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Sources of inorganic fertilizer in the growth of *Haematococcus pluvialis* Flotow (Chlorophyceae).

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Abstract

The influence of inorganic fertilizer media at different concentrations on the growth and biochemical composition of *Haematococcus pluvialis* microalgae was evaluated. Microalgae were placed in a bath culture with inorganic fertilizer (NPK) medium and WC medium during 28 days, Growth and biological aspects of microalgae between the media were compared. Mineral concentrations affected the physiological and growth rate of *H. pluvialis*, and the balance of nitrogen and phosphorus concentration favored higher algal biomass. High concentration of protein occurred when concentrations of nitrogen and phosphorus occurred in cultures NPK 20:5:20 and NPK 4:14:8. Amino acid profile were significantly higher ($p<0.05$) in culture media based on inorganic fertilizer when compared to that in commercial medium (WC). High cell density (4.6×10^5 cells.mL^{-1}) was obtained in NPK 10:10:10 and in WC medium (3.2×10^5 cells.mL^{-1}) after 16 days cultivation. Media inorganic fertilizer (NPK) was adequate and may replace the commercial medium WC for the cultivation of microalgae *H. pluvialis*, with high nutritional value and biomass concentration.
Keywords: NPK and WC media; biological aspects, nutritional value

Introduction

Nutrients are greatly relevant in the growth and development of microalgae. Supply from a single nutritional source influences physiological adaptation and the biochemical composition of microalgae (Liu et al., 2013). Although they may assimilate several sources of nutrients, absorption rate depends on culture conditions (Perez-Garcia et al., 2011). Phosphorus and nitrogen are the main elements that limit the growth of microalgae, which depends on the physiological requirements for each nutrient and species (Lagus et al., 2004). There are several types of basic nutrients for the growth of microalgae, such as calcium, magnesium, iron, sulfur and trace elements (Junying et al., 2013). Potassium is a macronutrient which is required for growth-related metabolic activities (Sivakumar and Arunkumar, 2009).

The choice of a culture medium for microalgae growth depends on the nutritional requirements of algae, product quality and production costs (Borowitzka, 2005). The supply of nutritional requirements contributes towards the efficient growth plus high biomass (Alkhamis and Qin, 2015). Culture costs and time decrease when alternative sources of nutrients are employed in the composition of culture media such as ammonium sulfate, urea, swine manure, water plants, inorganic fertilizers (NPK) and others (Sipaúba-Tavares et al., 2009; Rodrigues et al., 2010).

Haematococcus pluvialis Flotow is a green unicellular microalga with atypical life cycle since it modifies its cell metabolism from the vegetal state (rich in proteins and chlorophylls) to encysted cells (rich in astaxanthin) (Ba et al., 2016). Large-scale culture of *H. pluvialis* still has several operational impairments due to the species´s intrinsic characteristics, such as slow growth rate, contamination trends and high production costs of culture media. As a rule, *Haematococcus pluvialis* is cultivated in media WC, BM, BG11, M1B5 and other chemical additions such as iron and acetate ions with morphological alterations in vegetal cells (Solovchenko, 2014).

Although several studies focus on the N:P ratio in the commercial medium F/2 (Rasdi and Qin, 2015) and the use of agricultural fertilizers in the culture of microalgae (Dalay et al., 2007), very few research works have specifically dealt with the effect of different N and P concentrations with regard to development and biochemical composition, particularly the profile of amino acids, an essential compound to determine protein quality. Amino acids profile in algal biomass depends on several factors, such as alga species, environmental conditions and, mainly, the type of culture employed (Masojídek and Prášil, 2010).

Current study investigates the effect of the use of inorganic fertilizers at different concentrations of micro-minerals as the main factor in the culture medium for the cultivation of the microalga *Haematococcus*

pluvialis when compared to commercial medium WC, by assessing growth, biochemical composition, biological aspects and amino acid profile.

Materials and Methods

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. Algal were batch-cultured at 22±2°C and, exposed to light at 30 µmol m^{-2} s^{-1}. Two culture media was used, WC medium (Guillard and Lorenzen, 1972) as control, and inorganic fertilizer NPK at four different nitrogen, phosphorus and potassium concentrations; 20:5:20; 12:6:12; 10:10:10 and 4:14:8 (Sipaúba-Tavares 1999) and cells grown in batch culture (2L). Further, approximately 50g L^{-1} of inorganic fertilizer (at different concentrations) were added to 1L of distilled water and autoclaved at 1 atm during 30 minutes. The experiment started with 250 mL flasks, at microalgae density of 1 x 10^5 cells.mL^{-1} and cultured in WC media. When cultures reached the late exponential growth phase (14 day), approximately 280 mL of culture density (0.5 x 10^5 cells.mL^{-1}) were added in 2-L flasks with WC media, at density 0.5 x 10^5 cells.mL^{-1}; NPK (20:5:20) medium, 0.7 x 10^5 cells.mL^{-1} of NPK (12:6:12) medium, 0.4 x 10^5 cells.mL^{-1} of NPK (10:10:10) medium and 0.7 x 10^5 cells.mL^{-1} of NPK (4:14:8) medium. The experiments were performed in 2-L volumes with continuous air bubbling. Vitamin B12 complex was added to NPK media at the rate of 0.02 g.L^{-1} plus biotin (0.01 mg.L^{-1}). Growth performance and other physiological parameters and analytical methods were analyzed weekly (1, 7, 14, 21, 28 days), whereas the composition of amino acid was evaluated by the end of experiment. Only green cells from the exponential growth phase were used as inoculums for the experiment. Table 1 shows the composition of nutrients of different culture media.

Table 1. Composition of different inorganic fertilizers.

Culture medium	Chemical composition									
	mg L^{-1}			µg L^{-1}						
	N	P	K	S	Ca	Mg	B	Cu	Fe	Zn
20:5:20	213	52	198	0.1	71	4.4	0.1	0.03	0.5	-
12:6:12	121	62	118	0.01	104	6.5	0.1	0.1	0.6	0.01
10:10:10	105	107	107	0.01	135	8.4	0.2	0.1	0.6	0.02
4:14:8	42	143	79	0.01	335	21	0.1	0.1	0.5	0.03

- = not detected by method

Growth

Cell growth was monitored over a period of 28 days. Triplicate 1 mL aliquots were removed daily from the microalgae culture and a minimum of 2 x 1 μL^{-1} sub-sample was used for cell quantification by a Neubauer hemocytometer. Growth rate k (divisions per day) was calculated by k = (3.322/t2-t1 x log N2/N1) (t = time; N = number of cells. Subscripts denote rates 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 = $1k^{-1}$ (Td = duplication time, $1k^{-1}$ = days per division) (Guillard, 1973). Dry biomass was determined weekly, following Vollenweider (1974). Total length (µm), total organic carbon content (TOC) and cell volume were quantified weekly. Total length of 50 specimens was determined with microscope Leica DFC 295 by image analysis system Las Core (LAS V3.8), with a 400X micrometric objective. Calculation of cell volume was undertaken by mean cell size with the use of the most appropriate geometric form, or rather, 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 by Rocha and Duncan (1985).

Analytical methods

Nitrate, ammonia, total nitrogen and total phosphorus in the culture were evaluated weekly during the experiment and quantified spectrophotometrically according to techniques by Golterman et al.,(1978) and by Koroleff (1976). Chlorophyll *a* was extracted with 90% alcohol and quantified at 663 and 750 nm (Nusch, 1980). Conductivity, pH and dissolved oxygen were measured with a multiparametric probe YSI 556 MPS. Biomass was harvested weekly, centrifuged and lyophilized for the analyses of protein and lipids (A.O.A.C, 1990). At the end of the experiment, the biomass was harvested, centrifuged and amino acid profile were determined by acid hydrolysis and using ion-exchange chromatography by high-performance liquid chromatography (HPLC) method, modified by White et al. (1986).

Statistical analysis

All experiments were carried out in triplicate and expressed as Means ±Standard Deviation (SD). One-way ANOVA was applied for simple verification between culture media, with Statistica 10.0 (Statsoft, 2011). Differences were significant at 5% ($p<0.05$). The costs of culture media production include the prices of vitamin B complex, NPK fertilizer and different raw materials for commercial medium (WC). Costs were analyzed for a 50-L production of culture medium.

Results

Haematococcus pluvialis grew exponentially up to the 12th day in all culture media, with the exception of NPK medium (10:10:10) which grew up to the 20th day when cells totalled about 4.6 x 10^5 cells.mL^{-1}. Subsequently, the number decreased sharply to 3 x 10^5cells.mL^{-1}. From the 13th day, high cell density was obtained in NPK medium (10:10:10). Maximum cell density, 1.3 x 10^5 cells.mL^{-1} and 3.2 x 10^5 cells.mL^{-1} were obtained in WC medium during exponential growth curve (between the 4thand the 13th day). Lower cell density ($p<0.05$) occurred in NPK medium 4:14:8, the cell concentration reached maximum 1.7 x 10^5 cells.mL^{-1} on the 13th day of cultivation and started to decrease slightly(Figure 1). Great cell density was reached when N rate was higher than that of P (N>P), whereas the opposed ($p<0.01$) occurred in the inorganic fertilizer 4:14:8 (N<P). N:P balance in inorganic fertilizer medium 10:10:10 favoured the growth of *H. pluvialis* microalgae, ranging between 1.9 x 10^5 cells.mL^{-1} (10th day) and 4.6 x 10^5 cells.mL^{-1} (20th day) (Figure 1).

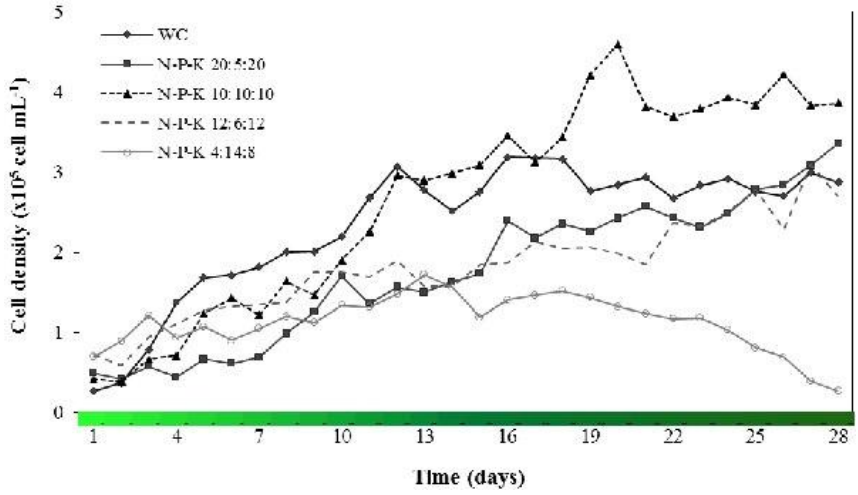

Fig 1.Daily concentration of *Haematococcus pluvialis* in different culture media (WC and NPK- 20:5:20, 12:6:12, 10:10:10, 4:14:8).

Concentrations of ammonia were higher ($p<0.05$) in the inorganic fertilizer medium compared to the commercial medium WC and decreased during the growing period. In commercial medium, nitrate was higher in initial days and consumed quickly during cultivation (Figure 2). Total phosphorus concentration were higher than total nitrogen in all media above 2.9 mg.L^{-1} (NPK 12:6:12), with highest levels obtained in NPK medium (4:14:8), between 2.9 and 3.2 mg.L^{-1}. Since total phosphorus and total nitrogen in all culture media were higher in the inorganic fertilizer (NPK), the factor demonstrated that there was high intake by *H. pluvialis* of the nitrogen compounds in WC medium. In the inorganic fertilizer medium, the higher intake was obtained by nitrate and phosphorus (Figure 2 and 3). Dissolved oxygen concentration were higher, over 7 mg.L^{-1}, whilst pH ranged between 6.4 and 8.4 during the study period. The conductivity was also highest but just in inorganic fertilizer media (> 800 µS.cm^{-1}) (Table 2).

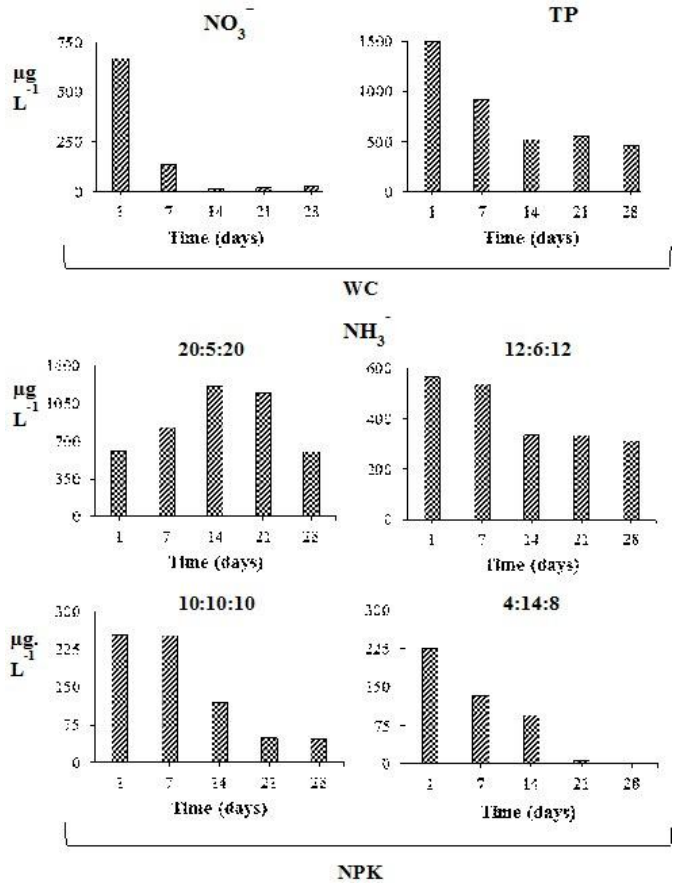

Fig 2. Concentrations of nitrate (NO_3^-) and total phosphorus (TP) in WC medium and, concentrations of ammonia (NH_3^-) in inorganic fertilizer NPK (20:5:20, 12:6:12, 10:10:10 and 4:14:8) of *Haematococcus pluvialis* in 2-L volumes.

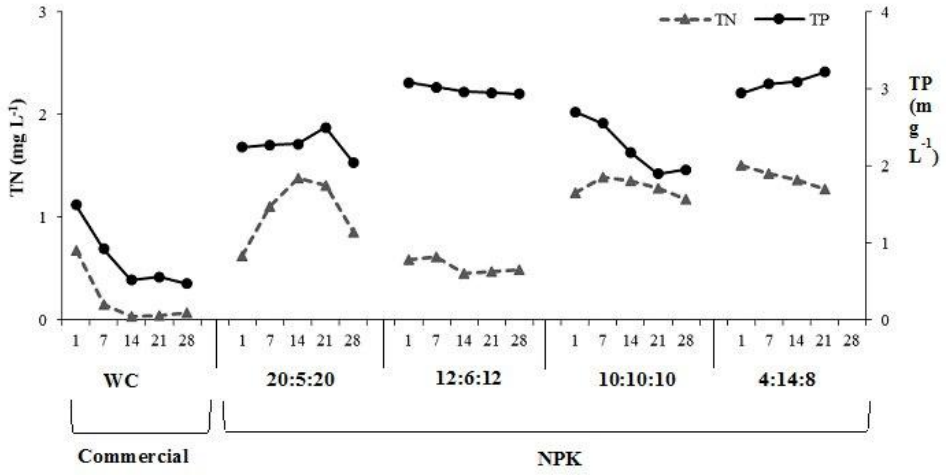

Fig 3. Weekly variations of total nitrogen (TN) and total phosphorus (TP) concentration in WC and inorganic fertilizer (NPK- 20:5:20, 12:6:12,10:10:10 and 4:14:8) culture media.

Total length of *H. pluvialis* was similar (p>0.05) in all cultured media, cell volume and total organic carbon was higher (p<0.05) in inorganic fertilizer medium (NPK 20:5:20) with values higher to the commercial medium WC. Doubling time was short in WC medium, with cell duplication approximately at 4.83 days, directly influencing growth rate (k=0.21) of *H. pluvialis*. Dry biomass was higher in all culture media, but the maximum cell

density was obtained by inorganic fertilizer 10:10:10 with 4.6 x 10^5 cells mL^{-1}. Other culture media was similar.Chlorophyll *a* was significantly higher ($p<0.05$) in NPK 20:5:20 (443 - 2,269 $\mu g.L^{-1}$) and lower in NPK 4:14:8 (319 - 885 $\mu g.L^{-1}$)(Table 2).

High concentrations of protein was observed in *Haematococcus pluvialis* except for inorganic fertilizer 12:6:12 ($p<0.05$) and the opposite was observed at the lipid concentration, below 10% in all culture media ($p<0.05$) (Table 2).

Amino acids profilewere higher ($p<0.01$) in inorganic fertilizer medium NPK 20:5:20, except hydroxyproline; the opposite occurred for WC medium. Glutamic acid and leucine had higher concentrationin all culture media. Higher leucine and glutamic amino acids profile were reached in all culture media used, and cysteine amino acid did not exceed 1% of total amino acid production (Table 3).

Table 3. Mean and Standard deviation of amino acids in *Haematococcus pluvialis* (g 100g dry biomass) cultured in WC and inorganic fertilizer (NPK- 20:5:20, 12:6:12, 10:10:10, 4:14:8) media.

Amino acid	Comercial	NPK			
	WC	20:5:20	12:6:12	10:10:10	4:14:8
Alanine	1.32±0.0 ^{e}	3.73±0.02 ^{a}	2.16±0.0 ^{d}	2.68±0.02 ^{c}	3.27±0.01 ^{b}
Arginine	1.09±0.0 ^{e}	3.51±0.04 ^{a}	2.11±0.01 ^{d}	2.86±0.01 ^{c}	3. 06±0.07 ^{b}
Aspartic acid	1.25±0.02 ^{d}	3.78±0.03 ^{a}	0.97±0.02 ^{e}	1.69±0.06 ^{c}	3.36±0.02 ^{b}
Cysteine*	0.11±0.0 ^{e}	0.41±0.0 ^{a}	0.17±0.0 ^{d}	0.21±0.0 ^{c}	0.36±0.0 ^{b}
Glutamic acid	1.72±0.0 ^{e}	5.2±0.05 ^{a}	2.07±0.0 ^{d}	3.17±0.02 ^{c}	4.37±0.0 ^{b}
Glycine	0.95±0.04 ^{e}	2.65±0.08 ^{a}	1.38±0.0 ^{d}	1.83±0.01 ^{c}	2.31±0.02 ^{b}
Hidroxiproline	0.45±0.01 ^{cd}	0.62±0.0 ^{ab}	0.37±0.0 ^{d}	0.54±0.04 ^{bc}	0.66±0.02 ^{a}
Histidine*	0.24±0.0 ^{e}	0.76±0.01 ^{a}	0.43±0.01 ^{d}	0.60±0.01 ^{c}	0.66±0.0 ^{b}
Isoleucine*	0.61±0.01 ^{e}	1.86±0.01 ^{a}	0.95±0.02 ^{d}	1.39±0.0 ^{c}	1.66±0.01 ^{b}
Leucine*	1.49±0.02 ^{e}	4.47±0.02 ^{a}	2.41±0.04 ^{d}	3.35±0.06 ^{c}	3.90±0.06 ^{b}
Lysine*	0.93±0.02 ^{e}	2.94±0.04 ^{a}	1.39±0.01 ^{d}	2.05±0.04 ^{c}	2.54±0.02 ^{b}
Methionine*	0.23±0.0 ^{e}	0.91±0.01 ^{a}	0.46±0.0 ^{d}	0.58±0.0 ^{c}	0.74±0.0 ^{b}
Phenylalanine*	0.89±0.01 ^{e}	2.59±0.08 ^{a}	1.40±0.02 ^{d}	2.01±0.01 ^{c}	2.27±0.02 ^{b}
Proline	0.85±0.01 ^{e}	2.45±0.02 ^{a}	1.34±0.02 ^{d}	1.83±0.04 ^{c}	2.12±0.01 ^{b}
Serine	0.82±0.01 ^{e}	2.21±0.03 ^{a}	1.17±0.01 ^{d}	1.53±0.0 ^{c}	2.01±0.01 ^{b}
Threonine*	0.70±0.01 ^{e}	2.12±0.06 ^{a}	1.18±0.0 ^{d}	1.56±0.01 ^{c}	1.91±0.03 ^{b}
Tyrosine*	0.45±0.01 ^{e}	1.69±0.0 ^{a}	0.89±0.01 ^{d}	1.20±0.12 ^{c}	1.40±0.0 ^{b}
Valine*	0.97±0.01 ^{e}	2.95±0.03 ^{a}	1.51±0.01 ^{d}	2.14±0.13 ^{c}	2.64±0.0 ^{b}
Total	15.14±0.04 ^{e}	44.91±0.5 ^{a}	22.44±0.1 ^{d}	31.28±0.3 ^{c}	39.32±0.2 ^{b}

* Essential aminoacid
Values in the same row with different superscripts are significantly difference ($p<0.05$).

Production cost of 50-L of *H. pluvialis* was low in all media, but WC had a higher cost than the inorganic fertilizer medium. The cost of media used were: WC= US$5.31; NPK 20:5:20= US$2.24; NPK 12:6:12= US$1.91; NPK 10:10:10= US$2.14 and NPK 4:14:8= US$2.03.

Discussion

High cell density in the culture of *H. pluvialis* was due to nutrient availability, light, levels of dissolved oxygen and pH. In fact, in the case of *H. pluvialis*culture, low luminosity between 30 and 60 µmol m^{-2} s^{-1}, dissolved oxygen over 4 mg $L^{-1}$and below 10 mg L^{-1}; pH between 7 and8accelerate photosynthetic processes and high production of *H. pluvialis´*s vegetal cells (Sarada et al., 2002).

In the case of *H. pluvialis*culture, Dominguez-Bocanegra et al. (2004) tested BBM medium and obtained a maximum cell density 3.5 x 10^5 cells mL^{-1}. Göksan et al. (2011) obtained a maximum cell density 2.6 x 10^5 cells $mL^{-1}$with BG-11 medium plus different nitrogen sources. Current study showed that maximum cell density in WC medium was corroborated by the above authors (3.2 x 10^5 cells mL^{-1}), with higher densities in inorganic fertilizing medium NPK 10:10:10 (4.6 x 10^5 cells mL^{-1}).

Microalga *Haematococcus pluvialis* requires greater concentrations of nitrogen sources due to greater intake of ammonia, nitrate and nitrite and urea (Göksan et al., 2011). In current analysis, ammonia in inorganic fertilizer media was preferentially consumed by *H. pluvialis*instead of nitrate.In inorganic fertilization-based media, the depletion of nitrite levels by *H. pluvialis* is commonly low (Tocquin et al., 2012). Since microalga *H. pluvialis* grew better in culture media with low N:P, high phosphorus levels favored the accumulation of biomass (Tocquin et al., 2012). However, when compared to nitrogen, phosphorus concentration over 50% caused growth decrease (Ashraf et al., 2011). The pattern has been reported in current study with regard to the culture medium NPK 4:14:8. In fact, differences in N:P concentrations may reduce algal growth for its adaptation to stress conditions (Juneja et al., 2013).

Nitrogen limitation in culture medium is a stress factor for *H. pluvialis*, whilst high phosphorus concentration foregrounds algal growth in the vegetal state which generally lasts between 9 and 20 days according to ratio between biomass and cell activity (Sun et al., 2016). High nitrogen and phosphorus availability content may enhance the production of green cells, as demonstrated in current study in which growth lasted till the 28thday of culture.

During the vegetal stage and when cultivated under low luminosity and low nitrogen and phosphorus availability, *Haematococcus pluvialis* is rich in proteins and has low lipid concentrations(Gacheva et al., 2015). In current analysis, the effect of luminosity on lipid accumulation was more effective than nitrogen concentrations, since lipid concentrationin medium NPK 10:10:10 were similar to those in WC commercial medium, whereas the lowest concentration occurred in medium NPK 4:14:8.The cultivation stage can also be correlated, since growing green *H. pluvialis* results in low lipids concentrations (Santos and Lombardi, 2017).

High nitrogen availability contents affects greatest chlorophyll *a* production in algal cells since nitrogen occurs in great amounts in the molecule (Rangel-Yagui et al., 2004). Results in current study demonstrate that concentrations of chlorophyllain the medium NPK 20:5:20 with high nitrogen concentrationwere higher when compared with those in other media.

High amino acid are an additional factor in the protection against biotic stress due to delay of cell degradation (Waqas et al., 2015). High amino acid were reported when *H. pluvialis* was cultivated in high nitrogen availability (NPK 20:5:20), whereas the contrary occurred when nitrogen decreased in fertilizer-based media. The limitation of nitrogen reduces amino acid synthesis, such as proline, leucine, methionine and tyrosine (Chia et al., 2015). However, the synthesis of amino acids may be stimulated again by increasing P:N ratio. However, when phosphorus concentrations are high, concentrations of amino acids should not be necessary high when compared to N-rich culture medium.

High production of glutamic acid and leucine and low production of cysteine in amino acids is common in most microalgae (Brown, 1991). The above was also reported in current study with regard to glutamic acid and leucine with high rates when compared to other amino acids. Kim et al. (2015) analyzed amino acids of *Haematococcus* sp. in commercial medium BG-11 and reported contents of total amino acids between 10g and 100g dry biomass. Result was close to that registered in current study for commercial medium WC in which the lowest amino acid occurred. Availability and greater assimilation of ammonia in media with inorganic fertilizers directly affected concentrations of amino acids due to direct relationship with the nitrogen compound (Bromke, 2013). The inorganic fertilizer NPK for *H. pluvialis* culture is low cost, approximately 65% cheaper than the commercial medium WC, coupled to high concentrations of cysteine, arginine, methionine and proline, especially in the inorganic fertilizer medium NPK 20:5:20, when compared to commercial medium WC.

Conclusion

Haematococcus pluvialis, cultivated in inorganic fertilizer medium NPK 10:10:10, featured high cell density. Microalga may have preferences in nutrient intake when compared to culture medium. Further, medium composition may also affect directly the microalga´s biochemical composition. The profile of amino acids was greater when *H. pluvialis* was cultivated in inorganic fertilizer-based media. The employment of inorganic fertilizer is a tool that improves algal development when compared to commercial medium WC by decreasing the costs in microalga culture, increasing productivity of biomass and compound of high commercial value. Results demonstrate that the use of inorganic fertilizers NPK, especially at 20:5:20 and 10:10:10, provide high nutritional and cell density rates for *Haematococcus pluvialis*.

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Table 2. Mean and Standard deviation of variables in *Haematococcus pluvialis* cultured in WC and inorganic fertilizer (NPK- 20:5:20, 12:6:12, 10:10:10 and 4:14:8) media.

Parameters	WC	NPK			
		20:5:20	12:6:12	10:10:10	4:14:8
Maximum cell density (cell mL^{-1})	3.2×10^{5b}	3.3×10^{5b}	3.1×10^{5b}	4.6×10^{5a}	1.7×10^{5c}
Growth rate (k)	0.21	0.10	0.08	0.18	0.11
Doubling time (days)	4.83	9.79	12.48	5.51	9.19
Dry biomass(pg $cell^{-1}$)	$1,966±1,086^{b}$	$1,597±297^{b}$	$1,720±842^{b}$	$2,798±735^{a}$	$2,272±635^{a}$
Total length (µm)	$23.7±2^{a}$	$23.5±2^{a}$	$20.5±2^{a}$	$21.7±3^{a}$	$21.5±1^{a}$
Cell volume ($µm^{3}$)	$7,707±1,976^{a}$	$8,039±2,336^{a}$	$4,994±1,196^{c}$	$6,051±2,685^{b}$	$5,861±567^{b}$
Total Organic Carbon (pg $cell^{-1}$)	$1,479±400^{a}$	$1,557±481^{a}$	$936±236^{c}$	$1,148±541^{b}$	$1,108±108^{b}$
Protein (% dry biomass)	$36.7±16^{ab}$	$45.5±1^{a}$	$25.2±3^{b}$	$31.6±5^{ab}$	$41.3±1^{ab}$
Lipids (% dry biomass)	$6.3±3^{a}$	$3.5±1^{ab}$	$3.1±2^{ab}$	$6±2^{ab}$	$2.1±1^{ab}$
Chlorophyll *a*(mg L^{-1})	$728±548^{b}$	$1,061±739^{a}$	$619±338^{b}$	$741±545^{b}$	$475±275^{c}$
Dissolved oxygen (mg L^{-1})	$8.1 ± 1^{a}$	$8.7 ± 1^{a}$	$7.5 ± 1^{a}$	$7.8 ± 1^{a}$	$7.4 ± 1^{a}$
pH	$7.7 ± 1^{a}$	$6.6 ± 1^{b}$	$8.4 ± 1^{a}$	$6.7 ± 1^{a}$	$6.4 ± 1^{b}$
Conductivity (µS cm^{-1})	$616 ± 93^{c}$	$1,950 ± 325^{a}$	$1,009 ± 147^{b}$	$797 ± 159^{c}$	$943 ± 219^{b}$

Values in the same row with different superscripts are significantly difference ($p<0.05$).