Effects of some parameters in upscale culture of *Haematococcus pluvialis* Flotow

Sipaúba-Tavares, LH.[a]**, Millan, RN.*[a,b] *and Berchielli-Morais, FA.*[a]

[a]*Centro de Aquicultura, Universidade Estadual Paulista - UNESP, Via de Acesso Prof. Paulo Donato Castellane s/n, CEP 14884-900, Jaboticabal, SP, Brazil*

[b]*Faculdade de Ciências Agrárias e Veterinárias, Programa de Pós Graduação em Microbiologia Agropecuária, Universidade Estadual Paulista - UNESP, Jaboticabal, SP, Brazil*

**e-mail: sipauba@caunesp.unesp.br*

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(With 3 figures)

Abstract

Growth rate and medium parameters between two bench scale volumes (13-L and 250-L) were compared. Experiments were maintained batch mode and culture parameters were periodically measured during a 13-day period. Culture growth during the cultivation of algae *Haematococcus pluvialis* was determined qualitatively by cell counting, optical density, dry weight, ash content, amount of chlorophyll-*a*, total organic carbon content and by direct measuring of medium nutrients and some abiotic aspects. Vegetative cell growth was higher when cultured in 13-L with 1.33 x 10^5 cells.mL^{-1} on the 12th day than when cultured in 250-L. Significant difference ($p < 0.05$) in the biology and water culture of *H. pluvialis*, with the exception of dry weight, ash, nitrite and ammonia, was reported between the volumes. Data obtained in current study for the upscale culture maintenance of *H. pluvialis* in laboratory conditions shows that it should be undertaken in a 13-L volume due to a greater time span of cells in a vegetative state, greater cell density, lipids and chlorophyll-*a* contents. Light was of paramount importance on the direct performance of *H. pluvialis* on the algal biological conditions.

Keywords: biological aspects, growth, nutritional content, microalgae.

Efeito de alguns parâmetros no cultivo em larga escala de *Haematococcus pluvialis* Flotow

Resumo

Foram avaliadas a taxa de crescimento e condições do meio de cultura em volumes de 13-L e 250-L em sistema estático, durante o período de 13 dias para a microalga *Haematococcus pluvialis*. Foi determinada qualitativamente a contagem de células, densidade, peso seco, teor de cinzas, clorofila-*a*, teor de carbono orgânico total e avaliação de nutrientes e fatores abióticos do meio de cultura. O crescimento foi mais elevado em volume de 13-L com 1,33 x 10^5 células.mL^{-1} no décimo segundo dia, do que em volume de 250-L. Em relação ao meio de cultivo e aspectos biológicos de *H. pluvialis*, foram observadas diferenças significativas ($p < 0,05$) entre os dois volumes com exceção do peso seco, cinzas, nitrito e amônia. Para cultivo em larga escale de *H. pluvialis* é recomendado nestas condições laboratoriais, o volume de 13-L devido ao maior tempo das células em estado vegetativo, maior densidade celular e elevados teores de lipídios e clorofila-*a*. A luz interferiu diretamente nas condições biológicas de *H. pluvialis*.

Palavras-chave: aspectos biológicos, crescimento, conteúdo nutricional, microalga.

1. Introduction

Microalgae culture studies are important for a deeper biological knowledge of different species. They are highly beneficent for production in controlled environments in which culture media provide the necessary nutrients for optimal growth of the species (McKim and Durnford, 2006).

Haematococcus pluvialis Flotow (Chlamydomonadales, Chlorophyceae), a unicellular green microalgae with biological and economical importance, is used as an additive pigment in aquaculture and a supplementary additive in the food industry. The microalgae exhibit some unfavourable characteristics when compared to other microalgae successfully cultivated at upscale. *H. pluvialis* has a complex life cycle which includes motile and non-motile cells and typical resting cell or cyst (González et al., 2009).

It is well known that the accumulation of astaxanthin in *H. pluvialis* is associated preferentially with the morphological transformation of green motile vegeta-

tive cells into deep-red non-motile cysts. The pattern of cell growth and carotenoid accumulation has generated two productive strategies for growing *H. pluvialis*: one, in a single step using a suitable medium (sub-optimum) for both biomass and astaxanthin production, where astaxanthin is accumulated while cells are growing, and the other, in two consecutive steps, the first under optimal conditions for vegetative growth followed by another for astaxanthin production in non-growing cells (Cifuentes et al., 2003). High productivity of vegetative cells requires the formulation of a sustainable culture medium since low cell productivity is obtained with the standard medium of freshwater microalgae (Fábregas et al., 2000).

In cells grown under excessive irradiance, a massive astaxanthin synthesis occurs and the efficiency of light utilization declines as cells become increasingly red (Torzillo et al., 2005). The growth of microalgae is affected by light (most microalgae upscale culture are limited by light since it is easily absorbed and scattered by the microalgae cells), by dissolved oxygen, carbon dioxide, pH, temperature, nutrients (mainly N and P), some trace elements and carbon (Jeon et al., 2005). Since most experiments with cells in the green stage have been carried out using relatively low irradiances, growth becomes saturated below 100 μmol $m^{-2}s^{-1}$. However, utilization and culture behaviour are strongly influenced by several parameters, such as cell concentration, light path, mixing rate, and geometry of the cultivation vessel (Torzillo et al., 2005).

Besides the parameters above, the microalgae *H. pluvialis* is characterized by low growth, susceptibility to contamination and preference for low temperature. These features require cultivation in closed systems which offer a number of advantages, including, better control of culture environment, protection from environment contamination and production of high cell density (Kaewpintong et al., 2007).

Current study comprises a comparison of the growth rate and the physiological and medium parameters between two upscale volumes (13-L and 250-L) in batch mode, and verifies whether simple laboratory conditions are viable for *H. pluvialis* culture. Batch culture systems are widely applied because of their simplicity and flexibility, allowing changing species and rapidly remedy defects in the system.

2. Materials and Methods

2.1. Microalgae strain and culture conditions

Haematococcus pluvialis (CMEA 227 C1) was obtained from the culture collection of the Biology Department of the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. So that the effects of different volumes on the growth of *H. pluvialis* could be examined, a WC medium (Guillard and Lorenzen, 1972) was used for the maintenance of cultures and the experiments were undertaken in 13-L and 250-L volumes with continuously air bubbled at 23 ± 2 °C. Vitamin B complex was added to the medium at the rate of 0.01 $g.L^{-1}$ (7 mg - B_1, 7 mg - B_2, 5 mg - B_6, 33 μg - B_{12}) plus vitamin H (0.1 mg). Continuous illumination was applied at an average light intensity of 94.7 μmol m^{-2} s^{-1} to 13-L and 184.8 μmol m^{-2} s^{-1} to 250-L volumes. Light was provided by cool white fluorescent lamps to observe the transformation from vegetative cells to cysts. Only the green cells from the exponential growth phase were used as inoculums for the experiment (Figure 1).

2.2. Culture experiment

Experiments were maintained in batch mode and the culture parameters periodically measured during 13 days, were done in two parts to study the influence of the two different culture volumes. The first experiment started with 2-L flasks, density 0.6 x 10^5 $cells.mL^{-1}$, containing WC medium. When cultures reached the late exponential growth phase (7 days), about 4-L, density 0.15 x 10^5 $cells.mL^{-1}$, they were transferred to 13-L sterilized recipients. The second experiment was similarly undertaken but 26-L, density 0.17 x 10^5 $cells.mL^{-1}$ of algae culture, were transferred to 250-L sterilized recipients (Figure 1).

2.3. Growth

Cell growth was monitored over a period of 13 days. Triplicate 1 mL aliquots were removed daily from the microalgae culture and a minimum of 2 x 1 μL subsample were used for cell quantification by a Neubauer hemocytometer. Growth rate k (divisions per day) was calculated by the formula: $k = (3.322/t_2\text{-}t_1 \times \log N_2/N_1)$ (t = time; N = number of cells. Subscripts denote values at different times) (Guillard, 1973). Doubling time (cell division time or generation time) was calculated from results obtained from the growth rate by the formula: $Td = 1\ k^{-1}$ (Td = duplication time, $1\ k^{-1}$ = days per division) (Guillard, 1973). Dry weight and ash content were determined on the 13th day, following Vollenweider (1974). Total length (μm), total organic carbon content (TOC) and cell volume were quantified on the 13th day. Total length of 50 specimens was determined with microscope Olympus BX 50 by the image analysis system Pro Plus 4.1, Media Cybernetics, USA, with a 400X micrometric objective. Calculation of cell volume was undertaken by mean cell size with the use of the most appropriate geometric form, which corresponds to the sphere formula (Hillebrand et al., 1999). TOC was calculated by $C = 0.1204.V^{1.051}$ (C = carbon content in $pg.cell^{-1}$; V = cell volume) using regression according to Rocha and Duncan (1985).

2.4. Analytical methods

Nitrate, ammonia, nitrite, orthophosphate and total phosphorus in the culture were evaluated every four days during the experiment and quantified spectrophotometrically according to techniques described by Golterman et al. (1978) and Koroleff (1976). Chlorophyll-*a*, quantified

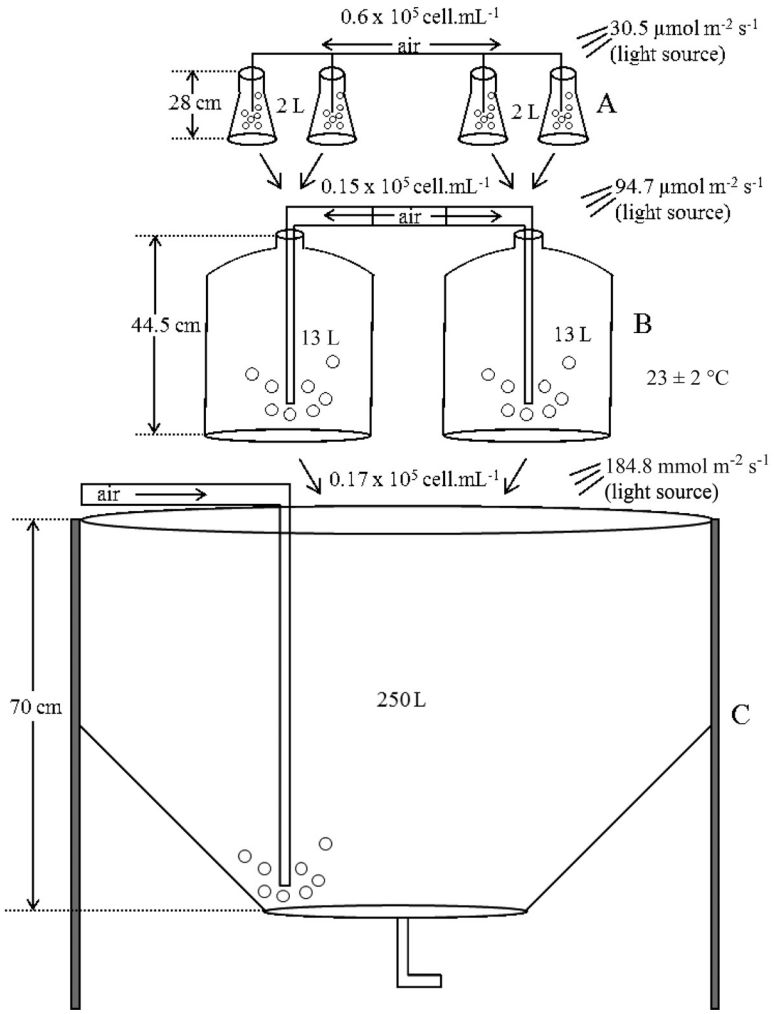

Figure 1 - Schematic diagram of batch culture mode of *Haematococcus pluvialis* algae in 2-L, 13-L and 250-L volumes, where: A = flasks, B = carboy and C = glass fiber.

every four days during the experimental period, was extracted with 90% alcohol and quantified at 663 and 750 nm (Nusch, 1980). The pH, conductivity and dissolved oxygen were measured with a multiparametric probe YSI 556 MPS and monitored twice a week. At the end of the experiment the biomass was harvested, centrifuged, and lyophilized for the analysis of proteins, lipids and fibre (AOAC, 1990). Astaxanthin was quantified on the 13th day and extracted with petroleum ether at 470 nm (Carvalho et al., 1992).

2.5. Statistical analysis

All experiments were carried out in triplicate. One-way ANOVA was applied for simple verification between volumes (Fowler et al., 1998). Differences were considered to be significance at a probability of 5% ($p \leq 0.05$). Statistical analyses were performed with Sigma Stat software program 2.03.

3. Results

H. pluvialis grew exponentially up to the 12th day and 7th day when cells totalled about 1.33 x 10^5 cells.mL^{-1} and 1.08 x 10^5 cells.mL^{-1} respectively. Subsequently, the number decreased sharply to 1.15 x 10^5 cells.mL^{-1} and 0.66 x 10^5 cells.mL^{-1} respectively in 13-L and 250-L volumes (Figure 1). Start of encystment first occurred in the 250-L volume on the 7th day, with high illumination rates 184.8 µmol m^{-2} s^{-1}, low nitrate rate and total phosphorus in the culture medium (Figures 2 and 3; Table 1). Chlorophyll-*a* rate were also lower in 250-L volume when associated to the two nutrients in the culture medium (Figure 3). Astaxanthin rate was low in both volumes (Table 2).

With the exception of nitrite and ammonia, nutrients in the culture medium were higher in the 13-L culture, with significant difference ($p < 0.05$) between the volumes. Constant air bubbling of medium produced an oxygen rate over 7.0 mg.L^{-1} in the 13-L volume and over

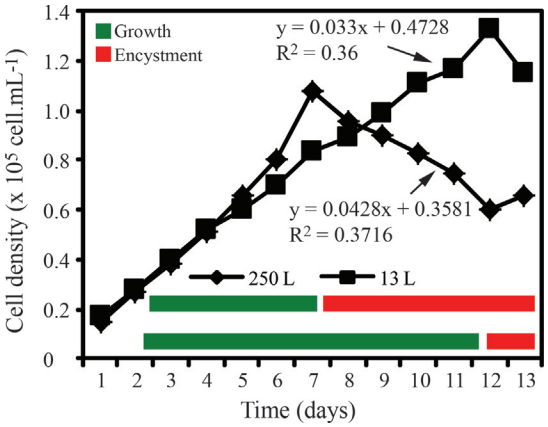

Figure 2 - Growth of *Haematococcus pluvialis* in 13-L and 250-L volumes.

4.0 mg.L^{-1} in the 250-L one ($p < 0.05$) (Table 1). Right rates of dissolved oxygen in 13-L volume provided lower nitrite concentration in this volume (Table 1). Ammonia concentrations in the medium culture showed an inversed relationship with the culture volumes under analysis between the first and ninth day of culture (Figure 3).

Due to low cell density, the dry weight was low with 1.82% and 1.80% for 13-L and 250-L, respectively. Growth rate was k = 0.14 and 0.36 with average density of 1.10 x 10^5 cells.mL^{-1} and 0.85 x 10^5 cells.mL^{-1} during exponential phase between the 8th and 12th day in the 13-L volume and between the 5th and 7th day in the 250-L volume. Doubling time was short, with cell duplication approximately 6.9 and 2.81 days for 13-L and 250-L volumes, respectively (Table 2; Figure 2).

Cell volume, TOC, protein content and total length showed significant differences ($p < 0.05$) between the volumes, or rather, they were higher in 250-L (Table 2). Lipid contents were very high ($p < 0.05$) in 13-L (8.18% dry weight) than in 250-L (2.68% dry weight) (Table 2).

4. Discussion

Cell growth in the 250-L volume occurred up to the 7th day, with 1.08 x 10^5 cells.mL^{-1}. Afterwards, cell decrease with cysts formation occurred. On the other hand, a higher proportion of cysts than that of motile cells in cultures indicate unfavourable environmental conditions for cells (González et al., 2009). Algae growth in the 13-L volume occurred up to the 12th day with 1.33 x 10^5 cells.mL^{-1}, with high doubling time than 250-L. Doubling time rates obtained were lower (greater number of

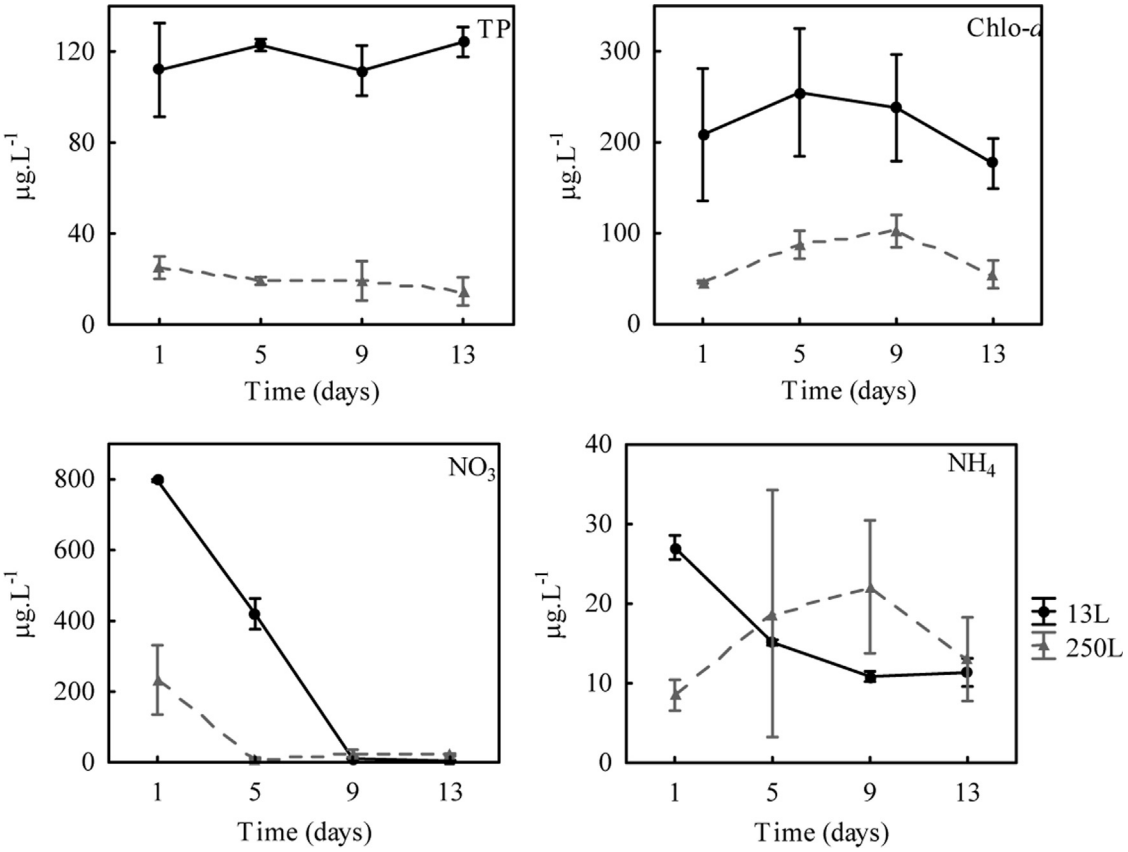

Figure 3 - Chlorophyll-*a* and nutrients concentrations in culture medium of *Haematococcus pluvialis* in 13-L and 250-L volumes. Vertical bars Standard Deviation (n = 12).

Table 1 - Estimated parameters of *Haematococcus pluvialis* medium cultured in 13-L and 250-L volumes. Values are the mean of three replications and the variation (±) is the standard deviation.

Parameters	13-L	250-L	ANOVA
Dissolved Oxygen ($mg.L^{-1}$)	7.5 ± 0.29	4.52 ± 0.18	$p < 0.05$
pH	6.5 ± 0.65	8.03 ± 0.08	$p < 0.05$
Conductivity ($\mu S.cm^{-1}$)	140.8 ± 7.88	307.5 ± 5.15	$p < 0.05$
Nitrate ($\mu g.L^{-1}$)	207.7 ± 132.37	72.75 ± 107.3	$p < 0.05$
Nitrite ($\mu g.L^{-1}$)	14.4 ± 8.09	21.64 ± 6.71	$p > 0.05$
Ammonia. ($\mu g.L^{-1}$)	13.9 ± 6.91	13.28 ± 6.19	$p > 0.05$
Orthophosphate ($\mu g.L^{-1}$)	46.54 ± 9.66	25.41 ± 7.01	$p < 0.05$
Total Phosphorus ($\mu g.L^{-1}$)	117.08 ± 6.87	19.52 ± 4.25	$p < 0.05$

Table 2 - Summary of some physiological parameters of *Haematococcus pluvialis* medium cultured in 13-L and 250-L volumes. The values are the mean of three replications and the variation (±) is the standard deviation.

Parameters	13-L	250-L	ANOVA
Chlorophyll-*a* ($mg.L^{-1}$)	205.53 ± 34.91	72.77 ± 26.44	$p < 0.05$
Astaxanthin ($\mu g.100\ g^{-1}$)	11.63 ± 10.17	11.83 ± 0.52	$p > 0.05$
Cell density ($x\ 10^5\ cell.mL^{-1}$)	1.1 ± 0.17	0.85 ± 0.21	$p > 0.05$
Growth rate (k)	0.14	0.36	-
Doubling time (days)	6.90	2.81	-
Total length (μm)	15.23 ± 3.05	25.14 ± 3.21	$p < 0.05$
Cell volume (μm^3)	2 073 ± 1 231	8 726 ± 3 460	$p < 0.05$
Dry weight ($pg.cell^{-1}$)	1.82 ± 0.62	1.80 ± 1.18	$p > 0.05$
TOC ($pg.cell^{-1}$)	371.7 ± 231.7	1 675 ± 700	$p < 0.05$
Ash contents (% dry weight)	3.24 ± 2.52	2.30 ± 1.06	$p > 0.05$
Proteins (% dry weight)	14.09 ± 0.38	17.53 ± 0.11	$p < 0.05$
Lipids (% dry weight)	8.18 ± 0.11	2.68 ± 0.66	$p < 0.05$

days) than those reported by González et al. (2009) with *H. pluvialis* cultures in 200 mL volumes. Moreover, the different culture conditions (light and nutrients), experimental designs and strains utilized are likely to provide the data differences obtained. The productivity of vegetative cells of *H. pluvialis* is a function of average irradiance, nutrients content of the medium, and to its sensitivity to changes on culture conditions (García-Malea et al., 2005).

In current study, low rates in cell density, growth rate and doubling time were directly associated with high irradiance (13-L = 94.7 μmol m^{-2} s^{-1} and 250-L = 184.8 μmol m^{-2} s^{-1}). In the case of algae growth, whereas irradiance is an important factor, *H. pluvialis* vegetative cells require a relatively low irradiance an irradiance ranging from 40 to 50 μmol m^{-2} s^{-1} is optimal (Cifuentes et al., 2003). Kaewpintong et al. (2007) found that cell growth was no longer observed when light intensity increased to 60 μmol m^{-2} s^{-1}. In fact, optimal light intensity for *H. pluvialis* growth was 20 μmol m^{-2} s^{-1}. Further, some studies have reported slow cell growth and low final cell densities when growth is carried out in batch mode (Hata et al., 2001; Cifuentes et al.; 2003 Kaewpintong et al., 2007). The effect of light intensity is dependent on the nutritional state of the culture (Fábregas et al., 2000).

Conditions of medium culture are important factor in algae yield. A sharp nitrate decrease on the 9th day in the 13-L volume had a direct effect on chlorophyll-*a* rates since it decreased in the medium culture. Whereas nitrate is preferred to ammonia for growth in *H. pluvialis*, the preference of nitrate in this species is controlled by the strain, age of inoculum and pH of medium (Cifuentes et al., 2003). According to Cifuentes et al. (2003), the best nitrogen source for growth and carotenoid accumulation is nitrate, since carotenogenesis was slightly inhibited in the ammonia and urea growth cultures. Astaxanthin rates

in current study were similar in both volumes due to the low concentration of nitrogenated compounds in the culture media.

As a rule, *H. pluvialis* strains preferred slight acid to alcaline pH, ranging between 6.0 and 8.7. Slight acid pH occurred in the 13-L volume in current study and during the exponential growth of *H. pluvialis* in this volume. It had a lower cell density than in the 250-L volume, although exponential growth occurred up to the 12th day with a cell density of 1.33 x 10^5 cells.mL^{-1}.

Some authors have found that biotin, thiamine and B_{12} have significant effect on growth rate and final dry weight for maximum growth (Fábregas et al., 2000). Kaewpintong et al. (2007) found that *H. pluvialis* cell density increased approximately 55% with the concentration of vitamin B when compared with cell growth in the medium without any vitamin addition. In this study, beside biotin and thiamin, vitamin B complex and vitamin H were added in the medium for *H. pluvialis* culture. Their presence improved the growth of *H. pluvialis*. The addition of vitamin B complex is routine in the laboratory and results showed its influence in the improvement of *Ankistrodesmus gracilis* growth, a Chlorophyceae easily found in the region, with high nutritional value as feed for fish larvae (Sipaúba-Tavares and Pereira, 2008).

Conductivity rates were higher in the 13-L volume and when associated to high rates in nitrate, total phosphorus and orthophosphate slightly, improved algae growth mainly as from the 9th day when total phosphorus increased in the medium. High concentrations of dissolved oxygen in the 13-L volume may have contributed to high chlorophyll-*a* (205.53 ± 34.91 mg.L^{-1}), cell density (1.33 x 10^5 cells.mL^{-1}) and doubling time (6.9 days) rates. This is due to adequate level of circulation in the system which may provide better growth rates (Issarapayup et al., 2009). Further, the 13-L volume warranted the better exposure of cells to light and provided more significant photosynthesis that with the culture of the 250-L volume.

Protein rates were low (between 14 and 17% dry weight). Since quantities were measured on the 13th day in both volumes, they may have been affected by the growth stage. Protein quantity and quality varied according to the phase culture. Likewise, dry weight had low rates in the 13-L (1.82 pg.cell^{-1}) and in the 250-L (1.80 pg.cell^{-1}) volumes, since dry weight varies according to the nitrogen source as much as the phase culture. High lipid contents (8.18% dry weight) were observed in the 13-L volume coinciding with a better cell growth and high levels of total phosphorus (124.2 µg.L^{-1}) on the 13th day. Lipids contents in this study were higher than those for the algae Chlorophyceae *A. gracilis* (7.48% dry weight) cultivated in an 850-L volume (Sipaúba-Tavares and Pereira, 2008).

Several studies on *H. pluvialis* have been focused on culture conditions that lengthen the active growing phase to improve yield per dry weight at the end of inductive phase (Fábregas et al., 2000; González et al., 2009).

The levels of TOC (pg.cel^{-1}), total length (µm) and cell volume (µm^3) quantified on the 13th day were high in the 250-L volume due to space and greater light penetration which warranted good growth conditions. In fact, a greater growth rate (k = 0.36) in the 250-L volume was the result. Ash contents (mineral composition) in the algae are probably due to the concentration of inorganic compounds in the medium in which the algae grew, although ash content varied within each group. In fact, average ash content in this study was lowest than that found by Sarada et al. (2006) with 4.6% dry weight for *H. pluvialis*.

Temperature is another factor that affects specific growth rate. The specific growth rate of *H. pluvialis* increases with a rise in temperature from 20°C to 28°C, but further increase in temperature decreases its specific growth rate (Wongsnanslip et al., 2007). In current study temperature rates were within standards for algal culture.

The physiological variability in the growth study of *H. pluvialis* in different culture conditions in batch mode provided a strong motivation for continuing research on the algae since the carotenoid is highly relevant to the aquaculture industry. Data from current study for the upscale maintenance culture of *H. pluvialis* in the laboratory showed that it should be undertaken with 13-L volumes. This is due to the greater time duration of cells in the vegetative stage and higher cell density, lipids and chlorophyll-*a* contents. Light was in fact highly relevant in *H. pluvialis* performance since it interfered directly in the algal biological conditions. Dissolved oxygen was low, with high pH and conductivity over 300 µS.cm^{-1} in culture medium in the 250-L volume. However in the case of 13-L volume, nitrate, total phosphorus and orthophosphate were higher, with greater nutrient availability for the algae, directly reflected in a higher doubling time and a faster growth rate. More research should be undertaken to assess laboratory conditions for the improvement of growth and lipid and dry weight rates of *H. pluvialis* cells.

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