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RESEARCH PAPERS

Effect of light/dark cycles on the growth of *Haematococcus pluvialis* in mixotrophic cultivation with alternative culture media

Lúcia Helena Sipaúba-Tavares[a*], Mayara Galatti Tedesque[a], Débora Cristina Fenerick[a], Rodrigo Ney Millan[b], Bruno Scardoeli-Truzzi[a]

[a]*Laboratory of Limnology and Plankton Production, São Paulo State University - UNESP, Aquaculture Center, Via de Acesso Prof. Paulo Donato Castellane s/n, Bairro Rural, 14884-900, Jaboticabal, SP, Brazil.*
[b]*Minas Gerais State University - UEMG, Department of Exact and Earth Science, Av. Escócia, 1000, Bairro Cidade das Águas, 38202-436, Frutal, MG, Brazil.*

Highlights

- Light/dark cycles variations were established for *Haematococcus pluvialis* growth
- NPK (10:10:10), WC and macrophyte extract (ME) were used as culture media to *H. pluvialis* growth
- Mixotrophic cultivation was evaluated to verify its influence on *H. pluvialis* growth rate
- Light/dark cycles increase cell density and compound synthesis, with economic benefits by saving light energy
- The culture media and mixotrophic cultivation play an important role on the production cost

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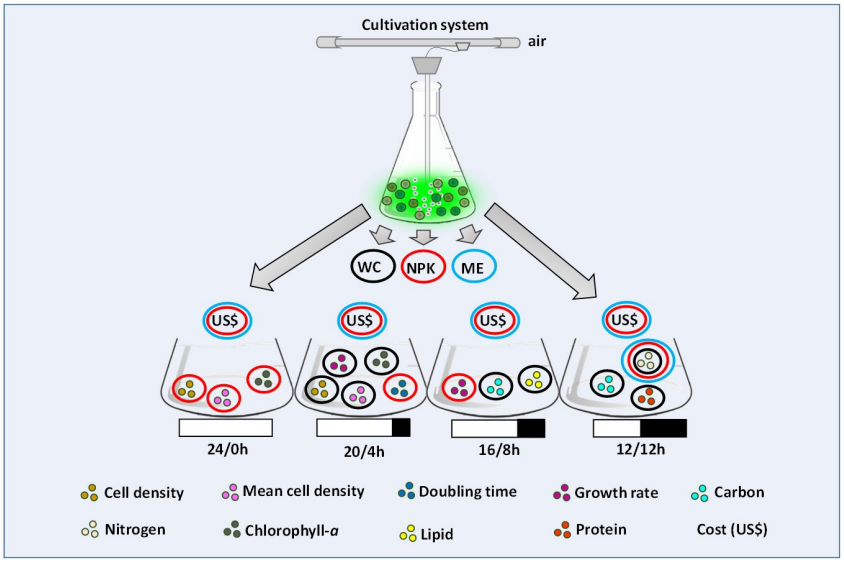
KEYWORDS
NPK (10:10:10); Macrophyte (*Eichhornia crassipes*); Biological parameters; Sugarcane molasses; Economic aspects.

Abstract: Current assay investigates the influence of light/dark cycles on the growth and chemical composition of *Haematoccocus pluvialis* in mixotrophic cultivation and different culture media: WC, NPK and ME (macrophyte extract). Four light cycles were used: 24/0 h; 20/4 h; 16/8 h and 12/12 h light/dark cycles. Highest cell density was reported at 24/0 h and 20/4 h light/dark cycle in NPK and WC culture media, reaching 3.8 x 10^5 cell mL^{-1}. Doubling time was faster in NPK culture medium with four days at 20/4 h light/dark cycle. Protein, lipid, carbon and nitrogen in the *H. pluvialis* biomass was higher in the absence of light in all culture media, mainly in WC culture medium at 12/12 h and 16/8 h light/dark cycles. Light/dark cycles may increase cell density and compounds synthesis, with economic benefits by saving electric power. Depending on the light cycle used the cost of the kw $year^{-1}$ ranged between US$ 760 (12/12 h) and US$ 1,519 (24/0 h). NPK culture media using sugar cane molasses as carbon source with dark period is a tool to be used in *H. pluvialis* cultivation with cheaper cost and high nutritional values. Although WC culture medium had good results for *H. pluvialis* growth, however it results in an expensive cultivation.

*Corresponding author.
E-mail: lucia.sipauba@unesp.br (L. H. Sipaúba-Tavares).

GRAPHICAL ABSTRACT



Introduction

Several factors such as culture medium, light availability and environmental conditions promote influence over microalgae growth, but light's role of is paramount. Light affects the microalga development and periods of darkness between light cycles may be adjusted to optimize the overall growth. Light supply is critical for photosynthetic growth and is, characterized by heterogeneous distribution of available light over the culture volume (Takache et al., 2015). The growth of different microalgae species has been enhanced by regulation of light irradiation and temperature, although determination of optimal light and temperature conditions is an effective control strategy for microalgae cultivation (Ma et al., 2017). The variation of light/dark cycles has been suggested as beneficial for photosynthetic efficiency but depends on cultivation purpose, microalgae species and the cultivation system type. The benefits promoted by light/dark cycles are microalgae faster growth and higher biomass concentration. This is due to the photoperiod being among the factors that influences microalgae's growth rate (Qin et al., 2018). Cell cycle progression is regulated by a circadian clock so that cell size increases during the light cycle and cell division occurs under the dark cycle affecting cellular composition. In addition, many central metabolites fluctuate throughout light/dark cycles (Kato et al., 2019). The quality, intensity and light period all have significant effects on the amount of energy received by microalgae, depending on species (Ma et al., 2017). *Haematococcus pluvialis* has a significant commercial value and has proven to be a good source of protein, pigmentation and biodiesel production. Low light intensity promotes *H. pluvialis* growth until the optimal cell density is reached, and high radiance is used as a stress condition to induce the accumulation of secondary carotenoids (Wong et al., 2016). For *H. pluvialis*, the best condition for cell growth, lipid and astaxanthin production occurred with 24/0 h light/dark cycle (Wong et al., 2016). The culture model of two stages has been successfully achieved in *H. pluvialis*. The first stage is called green stage, which has high cell yield and also play an important role in its further biosynthesis. The second stage can rapidly accumulate astaxanthin and is called red stage (Grujić et al., 2022).

Another tool to improve microalgae cultivation is the choice of condition (phototrophic, heterotrophic and mixotrophic). It will influence growth rate, biochemical composition and photosynthetic efficiency, albeit depending on the microalgae species. Mixotrophic cultivation, a variation of heterotrophic cultivation, involves assimilating both CO_2 and organic carbon simultaneously (Cecchin et al., 2018). Sugarcane molasses a low-cost by product, is an excellent source of carbon particularly in countries that produce sugarcane. Mixotrophic cultivation is promising approach to enhance biomass and obtain higher total lipid content. This method combines both phototrophic and heterotrophic modes of cultivation (Mondal et al., 2017). In mixotrophic culture, carbon sources such as glucose and fructose can be added as supplements and light/dark cycles can be used to alternate light availability. Under these conditions, algae cells can absorb organic and inorganic carbon for photosynthesis and respiration, respectively, mediated by carbon capture and ATP generation (Subramanian et al., 2016). Sugarcane molasses can be used in several ways, but hydrolysis of molasses increases the concentration of reducing sugar concentrations by converting sucrose into glucose and fructose (Yan et al., 2011).

An important aspect of technological development and improvement of microalgae growth is predicting of the process performance and optimizing culture-operating conditions (Gaurav et al., 2016). However, culture media can be highly specialized and sometimes difficult to obtain on local markets. To reduced production costs and increase algal biomass, alternative culture media for microalgae such as inorganic fertilizers and aquatic plants, are being used (Sipaúba-Tavares et al., 1999, 2009; Scardoeli-Truzzi & Sipaúba-Tavares, 2017). Inorganic fertilizer has a well-defined composition with high nitrogen and phosphorous contents, whilst macrophytes play dominant and supporting roles in

nitrogen and phosphorous recovery (Sipaúba-Tavares et al., 2018). The constitution of the culture medium influences growth and cell composition and is responsible for the high biomass and synthesis of other products.

Alternative strategies such as varying light cycles, carbon source and culture media can improve the biotechnological application of microalgae by increase biomass production in batch culture mode. In this study, *Haematococcus pluvialis* growth was investigated under four different light/dark cycles in mixotrophic cultivation using alternative culture media (NPK and ME) and a commercial culture media (WC).

Materials and methods

Culture media and growth conditions

Microalga strain *H. pluvialis* (CMEA 227 C1) was cultured in the laboratory in triplicate using three different culture media: WC (Guillard & Lorenzen, 1972), inorganic fertilizer, NPK (10:10:10) (Scardoeli-Truzzi & Sipaúba-Tavares, 2017) and macrophyte extract, ME (*Eichhornia crassipes*) (Sipaúba-Tavares et al., 2009) (Table 1). To produce the inorganic fertilizer medium (NPK 10:10:10), approximately 50 g L^{-1} of inorganic fertilizer was dissolved and added to 1 L of distilled water. Each flask contained 40 mL of the medium. Macrophyte extract (ME) culture medium was retrieved from the aquatic plant *Eichhornia crassipes*, as follows: approximately 5 kg (wet weight) of plant biomass was gently washed in tap water to remove detritus and epiphytes.

After sun drying the macrophytes were transferred to an oven at 60°C for 24 h. The plant material was homogenized in a grinder and boiled in distilled water for 1 h. The macrophyte extract (ME) was filtered and autoclaved at 120°C for 20 min. A 70 mL samples was collected, and diluted with distilled water up to 1.4 L, at which point 2.5 mL NPK was added. Vitamin B complex was added to NPK and ME culture media at rate of 0.02 g L^{-1} per culture flask (Sipaúba-Tavares et al., 1999, 2009). Molasses hydrolysis was prepared by adding 0.4% (w/w) hydrochloric acid (4 M HCL) with 20 min of incubation time at 80°C to convert sucrose into glucose and fructose. It was filtered to remove undissolved impurities, diluted in distilled water, and autoclaved at 1 atm for 30 min. Furthermore, 0.75 g L^{-1} of sugarcane molasses was added to the culture media as carbon source for mixotrophic cultivation (Scardoeli-Truzzi & Sipaúba-Tavares, 2017). The microalga was batch-cultured at 22 ± 2°C, with dissolved oxygen around 7.2±0.1 mg L^{-1} in continuous air bubbling and light intensity of 30 µmol photons m^{-2} s^{-1} provided by LED lamps (high CRI LED 6500K) on top of each culture in continuous lighting. The experiment started with 10 mL culture volume which was later cultured in 250 mL culture volume at a microalgae density of 0.2 x 10^5 cells mL^{-1} in WC culture medium. When cultures reached exponential growth phase (7^{th} growth day) approximately 10 mL with a density 1 x 10^5 cells mL^{-1} were added to 2 L WC culture medium. After the 7^{th} exponential growth day, the culture at a density of 1.3 x 10^5 cells mL^{-1} was transferred to experimental (2 L) sterilized recipients containing the culture media (WC, NPK and ME) (Figure 1). All culture media were autoclaved prior to use and transferred to flasks under aseptic conditions. This work was carried out with cells cultured in green stage.

Table 1. Nutrients composition of different culture media: NPK, WC and ME (macrophyte extract) (Sipaúba-Tavares et al., 2009; Scardoeli-Truzzi & Sipaúba-Tavares, 2017).

Constituints	NPK	WC	ME
	(g L^{-1})	(g L^{-1})	(g L^{-1})
P_2O_5	2.5	-	-
K_2O	10	-	-
N	10	-	0.095
P	-	-	0.091
C	-	-	0.199
Mn	-	-	0.06
Mg	-	-	0.003
Fe	-	-	2.13
K	-	-	0.056
Ca	-	-	0.001
Cu	-	-	0.05 mg
NaEDTA	-	8.7	-
KH_2PO_4	-	67.8	-
Trisaminomethane	-	200	-
Thiamine (B_1)	0.007	1.7	0.007
Vit B_2	0.007	-	0.007
Vit B_6	0.005	0.085	0.005
Vit B_{12}	33 µg	-	33 µg
Vit H	0.01 mg	-	0.01 mg
$NaNO_3$	-	470	-
$FeCl_3.6H_2O$	-	18.3	-
$CaCl_2.6H_2O$	-		-
$MnCl_2.4H_2O$	-	36	-
$CuSO_4.5H_2O$	-	2	-
$ZnCl_2$	-	2	-
$Na_2MoO_4.2H_20$	-	1.2	-
H_3BO_3	-	3.5	-
$NaHCO_3$	-	6.3	-
$MgSO_4.7H_2O$	-	18.5	-
$CuCl_2.2H_2O$	-	18.4	-

Light cycle variation

Illumination was provided by LED lamps (high CRI LED 6500K) programmed with an automatic on/off system to simulate light period. The light cycles were: 24/0 h; 20/4 h; 16/8 h and 12/12 h light/dark cycles. During the lighting period the light intensity was maintained at 30 µmol

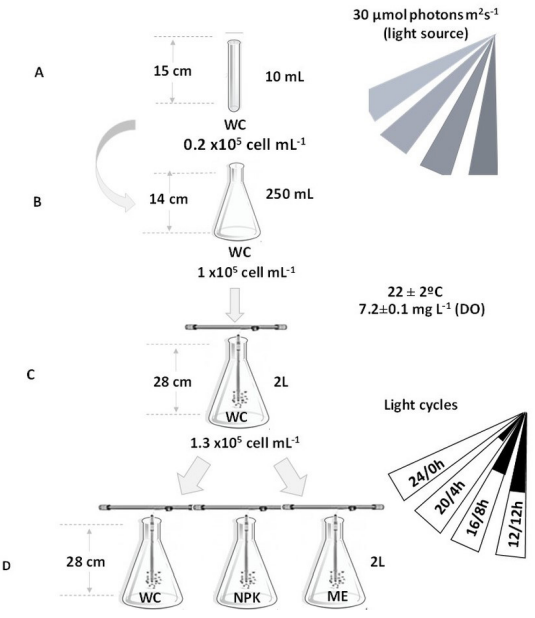


Figure 1. Diagram of *Haematococcus pluvialis* microalgae cultured in mixotrophic cultivation, where: A = maintenance of strain 10 mL; B = initial culture in 250 mL with WC culture medium; C = culture in 2L with WC culture medium; D = experiment treatments in 2 L using three culture media WC, NPK and ME (macrophyte extract) during four different light/dark cycles (24/0 h, 20/4 h, 16/8 h and 12/12 h).

photons m^{-2} s^{-1} and continuous aeration was provided by bubbling air from compressor (Figure 1).

Microalga growth

Cell density was monitored for consecutive 14 days and 1 mL aliquots were removed daily from the microalga culture in triplicate. A minimum of 2 x 1 μL^{-1} sub-sample was used for cell quantification, with a Neubauer counting chamber. Growth rate and doubling time were obtained according to Guillard (1973), where growth rate (k, divisions per day) was calculated as follows:

$$k = \frac{3.322}{t_2 - t_1} \times \log \frac{N_2}{N_1} \quad (1)$$

where, N_2 and N_1 are the number of cells on days t_2 and t_1, respectively. Doubling time (cell division time or generation time) was calculated from results obtained from growth rate by the formula:

$$Dt = 1k^{-1} \quad (2)$$

Culture media parameters and microalga biochemical composition

Analyses were performed weekly. Chlorophyll-*a* concentration was determined using a spectrophotometer, which extracted pigments with 90% alcohol (663 and 750 nm), following methodology by Nusch (1980). Conductivity, dissolved oxygen and pH were measured using a YSI 556 MPS multi-sensor probe. Total phosphorous and total inorganic nitrogen were quantified by spectrophotometer, following Golterman et al. (1978) and Koroleff (1976). At the end of each experiment, microalgae samples were collected for analysis of lipid, protein, nitrogen and carbon (% biomass dry weight). Total lipid content was extracted using petroleum ether and quantified by gravimeter (Association of Official Analytical Chemists, 2012). Microalgae´s protein, nitrogen and carbon contents, supplied by Leco (CN628), were measured following Dumas combustion method.

Economic considerations

The operational production costs were determined through an economic analysis of the components of the culture media, energy cost, water, required equipment and labor. The costs were analyzed for a volume of 2 L taking into consideration a production unit with a capacity of 100,000 L capacity and four light/dark cycles. The operational production cost was determined following the methodology of Matsunaga et al. (1976). Vitamin B, light, NPK and labor were considered upkeep of the fixed capital. The labor cost was calculated based on the employee's wage plus 74% of the social charge and benefits.

Statistical analysis

Two-way analysis of variance (ANOVA) was performed in the software Stat Soft STATISTICA 10 to test the effects between microalgae data and culture medium parameters. Tukey's test was applied when differences ($p < 0.05$) between treatments occurred. All experiments were carried out in triplicate.

Results and discussion

Effect of light cycles variations on the growth and chemical composition of *Haematococcus pluvialis*

The WC and NPK had best results for *Haematococcus pluvialis* growth than ME culture medium. The lighting periods had an influence on the growth parameters and biochemical composition of this microalgae. The highest cell density, 3.8 x 10^5 cells mL^{-1}, was observed in WC culture medium under 20/4 h light/dark cycle. Although in the NPK culture medium under 24/0 h light/dark cycle the cell density was also high with 2.9 x 10^5 cells mL^{-1}. Under 16/8 h light/dark cycle the cell density was similar to that a 24/0 h light/dark cycle, particularly from 10^{th} to 14^{th} growth day where it reached 3.1 and 3.0 x 10^5 cells mL^{-1} in WC and 2.8 and 3.0 x 10^5 cells mL^{-1} in NPK culture medium, respectively. For all culture media, the lowest cell density was reported when cultures exposed to 12/12 h light/dark cycle (Figure 2). The same trend was observed for the mean cell density (MCD) with the highest densities observed in WC and NPK culture media under 20/4 h and 24/0 h light/dark cycles, respectively. However, MCD remained high ranging from 2.1 x 10^5 cells mL^{-1} to 2.4 x 10^5 cells mL^{-1}, in both culture media under 24/0 h,

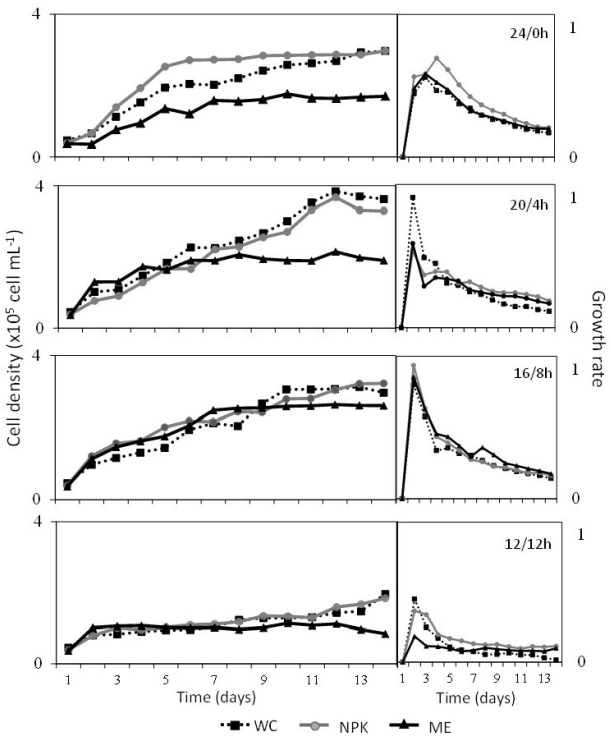


Figure 2. Cell density and growth rate (K) of *Haematococcus pluvialis* in mixotrophic cultivation at different light/dark cycles (24/0 h, 20/4 h, 16/8 h and 12/12 h) and culture media (WC, NPK and ME).

20/4 h, and 16/8 h light/dark cycles. Under 12/12 h light/dark cycle MCD was lower at 1.5 x 10^5 cells mL^{-1} and 1.2 x 10^5 cells mL^{-1} in WC and NPK culture media, respectively. In ME culture medium, the MDC under 24/0 h, 20/4 h, and 12/12 h light/dark cycles was lower compared to the other culture media. However, under 16/8 h light/dark cycle the MCD of *H. pluvialis* was similar to those obtained in WC and NPK culture media with 2.1 x 10^5 cells mL^{-1} (Figure 2; Table 2).

The growth rate was influenced by light/dark cycle, with high growth rate observed at 20/4 h (WC) and 16/8 h (NPK) light/dark cycles on the 2nd growth day with k=0.91 and 0.94, respectively. The lowest growth rate was observed in the ME culture medium under 12/12 h light/dark cycle, with a value below k=0.2 (Figure 2). The growth performance of *H. pluvialis* it was consistently worse in ME culture medium for all lighting cycles, particularly from the 7th growth day, except under 24/0 h light/dark cycle where the cell density in ME culture medium was always the lowest since the 2nd growth day. Consequently, the doubling time (DT) was slower in ME culture medium for all light/dark cycles with the longer time (10.5±0.2 days) under 12/12 h light/dark cycle, and the faster in NPK culture medium with 4±0.3 days under 20/4 h light/dark cycle (Table 2).

The light requirement for each species can vary and some do not tolerate long dark periods, with the consequent reduction of photosynthetic efficiency and cell density (Vejrazka et al., 2013). According to Wong et al. (2016) *H. pluvialis* had the best condition for cell density under 24/0 h light/dark cycle.

Table 2. Mean and standard deviation of variables (microalga and culture parameters) of *Haematococcus pluvialis* in mixotrophic cultivation at different light/dark cycles (24/0 h, 20/4 h, 16/8 h and 12/12 h) and culture media (WC, NPK and ME).

Light/dark cycles	Microalga		Culture Media				
	DT	MCD	Chlor-*a*	pH	Cond	TIN	TP
WC							
24/0 h	4.8±0.4^{Bb}	2.1±0.8^{Ab}	1±0.05^{Bb}	8.3±0.6^{Ab}	837±18^{Ba}	0.7±0.02^{Bb}	1.2±0.07^{Ba}
20/4 h	4.3±0.2^{Bc}	2.4±1.0^{Aa}	1.7±0.04^{Aa}	8.1±0.02^{Cc}	803±72^{Bc}	0.8±0.05^{Cb}	1.2±0.1^{Ba}
16/8 h	4.9±0.2^{Bb}	2.1±0.9^{Ab}	1.1±0.03^{Aab}	9.9±1.3^{Aa}	846±13^{Bab}	1.1±0.01^{Ba}	0.8±0.1^{Cd}
12/12 h	9.3±0.4^{Ba}	1.5±0.3^{Ac}	0.9±0.05^{Ab}	8.4±1.1^{Ab}	842±53^{Cd}	0.8±0.05^{Cb}	0.8±0.01^{Cd}
NPK							
24/0 h	4.4±0.2^{Cc}	2.4±0.8^{Aa}	1.5±0.1^{Aa}	8.1±0.6^{Ab}	953±209^{Ac}	1.5±0.03^{Aab}	3.3±0.07^{Ab}
20/4 h	4.0±0.3^{Cd}	2.1±1.0^{Ab}	0.9±0.07^{Bb}	8.8±0.04^{Aa}	975±93^{Ab}	1.4±0.05^{Abc}	3.6±0.1^{Ab}
16/8 h	5.2±0.3^{Ab}	2.2±0.8^{Ab}	1±0.02^{Bbc}	7.6±1.0^{Cc}	1,050±109^{Aa}	1.3±0.04^{Ac}	3.9±0.02^{Aa}
12/12 h	8.7±0.3^{Cd}	1.2±0.3^{Bc}	0.6±0.01^{Bc}	7.5±0.3^{Bc}	936±43^{Ac}	1.7±0.1^{Aa}	3.4±0.1^{Ab}
ME							
24/0 h	5.8±0.3^{Ac}	1.3±0.5^{Bc}	0.6±0.07^{Cb}	8.4±0.04^{Ab}	734±31^{Cb}	0.3±0.01^{Cc}	0.6±0.01^{Cd}
20/4 h	6.3±0.4^{Ab}	1.7±0.4^{Bb}	0.8±0.07^{Ba}	8.2±0.1^{Ba}	451±113^{Cd}	1.2±0.1^{Bb}	1.4±0.04^{Bc}
16/8 h	5.3±0.3^{Ad}	2.1±0.7^{Aa}	0.7±0.02^{Ba}	8.4±0.6^{Bc}	545±38^{Cc}	1±0.07^{Bb}	1.8±0.06^{Bb}
12/12 h	10.5±0.2^{Aa}	1.0±0.1^{Bc}	0.4±0.03^{Cc}	8.4±1.1^{Ab}	857±36^{Ba}	1.4±0.1^{Ba}	2.5±0.1^{Ba}

DT = doubling time (days); MCD = mean cell density (x10^5 cell mL^{-1}); Chloro-*a* = chlorophyll-*a* (mg L^{-1}); Cond = conductivity (µS cm^{-1}); TIN = total inorganic nitrogen (mg L^{-1}); TP = total phosphorus (mg L^{-1}). Difference uppercase letters above row indicate differences between culture medium within a determined light cycle, while lowercase letters indicate differences between light/dark cycles within each culture medium.

Current results revealed that mean cell density was higher under 24/0 h (NPK) and 20/4 h (WC) light/dark cycles, however high growth rate was found under 16/8 h light/dark cycle (NPK). Krzemińska et al. (2014) reported that species *Neochloris* presented the highest biomass production under 12/12 h light/dark cycle. The opposite was reported for *H. pluvialis* in mixotrophic cultivation under 12/12 h light/dark cycle with lower mean cell density and longer doubling time, consequently low growth rate. Jacob-Lopes et al. (2009) cultured *Aphonothece microscopica* and reported that the supply of light for periods longer than 22 h did not influence the growth rate.

Most microalgae require light for glucose uptake and fail to grow in total darkness or during long periods of dark (Subramanian et al., 2016). Sforza et al. (2012) verified that altering dark and light may promote growth inhibition with cell performance worse than those exposed to continuous light. Or rather, alternating light and dark may also reduce growth efficiency. However, *Tetradesmus obliquus* had faster growth rate under a 16 h:8 h light/dark cycle (0.09 h^{-1}) (León-Saiki et al., 2018). Similar was observed with *H. pluvialis* that was highest (k=0.94) in the same cycle.

According to Tran et al. (2019) in addition to the light cycle and nutrients present in the culture medium, the *H. pluvialis* growth is influenced by the interaction of some factors such as pH and temperature. For this microalgae species the optimum range of pH is from 6 to 8 and temperature between 20°C to 25°C at green stage.

The highest pH was reported in WC culture medium under 16/8 h light/dark cycle, reaching 9.9±1.3 (Table 2). In general, for microalgae high pH above 9 in culture medium is due to the rapid carbon consumption that is available and depends on the growth (Han et al., 2020). Besides daily cycles, pH usually increases at a higher microalgal cell density (Monica-Devi et al., 2020). In the current study, *H. pluvialis* cell density was lower under 12/12 h light/dark cycle and pH was above 8.2±0.1. In general, pH was alkaline above 7.5±0.3 (12/12 h, NPK) (Table 2). High carbon content in *H. pluvialis* was 52.4% and 52.2% of the biomass dry weigh under 12/12 h and 16/8 h light/dark cycles in WC culture medium when the pH was 8.4 and 9, respectively (Table 2, Figure 2). In microalgae culture decrease in the medium´s pH may inhibit the activity of carbonic anhydrase, triggering a decrease in growth rate and cell density (Tang et al., 2011). In the current study, low cell density and growth rate were also reported under 12/12 h light/dark cycle, but pH remained alkaline throughout the experiment period in all culture media used. According to Jacob-Lopes et al. (2009), the duration of the light/dark cycles is a criterion that has a direct relationship with carbon fixation capacity and consequently determines the productivity for biomass and cell growth rate.

High cell densities increased algae´s photosynthesis activity, with an increase in oxygen levels within the culture system (Zhao et al., 2011). Oxygen concentration was above 7 mg L^{-1} promoting adequate cell density and specific growth rate in WC and NPK culture media under 24/0 h, 20/4 h and 16/8 h light/dark cycles. The oxygen required for aerobic heterotrophic growth is supplied by photosynthesis and the CO_2 needed to carry out photosynthesis was provided by heterotrophic metabolism. The internal CO_2 recirculation converted 94% of substrate into biomass, making the process close to carbon neutrality (Abiusi et al., 2020).

Low cell density is a characteristic of *H. pluvialis* growth, and this directly reflected in chlorophyll-*a* content. Under 12/12 h light/dark cycle where occurred low cell density, chlorophyll-*a* ranged between 0.4±0.03 mgL^{-1} (ME) to 0.9±0.07 mgL^{-1} (WC). However, in the other culture media chlorophyll-*a* reached the highest content with 1.7±0.04 mgL^{-1} (20/4 h) in WC culture medium and 1.5±0.1 mgL^{-1} (24/0 h) in NPK culture medium. In ME chlorophyll-*a* was low than the other culture media and the highest content was under 20/4 h light/dark cycle with 0.8±0.07 mgL^{-1} (Table 2). Chlorophyll-*a* is a primary light harvesting pigments, transfers excitation energy directly to the photosynthesis, and a green stage is indicated with the highest chlorophyll-*a* content (Grujić et al., 2022). In the current study chlorophyll-*a* was above 9 mgL^{-1} where dark period was absent (24/0 h), the opposite occurred with long dark period (12/12 h). Chlorophyll content is very important to *H. pluvialis* due to a key indicator to evaluated astaxanthin (Fang et al., 2019).

Microalgae growth adds several minerals such as iron, nitrogen and phosphorous, which significantly involve in enhancing the conductivity (Patel et al., 2017). This confirms the high conductivity in NPK culture medium reaching 1,050±109 µS cm^{-1} (16/8 h), whilst the lowest conductivity in ME culture medium was between 451±113 µS cm^{-1} (20/4 h) and 857±36 µS cm^{-1} (12/12 h). High total nitrogen inorganic (TIN) and total phosphorus (TP) concentrations also was observed in NPK culture medium where, TP concentrations ranged between 3.3±0.07 mg L^{-1} (24/0 h) to 3.9±0.02 mg L^{-1} (16/8 h). Highest TP concentrations were reported in NPK culture medium above 3.3±0.07 mg L^{-1} (24 /0 h). For TIN concentrations ranged from 1.3±0.04 mg L^{-1} (16/8 h) to 1.7±0.01 mg L^{-1} (12/12 h) (Table 2). In generally, TIN concentrations were lower than TP in all culture media. Microalgae utilize N and P in various biochemical reactions to produce several primary and secondary metabolites that alternatively determine the overall biochemical composition of biomass (Patel et al., 2017).

Sforza et al. (2012) observed that large lipid production was found in a culture medium where nitrogen was provided in excess and confirmed that light stress alone did not induce lipid biosynthesis, but its influence is integrated with other metabolic signals. In the current experiment lipid content was higher under 16/8 h light/dark cycles with 5.75%(WC), 4.8% (NPK), and 4.95 (ME) biomass dry weight (Figure 3). *Chlorella vulgaris* achieved the highest lipid content under 12 /12 h and 18 /6 h light/dark cycles, *Nanochloropsis* sp. under 18/6 h light/dark cycle and *N. gaditana* under 16/8 h light/dark cycle. However, the precise metabolic mechanisms that influence light cycles on lipid accumulation and biomass production are still not well understood (Kato et al., 2019). Nitrogen concentrations were higher in WC culture medium ranging between 7.1% (24/0 h) to 9.5% (12/12 h) biomass dry weight in WC culture medium. The other culture media the highest nitrogen concentration was 8.8% and 8.6% biomass dry weight in NPK and ME culture media, respectively (Figure 3).

Nutrients availability influences the biochemical composition of microalgae biomass that alternatively provokes the biosynthesis of some primary and secondary metabolites (Patel et al., 2017). Protein content also higher under 12/12 h

light/dark cycle with 59.5% biomass dry weight (WC), 54.9% biomass dry weight (NPK) and 54% biomass dry weight (ME), and lowest content under 24/0 h light/dark cycle with 44% biomass dry weight (WC), 45.7% biomass dry weight (NPK) and 35.4% biomass dry weight (ME) during the experimental period (Figure 3). Colusse et al. (2019) reported maximum protein of 41% biomass DW under 12/12 h light/dark cycle occurred in *H. pluvialis*. Except under 24/0 h light/dark cycle the protein content of *H. pluvialis* was above 48% biomass dry weight (Figure 3).

Economic considerations

The production cost with culture media were the highest ranging between 10% (ME) and 60% (WC) of total cost including ingredients only. Production cost of labor was the second most expensive item, corresponding to 44% of the production cost. Electricity represented 30% of total cost including, light, air conditioner, air-pump, water pump, centrifuge and aseptic chamber. The cost of energy kw year^{-1} ranged between US$ 760 (12/12 h light/dark cycle) and US$ 1,519 (24/0 h light/dark cycle) (Table 3). Water cost for microalgae production was US$ 1,657. Total operational cost *H. pluvialis* biomass in laboratory ranging between US$ 11,726 (12/12 h, ME) to US$ 30,609 (24/0h, WC) (Table 3). The production cost per treatment of *H. pluvialis* showed that alternative culture media (NPK and ME) were cheaper than commercial medium (WC), or rather, it was more than double the cost in all light/dark cycle treatments. Macro and micronutrients from each culture medium play an important role on the production cost, mainly regard to WC that has major cost participation representing 93.9% of total culture media cost. According Colusse et al. (2019), the cost of each component is highly related with the regional market. The cultivation conditions used for *H. pluvilis* using the three cultures media (WC, NPK and ME) and the four lighting cycles (24/0 h, 20/4 h, 16/8 h, and 12/12 h light/dark cycles) presented different operating costs, with the commercial WC being the most expensive. However, despite the ME having a cheaper operating cost, the use of NPK is the most appropriate in relation to growth rate, protein and lipid using a lighting period of 20/4 h and 12/12 h.

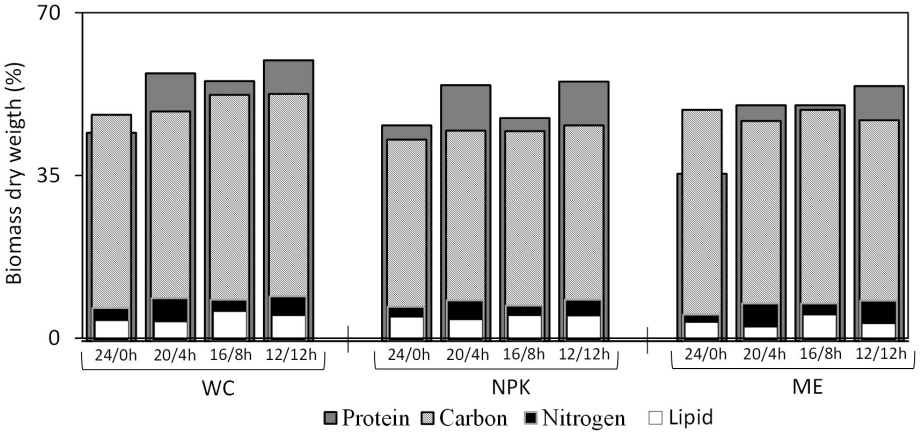


Figure 3. Chemical composition of *Haematococcus pluvialis* in mixotrophic cultivation at different light/dark cycles (24/0 h, 20/4 h, 16/8 h, 12/12 h) and culture media (WC, NPK and ME).

Table 3. Estimation of production costs (in US$ dollar)* producing 100,000L year^{-1}, of *Haematococcus pluvialis* in mixotrophic cultivation at different light/dark cycles (24/0 h, 20/4 h, 16/8 h and 12/12 h) and in three culture media (WC, NPK and ME).

Production input	Assumptions	Cost (US$)
(1) Labor	Cost of labor (2 to 4 hours)	
a) Preparing of nutrient medium, harvesting, cleaning of algae tanks, simple technical maintenance, 1 hour day^{-1} (365)	-	-
b) Laboratory work ½ hour day^{-1}, counting and checking cells under microscope (182.5)	-	-
c) Basic technical and replacing parts fixing and replacing parts ½ hour day^{-1} (182.5)	-	-
Total labor 730 hours year^{-1}		2,258

*Average exchange rate (November 2021) US$ 1.00 = R$ 5,49. - = not available.

Table 3. Continued...

Production input	Assumptions	Cost (US$)
2) Energy in Kw hour year^{-1}	Cost of electricity US$ 0.17 kW/h	
Air pump	0.18 kW x 24 hours day^{-1} x 365 days year^{-1}	268
Water pump	0.70 kW x 24 hours day^{-1} x 365 days year^{-1}	1,042
Air-conditioner	3.52 kW x 24 hours day^{-1} x 365 days year^{-1}	5,241
Centrifuge algae	0.70 kw x 1 hour day^{-1} x 365 days year^{-1}	44
Aseptic chamber	0.3 kw x 1 hour day^{-1} x 365 days year^{-1}	19
Culture room lamps (light/dark cycle 24/0 h)	1.02 kw x 24 hours day^{-1} x 365 days year^{-1}	1,519
Culture room lamps (light/dark cycle 20/4 h)	1.02 kw x 20 hours day^{-1} x 365 days year^{-1}	1,266
Culture room lamps (light/dark cycle 16/8 h)	1.02 kw x 16 hours day^{-1} x 365 days year^{-1}	1,012
Culture room lamps (light/dark cycle 12/12 h)	1.02 kw x 12 hours day^{-1} x 365 days year^{-1}	760
(3) Culture medium	-	
WC		18,561
NPK		769
ME		437
(4) Water (730 m^3 year^{-1})	Cost of water US$ 2,27 m^3	1,657
(5) Total operational cost		
WC	light/dark cycle 24/0 h	30,609
	light/dark cycle 20/4 h	30,356
	light/dark cycle 16/8 h	30,102
	light/dark cycle 12/12 h	29,850
NPK	light/dark cycle 24/0 h	12,817
	light/dark cycle 20/4 h	12,564
	light/dark cycle 16/8 h	12,310
	light/dark cycle 12/12 h	12,058
ME	light/dark cycle 24/0 h	12,485
	light/dark cycle 20/4 h	12,232
	light/dark cycle 16/8 h	11,978
	light/dark cycle 12/12 h	11,726

*Average exchange rate (November 2021) US$ 1.00 = R$ 5,49. - = not available.

Conclusion

Culture medium had a direct impact on the production cost of *H. pluvialis* in mixotrophic cultivation. WC and NPK were better than ME culture medium regard to biological parameters and biochemical components. Dark periods were also a significant factor in the culture of the microalga regard to biochemical compounds and to growth. However, longer dark periods (12/12 h light/dark cycle) negatively affected growth rate. On the other hand, protein, nitrogen, carbon and lipid were high in long dark period (16/8 h, and 12/12 h light/dark cycles). Throughout the growth period of *H. pluvialis* cell density varied according to culture medium: ME culture medium had the lowest cell density than NPK and WC culture media. Alternative medium NPK may be suitable for *H. pluvialis* cultivation in different lighting cycles, particularly under 20/4 h light/dark cycle. However, cell density, protein, lipid, carbon and nitrogen were highest in the commercial medium (WC). Production costs reduction with high biomass and nutritional rates are alternatives which may be achieved at laboratory scale in green stage of *H. pluvialis* growth. WC and NPK culture media showed the best results for the *H. pluvialis* growth in mixotrophic cultivation, however, if economic aspects are considered NPK is a good option for to use in *H. pluvialis* cultivation.

Conflict of interests

The authors declare that there is no conflict of interest.

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