Journal of Algal Biomass Utilization
J. Algal Biomass Utln. - Copyright © PHYCOSPECTRUM
ISSN: 2229 – 6905

Growth, photosynthesis and biochemical composition of *Haematococcus pluvialis* at various pH

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ABSTRACT
Astaxanthin is a carotenoid with potent antioxidant properties. In nature, the freshwater microalgae *Haematococcus pluvialis* shows the highest astaxanthin concentrations. When subjected to environmental perturbations, such as temperature, light intensity and/or pH variations, astaxanthin accumulates. This study aimed at analyzing the influence of pH in the vegetative growth, photosynthetic efficiency and biochemical composition (lipids, proteins and carbohydrates) of *H. pluvialis*. It was accomplished using MES, PIPES and HEPES to buffer culture medium that was inoculated with cysts. The highest growth rate was obtained for pH 6.3 (0.45 d^{-1}; MES buffer), while the lowest was 0.18 d^{-1} for not-buffered cultures at initial pH 6.3. Photosynthetic activity, expressed as the maximum quantum yield of photosystem II. The results showed that pH 6.3 buffered system provided the best cysts germination and growth rate and, consequently, higher biomass at the shortest period of time (5 days). Photosynthetic activity was highest in the vegetative green flagellates (days 1 to 12) and lower at the end of the experiments, dominated by palmelloid cells. The biochemical composition of green flagellates showed that controls had the lowest proteins:carbohydrates (P:C) ratio compared to buffered treatments, indicating physiological stress. No effect of pH was observed on total lipids.

Keywords: *Haematococcus pluvialis*, vegetative growth, buffered cultures.

INTRODUCTION

Microalgae represent a promising source of new metabolites with applications in agriculture, chemistry, food, pharmaceutical and cosmetic industries, among others (Borowitzka and Moheimani, 2013). *Haematococcus pluvialis* Flotow is a microalgae that presents vegetative growth in photoautotrophic (García-Malea *et al*., 2005; Kang *et al*., 2007; Ranjbar *et al*., 2008), mixotrophic (Borowitzka *et al*., 1991; Hata *et al*., 2001) and heterotrophic metabolisms (Kobayashi *et al*., 1992; Moya *et al*., 1997; Hata *et al*., 2001). Under favorable growth conditions, *H. pluvialis* is a green biflagellate swimmer that under unfavorable conditions, it starts carotenogenesis transforming itself into still astaxanthin rich aplanospores (Nagaraj *et al*., 2012).

To successfully commercialize *H. pluvialis* astaxanthin it is necessary to achieve high biomass. This has lead to increasing the investigations onto the optimization of its vegetative growth. Usually this is performed through variation of the physical and chemical growth conditions, such as nutrient concentration, photoperiod, light intensity, temperature and pH (Fábregas *et al*., 2001; García-Malea *et al*., 2005; Imamoglu *et al*., 2007; Devgoswami *et al*., 2011). Once with elevated biomass, the induction of astaxanthin biosynthesis is promoted through cellular stress, such as high temperature (Tjahjono *et al*., 1994), high irradiance (Boussiba *et al*., 1992; Harker *et al*., 1996) and/or partial oxygen pressure increase (Lee and Ding, 1995). Thus, astaxanthin production in *H. pluvialis* is usually achieved through a two-phase process, e.g., green vegetative and red cysts phases. Despite the huge investment to increase *H. pluvialis* biomass production, yet this procedure has not been completely optimized.

Growing *H. pluvialis* is a difficult task due to its complex life cycle, in which aplanospores originate 4, 8, 16 or 32 green biflagellate cells. Usually, in laboratory cultures it shows low growth rates (0.30 – 0.60 d^{-1}), and consequentely low biomass yield (5×10^4 – 6×10^6 cells ml^{-1}) (Fan *et al*., 1994; Fábregas *et al*., 2000; Cifuentes *et al*., 2003; García-Malea *et al*., 2005)

The photosynthetic apparatus is composed of two photosystems, PSII and PSI. PSII is formed by a pigment-protein complex that catalyses light-induced transfer of electrons from water to plastoquinone. It has been shown that PSII is sensitive to low concentrations of pollutants, nutrient starvation and other environmental factors (Baumann *et al*., 2009; Lombardi and Maldonado, 2011) and pulse amplitude-modulated (PAM) fluorescence has been used to assess the effects of environmental factors on PSII (Juneau *et al*., 2002; Miao *et al*., 2005; Perales-Vela *et al*., 2007; Baumann *et al*., 2009; Lombardi and Maldonado, 2011). This technique is well established for the characterization of the state of the photosynthetic apparatus, e.g., the performance of photochemical processes in photosystem II (PSII) (Kromkamp and Forster, 2003).

In cultures, the pH is important because it can affect both nutrients speciation, acting upon their availability and the microalgae surface charge (Wilde *et al*., 2006; Borowitzka *et al*., 1991; Kobayashi *et al*., 1993). As a photoautotrophic organism, *H. pluvialis* incorporates CO_2 and consumes inorganic nutrients that lead to pH increase in the culture, what can further inhibit microalgae growth. Thus, the use of pH buffers to avoid such problems and allow vegetative growth for longer periods is a promising tool to optimize *H. pluvialis* growth. Stross (1963) investigated the effects of initial pH on nitrogen

metabolism of *H. pluvialis* and observed that the assimilation of nitrate or ammonium ions is influenced by culture pH. Sarada *et al*. (2002) used BBM (Bold's Basal Medium) culture medium under different initial pHs and observed maximum cellular density in pH 7.0, no growth in pH 5.0 and the lowest cellular density in pH 9.0. Borowitzka and Huisman (1993) investigated the formation of aplanospores in *Dunaliella salina*, using several organic pH buffers in a pH range from 6.0 to 9.0 and obtained high growth and biomass in the 6.0 < pH < 7.5 range. However, most of these investigations focus on initial pH and not onto the pH behavior through the experimental time at the same time that the photosynthetic response is monitored. Aiming at a improving *H. pluvialis* growth during its green vegetative phase as to obtain higher biomass, the current study analyzed the influence of the pHs 6.0, 6.3, 6.7 and 7.2 in the vegetative growth, maximum quantum yield of photosystem II and the biochemical composition (total proteins, carbohydrates) of *H. pluvialis*.

MATERIALS AND METHODS

Cultures

Haematococcus pluvialis (strain 7072) was obtained from the Canadian Center for the Culture of Microorganisms (UBC, Vancouver, Canada). In the Laboratory of Algae Biotechnology, at Federal University of São Carlos (SP, Brazil), the strain was grown in modified L. C. Oligo medium (AFNOR, 1980), according to the composition shown in Table 1 and prepared with distilled water. Prior to inoculation culture medium was sterilized in autoclave (121 °C, 20 min). Due to the life cycle complexity of *H. pluvialis*, the inoculation of cysts instead of green flagellate cells has been used to induce culture synchronization (Fábregas *et al*., 2003). Doing this, high percent germination was achieved.

Table 1: Composition of the culture medium LC Oligo (AFNOR, 1980) modified.

Nutrients	Final concentration ($\mu mol\ l^{-1}$)
KNO_3	2000
$Ca(NO_3)_2 . 4H_2O$	349
$MgSO_4.H_2O$	240
K_2HPO_4	460
$CuCl_2.2H_2O$	0.12
$(NH_4)_6Mo_7O_{24} . 4H_2O$	0.048
$ZnSO_4.7H_2O$	0.2
$CoCl_2.6H_2O$	0.252
$Mn(SO_4)_2 . H_2O$	0.24
H_3BO_3	0.98
$C_6H_5FeO_7 . H_2O$	5.8
$FeCl_3 . 6H_2O$	6.28
$FeSO_4 . 7H_2O$	4.48
$C_6H_8O_7 . H_2O$	0.286
NH_4HCO_3	358

The experiments were performed in 500 ml borosilicate Erlenmeyer flasks containing 200 ml of culture, had initial density of 10^4 cysts ml^{-1}. This represents a proportion of approximately 10% in relation to the final culture volume. All cultures were daily shook and kept under fluorescent lamps at a irradiance of 65 µmol photons $m^{-2}\ s^{-1}$, photoperiod 12:12 h (light:dark cycle) and temperature of 23 ± 2 °C. No aeration was supplied.

H. pluvialis was cultivated at 6.0, 6.3, 6.7 e 7.2 pH with the use of organic pH buffers. Controls did not receive pH buffer, but its initial pH was adjusted with HCl (analytical grade) to 6.3 ± 0.5 (Suh *et al*., 2006). All buffers were used at a final concentration of 1.0 mM and consisted of acid 2-morfolinoetanosulfonic monohydrate (MES) buffering at pH 6.0 and 6.3; piperazine-1,4-bis(acid 2-ethanesulfonic) (PIPES) for pH 6.7, and acid 2-[4-(2-hidroxyethyl)1-piperazinyl]-ethanesulfonic (HEPES) for pH 7.2. All treatments were performed with 3 experimental replicates.

Experiments lasted 20 days and growth parameters were monitored on alternate days. Samples were obtained for cell counting in a Fuchs-Rosenthal chamber under optical microscope (Leica, Germany) and absorbance determination at 680 nm (Katsuda *et al*., 2004) in spectrophotometer (FEMTO Model 800 XI, Brazil). The specific growth rates (μ) were calculated through graphic representation of the natural logarithm of the number of cells per ml as function of time. The linear regression from the straight line was calculated for the exponential growth phase that usually lasted 5 days. In this case, the angular coefficient represents the specific growth rate. Chlorophyll *a* concentration (mg L^{-1}) was determined through *in vivo* fluorescence using a Turner Designs fluorometer (*Trilogy* model, U.S.A.) after the instrument calibration for chlorophyll *a* concentration *vs* fluorescence intensity.

The kinetic parameters of fluorescence induction of chlorophyll were determined for each treatment in the 5th culture day according to that described in Roháček (2002) using a PHYTO-PAM® Fluorometer Analyser (Walz, Germany) equipped with an ED-101US/MP optical unit. Culture samples were obtained 2 - 3 h after the beginning of the photoperiod. Cells were dark adapted during 15 minutes before determination of the fluorescence parameters, F_o – fluorescence at low intensity of modulated light (< 0.3 μmol photons m^{-2} s^{-1}) according to Genty *et al*. (1989), and F_m – maximum fluorescence at saturating light (2.600 μmol photons m^{-2} s^{-1} for 0.3 s, reproduced at the frequency of 600 Hz). Variable fluorescence (F_v) was determined by the difference between F_o and F_m, and maximum quantum yield given by F_v/F_m (Schreiber *et al*., 1986; Schreiber and Bilger, 1993) in dark adapted cells. For the light-adapted state, when the maximum effective fluorescence (F_m') and initial effective fluorescence (F_o') were obtained, cells were exposed to actinic light at 64 μmol photons m^{-2} s^{-1} for 10 minutes. Variable fluorescence ($F_v' = F_m' - F_o'$), photochemical quenching (qP), non-photochemical quenching (NPQ) and Φ_{PSII} (effective photochemical quantum of PSII F_v'/F_m') (Roháček, 2002) were obtained. The fluorescence parameters were normalized in relation to F_o according to Roháček e Barták (1999). Background fluorescence in the culture was determined using a blank sample that was obtained filtering the culture (0.22 μm membrane filter, Millipore) immediately before analysis. F_o represents chlorophyll *a* fluorescence emission produced by the excitation of the light harvesting complex (LHC_{II}) before energy transference to the PSII reaction center (Krause and Weis, 1991), while F_m represents the maximum fluorescence when primary electron acceptor of PS II (Q_A) is reduced and the reaction center remains unable for charge separation (Nedbal *et al*., 2000).

Biochemical composition

The biochemical composition of the biomass was based in lipids (total and classes), total intracellular carbohydrates, and total intracellular proteins. Carbohydrates were determined according to the methodology described in Liu *et al*. (1973) with glucose as standard. Proteins were determined according to Bradford (1976) using bovine albumin fraction V serum (BSA) as standard. Protein extraction followed Rausch (1981) protocol that is based in alkaline digestion. For carbohydrates and proteins, culture samples were centrifuged at 385 *g* during 10 min (Sorvall Legend XTR, USA) at 21 ºC and the pellet was kept at -20 °C until analysis, while the supernatant was discarded.

Lipids were extracted and determined according to the procedure described in Parrish (1999) through thin-layer chromatography with flame ionization detection (TLC/FID) using an Iatroscan™ analyzer TLC/FID MK-6S model (Iatron Laboratories Inc., Tokyo, Japan). For lipids extraction, algal suspensions were filtered through previously baked (400 °C, 12 h) glass fiber filters (WHATMAN GF/C) and sonicated in chloroform:methanol (4:1, v/v). Ketone (3-Hexadecanone), used as internal standard (20 μL), was spiked onto the filters immediately after filtering. After extraction, the filters were stored immersed in chloroform and kept in glass tubes with a Teflon® lid, in a freezer at a -20 °C until the moment of the analysis. Extracted samples were stored for a maximum of 90 days.

The chromatography (TLC/FID) was processed in samples that were previously concentrated in a rotary evaporator at 25 °C and further in N_2. The system was calibrated using lipid standards commercialized by Sigma-Aldrich (E.U.A.). The analytical conditions used for the Iatroscan operation were: hydrogen flow of 173 ml min^{-1}, air flow of 2 L min^{-1} and scanning speed of 4 mm s^{-1}. The chromatography was processed in 3 distinct solvent mixtures resulting in different polarities that fractionated the samples into 9 lipid classes. The first and least polar solvent mixture consisted of hexane:diethyl ether:acid formic (98.95:1:0.05) that separated aliphatic hydrocarbons (HC), wax esters (WE) and ethyl ketone (KET), the internal standard. The second mixture, of intermediate polarity, was elaborated with hexane:diethyl ether:formic acid (79:20:1), in which triglycerides (TG), free fatty acids (FFA), free aliphatic alcohols (ALC) and free sterols (ST) were separated, and the third and most polar solvent mixture among the three was made of chloroform:methanol:water (5:4:1). Chromatographic rods were placed into 100% acetone for 15 min to focus and then inserted into the most polar mixture where the mobile polar lipids (AMPL), mostly related to pigments, and phospholipids (PL) were separated.

All glass materials that would get into contact with samples for lipid analysis were previously baked at 400 °C for 12 h. Teflon® flasks, glass materials, and metals were rinsed with chloroform and methanol immediately before use to reduce contamination. All chemicals used for lipid analysis were HPLC (High Performance Liquid Chromatography) grade or higher.

Statistical Analysis

Statistical analysis consisted of ONEWAY ANOVA, and when significant differences were present ($p < 0.05$), '*a posteriori*' Tukey (Snedecor and Cochran, 1980) test was applied. All variances were checked for normality and

homogeneity. The resulting data was analyzed in the statistical programs contained in OriginPro, version 9.0 and Sisvar, version 5.3.

RESULTS

Figure 1 reports pH variation as function of time. It shows that pH buffering was important for daily maintenance of culture pH within minor oscillations. In the controls, where no pH buffer was added, pH varied from 6.3 to 10.3, while in the buffered systems pH variation never exceeded 1.4 in pH scale. The lowest pH variation was obtained for the pH 6.3 buffered cultures. Figure 2 shows the specific growth rates (μ), which were different among treatments ($p < 0.05$) and followed the decreasing order $\mu_{pH\ 6.3} > \mu_{pH\ 7.2} = \mu_{pH\ 6.7} = \mu_{pH\ 6.0} > \mu_{control}$.

Fig 1: Variation of pH in *H. pluvialis* buffered cultures as function of experimental time. Symbols: ■ Control; ◊ pH 6.0; ▼ pH 6.3; Δ pH 6.7; ● pH 7.2. Error bars represent the standard deviation of n = 3.

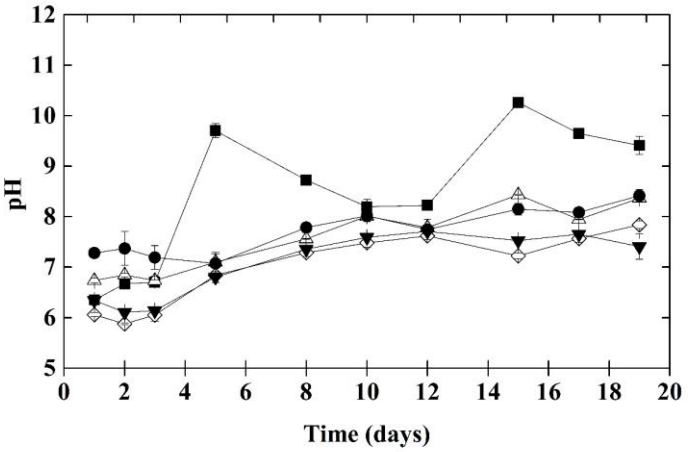
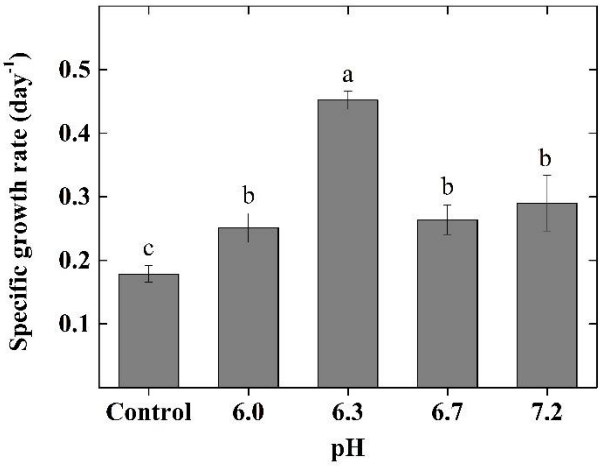
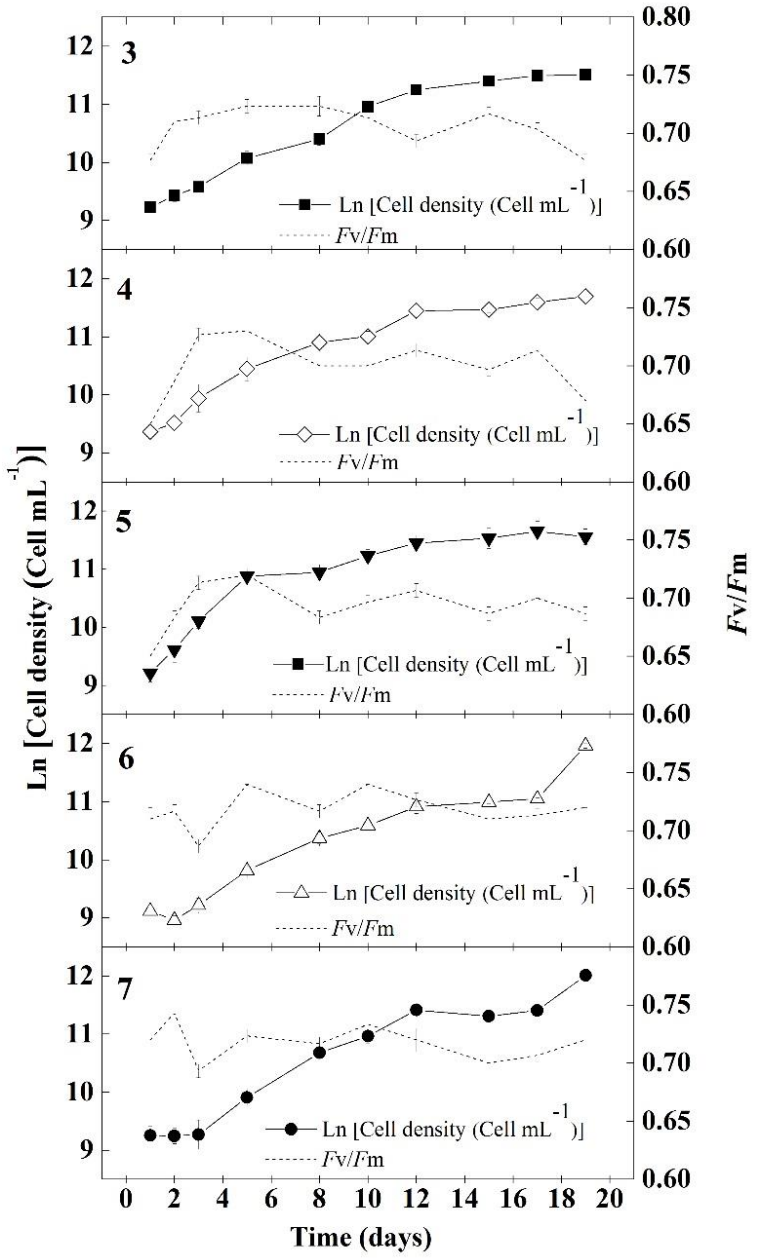


Fig 2: Specific growth rates obtained for exponentially growing *H. pluvialis* at the several pH treatments. Values represent the mean of n = 3; means followed by same letter do not differ statistically by Tukey's test at 5% probability.



Growth curves and maximum quantum yields of PSII are shown in Figs 3 to 7. The germination phase was marked by an increase in PSII quantum yield up to a maximum value in the exponential growth phase, where it stabilized in 0.7. The pH 6.3 buffered treatment presented the typical *H. pluvialis* growth pattern in batch cultures, with two exponential growth phases, the first one from 1 -5 culture day, and the second from 9-17 day. In the pH 6.3 buffered culture there was no lag phase of growth, but this was detected in the pH 6.7 and 7.2 buffered cultures.

Figs 3-7: Population density as the natural logarithim (Ln) of cell ml^{-1} and F_v/F_m in *H. pluvialis* pH buffered cultures as function of experimental time. Symbols: ■ Control (a); ◊ pH 6.0 (b); ▼ pH 6.3 (c); ∆ pH 6.7 (d); ● pH 7.2 (e) Error bars represent the standard deviation of n = 3.



Photosynthetic responses for cultures in exponential growth (day 5) are shown in figure 8 (maximum quantum yields, F_o, F_m) and 9 (qP, NPQ). The highest NPQ and F_m value were obtained for the pH 6.0 buffered cultures. NPQ differed significantly ($p < 0.05$) among treatments, but qP was similar throughout, except for the pH buffered culture, which was lower ($p < 0.05$).

Fig 8: Maximum PSII quantum yield (F_v/F_m – black bar; left Y axis), maximal fluorescence (F_m – white bars; right Y axis), and initial fluorescence (F_o – gray bars; right Y axis) for exponentially growing *H. pluvialis* cultures. Error bars represent standard deviation (n = 3). Means followed by same letter do not differ statistically by Tukey's test at 5% probability.

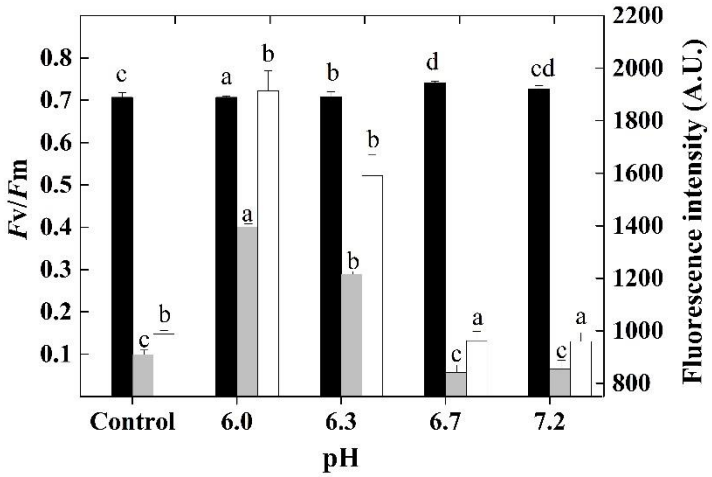
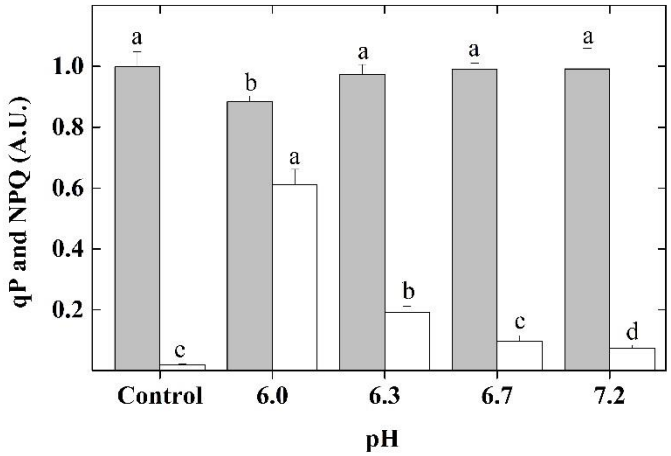


Fig 9: Photochemical quenching (*q*P – gray bars) and nonphotochemical quenching related to heat dissipation (NPQ – white bars) for exponentially growing cells. Values are mean of n = 3 and error bars the standard deviation. Means followed by same letter do not differ statistically by Tukey's test at 5% probability.



The biochemical composition (total proteins, carbohydrates and lipids) are presented in Fig 10, and proteins:carbohydrates (P:C) ratios in figure 11. The greatest proteins (1530 pg cel^{-1}) and carbohydrates (395 pg cel^{-1}) contents were obtained in cultures buffered at pH 6.7 ($p < 0.05$). These values were 145% and 16% higher in comparison with the controls, respectively. The P:C ratio, which tells about the health status of microalgae, were higher in the buffered treatments in comparison with the controls (ANOVA $p < 0.05$). This means that pH variation affects protein and carbohydrate synthesis, decreasing the first in relation to the second. The pH 6.7 buffered culture had a 47.5% higher P:C ratio than controls.

Fig 10: Concentration of total proteins (black bars), total carbohydrates (gray bars) and total lipids (white bars) for the different pH buffered cultures in exponentially growing *H. pluvialis*. Values are mean of n = 3 and error bars represent standard deviation. Means followed by the same letter do not differ by Tukey test at 5% probability.

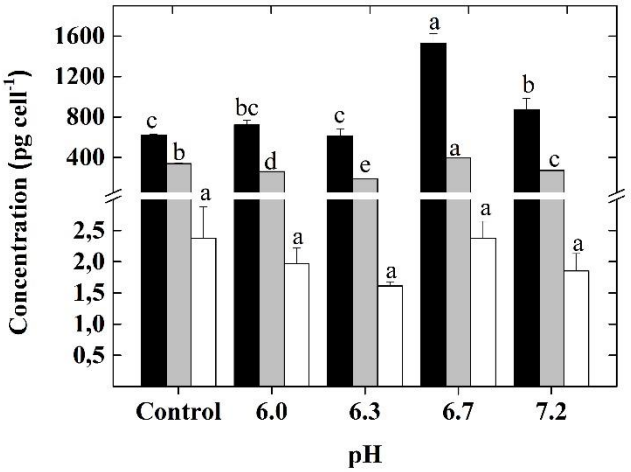
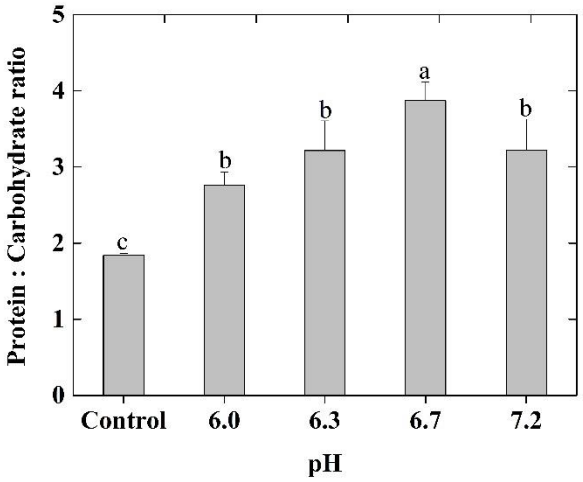
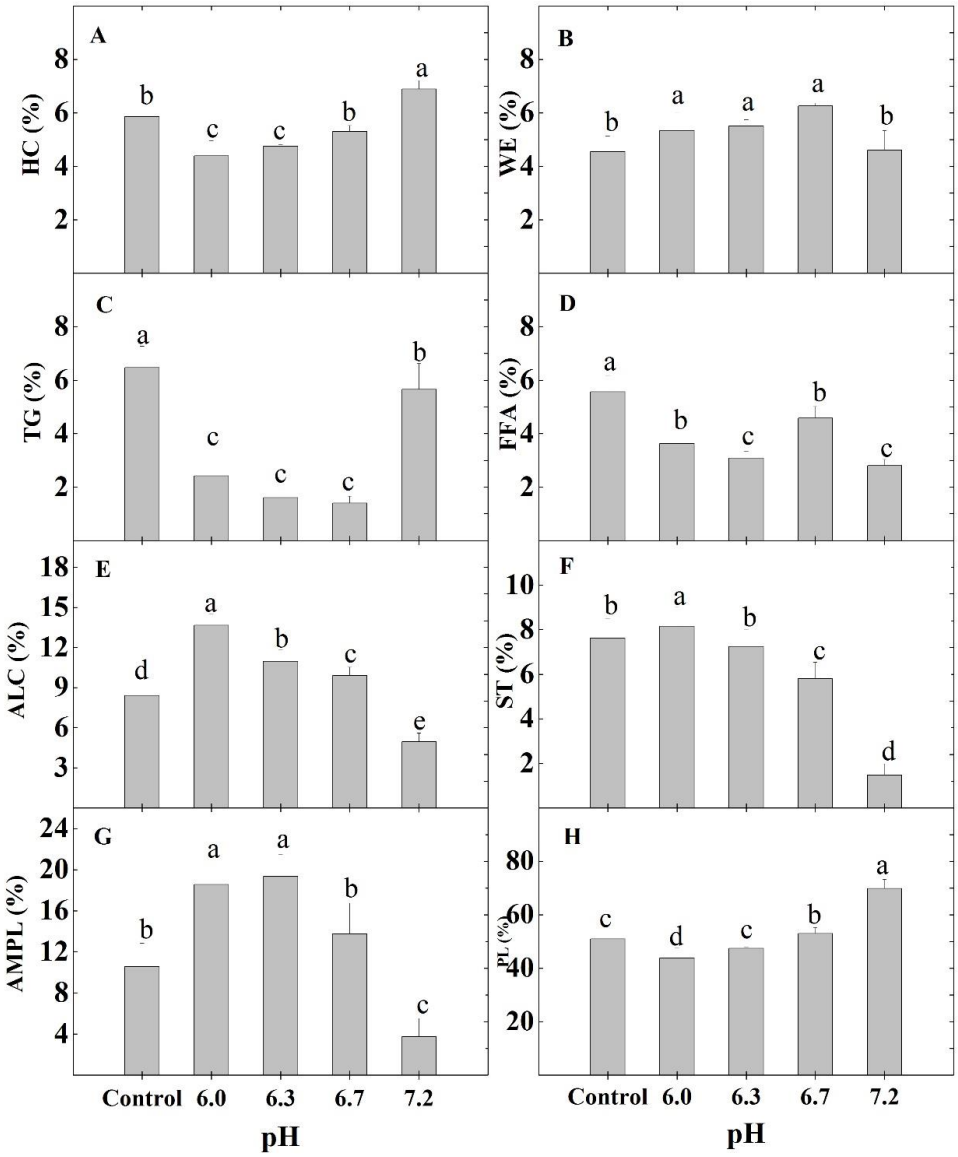


Fig 11: Proteins:carbohydrates (P:C) ratio for the different culture pH treatments in exponentially growing *H. pluvialis*. Values are mean of n = 3 and error bars represent standard deviation. Means followed by the same letter do not differ by Tukey test at 5% probability.



Considering total lipids, no significant difference was observed among treatments and controls ($p > 0.05$). Lipid classes (Fig 12) with higher representation in the vegetative *H. pluvialis* biomass were AMPL (~ 20%) and PL (~ 70%), pigment and cell wall related lipids, respectively. Aliphatic hydrocarbons (HC) were present at 6.9% of total lipids and TG at 5.6%, both in cultures at pH 7.2. Wax esters were mostly present in the control and at pH 6.0, 6.3 and 6.7 treatments. In average, the WE amounted ~ 5.7% of the total lipids. Free fatty acids (6.7%) and ST (8.7%) and were generally lower than the control treatment. The free aliphatic alcohols (ALC - 13.7%) were higher in cultures at pH 6.0 (ANOVA $p < 0.05$) in comparison with the other treatments and controls.

Fig 12: Relative composition of lipid classes during exponential growth of *H. pluvialis* buffered cultures at different pH. Abbreviations: HC, aliphatic hydrocarbon(s); WE, wax ester(s); TG, triacylglycerol (s); FFA, free fatty acid(s); ALC, free aliphatic alcohol(s); ST, free sterol(s); AMPL, acetone mobile polar lipid(s); PL, phospholipid(s). Error bar represents the standard deviation. Mean comparison test: means followed by the same letter do not differ by Tukey test, at 5% probability.



DISCUSSION

The inoculation of *H. pluvialis* cysts resulted in approximately 100% germination into green vegetative flagellates, and cultures synchronization at similar growth phases, were obtained. This decreased experimental errors among replicates. This confirms literature results that show the importance of cycts as inoculum in cultures of *H. pluvialis* (Vidhyavathi *et al*., 2007). Inoculating green flagelates can led to different growth patterns within replicates, increasing the errors and impairing comparisons (Imamoglu, Sukan and Dalay, 2007; (Sipaúba-Tavares *et al*., 2013).

The present results showed that pH variation in buffered treatments was minor in contrast to pH variation in unbuffered controls. These results are in general agreement with literature data. Lababpour *et al*. (2004) investigated the growth of *H. pluvialis* in unbuffered cultures at initial pH 6.8, and after 150 h (~ 6 days), culture pH had raised to 10. However, the authors did not observe any significant difference in *H. pluvialis* vegetative growth that could be attributed to pH. We can rationale that in our conditions, HCO_3^- and CO_2 (aq) were the predominant species throughout the experiments, while in studies where pH reach values equal to or higher than 10, CO_3^{2-} predominates. In this situation, *H. pluvialis* can possibly be limited by inorganic carbon. In the present study, cultures were not inorganic carbon limited because of the pH range in which they developed; we can further hypothesize that such limitation may cause *H. pluvialis* red aplanospore formation, not observed in our study, but common in the literature (Kang *et al*., 2007; Minyuk et al., 2016).

Although we used buffered systems to grow *H. pluvialis*, the density in our cultures was lower than that in Nagaraj *et al*. (2012) but within the range of cell density that can be found in literature (Harker *et al*., 1996; Tripathi *et al*., 1999; Kaewpintong *et al*., 2007). In our case, there was a significant difference in cell density ($p > 0.05$) for in the 5th culture day (representative of exponentially growing green vegetative *H. pluvialis*) with the highest value obtained at pH 6.3. The influence of pH in the vegetative growth of *H. pluvialis* without addition of buffers was assessed by Sarada *et al*. (2002) and Nagaraj *et al*. (2012). Sarada *et al*. (2002) obtained maximum biomass in cultures at initial pH 7.0, but no growth in pH 5.0, while Nagaraj *et al*. (2012) obtained maximum biomass (6.44×10^6 cells ml^{-1}) in pH 7.0 after 18 days of incubation

The present results confirmed literature data that found pH 6.3 to 6.5 to be the best ones for *H. pluvialis* growth (Kobayashi *et al*., 1993; Harker *et al*., 1996; Cifuentes *et al*., 2003). In these pH, growth rates ranged from 0.31 (unbuffered controls) to 0.45 d^{-1} (pH 6.3 buffered cultures). The highest value was approximately 50% higher than the controls and 40% higher than the other treatments. Hata *et al*. (2001) tested different initial pH 5.0 – 9.0 for *H. pluvialis* in heterotrophic metabolism and obtained growth rates of 0.20 d^{-1} and lower. This is nearly half of the values we obtained. Higher growth rates were obtained by Orosa *et al* (2005) when investigating the effects of nitrate, malonate and acetate in the growth and astaxanthin accumulation in *H. pluvialis*. They obtained growth rates of 0.64 d^{-1} and final biomass yield of ~ 2×10^6 cells mL^{-1}. In our best growth condition, final biomass was 2×10^5 cells mL^{-1}. Thus, controlling the chemical environment through pH maintenance can increase growth rates in *H. pluvialis*, but furnishing an organic source, such as acetate that can be a nutrient and pH buffer, higher growth rates will be obtained. Acetate as nutrient was investigated by Borowitzka *et al*. (1991). The authors concluded that the use of acetate buffer increased the growth rate and induced the formation of red aplanospores and palmelloid *H. pluvialis* cells. In relation to cell morphology, our results differ from those of Botowitzka *et al*. (1991), since we obtained no effect of buffering systems on aplanospores or palmelloid formation. In the present research, we obtained a rate variation for H^+ ions concentration of 2.3×10^{-8} mol l^{-1} d^{-1}, similar to that obtained by Botowitzka *et al*. (1991) with acetate buffer.

Literature data show that despite the variation in chlorophyll *a* content known to occur during the growth of *H. pluvialis* (Gu *et al*., 2013) in reason to its life cycle, quantitation of the photosynthetic efficiency can indicate the beginning of the carotenoid synthesis, thus the end of vegetative growth. We showed higher maximum quantum yields (~ 0.70 – 0.74) during the vegetative growth phase that in this research occurred from the 4th to 12th day, compared to palmelloid cells, where carotenoid synthesis dominates (Collins *et al*., 2011). Palmelloid cells were present from the 13rd to the 20th culture day in the present research, showing lower maximum quantum yield values. The values of both maximum and operational F_v/F_m we obtained suggested healthy cells and agree with previous literature (Herrmann *et al*., 1996; Vonshak and Torzillo, 2004; Kumar *et al*., 2014). For *H. pluvialis*, Chen *et al*. (2012) found maximal F_v/F_m values ~ 0.70, in agreement with this research, but higher than those reported in Wang *et al*. (2011) (F_v/F_m ~ 0.4 – 0.5).

We observed a 3 day delay for inoculated cysts to began transformation into green flagellate vegetative cells in cultures buffered at pHs 6.7 and 7.2 in contrast to pH 6.3 buffered cultures that entered vegetative growth immediately after inoculation, exhibiting the highest growth rate. These results agree with those of Gu *et al*. (2013) that investigated *H. pluvialis* in different growth phases and showed an extensive acclimation mechanism during its aging process. The authors proposed that both green vegetative cells and astaxanthin that dominates in resting cells can affect the final astaxanthin yield in industry. According to Boussiba and Vonshak (1991), and Nagaraj *et al*. (2012), when *H. pluvialis* cells are exposed to perturbations, there is a reduction in F_v/F_m, indicating damages to PSII in the green mobile cells. This may prevent energy transfer to PSII reaction center by disconnection of the light harvesting center (LHC_{II}). The present results showed lower F_m for controls and cultures at pH 6.7 and 7.2 in comparison with cultures at pH 6.0 and 6.3, suggesting a higher stock of electron acceptors in photosystem II due to an imbalance between the flow of electrons in the donor side in comparison with the flow in the acceptor side. According to Strasser (1997), this can cause a reduction of the maximum fluorescence (F_m).

In the present research, *q*P values were within 0.88 – 0.98 for all treatments, the lowest one for pH 6.0 buffered culture. This indicates that it is possible that the pH range we used did not affect light absorption by the PSII reaction centers, charge separation, nor electron transfer to Q_A. In fact, only small differences were observed for maximum quantum yields among treatments.

NPQ is a mechanism that protects PSII from the harmful effects of high light through the dissipation of excess excitation energy, thus minimizing photo-damage (Krause and Jahns, 2003). It is mostly related to heat loss. In the present research NPQ increased in pH 6.0 buffered cultures, suggesting that at this pH, PSII could have been facing problems and not using the majority of incident light for photochemistry. According to Roháček and Barták (1999), NPQ can be correlated with the formation of zeaxanthin in cells of *H. pluvialis*. Torzillo *et al*. (2003) and Chen *et al*. (2012) correlated increased NPQ with morphologic transformation in mobile green cells into fully red cysts in cultures at pH 7.0. This did not happen in the present research, where cultures kept at pH 7.2 had NPQ as low as the controls or pH 6.3, our best condition. This difference can be due to the buffer system, which kept the pH under low variation, in contrast to both authors that did not use buffer, so pH should have varied.

The protein concentration of 1.53 ng cell^{-1} obtained in exponentially growing flagellates from pH 6.7 buffered cultures was nearly five times greater than that reported in Kobayashi *et al*. (1997). However, it is 30% lower than that reported by the same authors 4 years before (Kobayashi *et al*., 1993) with Fe^{2+} supplemented *H. pluvialis* cultures at initial pH 6.8.

Tripathi *et al*. (2002) obtained protein concentration of 3.5 ng cell^{-1} for *H. pluvialis*, 2.3 times higher than what we obtained. Kakizono *et al*. (1992) cultured *H. pluvialis* in BBM medium supplemented with sodium acetate in pH 6.8 and on the 6th day they obtained a protein content of 0.38 ng cell^{-1}. It is known that proteins are part of structural materials and despite its presence in considerable amounts in exponentially growing and healthy cells if compared with stationary ones (Rocha *et al*., 2014), its concentration can vary according to culture medium composition. We used LC Oligo culture medium that compared with BBM has less nitrogen, what may be responsible for the lower protein content in our cultures.

Despite its importance for cell metabolism, few studies have focused on the carbohydrate synthesis in *H. pluvialis*. The range of 0.35 – 0.40 ng cell^{-1} of carbohydrates present in all culture conditions we studied, independent of its pH, is similar to the results presented in Recht *et al*. (2012), who obtained 0.30 ng cell^{-1} of carbohydrates in *H. pluvialis* cultures at pH 7.6. Intracellular carbohydrates are energy compounds that are usually detected in higher concentrations in stationary growth phase or under stressing conditions. Low amounts of carbohydrates in relation to proteins indicate healthy cells (Healey and Hendzel, 1980; Kilham *et al*., 1997; Rocha *et al*., 2014). In the present research, the lowest P:C ratio was obtained for the controls. This supports the hypothesis that pH variation in the cultures may have been a problem for *H. pluvialis* and that keeping pH within narrow range resulted in better growing conditions.

The low total lipids concentrations (~1.8 – 2.5 pg cell^{-1}) we obtained can be expected for exponentially growing green *H. pluvialis.* According to Saha *et al*. (2013), the accumulation of neutral lipids and astaxanthin esters in this microalgae is correlated with stressing conditions and cysts formation. The dominance of structural lipids in *H. pluvialis* vegetative cells is in accordance to literature results, which show that these are, in fact, dominant classes in healthy phytoplankton cells (Parrish and Wangersky 1987; Lombardi and Wangersky, 1991; 1995; Chia *et al*., 2013). Phospholipids were present in 69% in *H. pluvialis*, while Chia *et al*. (2013) found it to be present in 45% in *Chlorella vulgaris*. In cells of *Dunaliella tertiolecta*, Lombardi and Wangersky (1995) obtained 25% of the total lipids to be phospholipids.

CONCLUSION

We showed that maintaining pH variation to a minimum, so keeping more stable the chemical environment healthier *H. pluvialis* will be generated and better conditions provided for cysts germination, decreasing the lag phase in the beginning of cultures and exponential growth. The highest growth rate was obtained for pH 6.3 buffered culture compared to pH 6.0, 6.7, and 7.2 cultures. Cultures buffered at pH 6.3 also had the highest cell density and best F_v/F_m values for exponentially growing cells compared to unbuffered cultures. Protein:carbohydrate ratios confirmed healthy cells in buffered cultures. Total intracellular lipid synthesis were not affected by culture pH in *H. pluvialis* cells.

ACKNOWLEDGMENTS

The authors are grateful to São Paulo State Research Foundation (FAPESP-Brazil) for financial support through grant no. 2014/15894-0 and to CNPq - Brazil through grant no. 302837/2012-4.

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