ISNN 1517-6770

Optimized culture and growth curves of two ciliated protozoan strains of *Paramecium caudatum* Ehrenberg, 1833 to use in ecotoxicologycal assays

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Abstract. Protozoans are microbial eukaryotes known to be ideal for aquatic ecotoxicological testing. Therefore, aiming to evaluate their possible use in standardized ecotoxicity assays we determined the best cultivation conditions and growth curves for two strains of the ciliated protozoan *Paramecium caudatum* Ehenberg 1833. One strain (PC1) was isolated from the Monjolinho reservoir-SP (22° 01' S and 47° 53' W) where the mean dissolved O_2 is 5.205 mgL^{-1}, pH 6.43 and electrical conductivity 34.75 µS cm^{-1}. The other strain (PC2) from the Óleo lagoon in Jataí Ecological Station- SP (21° 36' S and 47° 49' W), with mean dissolved of 4,42 mgL^{-1}, pH 4.90 and electrical conductivity 10.96 µS cm^{-1}. The higher mean cellular biovolume and density obtained were used to evaluate different cultivation parameters such as temperature, pH, light exposition, stirring regimen and cultivation flask size. Considering protozoan densities and cell biovolume, the best culture conditions for PC1 strain was pH 9.0 at 30 °C and for PC2 was pH 7.0 at 27.5 °C. For both strains and evaluated parameters (biovolume and density), the bottle size did not interfered in the results and absence of light resulted in better ones. Concerning agitation, for PC2, there was no significant difference for this parameter and for PC1 in the absence of shaking we obtained better results. Therefore we decided to make the tests in the absence of light and without shaking. Regarding cell biovolume, the best condition was pH 9.0 at 25 ° C for the PC1 strain and pH 8.0 at 20 ° C for PC2 strain. The generation time calculated in a 96 hours growth curve was 8.35 hours for PC1 and 7.6 hours for PC2. In the end of the experiment, the number of generations (*n*) and maximum population (N) were higher for PC2 than for PC1 and were, 11.02 and 10.78, respectively, for *n* values and 7548 and 6400 for N ones. The growth strategies of the two strains were quite different but both were considered suitable for laboratorial growth and use for ecotoxicity tests in standardized conditions.

Keywords: ciliate, culture, freshwater, generation time.

Introduction

Having environmental health as main concern, the "*in vitro*" ecotoxicological evaluation can be made up using test-organisms, which are considered as living responses models for chemical compounds. "*In vitro*" toxicity experiments can be made through direct exposure of the test organisms to different concentrations of pure chemical reagents, mixtures of defined reagents to check for possible synergisms/antagonisms or even undefined chemical mixtures such as crude samples dumped

from different industries.

According to Niemeyer *et al.* (2009), toxicity tests require easily obtained organisms with short life cycle, high number of descendants and well adapted to the laboratory conditions. Moreover, according to Domingues & Bertoletti (2008), it is necessary high and constant sensitivity to a variety of chemicals to obtain accurate results, good repeatability and reproducibility.

For laboratory cultivation and testing, these authors, pointed that is necessary prior knowledge of some aspects of the species biology, such as reproduction, feeding habits, physiology and behavior. Protozoans are eukaryotic organisms known to be very sensitive to environmental changes (Madoni, *et al*., 1995; Madoni & Romeo, 2005) and ideal as early warning indicators of aquatic ecosystems deterioration and for toxicological testing (Madoni, 2000; Logar & Vodovnik, 2007; Gomiero *et al.,* 2013). They can be important links and mediators of substances and energy flow in trophic chains (Sherr *et al.,* 1988). The ciliates are basic components of protozoan microplanktonic and microbentonic communities in aquatic environments, playing qualitative and quantitative critical roles (Fenchel, 1987). Protozoans have been used in toxicological studies and proposed as biological indicators of water pollution due to their sensitivity to environmental changes, short life cycle and ease of cultivation and maintenance in the laboratory (Nicholas *et al.*, 1999; Nalecz -Jawecki & Sawicki, 2002; Dias, Mortara & Lima, 2003; Nalecz-Jawecki, 2004; Rao, 2006).

The protozoans frequently used in toxicity experiments are ciliates, especially *Tetrahymena*, in axenic culture. This genus was tested against 57 different chemical reagents (Yoshioka *et al.*, 1985), heavy metals, drugs and certain surfactants (Nilsson, 1989; Nicholas, & Mota Lima, 1999; Dias *et al*., 2003), mycotoxins (Benitez *et al.,* 1994); xenobiotics (Sauvant *et al*., 1995), inorganic elements such as Ba, Cd, Co, Cr, Cu, Fe, Ge, Hg, Mn, Nb, Pb, Sb, Sn, Ti, and Zn (Sauvant, 1997) and titanium dioxide nanoparticles (Ud-Daula *et al*., 2013).

Ciliates are also used in monoxenic cultures to assess the pollutants toxicity such as detergents (Dryl & Mehr, 1976), heavy metals (Martin-Gonzalez *et al*., 2006; Miyoshi *et al.,* 2003), carcinogenic substances, herbicides, insecticides, fungicides, antibiotics and organic solvents (Miyoshi *et al.,* 2003) However, ciliates were also used in holoxenic cultures to assess the toxicity of heavy metals, pesticides, benzene and phenol derivatives (Twagilimana *et al.,* 1998; Nalecz-Jawecki & Sawicki, 1999 2002; Nalecz-Jawecki, 2004).

The knowledge of the ciliates sensitivity to a large number of toxic substances will help to identify the strength and potential of ecological damage caused by anthropogenic pollutant discharges to surface waters (Madoni & Romeo, 2006). The ciliate *Paramecium caudatum,* used in this study, is one of the best known protozoans, usually found in the hypolimnion or associated with macrophytes and, according to Miyoshi *et al.* (2003), is one of the most commonly used ciliate species in laboratory research.

As *P. caudatum* is easily cultivable in the laboratory (Rao *et al.,* 2006, 2008) and has high tolerance to organic matter (considering its natural habitats and high value of saprobic index), we believe it has potential to be used as test organism to evaluate pure chemical pollutants and in mixture in environmental samples. So, in this study we compared the cultivation parameters for two *Paramecium caudatum* strains, aiming to evaluate

the most appropriate for use in ecotoxicological tests.

This work evaluated the best culture conditions of two strains of *P. caudatum*, a cosmopolitan ciliate protozoan originated from different sources, based on parameters of its life cycle, aiming the utilization of this microorganism as a model to ecotoxicological bioassays.

MATERIAL AND METHODS

Strains and sampling sites

The two strains of *P. caudatum* used in this study were isolated from environmental samples that were screened in laboratory. Isolation and introduction of the organisms into culture media were made using glass microcapillaries under an optical microscope. After confirming the protozoan growth and cultures purity, a new culture was started, for each strain, using only one individual.

One strain, which was assigned with the code PC1, was isolated several years ago from Monjolinho Reservoir (São Carlos-SP), located at the campus of the Federal University of São Carlos, that has no history of pesticide contamination. The other strain, which received the code PC2, was isolated 23 months before the start of this work from water samples containing macrophytes of the Oil lagoon (Jataí Ecological Station- SP), a location with reports on pesticide contamination (Peret *et al.*, 2010) from sugarcane plantations. The sites of isolation of *P. caudatum* strains were chosen because of the differences on the pesticide contamination history of both.

Protozoan cultures

According to protocols established by *e.g.* Packroff *et al.*, 2002; Bass *et al.*, 2007; Wickham & Gugenberger, 2008, the protozoans were cultivated in 25 mL bulk test tubes with a 2% agar slant. In each tube, it was added a rice grain with husk (previously boiled and autoclaved) and 10 mL of an *Enterobacter aerogenes* bacterial suspension (10^6 cells mL^{-1}) made with mineral water Minalba® (Figure 1). Bacteria is the food for this ciliated protozoan and we used this concentration (10^6 cells mL^{-1}) because it is the most common value found in plankton. Based on our laboratory observations, for culture maintenance, subcultures were done once a month from the cultures started with one organism.

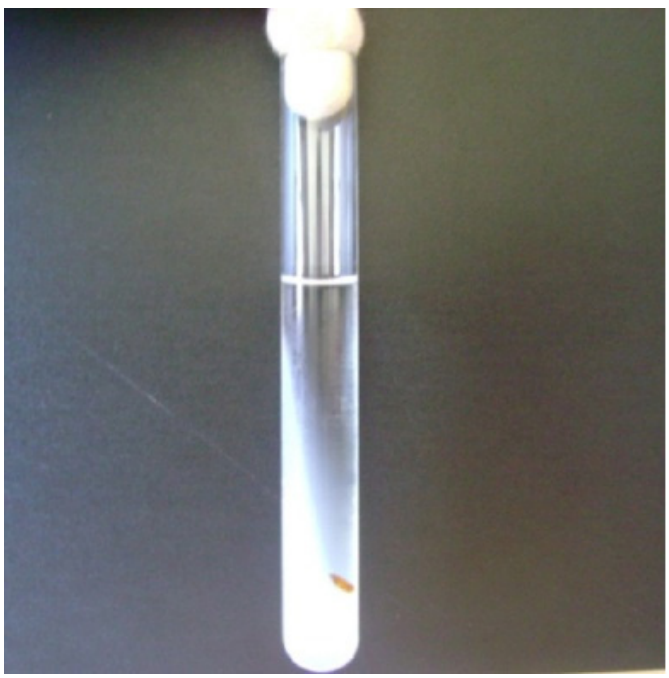

Figure 1. Test tube with protozoan culture media.

Cultivation experiments of *Paramecium caudatum*

The two strains of *Paramecium caudatum* (PC1 and PC2) were tested for their best growth conditions related to the physical-chemical parameters (pH, temperature, light, size of culture flasks and shaking). The first experiments were

performed in glass test tubes with 10 mL of culture medium, varying the pH (6, 7, 8, 9) and incubation temperatures (20 ° C, 25 ° C, 27.5 ° C and 30 ° C), based on the most common natural environmental conditions. After determining the best conditions for these two variables, tests were performed in larger scale using milk dilution bottles (total volume of 140 mL) containing 120 mL of bacterial suspension (10^6 cells mL^{-1}), twelve rice grains with husk and a base of 2 % agar. In these flasks, using the best pH and temperatures defined, it was tested different photoperiods, because they can live in shadow or exposed to solar light. In the third experiment we tested the effect of agitation in the cultures, trying to simulate natural conditions which were also performed in milk dilution bottles using the best pH, temperature and photoperiod for each strain. For each of the sixteen different conditions (combinations between pH and temperature) three replicates were prepared, with a total of forty-eight tubes. The duration of this experiment was pre-set to 72h.

Determination of the best temperature and pH values for the protozoan growth

The mineral water used to prepare the bacterial suspension was sterilized at an autoclave and subsequently had the pH aseptically adjusted to the desirable value according to the experiments, because it was verified that this sterilization process significantly raises the mineral water pH. For these experiments, cultures were prepared in the cited test tubes containing agar slant at 2% as described previously. Five protozoans were inoculated in each cultivation tube.

At the end of the cultivation period, three 1 mL aliquots from each tube were fixed with a saturated $HgCl_2$ solution and stained with 0.04% bromophenol blue (Pace & Orcutt, 1981). For each sample, the fixed protozoans were counted and measured under a light microscope at 100X using a Sedgewick-Rafter chamber and the mean linear measurements (length and width, measured with a microscale inserted in the ocular) of 40 individuals were used for the volume estimations. In samples with less than 40 individuals, all the organisms were measured. For the volume estimations we used the prolate spheroid geometric formulae whose shape resembles more *Paramecium caudatum*. The formula was: 4/3. ¶. $a.b^2$, where "a" corresponded to the length of the higher semi-axis and "b" to the length of the lower semi-axis.

The optimum conditions were considered those who had shown the greatest number of cells with bigger biovolumes, as parameters. They were chosen because, these parameters are used for biomass calculations, which ultimately is used to evaluate how much these populations are important and representative in terms of organic matter in the environment.

Influence of illumination on *P. caudatum* strains growth

Three illumination conditions (12:12 hours light/dark photoperiod, 24 hours dark and 24 hours light) were used. For each strain (PC1 and PC2), sixty organisms were inoculated in the cultivation bottles (capacity 140mL) containing 120 mL of the bacterial suspension (10^6 cells.mL) and a base agar. The flasks were incubated for 72h at pH and temperature determined in the previous experiment. This experiment was performed in triplicate and, after incubation, three 1 mL aliquots from each flask were fixed, stained and counted as

described above. Based on the highest number of cells obtained at the end of the experiment, the most favorable illumination condition for each strain was defined.

Influence of culture agitation on the *P. caudatum* strains growth

It was tested a moderate agitation effect (70 RPM), compared to static cultivation on the growth of the two *P. caudatum* strains. For each strain, sixty protozoan cells were inoculated in three bottles (triplicates) with the bacterial suspension (120 mL) with an agar base. The cultivation flasks were incubated, at the temperature, pH and light conditions determined in the previous experiments.

Protozoans growth curves and generation time

Each strain was cultivated in three bottles (triplicates) with 120 mL of bacterial suspension, as described above, with sixty protozoan cells. These experiments were incubated at the temperature, pH, light and shaking conditions determined in the previous experiments. Cultures were monitored for 96 hours, removing aseptically 1 mL aliquots at time intervals of 6 hours, that is a lower value than its generation time in literature. The aliquots were immediately fixed with saturated solution of $HgCl_2$, stained with bromophenol blue solution of 0.04% according to Pace & Orcutt (1981), and subsequently counted in Sedgewick-Rafter chamber at optical microscopy (100X).

The mean protozoan density of the triplicates was plotted with time and the generation time was calculated from the maximum growth phase according to Pelczar Jr. *et al*. (1997). The number of generations was calculated using the formulae: $n = 3.3 (\log N - \log N_0)$ - where n is the number of generations, N corresponds to the total population maximum and N_0 is the number of microorganisms inoculated. Then, the generation time (g) was calculated by the following formulae: $g = t / n$ - where: t corresponds to the time in wich N was obtained.

Statistics

The experiments were designed by factor analysis and the results, after obtaining the data, were plotted to obtain Pareto charts using Estatistica® 9.1 software. Such charts were not shown here but used to define if the variable influence or not the studied parameters.

RESULTS

Determination of the best temperature and pH values for the protozoan growth

***Paramecium caudatum* strain from Monjolinho reservoir (PC1)**

Figure 2 shows the average densities of organisms (PC1 strain), obtained after 72 hours of culture in tubes, under different conditions of pH and temperature. At 20 ° C, the condition that showed the highest densities was pH 8 and the lowest was pH 7 (Figure 2A). At 25 ° C, in all pHs tested, the average number of organisms were much lower than those obtained at other temperatures (Figure 2B). At 27.5 ° C, the condition that showed highest densities was again pH 8 and the lowest was at pH 6 (Figure 2C) and at 30 ° C (Figure 2D), the best growth condition was at pH 9 and the worse was at pH 6.

Comparing the density obtained for PC1

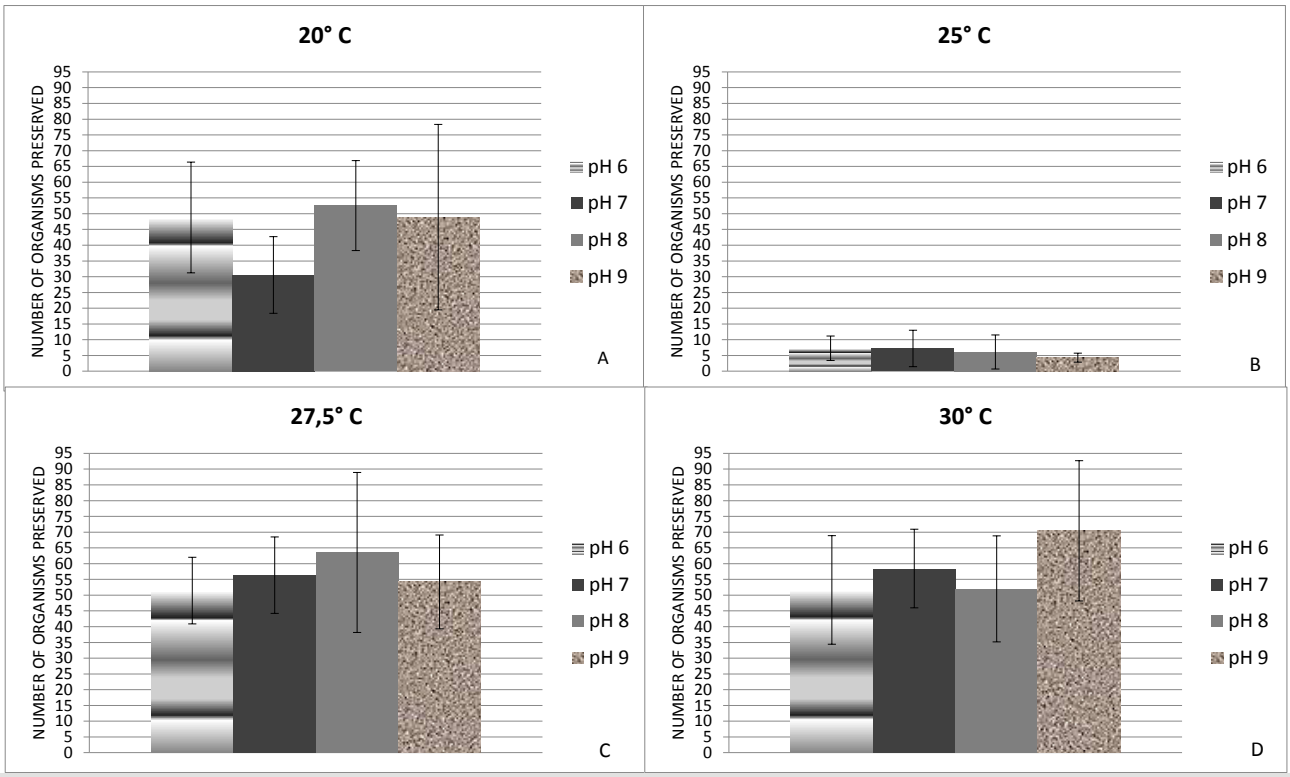

Figure 2. Mean densities (mL^{-1}) of *P. caudatum* PC1 strain incubated at 6, 7, 8 and 9 pHs for 72h at 20° C (A), 25° C (B), 27,5° C (C) and 30° C (D).

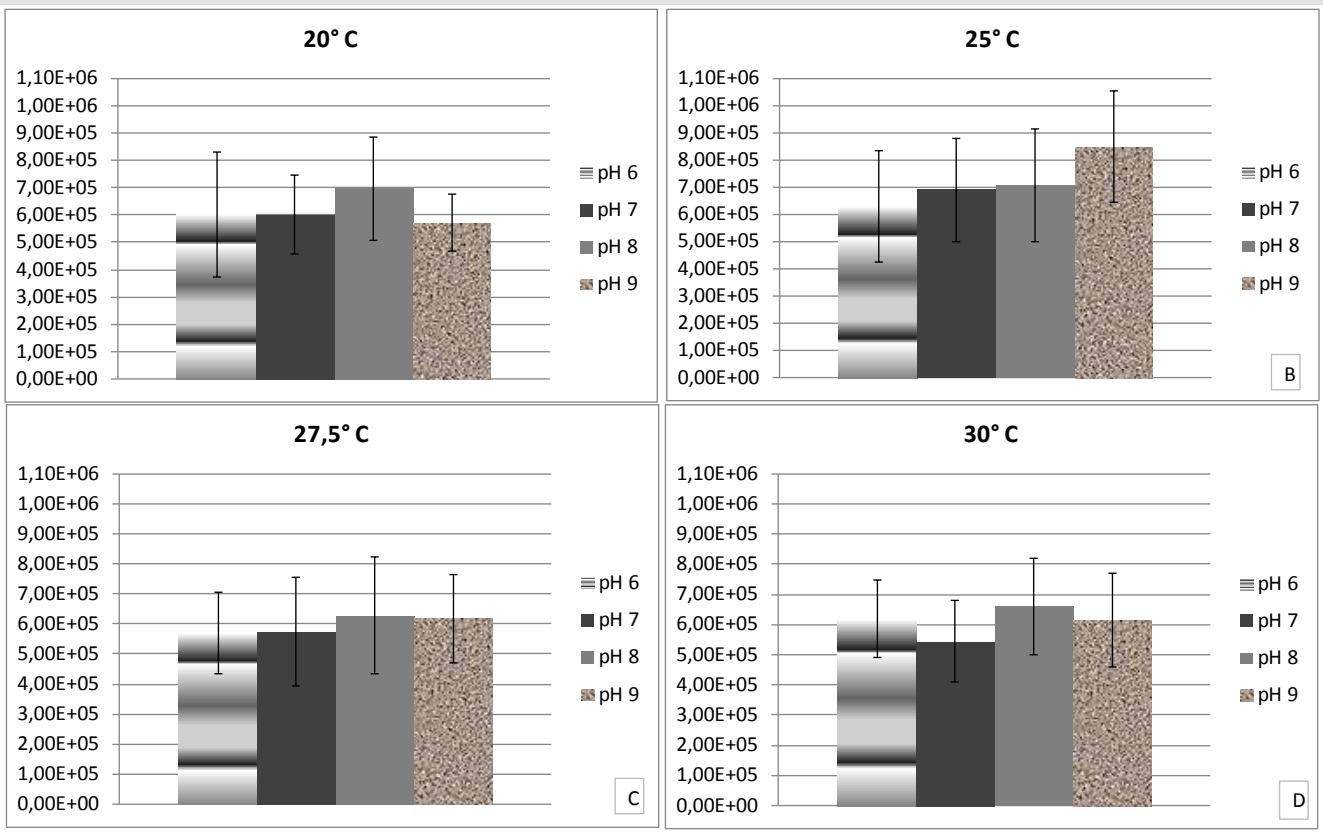

Figure 3. Mean cell biovolumes of *P. caudatum* (PC1) strain after 72h growth at 20°C (A), 25°C (B), 27.5°C (C) and 30° C (D).

strain at the four temperatures (20, 25, 27.5 and 30 ° C) and pHs (6, 7, 8 and 9) tested, the best conditions, i.e. the highest densities obtained, were: at 30 ° C and pH 9 (Figure 2D), with mean values of 70.44 (±22.27) cells mL^{-1} and at 27.5 ° C and pH 8 (Figure 2C), with mean values of 63.56 (±25.38) cells mL^{-1}. On the other side, the worst condition was at 25 ° and pH 9 (Figure 2B), with mean values of only 4.33 (±1.41) cells mL^{-1}.

For PC1 strain, the biovolume and density data at the same pH and temperature conditions (25° C and pH 9) had opposite trends, as we can see at Figures 3B and 2B, where we found the lowest population density and the highest mean cell biovolume.

Figure 3 shows the mean cell biovolumes obtained for PC1 strain, after 72 hours of cultivation in tubes, under the same conditions of pH (6, 7, 8 and 9) and temperature (20, 25, 27.5 and 30ºC). Although the best pH was 8 for the three temperatures (20°C, 27.5°C and 30°C, respectively, Figures 3A, 3C and 3D) the result at pH 9 at 25°C presented the highest mean biovolume 8.486 ±2.023 x 10^5 μm^3. In contrast, the worst condition, where the mean biovolume (5.430 ± 1.351 x 10^5 μm^3) was lower, was at pH 7 and 30 ° C (Figure 3D).

Paramecium caudatum strain from Óleo Lagoon (PC2)

Figure 4 shows the mean densities obtained for PC2 strain, after 72 hours of cultivation in tubes, under different conditions of pH and temperature.

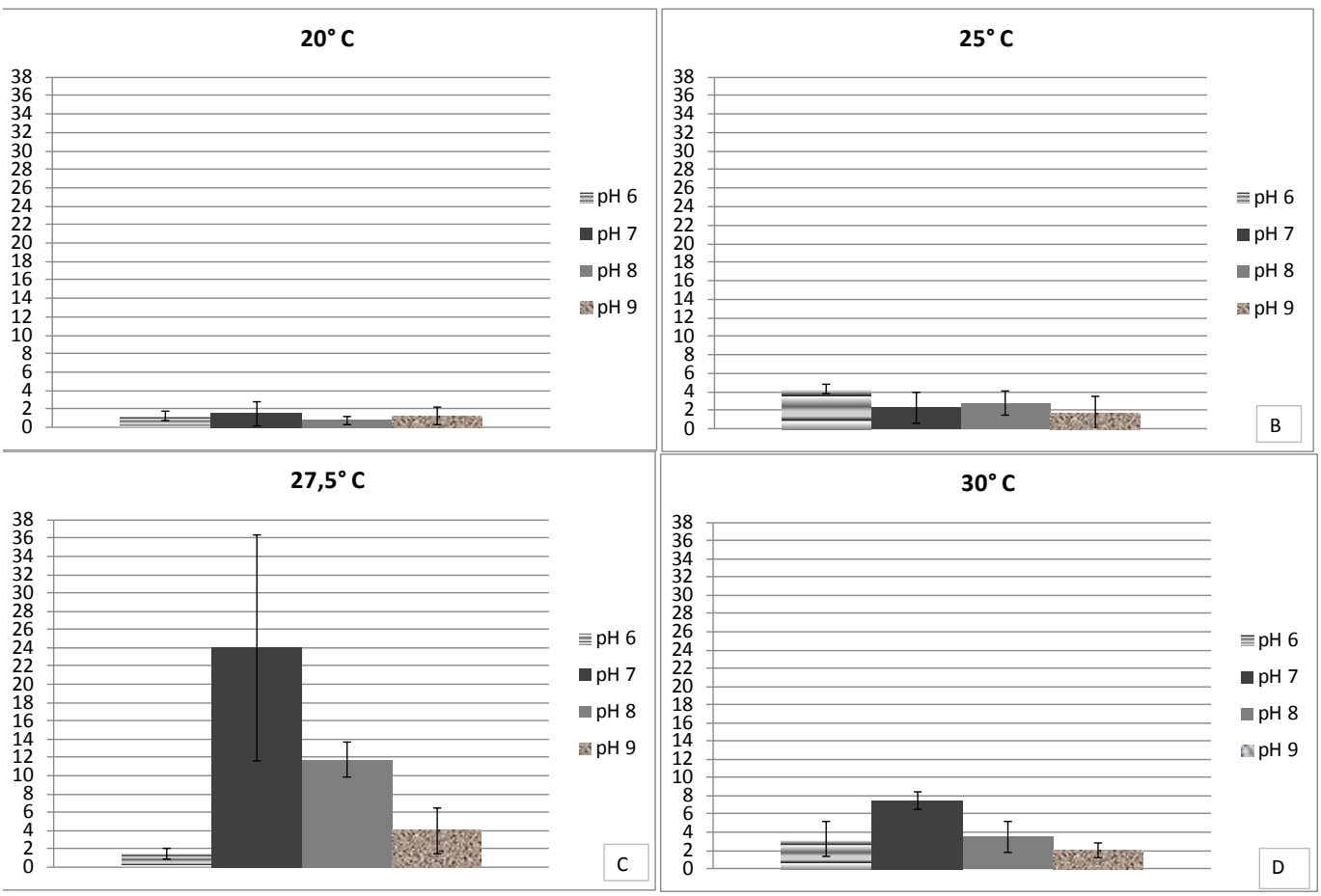

Figure 4. Mean densities (mL^{-1}) of *P. caudatum* PC2 strain incubated at 6, 7, 8 and 9 pHs for 72h at 20° C (A), 25° C (B), 27,5° C (C) and 30° C (D).

When comparing all conditions, we found at 27.5 °C (Figure 4C) the highest densities of protozoa (pH 7) and also the lowest (pH 6). On average, temperature 20 °C (Figure 4A) showed lower densities at all pHs. In pH 6 the best results (highest density of protozoa) was found at 25 °C, in pH 7 at 27.5 °C, and in pH 8 and 9 at 30 °C.

Figure 5 shows the mean cell biovolumes found for PC2 strain after 72 hours of cultivation in tubes, under the same conditions of pH and temperature. At 20 ° C (Figure 5A) and 25 ° C (Figure 5B) the higher mean biovolumes were obtained in pH 8, whereas at 27.5 ° C (Figure 7C) in pH 7 and at 30 ° C (Figure 5D) in pH 6. The best condition was in pH 8 at 20 ° C (Figure 5A) that resulted in a mean cell biovolume of 7.973 ± 2.170 x 10^5 µm^3. In contrast, the worst was in pH 9 at 30 ° C (Figure 5D), with a mean cell biovolume of 4.538 ± 1.612 x 10^5 µm^3.

Influence of illumination on the growth of *P. caudatum* strains

For PC1 strain, the most favorable cultivation condition was pH 9 at 30 ° C, considering the mean cell biovolume and density in flask cultures. Therefore, the best condition with larger culture flasks was in the dark (average density of 67.833 cells mL^{-1}) as shown in Figure 6A.

For PC2 strain, the most favorable cultivation condition was pH 7 at 27.5 ° C. The higher density for this strain with larger culture flasks was also obtained in the dark (mean density of 20.833 cells mL^{-1}), as can be seen in Figure 6B.

Influence of culture agitation on the *P. caudatum* strains growth

These experiments were performed at the

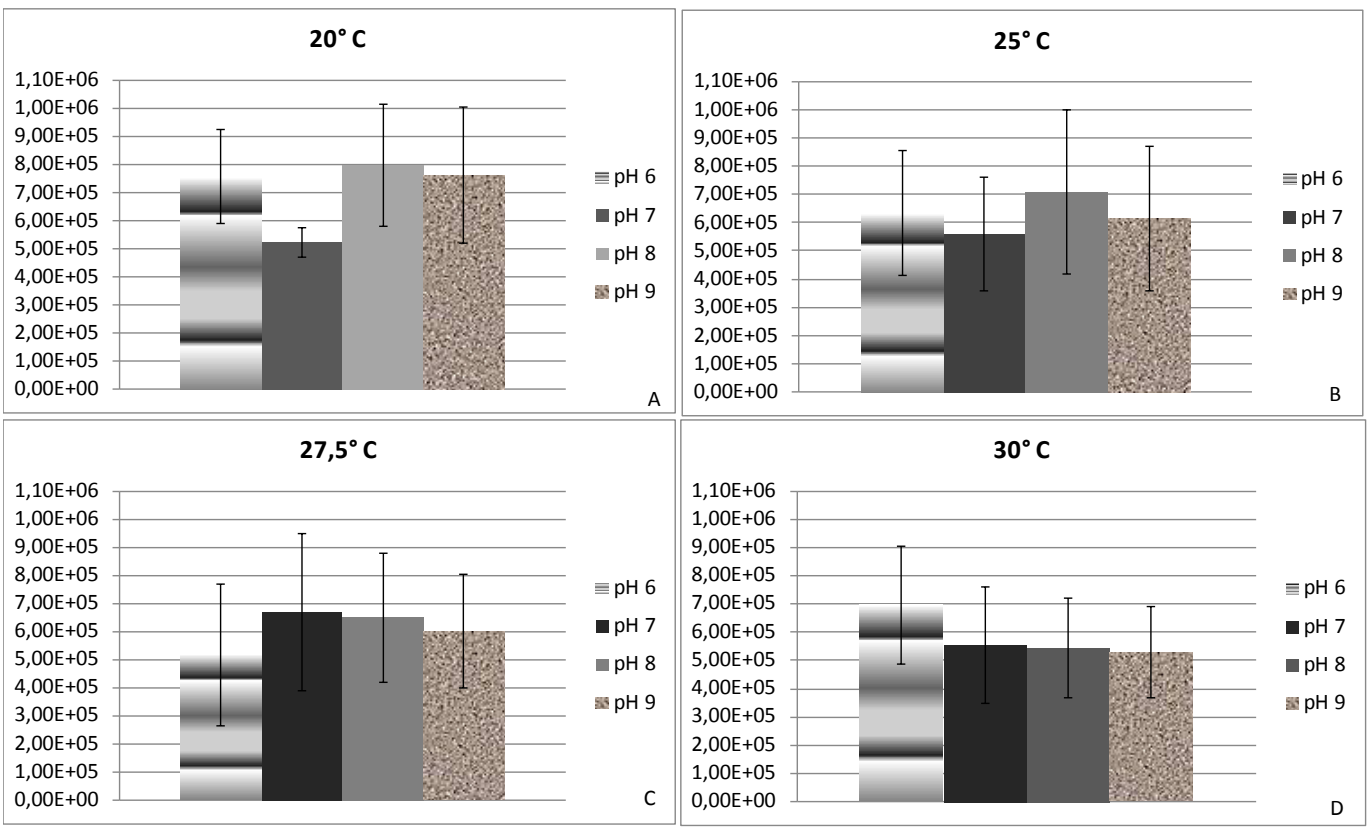

Figure 5. Mean cell biovolumes of *P. caudatum* (PC2) strain after 72h growth at 20°C (A), 25°C (B), 27,5°C (C) and 30° C (D).

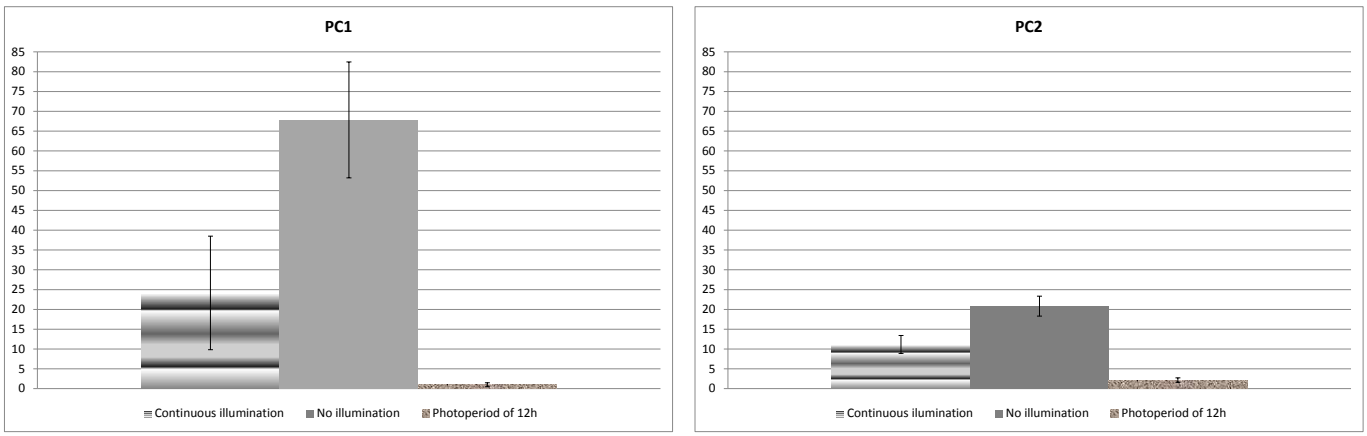

Figure 6. Mean density (cells mL-1) of *P. caudatum* strains (PC1 and PC2) in different illumination conditions.

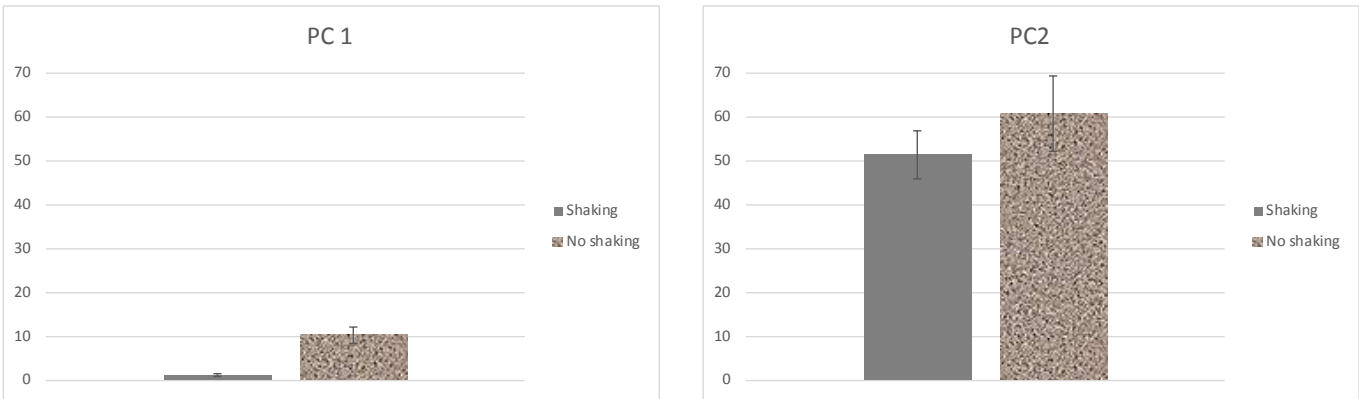

Figure 7. Mean density (cells mL^{-1}) of *P. caudatum* strains (PC1 and PC2) with and without shaking.

same conditions used to evaluate the illumination effect. Here, flasks with three replicates for each strain were incubated with agitation and three under static conditions. For PC1 the mean density obtained with shaking was 1.16 cells mL^{-1}, almost 9-fold lower than without shaking (10.33 cells mL^{-1}). For PC2 the mean values with and without shaking were similar: 51.5 cells mL^{-1} and 60.75 cells mL^{-1}, respectively.

Growth curves and generation time

Figures 8 and 9 shows, respectively, the growth curves of strains PC1 and PC2, both constructed using mean cell density data obtained from three replicates. PC1 strain was incubated in the dark at 30°C and pH 9. PC2 strain was incubated in the dark at 27.5 ° C and pH 7.

For PC1 strain, the maximum mean density was 6400 cells mL^{-1} in 90h with 10.782 generations (*n*) approximately and a generation time (*g*) of 8.35 hours. Otherwise, for PC2 strain, the maximum mean density was 7548 cells mL^{-1} in 84h with 11.019 generations (*n*) approximately and a generation time (*g*) of 7.62 hours.

The lag phase of PC1 strain growth curve initiated before 36h (Figure 8) and finished around 60h. Following, the stationary phase ended at 90h, with the beginning of the culture death phase. The lag phase of the PC2 strain growth curve (Figure 9) lasted until around 36 hours from the experiment start. Then the logarithmic phase started and finished around 78h, with the stationary phase. The culture decline started between 90 and 96h.

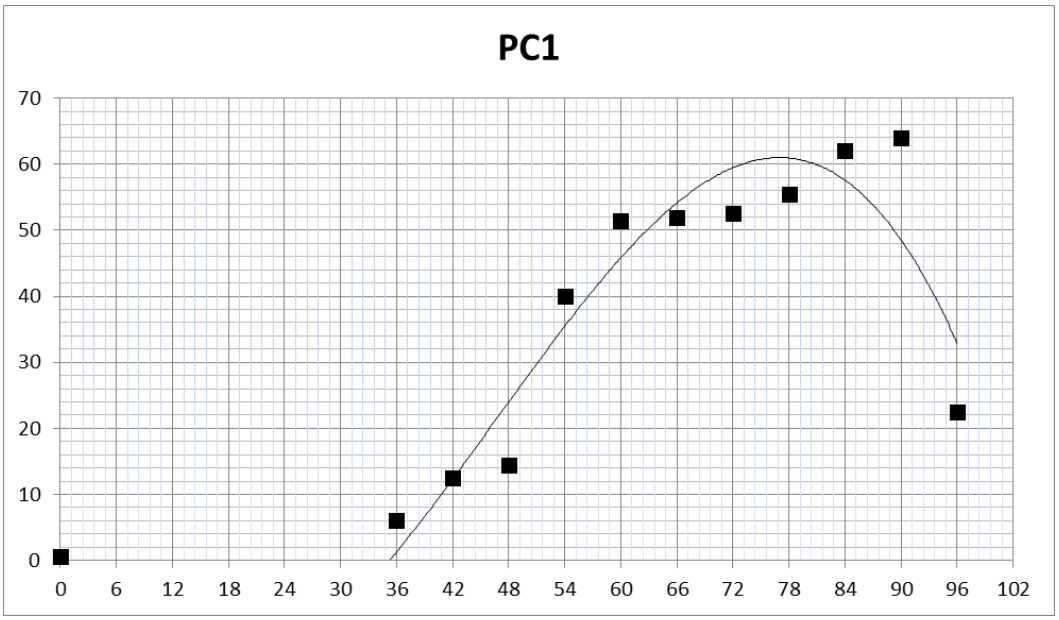

Figure 8. Mean density (cells mL^{-1}) at 6 hours interval of *P. caudatum* PC1 strain cultivated for 96h at 30 °C and initial pH of 9.

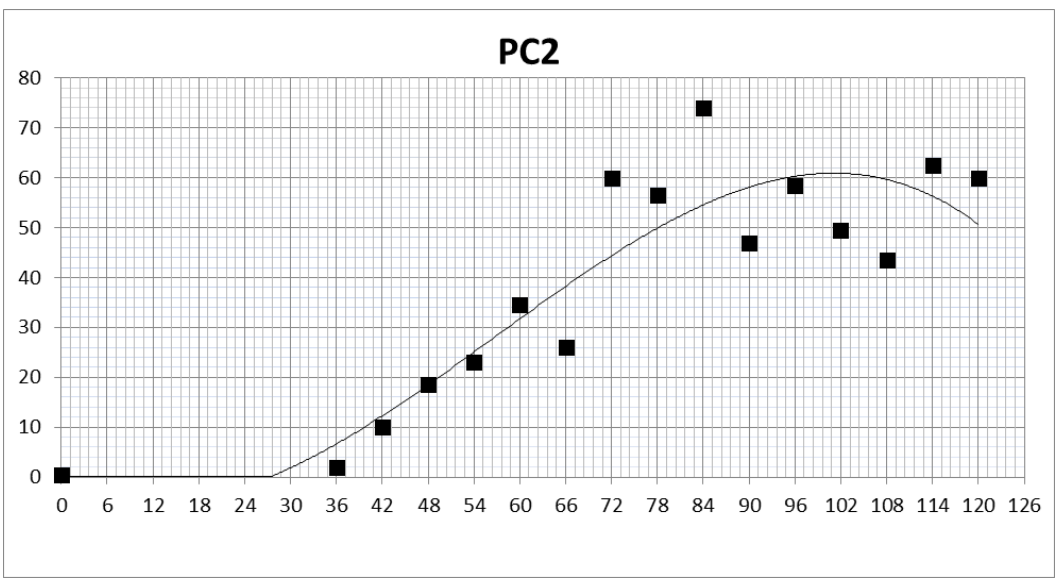

Figure 9. Mean density (cells mL^{-1}) at 6 hours interval of *P. caudatum* PC2 strain cultivated for 120h at 27.5 °C, and initial pH of 7.

DISCUSSION

Determination of the best pH and temperature for both strains

For PC1 strain, the biovolume and density data at the same pH and temperature conditions (25° C and pH 9) had opposite trends, as we can see at Figures 3B and 2B, where we found the lowest population density and the highest mean cell biovolume. Probably, at this condition, the energy obtained by the organisms was invested into the cell development, resulting into larger cells and less divisions.

It was also noted, for PC1 strain, that biovolume and cell densities were higher at pH between 8 and 9. With regard to temperature, the lowest ones (20 ° C and 25 ° C) provided higher

biovolumes while for the higher temperatures (30 ° C and 27.5 ° C) higher densities were verified. Due to metabolic acceleration at higher temperatures, the PC1 strain accelerate reproduction, whereas the increase in cell size indicate a strategy to energy storage for overcoming the most stressful conditions caused by lower temperatures.

So, it is clear that the best growth condition for strain PC1 was pH 9 at 30 ° C, although the mean cell biovolume obtained for this condition (6.157 x 10^5 µm^3) was not the best but slightly lower than the best condition for this parameter.

For PC2 strain, considering density for all tested conditions, the best was 27.5 ° C at pH 7 (Figure 4C), with mean values of 24.00 cells mL^{-1}. The worst was 20 ° at pH 8 (Figure 4A) with mean of only 0.75 cells mL^{-1}. The same pattern found in PC1 strain was observed for PC2: at the same pH and temperature conditions, biovolume and cell density data had opposite trends, as we could observe at 20 ° C where we found the lowest population growth and the highest mean cell biovolume at all pHs tested (Figure 4A), with exception of pH 7. Probably, the energy obtained by the organisms was invested into the cell development, resulting into larger cells and less cell divisions. This pattern was observed for both studied strains.

Comparing the performance of the two *P. caudatum* strains (PC1 and PC2) in their best growing conditions, we could see that the mean cell density of PC1 (70.44 cells mL$^{-1)}$ is more than 2.9 times higher than for PC2 strain (24.00 cells mL^{-1}) . While considering the mean cell biovolume, PC1 strain (6.157 x 10^5 µm^3) is approximately 8.51% lower than PC2 (6.681 x 10^5 µm^3). This leads us to believe that these strains, isolated from different environments, have different strategies in terms of reproduction: PC1 invests more energy to cell multiplication, as a *R*-strategist, and PC2 to cell growth, as a *K*-strategist. We hypothesize that these differences found among the strains were selected by their origin environments and the PC1 strain can be more adapted to laboratorial conditions, as indeed it is kept in culture for several years, and the PC2 strain for only 23 months.

Influence of ilumination conditions on growth of both *P. caudatum* strains

For PC1 strain, at the three illumination condition tested, the dark one showed the best results, considering cell densities. The mean cell density obtained at the dark in large bottles was statistically equal (considering standard deviations) to that obtained in test tubes (70.44 against 67.83 cells mL^{-1}). This is very interesting bearing in mind that the scale cultivation was increased by 12 times. Similar results were obtained for PC2 strain: at milk dilution bottles, the best growth was in the dark, yielding 20.83 cells mL^{-1}, value statistically similar to that obtained in test tubes (24 cells mL^{-1}).

Evaluation of agitation effects

On average, the PC1 strain showed no virtual growth with stirring (70RPM), since maximum mean population was 1.16 cells mL^{-1}, almost the same number of the beginning of the experiment. The results lead us to believe that this protozoan strain does not like water agitation and may not be adapted to lotic environment. On the other hand, for the PC2 strain, the difference between the cultures with and without shaking was much smaller, with almost similar values (51.5 cells mL^{-1} and 60.75 cells mL^{-1}). We can assume, therefore, that this strain would not have problems filling a

lotic environment with low water flow, reproducing normally, but the results shows that it still prefers more stagnant waters.

Growth curves

Although the number of generations (n) and maximum population (N) were higher in PC2 than PC1 (n = 11.02 and 10.78; N = 7548 and 6400), the PC1 strain (Figure 8) had a late, but more intense and shorter logarithmic growth phase than PC2 (Figure 9), reaching also higher average densities (36.125 against 34.208 cell mL^{-1}) since the first sampling (36h) to the last (66h), with one exception that was at 48h of cultivation. On the contrary, the PC2 started earlier the logarithmic phase, but it was more enduring than in PC1. The stationary phase was shorter by a decline of less intense than PC1 and the death phase was less intense than for PC1. Therefore, the growth strategies of the two strains were quite different.

The generation time (g) obtained for PC2 at 27.5 ° C (7.62 h) was lower, and for PC1 at 30 ° C was higher (8.35 h), than the value obtained by Rao (2006) for *Paramecium caudatum* (8h at 27 ° C). However, the differences were small comparing with Rao: about 5% considering PC2 and approximately 4.4% considering PC1. So, the values obtained seem to be very satisfactory because they are relatively similar and consistent with the optimal temperatures used for *Paramecium caudatum* cultivation.

Conclusion

Some differences on the most suitable growth conditions, especially temperature and pH, were found for the two different strains of the ciliated protozoan *P. caudatum* studied. Differences on their growth traits were also found and these are probably due to the selection pressure at their origin environments and the adaptation to growth in culture. Both are considered suitable for laboratorial growth, especially PC1 strain, and can be used for toxicity tests in standardized conditions.

AKNOWLEDGMENTS

We thanks to FAPESP to the MSc grant awarded to HCA (Process n° 2008/02217-9) and to CNPq to the grant awarded to MHRS (Process n° 301707/2010-3).

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