Growth kinetics of the microalga *Pseudokirchneriella subcapitata* (Korshikov) Hindak (Chlorophyceae) in natural water enriched with NPK fertilizer

Cinética de crescimento da microalga Pseudokirchneriella subcaptata (Korshikov) Hindak (Chlorophyceae) em água natural enriquecida com o fertilizante NPK

Títulos abreviados:
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Cinética de crescimento de *P. subcaptata* em água natural enriquecida com NPK

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ABSTRACT

Microalgae can have multiple functions in biotechnological studies, but it is necessary to encourage research to bioprospect and know the intrinsic characteristics of these microorganisms. Thus, the aim of this study was to evaluate the growth kinetics of *Pseudokirchneriella subcapitata* in an alternative culture medium prepared with natural water that was enriched with the chemical fertilizer NPK (20-5-20). Three treatments were designed to evaluate growth kinetics: (T1) 200 mL of autoclaved distilled water, (T2) 200 mL of autoclaved natural water, and (T3) 200 mL of natural water that was not autoclaved. All of the treatments were performed in triplicate and 200 mL of NPK and 100 mL of *P. subcapitata* culture were added. With this design, we expected to observe differences in the population growth of the microalga that were a function of the competition with the native species present in the test treatments. However, this was not observed, and the treatments did not result in significant differences in algal density (analysis of covariance [ANCOVA] $F_{2,50}$ = 1.43, p = 0.25). Instead, all cultures displayed high productivity, tolerance to fluctuations in the pH of the medium, and growth in an alternative low-cost medium (NPK). Thus, it was possible to conclude that microalgae have great potential for biotechnological studies, both in bench and industrial scales.

Keywords: bioassays, algal density, biotechnological processes, exponential growth rate

RESUMO

As microalgas podem apresentar múltiplas funcionalidades em estudos biotecnológicos, porém torna-se necessário o incentivo a pesquisas no sentido de bioprospectar e conhecer as características intrínsecas destes micro-organismos. Assim, o objetivo do presente estudo foi avaliar a cinética de crescimento de *Pseudokirchneriella subcapitata* em meio de cultivo alternativo preparado com água natural enriquecida com adubo químico NPK (20-5-20). Para preparação dos ensaios foram delineados três tratamentos: (T1) adicionado 200 mL de água destilada autoclavada; (T2) adicionado 200 mL de água natural autoclavada; (T3) adicionado 200 mL de água natural sem autoclavar. Todos os tratamentos foram realizados em triplicatas e adicionados 200 mL da solução NPK e 100 mL da cultura de *P. subcapitata*. Com isso, esperava-se observar diferença no crescimento populacional da microalga em função da competição com as espécies autóctones presentes no tratamento T3. Porém, este fator não foi observado e os tratamentos não apresentaram diferença significativa na densidade algal (ANCOVA $F_{2,50}$ = 1,43; p = 0,25), apresentando elevada produtividade, tolerância às oscilações de pH do meio e crescimento em meio alternativo de baixo custo (NPK). Assim foi possível inferir que a microalga apresenta grande potencial para ensaios biotecnológicos, tanto em escala de bancada como em escala industrial.

Palavras-chave: bioensaios, densidade algal, processos biotecnológicos, taxa de crescimento exponencial.

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INTRODUCTION

Microalgae are unicellular organisms that are capable of faster and more efficient photosynthesis than terrestrial plants. In this sense, studying the cultivation of microalgae is important to increase the knowledge of the biology of the various species. This understanding would favor further production in controlled environments where the culture media can provide the nutrients that are required for the optimum growth of each species (SIPAÚBA-TAVARES et al., 2009).

Among the different applications and encouragement of the production of microalgae in Brazil, feeding plankton and fish larvae are the best known (HARDY; CASTRO, 2000; SIPAÚBA-TAVARES et al., 2009). These microorganisms may also serve multiple functions in biotechnological studies, such as bioremediation of metals and other pollutants, toxicology testing (MOREIRA-SANTOS et al., 2004), solar energy bioconservation (energy storage), ingredients for feed, fermentation (methane production), extraction of various products such as pigments and algins (SIPAÚBA-TAVARES; ROCHA, 2003), biodiesel production, and food supplementation.

However, it is necessary to encourage research to bioprospect and gain knowledge about the intrinsic and functional characteristics of these species. Among the grown freshwater microalgae, unicellular algae of the class Chlorophyceae have been widely used (SIPAÚBA-TAVARES et al. 2009). From the several existing species of unicellular algae, the Chlorophyceae *Pseudokirchneriella subcapitata* has been widely used for both feeding microcrustaceans and performing toxicity tests (DOMINGUES; BERTOLETTI, 2008). This microalga is able to tolerate extreme conditions of salinity and nutrient-poor medium, is easily found (from culture collections), and can be maintained in reproducible laboratory culture conditions (MOREIRA-SANTOS et al., 2004).

Studies suggested that temperature, nutrients, light availability and culture management were determining factors on microalgae productivity (SIPAÚBA-TAVARES; PEREIRA, 2008). Thus, the use of alternative media, such as NPK for the development and growth of algal biomass has been implicated as an important increase in production of microalgae in large scale (HARDY; CASTRO, 2000; SIPAÚBA-TAVARES; PEREIRA, 2008; SIPAÚBA-TAVARES et al., 2009, 2011). According to Sipaúba-Tavares and Rocha (2003) the NPK fertilizer is an alternative media cheaper and as efficient as the standard synthetic medium CHU_{12}.

However, many aspects regarding the growth kinetics of this microalga need to be studied both for large-scale production and for bench studies. The objective of this study was to evaluate the growth kinetics of *P. subcapitata* in an alternative culture medium prepared with natural water and enriched with chemical fertilizer NPK (20-5-20).

MATERIAL AND METHODS

A strain of *P. subcapitata* was received courtesy the Laboratory of Limnology, Department of Ecology and Evolutionary Biology, Universidade Federal de São Carlos (UFSCar). The microalga was subsequently cultured in the synthetic medium CHU12 in the laboratory of the Research Center for Biodiversity (CPBio), Universidade Estadual de Mato Grosso do Sul (UEMS) in a non-axenic static culture system with constant aeration, room temperature, and photoperiod (12 h light/12 h dark).

For the tests, a stock of synthetic medium was prepared by adding 0.70 g of chemical fertilizer N:P:K (20:5:20 g/L) in 1000 mL of distilled water, and the solution was autoclaved at 121 ºC for 20 min (SIPAÚBA-TAVARES; ROCHA, 2003). All of the treatments were performed in triplicate and kept in a 500 mL Erlenmeyer flask and its intrinsic characteristics are described in Table 1. The tests were kept in a BOD incubator with controlled photoperiod of 2.500 lux provided by white fluorescent daylight tubes (12 h light/12 h dark), temperature (22 ± 2.0°C), and constant aeration.

Table 1. Description of components and intrinsic characteristics of each treatment

Treatments	NPK medium (200 mL)	*P. subcapitata* culture (100 mL)	Additional media (200 mL)
T1	Present	Present	autoclaved distilled water
T2	Present	Present	autoclaved Dourado River water (natural sterile water)
T3	Present	Present	Dourado River water without autoclaving (natural water)

The water from the Dourado River was collected on the day before the start of the tests by using Van Dorn bottles, and presented the physicochemical

parameters: pH 7.32; turbidity 19.3 NTU; conductivity 39.51 µS.cm-1; total solids 0.24 mg.L-1; alkalinity 23.66 mg.L-1; organic matter 18.83 mg.L-1; total dissolved solids 20.97 ppm. Water samples were directly transported to the laboratory and maintained under refrigeration. Every 3 days, the algal density was measured using a hemocytometer (Neubauer chamber), and the pH of the medium was measured with a bench pH meter for 15 days. Each treatment was performed in triplicate, and an average of 5 samples was collected from each treatment. To evaluate the difference in the curves of algal growth, we used the analysis of covariance (ANCOVA) (ZAR, 1999), and we applied a regression analysis (r2) to obtain the exponential growth rate (k). We used linear regression analysis (r2) and Spearman's correlation to analyze the correlation between the algal density and the pH of the medium. The daily growth rates were obtained by the dividing the difference between the algal density of the first and last days of testing by the elapsed time in days (15 days). The values in the daily growth rate were arcsine transformed (ZAR, 1999) to test for possible differences between the days of sampling for analysis and simple variance (one-way analysis of variance, ANOVA). The software used for data analysis was Statistica 7.0 (Statisoft Inc, Tulsa, OK, USA).

RESULTS AND DISCUSSION

The microalga *P. subcapitata* grew about seven times of its initial density in just 15 days during the experiment. This percentage represents an initial density of 18.1 ± 0.9 and a final density of 119.4 ± 22.2 ´ 10^5 cells/mL (mean ± standard deviation). However, no significant difference was observed between treatments by ANCOVA ($F_{2, 50} = 1.43$, $p = 0.25$). This indicates that, despite the use of water in the cultivation media with intrinsic characteristics, the treatment conditions were not sufficient to interfere with the population growth of the microalga. It is important to note that the intention of the T2 and T3 conditions was to investigate whether water could interfere with the natural growth of algae compared to the T1 condition, which used distilled water.

Natural water is more common in large-scale productions, while distilled water is used in most toxicity tests (SIPAÚBA-TAVARES; ROCHA, 2003; DOMINGUES; BERTOLETTI, 2008). Likewise, the NPK fertilizer, which was used in all of the treatments, has been employed as an alternative way to produce microalgae because of its low cost (DOMINGUES; BERTOLETTI, 2008; HARDY; CASTRO, 2008; SIPAÚBA-TAVARES; ROCHA, 2003; SIPAÚBA-TAVARES; PEREIRA, 2008; SIPAÚBA-TAVARES et al., 2009, 2011). However, the average algal density of this assay was below the 333 ´ 10^5 cells/mL (SIPAÚBA-TAVARES et al., 2009) and above the 144 ´ 10^4 and 74.16 ´ 10^5 cells/mL (SIPAÚBA-TAVARES; PEREIRA, 2008; SIPAÚBA-TAVARES et al., 2011; respectively) observed for Chlorophyceae *Ankistrodesmus gracilis* cultivated in NPK. However, these aforementioned experiments also included the use of an additional macrophyte preparation to complement the NPK fertilizer, which was not used in this experiment.

Both the T2 and T3 treatments used natural water. However, the water in the T3 treatment was not sterilized, which possibly suggests the competition of native microscopic organisms for resources. Among the possible microorganisms present in natural water, only the cyanobacteria *Anabaena* and *Microcystis* could be visualized by microscopy in non representative quantities. However, none of the hypotheses were confirmed because the treatments showed no significant difference in the growth of *P. subcapitata*. Nevertheless, the data that will be discussed below suggest that microalgae develop better in natural non-sterile water (T3) than in sterile water.

In a more detailed temporal analysis of the algal density, a steady growth (exponential phase) was observed from the 1st to the 6th day and from the 9th to the 15th day of the experiment. Only between 6 and 9 days was a lag phase observed, in which a small decrease in algal density was observed for treatments with distilled water (T1) and sterilized natural water (T2) (Figure 1). In this same period, the pH stabilized, starting from pH 7.23 ± 0.48 (mean ± standard deviation) and finishing at pH 5.63 ± 0.44 on the last day (Figure 1).

A linear regression analysis also demonstrated a significant correlation, but negative between the values of pH and the algal density (Figure 2); that is, an increase in the algal density was associated with a drop in the pH. Conversely, Sipaúba-Tavares et al. (2011) observed a decrease in the growth of Chlorophyceae *A. gracilis* in an acidic pH, which was associated with the ability of microalgae to metabolize inorganic carbon. Despite the observation that some chlorophytes cannot grow in an acidic pH (SIPAÚBA-TAVARES et al., 2011), *P. subcapitata* showed high growth even with pH variations.

Figure 1. Cell density (mean ± standard error) of the microalga *P. subcapitata* and pH of the medium from 3 treatments: T1, sterile distilled water; T2, sterile natural water; and T3, natural water that was non-sterile.

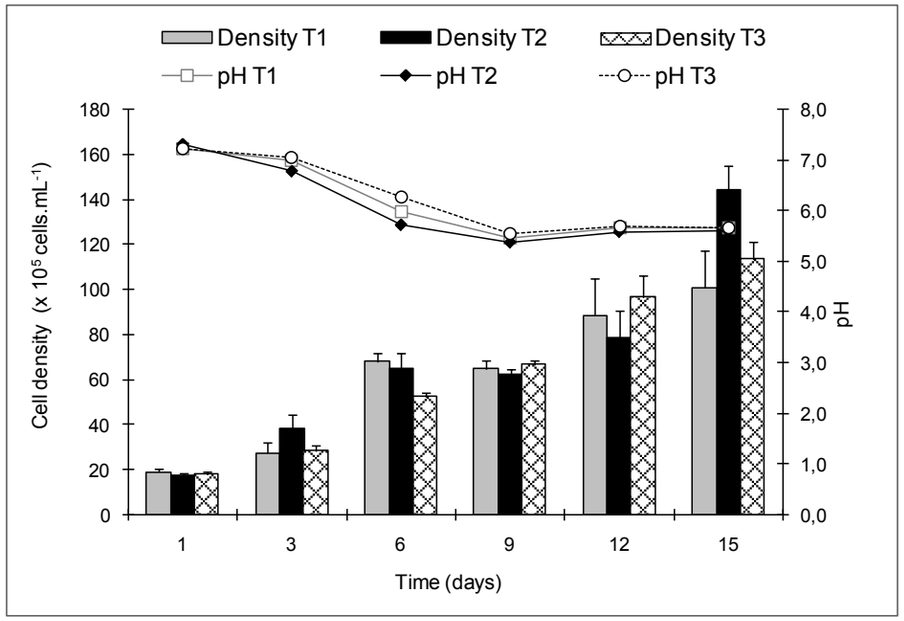
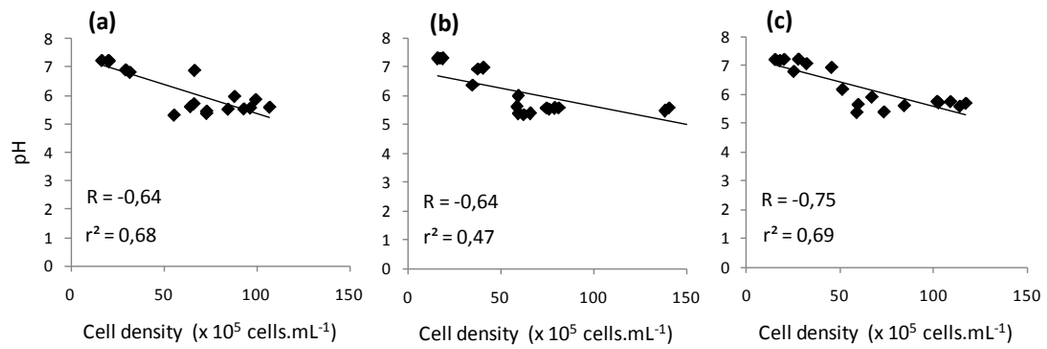


Figure 2. Linear regression analysis (r2) test and Spearman's correlation (R) between the density of *P. subcapitata* and pH in treatments T1 (a), T2 (b), and T3 (c).



Confirming what was observed for algal density, the regression analysis showed that the treatment T3 had the best exponential growth (k = 0.13 and r^2 = 0.93), followed by T2 (k = 0.12 and r^2 = 0.86) and T1 (k = 0.12 and r^2 = 0.86) (Figure 3). This result confirmed that the microorganisms that were naturally present in the water did not inhibit algal growth. However, exponential growth rates were similar to the lower values observed for Chlorophyceae *A. gracilis*, with rates that ranged from k = 0.14 to 0.56, depending on the medium and container used for cultivation (SIPAÚBA-TAVARES et al., 2009, 2011). However, a photoperiod of 24 h was used in these assays, whereas a 12 h photoperiod was used in our work. According to Sipaúba-Tavares et al. (2011), the higher exponential growth phase that was observed was associated with 24 h of light. In this regard, increasing the photoperiod can elevate the exponential growth rate of *P. subcapitata*.

Figure 3. Regression analysis (r2) and exponential growth (k) of *P. subcapitata* in treatments T1 (a), T2 (b), and T3 (c).

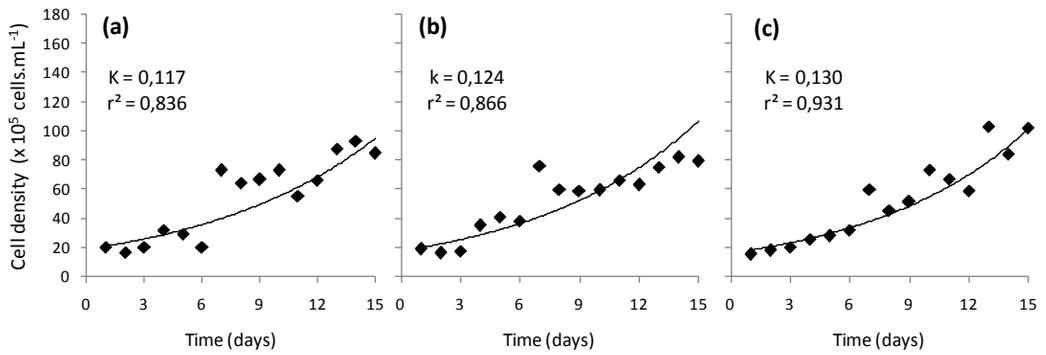
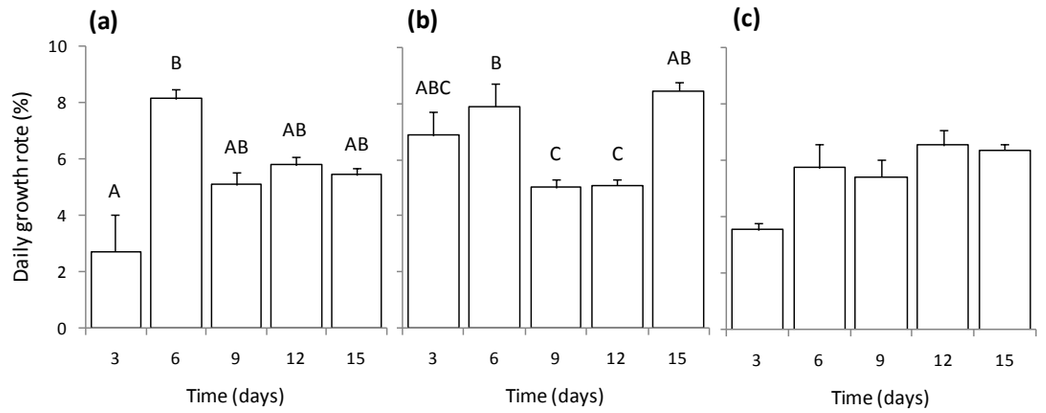


Figure 4. Daily growth rate (mean ± standard error) of the microalga *P. subcapitata* in treatments T1 (a), T2 (b), and T3 (c). The letters above the columns indicate significant differences by Tukey's test.



It has been previously mentioned that T3 was the only treatment that did not show an induction or stationary phase. The growth was continuous until the last day of the experiment. Even in the daily growth rate, the growth in T3 was relatively continuous, with no peaks or significant decreases (Figure 4). The ANOVA comparing the sampling days per treatment corroborated these results by reporting a significant difference only for T1 and T2 (ANOVA, F4, 10 = 4.00 and 8.37, P = 0.034 and 0.003, respectively). The growth in the T1 and T2 treatments, however, peaked between day 3 and 6, whereas growth in the T2 treatment also peaked between day 12 and 15 (Figure 4). Hardy and Castro (2000) also observed a peak in the growth of Chlorophyceae *Scenedesmus quadricauda* between day 5 and 7, with a trend of better growth in the medium NPK.

The microalga *P. subcapitata* showed high plasticity in the treatments used in this study and illustrated its representative production in natural waters, where competition can occur with other organisms. This plasticity was also verified with respect to changing pH: we observed high algal growth in an acidic medium. Furthermore, our results indicate that a culture medium enriched with natural water and NPK fertilizer can be used directly as an alternative to culture media with higher costs. However, further research is needed to study the physiological aspects involved in the growth of *P. subcapitata* and its biochemical composition to understand its many functions in biotechnology.

CONCLUSION

Considering the use of an alternative and low-cost culture medium, the microalga *P. subcapitata* showed satisfactory growth when compared with previously published growth data. Moreover, compared to other species of Chlorophyceae that were grown in a medium rich in nutrients and with a longer photoperiod, the microalga *P. subcapitata* showed growth rates within the observed patterns. Thus, we conclude that *P. subcapitata* has high productivity, tolerance to fluctuations in the pH of the medium, and growth in a low-cost alternative medium (NPK). Thus, according to the results of other studies (GUÉGUEN et al., 2003; MOREIRA-SANTOS et al., 2004), we can infer that *P. subcapitata* has great potential for biotechnological tests including bioremediation and toxicity studies at both the bench level and the industrial level.

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