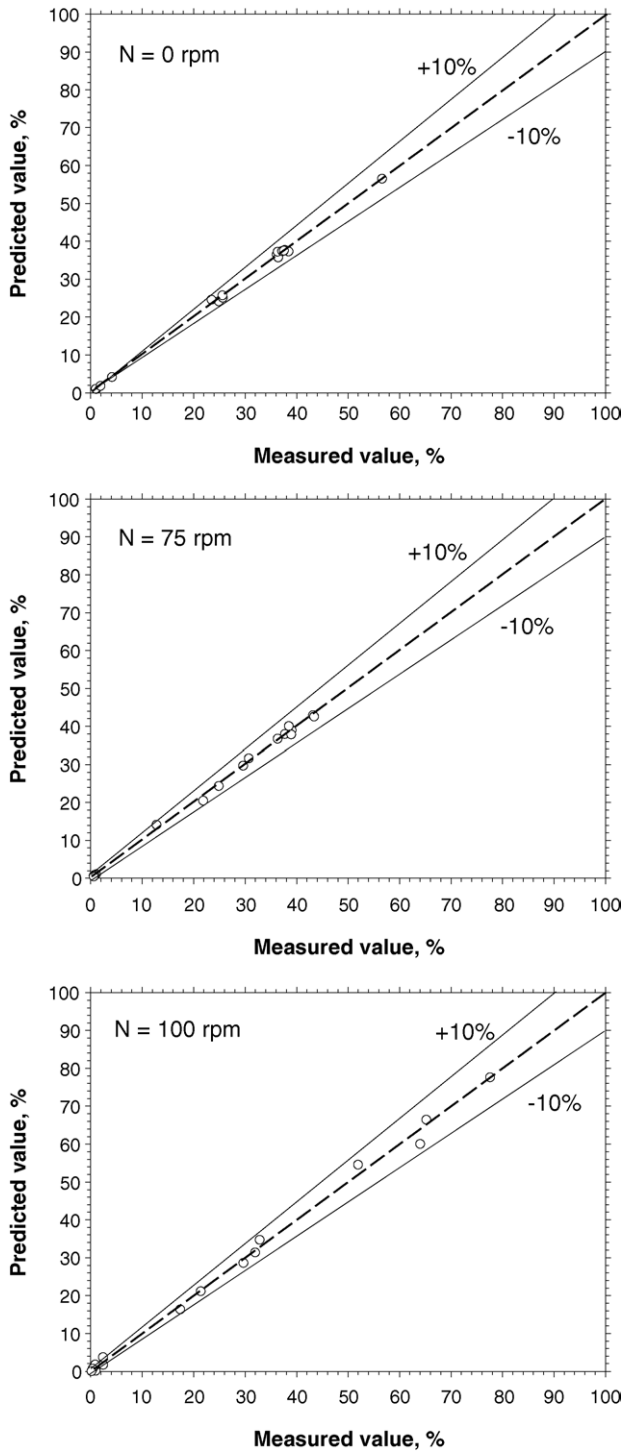


Fig. 5. A comparison of the measured and model predicted values of the viable cell fraction V at various agitation speeds (0, 75 and 100 rpm).

This substantiates the general cell damage and debris generation mechanisms proposed in Fig. 1b and the following significant assumptions of model: (1) A first-order transformation of the viable cells to apoptotic or irreversibly damaged cells and dead cells; (2) a long-lived apoptotic or irreversible damage state that was essentially independent of the hydrodynamic conditions; (3) a first-order disintegration of the dead whole cells to fragments; (4) an absence of cell–cell interactions as a factor contributing to cell damage.

4.1. Effects of Pluronic® F68

The effects of supplementing the cells with Pluronic® F68 in suspensions agitated at 100 rpm, are shown in Fig. 6. In PF68-free suspension, the viable fraction (V) of the populations declined dramatically within 1 h of agitation and the fraction of the debris D rose with time. In contrast, the viable fraction and debris levels remained stable for up to 6 h in PF68-supplemented suspensions. Clearly, PF68 had a protective effect on cells.

Pluronic® F68 is a nonionic surfactant polyol with a molar mass of 8.3 kDa. The surfactant is not metabolized by animal cells and has no effect on the viscosity of suspensions in the

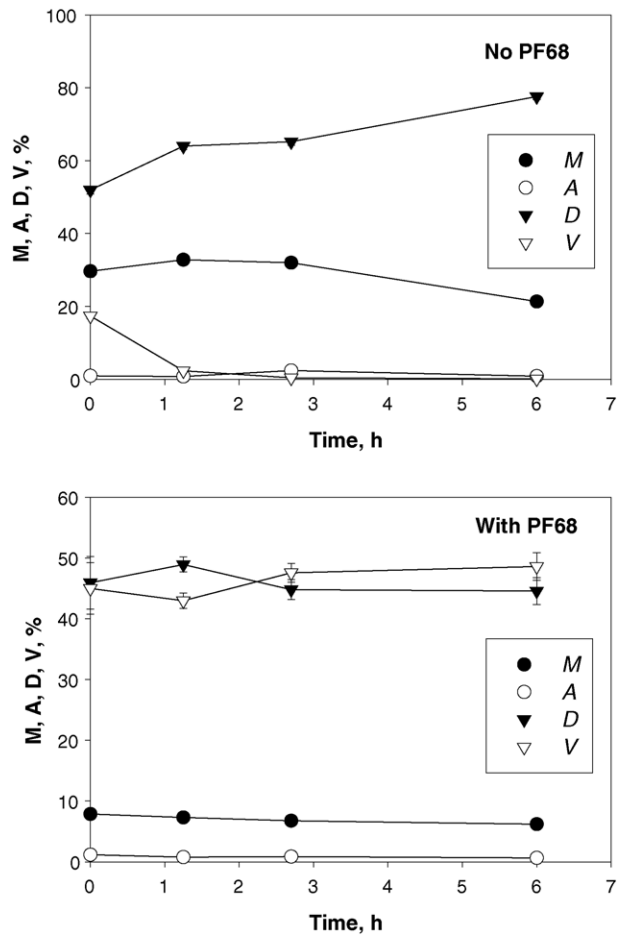


Fig. 6. The variables M , A , D and V vs. time at 100 rpm. Effect of Pluronic® F68.

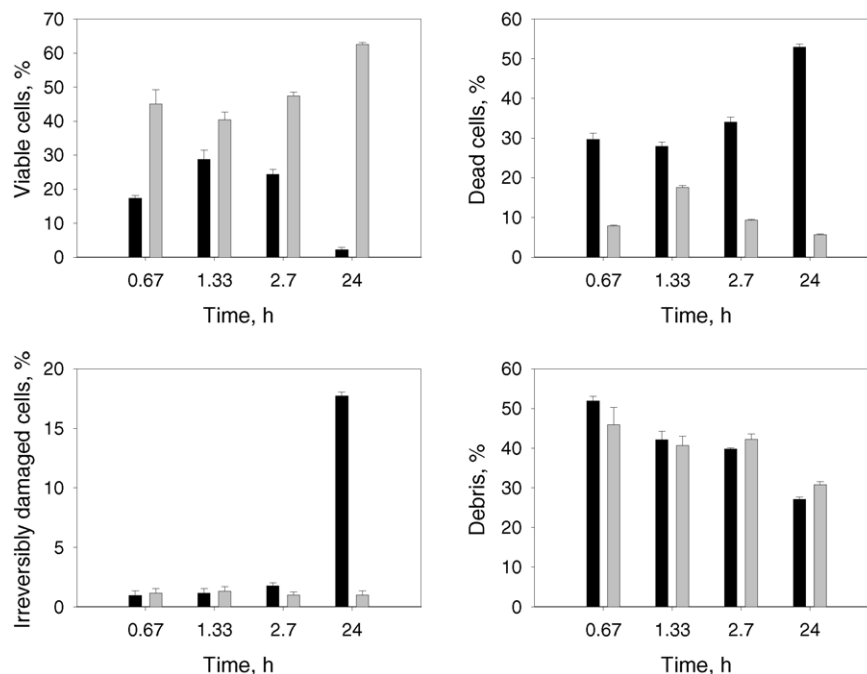


Fig. 7. Percent of viable cells, dead cells, irreversibly damaged cells and debris in samples at various times. Solid bars are for controls (no. PF68). Grey bars are for samples supplemented with PF68.

concentration used. PF68 has been shown to have a protective effect on a great variety of animal cells in both aerated and unaerated cultures. Because it does not affect viscosity, a possible dampening of turbulence in PF68-supplemented media cannot be an explanation for the observed cell protective effect. PF68 is known to biochemically associate with the cell membrane to reduce the plasma-membrane fluidity and this has been suggested as a possible mechanism of protection [6,10–14]. A physical strengthening of the cells will almost certainly reduce the values of the first-order rate constants K_{1-3} in Fig. 1, to reduce the cell damage.

The Müller protocol for producing primary cell suspensions from sponge tissue, typically requires agitation of the tissue in CMFSW with a change of the suspending medium at 40, 80, 160 min and 24 h intervals [3]. The protocol as used currently, does not supplement the medium with PF68. Results presented in Fig. 7 show that in comparison with the current protocol, supplementation with PF68 greatly improves the yield of the viable cells and reduces the fraction of the dead whole cells at each of the transfer stages (0.67, 1.33, 2.7 and 24 h) of the protocol. Thus, PF68 reduces damage to cells as they are produced by dissociation from the parent sponge tissue.

4.2. Effects of incubation temperature

The effect of incubation temperature (3 and 17 °C) on survival of suspended cells in nonagitated medium was examined in some experiments. The data on viable fraction V and the ratio of the viable to dead whole cells (V/M), are

shown as a function of incubation time and temperature in Fig. 8. Although in the first 2.5 h, the effect of temperature on decline in the viable population is not quite obvious, the measurements at 96 h clearly reveal that the viable fraction of the population declined much slower at 3 °C than at 17 °C (Fig. 8). Furthermore, at the lower temperature the viable cells constituted a much bigger proportion of the intact cells than at the higher temperature (Fig. 8). The results confirm that the rate constants K_{1-4} are temperature dependent in addition to being influenced by the hydrodynamic environment.

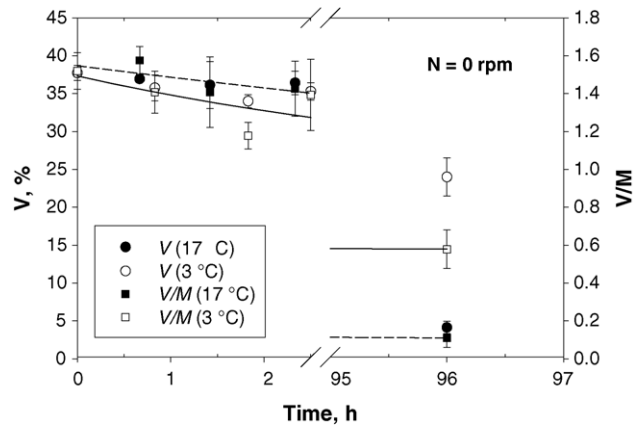


Fig. 8. Effect of operating temperature (3 and 17 °C) on viable fraction V and the ratio V/M . No agitation.

5. Concluding remarks

The following specific conclusions can be drawn:

1. The well-known Müller protocol [3] for producing viable sponge cells from explants, damages cells. The damage can be prevented by supplementing the artificial seawater medium with Pluronic® F68 at a concentration of 0.1 (w/v). Consequently, supplementation with PF68 can enhance the supply of cells for producing primmorphs and whole sponges from single cells.
2. Potentially, the damage to cells can be reduced by reducing the operating temperature.
3. Only a few apoptotic or irreversibly damaged cells are formed from viable cells and any apoptotic or irreversibly damaged cells formed, slowly progress to the dead cell stage.
4. A six-step mechanistic model was reduced to one of four-step for cell damage in orbital shakers, which explains exceptionally well the observed kinetics of damage. The rate of damage increases strongly with increasing intensity of agitation. The damage does not depend on cell–cell interactions and the individual steps of the model obey first-order kinetics.

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