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ORIGINAL ARTICLE

Haematococcus pluvialis cultivation and astaxanthin production using different nitrogen sources with pulse feeding strategy

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Abstract

The microalga *Haematococcus pluvialis* has astaxanthin as the most economically valuable compound. However, there are challenges related to its cultivation and low biomass productivity. Therefore, the present study proposes a change in the nitrogen supply mode, through the pulse feeding strategy, and the use of different N sources to increase the biomass of *H. pluvialis* and astaxanthin production. The two-factor experimental design had factor 1 (three sources of nitrogen—$NaNO_3$, NH_4NO_3, and $(NH_2)_2CO$) and factor 2 (nitrogen feeding strategy—pulse feeding (PF) and without pulse feeding (WPF)). Nitrogen source of the BBM ($NaNO_3$) was replaced by NH_4NO_3 or $(NH_2)_2CO$, maintaining the original [N] in WPF, while in PF, N sources were added by pulses. The results of growth variables showed a significant difference for maximum cell density, with higher values for $NaNO_3$-PF (176×10^4 cell mL^{-1}) and $(NH_2)_2CO$-PF (165×10^4 cell mL^{-1}). Yield and biomass productivity in the vegetative and cystic phases were higher for cultures in PF. Higher N content was found in the PF medium, providing greater cell reproduction; however, excess of nitrogen after the exponential growth phase limits carotenogenesis. The contents and concentrations of total carotenoids and astaxanthin, in general, were higher in NH_4NO_3-WPF (astaxanthin content ~ 23 mg g^{-1}) being influenced by nitrogen depletion and pH variation. Thus, nitrogen pulse feeding strategy provided higher biomasses of *H. pluvialis*, and the decrease in the pulses concentrations can result in higher astaxanthin production due to the lower N residue.

Keywords Microalgae · Culture medium · Nitrogen supply · Biomass yield · Carotenoid

1 Introduction

Haematococcus pluvialis (Chlorophyta) has the ability to produce astaxanthin (3,3′-dihydroxy-β,β-carotene-4, 4′-dione), and it is commonly cultivated for this purpose [1]. This microalga's market is estimated at US$ 240 million, and the world market for astaxanthin, from different origins, is constantly growing and is valued at approximately US$ 600 million, showing the importance of increasing the production of *H. pluvialis* [2, 3]. Astaxanthin is a carotenoid widely used in the pharmaceutical, nutraceutical, cosmetic, and food industries [4], as it has antioxidant (10 times more than β-carotene), anti-inflammatory, antitumor, antidiabetic, and immunomodulatory properties [5], in addition to being widely used in aquaculture, both for pigmentation and to improve the immune response and zootechnical performance of shrimp and fish [6].

Morphological, physiological, and biochemical changes in cells are characteristics of astaxanthin production in this microalga, which are a result of the influence of environmental factors such as high luminosity and the presence/absence of nutrients [7]. In relation to other astaxanthin-producing organisms, such as bacteria, yeasts, plants, and other microalgae, *H. pluvialis* is considered to be the species with the greatest capacity to accumulate this carotenoid (up to 5% of dry biomass) [4]. However, feasibility of obtaining this molecule from

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H. pluvialis depends on the cultivation conditions, such as temperature, pH, salinity, luminosity, and culture medium. Therefore, for production, these conditions are manipulated to achieve optimal conditions and promote cell growth as well as astaxanthin synthesis [8].

The culture medium influences cell growth, being the main responsible for the productivity obtained, since it stimulates or inhibits growth according to the availability of nutrients. Besides this, it is also able to influence and determine the cellular composition of microalgae [9]. Among the nutrients used for the preparation of culture media, nitrogen is one of the main responsible for the growth and development of microalgae and can be made available in the form of nitrate, urea, or ammonia [10]. In addition to variations in nitrogen sources and concentrations, feeding strategies (e.g., continuous, staged, and pulsed) can also be modified. Different feeding strategies of nitrogen sources, as well as of carbon and phosphorus, modify the physiological metabolism of microalgae and can provide greater productivity in biomass or metabolites of high economic value [11, 12]. Pulse feeding strategy has already been used for several species of microalgae, such as *Scenedesmus acuminatus*, *Chlorella sorokiniana*, *Chlamydomonas reinhardtii*, and *Nannochloropsis gaditana* [13–16], and according to Devasya and Bassi (2021), it significantly increases biomass and biomolecules production in microalgae cultivation [14].

In *H. pluvialis*, the availability of nitrogen in the medium promotes cell division, while its deprivation stimulates the synthesis of astaxanthin [17]. Therefore, one-step cultivation can occur through moderate nitrogen starvation and moderate light, promoting growth and carotenogenesis, simultaneously. Meanwhile, two-stage cultivation works as follows: first, conditions are controlled to intensify cell growth, vegetative phase (e.g., high nitrogen availability and low light intensity), and then, when maximum cell density is reached, carotenogenesis is stimulated, cystic phase (e.g., nitrogen starvation and high light intensity) [8].

Compared to other commercially cultivated species, *H. pluvialis* has low biomass productivity; hence, changes in the culture medium are usually used to increase the production of this biomass [18]. Based on this, an adequate feeding strategy with nitrogen sources can be a promising alternative to achieve this goal. Also, since the biomass productivity of *H. pluvialis* is extremely important to obtain a high amount of astaxanthin, finding optimal cultivation conditions for the production of biomass has been the subject of numerous studies.

Thus, in the present study, the use of different nitrogen sources under the pulsed feeding strategy was investigated in the cultivation of the microalga *H. pluvialis* to increase cell multiplication, biomass, and carotenoids and astaxanthin production.

2 Material and methods

2.1 Strain and culture conditions

H. pluvialis was obtained from the Live Food Production Laboratory, at the Fisheries and Aquaculture Department of the Federal Rural University of Pernambuco. Cultures were developed in 500-mL bottles, with fresh water previously treated with chlorine (3 ppm), filtered (22 µm) and autoclaved (120 °C), and then enriched with modified Bold's basal medium - BBM (Table 1) [19].

2.2 Experimental design

The experiment was carried out on a laboratory scale using a bifactorial design (3 × 2), with factor 1 (three nitrogen sources—sodium nitrate ($NaNO_3$), ammonium nitrate (NH_4NO_3), and urea ($(NH_2)_2CO$)) and factor 2 (nitrogen supply strategy—with pulse feeding (PF) and without pulse feeding (WPF)).

The nitrogen source of the BBM ($NaNO_3$) was replaced by NH_4NO_3 or $(NH_2)_2CO$, maintaining the original N concentration (3 mM) in the combinations without the addition of pulses (WPF). Pulse feedings were carried out on the 1st, 6th, and 9th days by adding 1.5, 3.0, and 3.0 mM of nitrogen, respectively.

Table 1 Composition (mg L^{-1}) of Modified Bold's basal medium used in the *Haematococcus pluvialis* culture

Compound	Concentration (mg L^{-1})
$NaNO_3$	255
$CaCl_2.2H_2O$	25
NaCl	25
KOH	31
EDTA $Na.2H_2O$	50
K_2HPO_4	75
KH_2PO_4	175
$FeSO_4.7H_2O$	49.8
$MgSO_4.7H_2O$	75
H_3BO_3	11.42
$ZnSO_4.7H_2O$	1.412
$MnCl_2.4H_2O$	0.232
$CuSO_4.5H_2O$	0.252
$Na_2MoO_4.2H_2O$	0.192
$Co(NO_3)_2.6H_2O$	0.08

H. pluvialis was inoculated with an initial concentration of 2×10^4 cells mL^{-1}, at a temperature of 22 °C, photoperiod 12 h:12 h, and light intensity of 40 µmol photons m^{-2} s^{-1}, under constant aeration. Cultivation occurred under photoautotrophic conditions until the stationary growth phase (vegetative phase), when organic carbon (sodium acetate, 1.98 mg L^{-1}) was introduced and there was an increase in light intensity (100 µmol photons m^{-2} s^{-1}). These modifications were used as factors to stimulate the process of carotenogenesis (cystic phase) as well as natural deprivation of nitrogen. The temperature and pH variables were measured on days 1, 6, 9, 14, and 24 (based on key moments of change in growth and carotenogenesis phases), using a digital pH meter (Kmoon pH/EC-983).

2.3 Growth analysis and biomass harvesting

To evaluate, growth samples were taken daily and fixed in formaldehyde (2%) for quantification using a hemocytometer (Neubauer chamber). With this data, the following parameters were calculated: maximum cell density (MCD); growth rate (K, Eq. 1), which represents the number of cell divisions per day performed during the total culturing time, expressed as "division day^{-1}"; specific growth rate (µ, Eq. 2), which represents the cell growth rate during the exponential phase of the growth curve as a function of time, expressed as "day^{-1}"; and doubling time, which corresponds to the time required for doubling the initial density, expressed as "day $division^{-1}$" (DT, Eq. 3) [20].

$$K = \frac{3.322}{\left(t - t_0\right) \,.\, \mathrm{Log}(N \div N_0)} \tag{1}$$

where t is the last incubation day (days); t_0 is the beginning incubation day (days); N is the final cell number (cell mL^{-1}); and N_0 is the initial cell number (cell mL^{-1}).

$$\mu = \frac{\ln(\mathrm{N(t)} \div N_0)}{\left(\mathrm{t} - t_0\right)} \tag{2}$$

where $N(t)$ is the cell number (cell mL^{-1}); N_0 is the initial cell number (cell mL^{-1}); t is the time (days); and t_0 is the beginning incubation day (days).

$$\mathrm{DT} = \frac{1}{\mathrm{K}} \tag{3}$$

The growth curves with the average daily cell density were fitted by approximating the logistic curve: $Y = P_1/1 + (P_2 - N_0/N_0.e^{-kt})$ [21].

The dry biomass yield (g L^{-1}) and biomass productivity (g L^{-1} day^{-1}) for vegetative and cystic phases were determined by filtering 10-mL aliquots of suspended cells through a Whatman GF/C glass microfiber filter (1.2 µm) and by drying at 75 °C for 24 h [22].

2.4 Nitrogen analysis

Nitrate–N (NO_3-N) and ammonia-N (NH_3-N) were analyzed in the culture medium on days 1, 6, 9, 14, and 24 (based on key moments of change in growth and carotenogenesis phases). Samples of 10 mL were filtered through a Whatman GF/C glass microfiber filter (1.2 µm), and then, the filtered volume was collected for analysis of NO_3-N [23] and NH_3-N [23]. The concentrations found were converted into total nitrogen concentration (mM).

2.5 Quantification of total carotenoids and astaxanthin

The concentrations of total carotenoids and astaxanthin were determined at the end of cultivation using a spectrophotometer. Carotenoid analysis was performed from a 10-mL aliquot of the algae suspension that was centrifuged (1700 × *g*, 10 min) and the precipitate incubated (70 °C, 10 min) in 10 mL of dimethylsulfoxide (DMSO). This suspension was analyzed in a spectrophotometer (480 nm), and the concentration of total carotenoids was calculated: 4 × OD480 [18]. To obtain the concentration of astaxanthin, 1 mL of algal suspension was centrifuged (1700 × *g*, 10 min), and the precipitate was treated with a solution of KOH (5% (w/v)) in methanol (30% (v/v)) and then incubated at 70 °C for 10 min to denature chlorophyll. Then, the suspension was centrifuged (1700 × *g*, 10 min), and glacial acetic acid (100 µL) and DMSO (5 mL) were added to the pellet and maintained at 70 °C for 15 min. After final centrifugation (1700 × *g*, 10 min), the supernatant was analyzed in a spectrophotometer (490 nm) [18]. Astaxanthin concentration and content were calculated from the expressions [4.5 × OD490 × (*Va*/*Vb*)] and *P*/*W*, respectively, where *Va* (mL) was the volume of DMSO, *Vb* (mL) was the volume of microalgae samples, *P* (mg L^{-1}) was the concentration of astaxanthin, and *W* (g L^{-1}) denoted the dry biomass of microalgae per unit volume of medium [18].

2.6 Statistical analysis

The experiment was performed with 6 combinations and 4 replicates, totaling 24 experimental units. All data were presented as mean ± standard deviation ($n = 4$). The response variables were submitted to the Shapiro–Wilk normality test

and to the Bartlett's test of homogeneity of variances. The pH and N data were transformed by $\log(x+1)$. All data were submitted to a two-way ANOVA followed by Tukey's test when a significant difference was observed. Pearson's correlation test and principal component analysis (PCA) were applied to the physicochemical and growth variables. p values < 0.05 were considered statistically significant for all tests. Statistical analysis was performed using the R Core Team software [24].

3 Results and discussion

3.1 Cell growth and biomass production of *H. pluvialis*

H. pluvialis is characterized by achieving low growth rates, cell density, and biomass, compared to other microalgae species [18]. The results of growth variables showed a significant difference for MCD (Table 2), where the combinations with pulse feeding strategy (PF) obtained higher densities, demonstrating that high concentrations of nitrogen in PF result in increased cell growth. The MCD can vary between 11 and 120×10^4 cell mL^{-1} [25–27], and in this study, a MCD of up to 195×10^4 cell mL^{-1} was obtained. Orosa et al. [26], using 3–12 mM of nitrate, reached a MCD of $110–120 \times 10^4$ cell mL^{-1}, in 15 days of culture and μ of 0.47–0.50 day^{-1}, corroborating this study. The specific growth rate (μ) of this species varies between 0.10 and 0.64 day^{-1} [25, 26, 28], and in the present study, the highest μ value was 0.5 day^{-1}, with a mean of 0.3 for all combinations (Table 2). As regard to DT, it can vary from 2.8 to 6.9 day $division^{-1}$ [10, 27, 28], a higher value than that found in the present study (2.7–2.8 day $division^{-1}$). Furthermore, the availability of nitrogen allows a longer duration of the cell division phase (vegetative phase); consequently, the day of MCD was later for the PF cultivation units, regardless of the nitrogen source (Table 2).

This trend can be seen in the growth curves (Fig. 1), with a longer duration of the exponential phase (10 to 12 days, approximately) for treatments in which nitrogen pulses were applied. WPF, regardless of the N source, had a shorter exponential phase (between the 5th and 11th day of cultivation). On the other hand, MCD was influenced by N sources, obtaining higher densities with $NaNO_3$ and $(NH_2)_2CO$ under PF (Table 2). Different N sources affect microalgae growth and metabolite production [29]. Generally, for microalgae, the order of preference for nitrogen utilization is $NH_4^+ > NO_3^- > NO_2^- > (NH_2)_2CO$ [30], being ammonia the one that requires less energy cost to be assimilated [31]. For *H. pluvialis*, lower MCD were achieved with NH_4NO_3, possibly because in the presence of NH_4^+, many genes involved in the assimilation of NO_3^- and NO_2^- are inhibited [30]. In a study carried out by Göksan et al. [32], $NaNO_3$ also provided the highest MCD (25.3×10^4 cell mL^{-1}), while $(NH_2)_2CO$ resulted in the lowest MCD (17.4×10^4 cell mL^{-1}).

As stated by Devasya and Bassi [14], an increase in the number of cell populations due to the progression of the growth stage demands an increase in the level of substrates; hence, pulse feeding ensures the continuous availability of nutrients in the media and promotes the assimilation of nutrients that enhance cellular activities. Also, according to the same authors, pulse nitrogen feeding significantly increases biomass and lipid production in fed-batch cultures of *Nannochloropsis gaditana*. Similarly, Figueroa-Torres et al. [16] observed that fed-batch pulse feeding regime with organic carbon (acetate) in the cultivation of *Chlamydomonas reinhardtii* increased the production of biomass (94%), starch (676%), and lipids (252%).

WPF cultivation units reached the stationary growth stage earlier, approximately on the 12th day (Fig. 1), which resulted in a shorter vegetative phase. The cells of *H.*

Table 2 Growth variables of *Haematococcus pluvialis* cultivated with different nitrogen sources and feeding strategies

Feeding strategy	Nitrogen sources	K (day^{-1})	μ (day^{-1})	DT (days)	MCD ($\times 10^4$ cell mL^{-1})	MCD day
WPF	$NaNO_3$	0.348 ± 0.01	0.285 ± 0.19	2.73 ± 0.26	122.03 ± 18^{Ab}	15
	NH_4NO_3	0.355 ± 0.02	0.267 ± 0.17	2.73 ± 0.08	106.50 ± 20^{Bb}	17
	$(NH_2)_2CO$	0.363 ± 0.02	0.272 ± 0.18	2.66 ± 0.01	109.22 ± 17^{Bb}	17
PF	$NaNO_3$	0.375 ± 0.01	0.282 ± 0.19	2.61 ± 0.01	175.92 ± 20^{Aa}	19
	NH_4NO_3	0.363 ± 0.01	0.266 ± 0.17	2.75 ± 0.08	140.67 ± 13^{Ba}	19
	$(NH_2)_2CO$	0.356 ± 0.03	0.273 ± 0.18	2.77 ± 0.23	164.67 ± 10^{Aa}	19
	S	ns	ns	ns	*	-
	F	ns	ns	ns	***	-
	S × *F*	ns	ns	ns	ns	-

Mean ± standard deviation ($n = 4$). Mean values for the same column with different letters indicate significant differences by a two-way ANOVA followed by Tukey's test (uppercase, nitrogen sources factor; lowercase, feeding strategy factor). *S*, factor 1 (nitrogen sources); *F*, factor 2 (feeding strategy); *S x F*, interaction between the two factors. *ns*, not significant; *** ($p < 0.001$); * ($p < 0.05$). *WPF*, without pulse feeding; *PF*, pulse feeding strategy; *K*, growth rate; *μ*, specific growth rate; *DT*, doubling time; *MCD*, maximum cell density

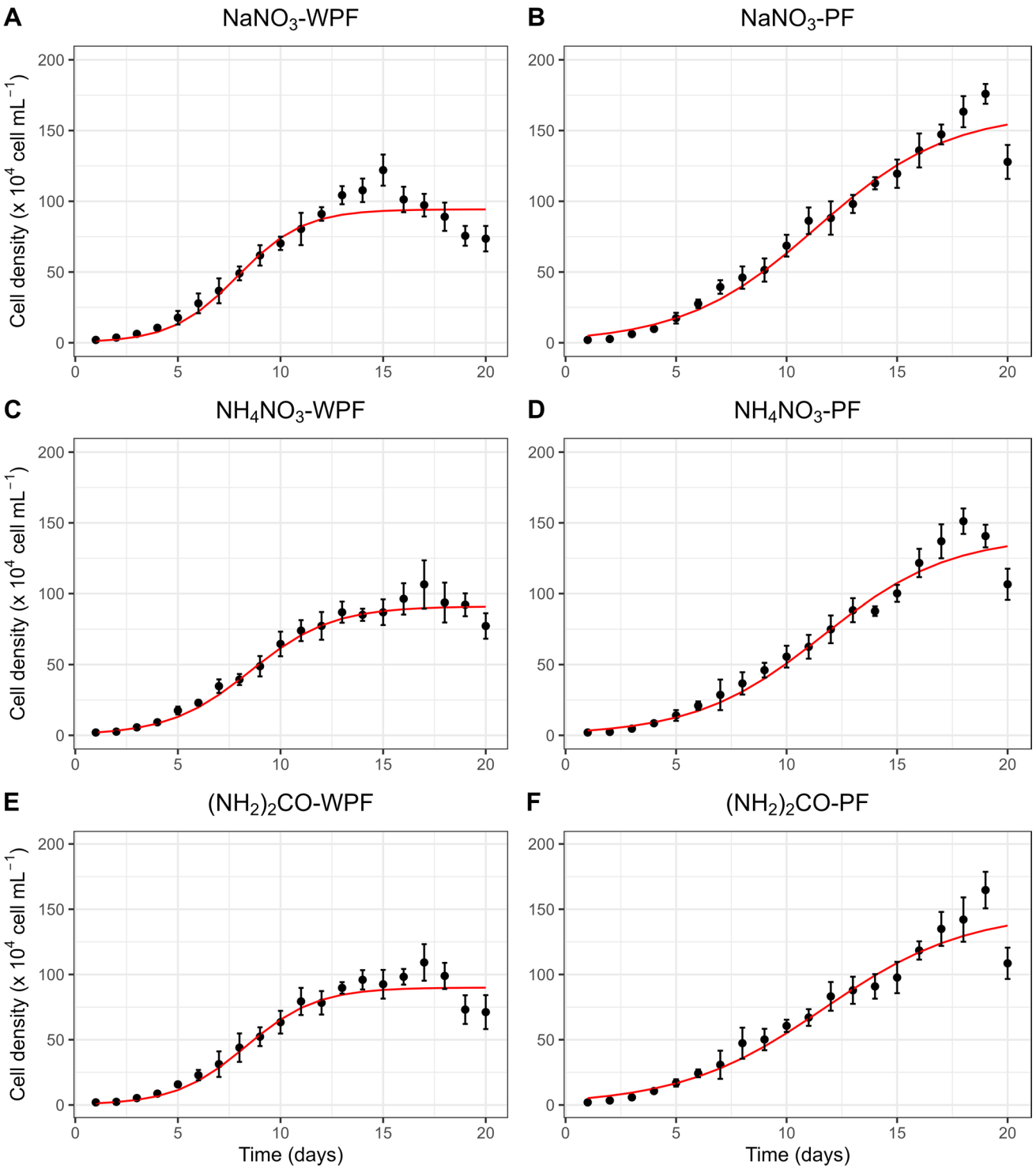

Fig. 1 Growth curves of *Haematococcus pluvialis* cultivated with different nitrogen sources and feeding strategies

pluvialis in the vegetative phase are undergoing intense division, and favorable conditions for growth must be provided, such as high nitrogen availability. Thus, a high concentration of nitrogen in the culture (up to approximately 18 mM) should provide a greater amount of biomass, mainly due to the increase in cell density [17]. Therefore, biomass yield in the vegetative phase was also influenced by the nitrogen feeding strategy, obtaining higher values in PF (Table 3).

Relative to dry biomass and biomass productivity, *H. pluvialis* can reach up to 0.5–2.8 g L^{-1} and 0.07–0.7 g

Table 3 Yield (g L^{-1}) and productivity (g L^{-1} day^{-1}) of *Haematococcus pluvialis* biomass in the vegetative and cystic phases cultivated with different nitrogen sources and feeding strategies

Feeding strategies	Nitrogen sources	Vegetative yield (g L^{-1})	Cystic yield (g L^{-1})	Vegetative productivity (g L^{-1} day^{-1})	Cystic productivity (g L^{-1} day^{-1})
WPF	$NaNO_3$	0.71 ± 0.04^{Ab}	0.91 ± 0.02^{Ab}	0.050 ± 0.004^{Ab}	0.038 ± 0.001^{Ab}
	NH_4NO_3	0.75 ± 0.07^{Ab}	0.94 ± 0.03^{Ab}	0.054 ± 0.005^{Ab}	0.039 ± 0.001^{Ab}
	$(NH_2)_2CO$	0.65 ± 0.02^{Ab}	1.0 ± 0.04^{Ab}	0.047 ± 0.002^{Ab}	0.042 ± 0.002^{Ab}
PF	$NaNO_3$	086 ± 0.05^{Aa}	1.34 ± 0.06^{Aa}	0.061 ± 0.004^{Aa}	0.056 ± 0.003^{Aa}
	NH_4NO_3	0.89 ± 0.05^{Aa}	1.30 ± 0.14^{Aa}	0.063 ± 0.003^{Aa}	0.054 ± 0.006^{Aa}
	$(NH_2)_2CO$	0.82 ± 0.09^{Aa}	1.22 ± 0.04^{Aa}	0.059 ± 0.007^{Aa}	0.051 ± 0.002^{Aa}
	S	ns	ns	ns	ns
	F	***	***	***	***
	S×*F*	ns	ns	ns	ns

Mean ± standard deviation ($n = 4$). Mean values for the same column with different letters indicate significant differences by a two-way ANOVA followed by Tukey's test (uppercase, nitrogen sources factor; lowercase, feeding strategy factor). *S*, factor 1 (nitrogen sources); *F*, factor 2 (feeding strategies); *S x F*, interaction between the two factors. *ns*, not significant; *** ($p < 0.001$). *WPF*, without pulse feeding; *PF*, pulse feeding

Table 4 Physicochemical variables of *Haematococcus pluvialis* cultivation with different nitrogen sources and feeding strategies

Feeding strategies	Nitrogen sources	pH	T (°C)	Nitrogen concentration (mM)
WPF	$NaNO_3$	8.11 ± 0.07^{Aa}	22.34 ± 0.10^{Aa}	1.07 ± 0.12^{Ab}
	NH_4NO_3	7.03 ± 0.13^{Ca}	22.17 ± 0.03^{Aa}	1.39 ± 0.28^{Ab}
	$(NH_2)_2CO$	7.37 ± 0.01^{Ba}	21.98 ± 0.09^{Aa}	1.20 ± 0.15^{Ab}
PF	$NaNO_3$	8.39 ± 0.10^{Aa}	22.24 ± 0.02^{Aa}	2.64 ± 1.59^{Aa}
	NH_4NO_3	6.86 ± 0.09^{Ca}	22.07 ± 0.09^{Aa}	3.17 ± 1.82^{Aa}
	$(NH_2)_2CO$	7.70 ± 0.02^{Ba}	22.07 ± 0.13^{Aa}	3.02 ± 1.69^{Aa}
	S	***	ns	ns
	F	ns	ns	***
	S×*F*	*	ns	ns

Mean ± standard deviation ($n = 4$). Mean values for the same column with different letters indicate significant differences by a two-way ANOVA followed by Tukey's test (uppercase, nitrogen sources factor; lowercase, feeding strategy factor). *S*, factor 1 (nitrogen sources); *F*, factor 2 (feeding strategies); *S x F*, interaction between the two factors. *ns*, not significant; *** ($p < 0.001$); * ($p < 0.05$). *WPF*, without pulse feeding; *PF*, pulse feeding

L^{-1} day^{-1} [33–36]; in the present study, the highest average biomass was 1.34 g L^{-1}, and the maximum productivity was 0.18 g L^{-1} day^{-1}. According to the N-nitrate concentration, the maximum biomass productivity can vary between 0.27 ([N] = 4.4 mM) and 0.30 g L^{-1} day^{-1} ([N] = 17.6 mM) [17]. When comparing different nitrogen sources, Göksan et al. [32] obtained higher dry biomass (1.2 g L^{-1}) with NH_4NO_3 ([N] = 3 mM) and lower biomass (0.65 g L^{-1}) with $(NH_2)_2CO$ ([N] = 4 mM).

The nitrogen content of the culture medium was evaluated over time, finding a higher mean concentration of N in PF (Table 4). Regarding the profile of the concentration of N during the cultivation (Fig. 2), it is possible to observe in PF greater availability of N from the 6th day onwards, due to the pulses. The feeding strategies created varied cellular activities that resulted in a heterogeneous cell growth status, resulting in variations in nitrogen depletion and residual nitrogen levels [14]. In WPF, N was significantly consumed until day 14, coinciding with the beginning of the stationary phase and, consequently, the transition to the cystic phase. Pulse feeding mode provided a short exposure to nitrogen starvation condition compared to WPF cultures, and this condition delayed the stationary phase of growth in PF. In the exponential phase of cultivation, the consumption of N is strongly linked to growth, and from the stationary phase, the cells start to absorb N, modifying the cellular composition [37]. Therefore, with the arrival of the stationary growth stage and the decrease of N concentration by the consumption of microalgae, the cultivation conditions were directed to stimulate carotenogenesis, with the insertion of an organic carbon source (sodium acetate) and an increase in light intensity. Thus, nitrogen started to participate in the metabolism of carotenogenesis, with total N consumption up to day 24 for all sources in WPF (Fig. 2).

In addition to the availability of nutrients, such as nitrogen, other physical–chemical factors are important for the functioning of cellular metabolism, such as pH. Keeping the pH variation to a minimum ensures a more stable chemical environment and, consequently, better conditions for *H. pluvialis* cell division [38]. In this study, the initial pH ranged between 6.5 and 7.5; generally, at an initial pH of 7, greater growth of *H. pluvialis* is observed, while at a pH of 9 and 5, less growth and inhibition are observed, respectively [39]. Lower pH was found with NH_4NO_3 (Table 4) due to the release of H^+ in the medium [40]. In addition, pH decrease was observed after NH_4NO_3 inputs, generating pH

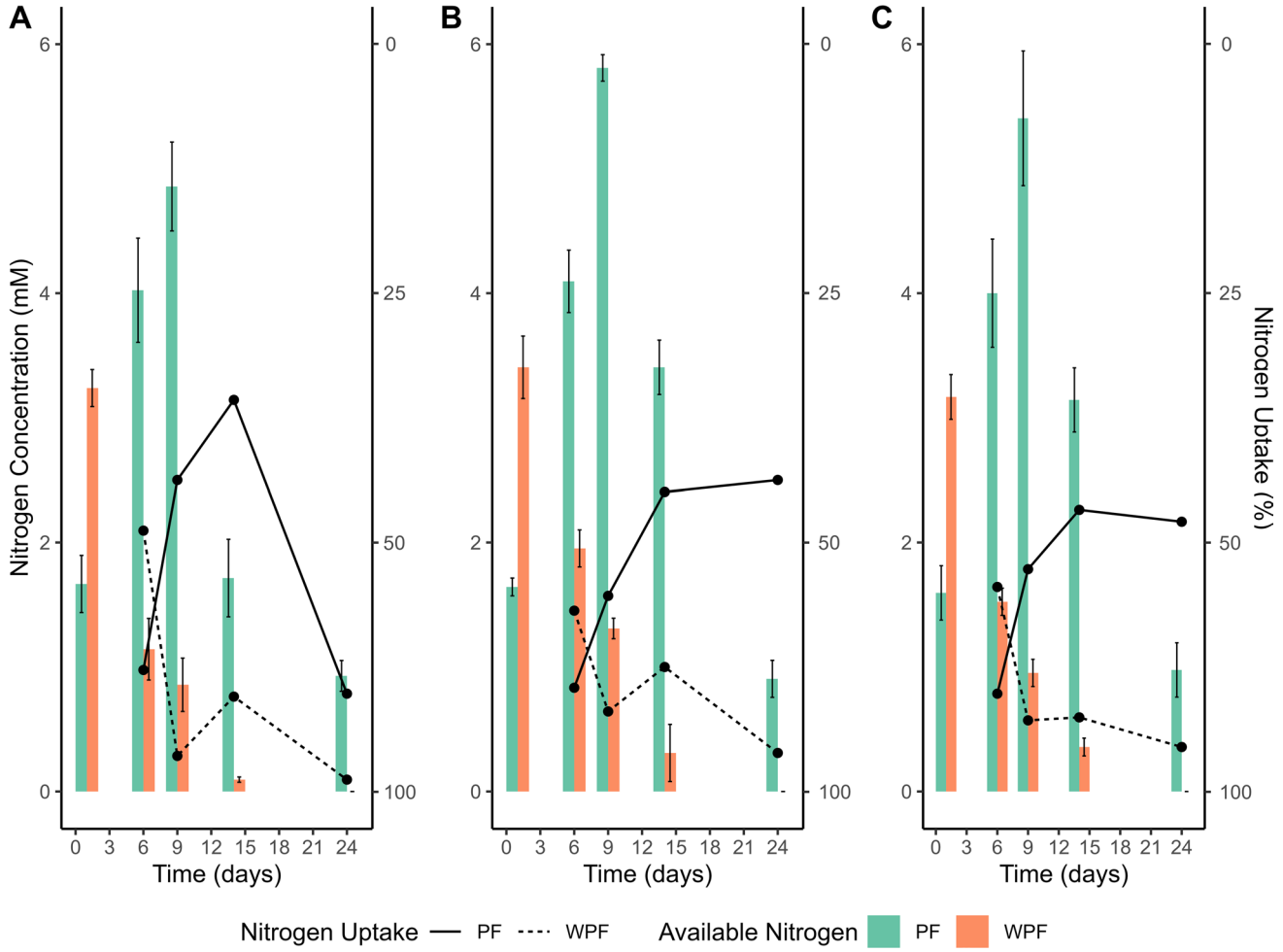

Fig. 2 Nitrogen uptake profile (rows) *versus* available nitrogen (columns) in *Haematococcus pluvialis* cultivation under different feeding strategies (PF and WPF) and nitrogen sources ($NaNO_3$ (**A**), NH_4NO_3 (**B**), $(NH_2)_2CO$ (**C**))

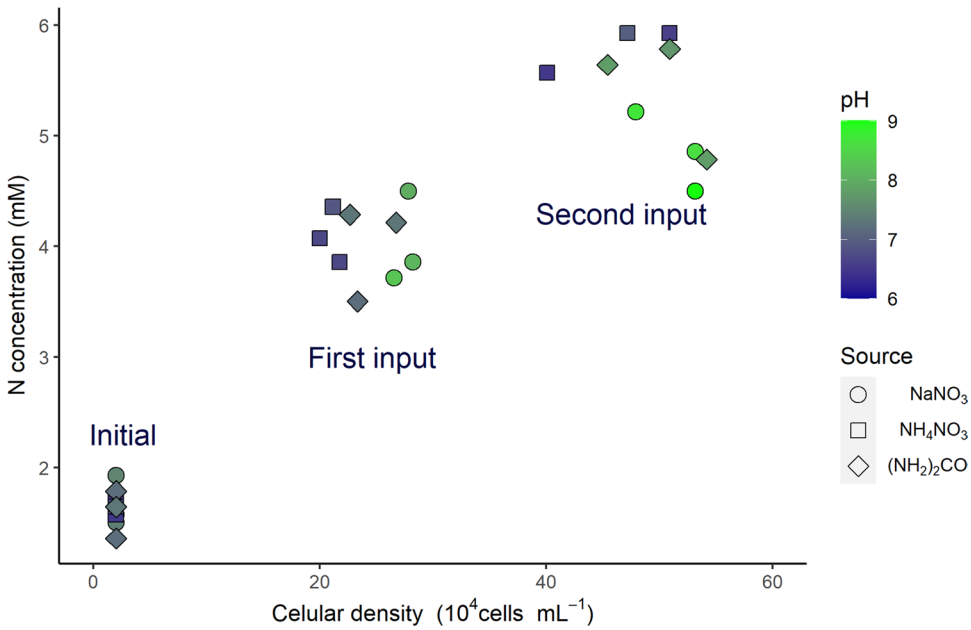

Fig. 3 Correlation among N concentration, cellular density, and pH in *Haematococcus pluvialis* culture under pulse feeding strategy with different N sources

fluctuations (Fig. 3). The stress caused by these variations may have negatively influenced the growth in NH_4NO_3-PF, reaching a lower MCD than the other sources. However, a smaller variation was observed between the initial and final pH of NH_4NO_3-PF culture due to the neutralization of OH^- (released in photosynthesis) by the release of H^+ from the N inputs, as well as by the preference of microalgae to consume ammonium ions, avoiding acidification of the medium [40].

Pulse feeding strategy increased the availability of N throughout the culture and provided an increase in cell density (CD), influencing the pH variation of the medium as a function of the N source (Fig. 3). The inputs with $NaNO_3$ and $(NH_2)_2CO$ reflected an increase in pH due to the release of OH^- through photosynthesis and the consumption of nitrate [40, 41], while the inputs with NH_4NO_3 neutralized the pH of the medium, through the production of H^+ [40]. In this scenario, pH did not correlate with the concentration of N and CD ($0.29 < r < 0.42$), differently of inputs where N concentration and CD are strongly correlated ($r > 0.97$).

In the principal component analysis (PCA), degrees of explanation of 85.8% for PF and 89.58% for WPF were found, divided between two principal components, PC1 and PC2 (Fig. 4). For the combinations in PF (Fig. 4A), all variables were positively correlated. Higher pH values were recorded for the source $NaNO_3$, higher concentrations of N for NH_4NO_3, and higher CD and μ for $(NH_2)_2CO$. As for the WPF combinations (Fig. 4B), the variable N is inversely correlated with the other variables. $NaNO_3$ source had the highest pH values, while NH_4NO_3 and $(NH_2)_2CO$ had the highest CD, μ, and N.

Our results confirmed that the nutritional management of nitrogen sources during the growth period in fed-batch culture systems using pulse feeding mode is essential to achieve greater cell density and biomass of *H. pluvialis*.

3.2 Production of carotenoids and astaxanthin in *H. pluvialis*

In the cystic phase, *H. pluvialis* cells begin to synthesize and accumulate carotenoids, mainly astaxanthin. In addition, there are morphological changes—increased cell wall thickness, size, and weight [42]. In this phase, higher biomass was obtained for PF (1.22 to 1.34 g L^{-1}), regardless of the nitrogen source (Table 3). The increase in biomass in the cystic phase occurred both due to reproduction, observing an increase in cell density until the 15th (WPF) and 20th (PF) days of culture, and to the increase in cell size. When there are high concentrations of N in the medium, as in the case of PF cultures, cells can reproduce, whereas when these concentrations are low, they synthesize carotenoids [17]. As for the content of total carotenoids obtained in the cystic phase, a significant effect was observed for the feeding strategy factor ($p = 0.00393$), with higher concentrations for the

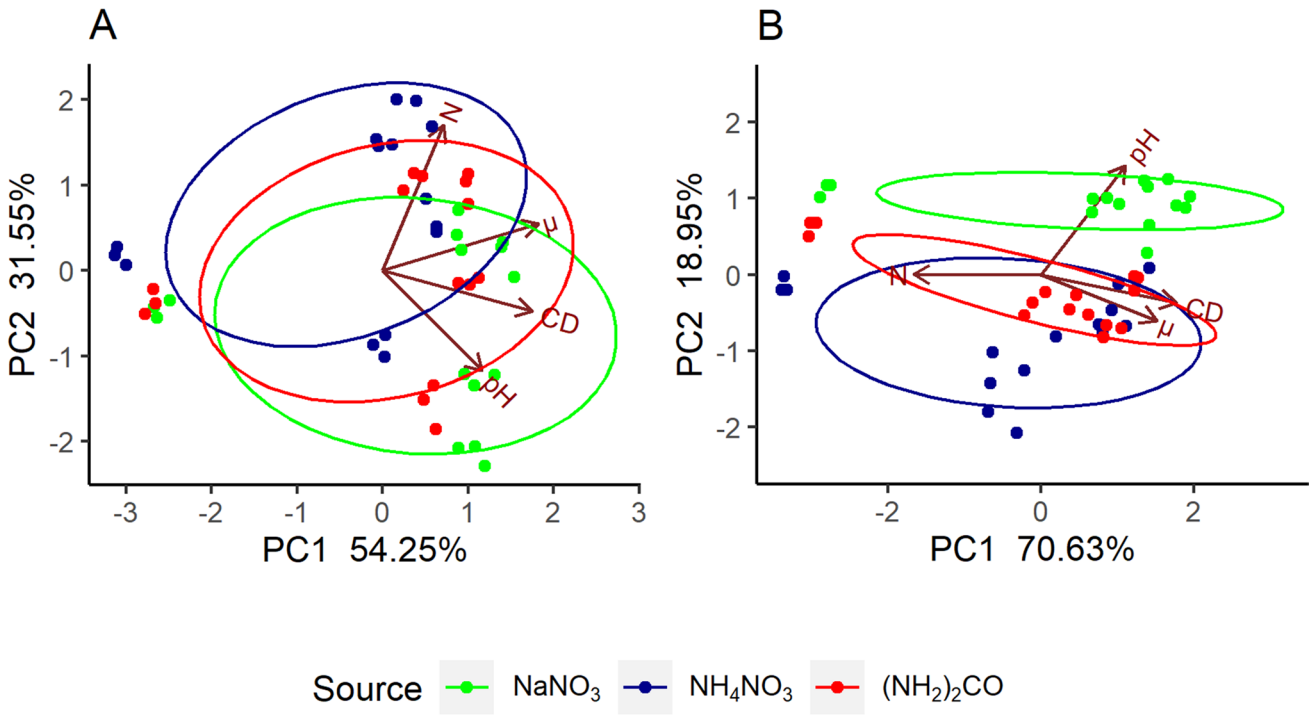

Fig. 4 Principal component analysis for physicochemical and growth variables of *Haematococcus pluvialis* culture using different nitrogen sources under pulse feeding, PF (**A**), and without pulse feeding, WPF (**B**)

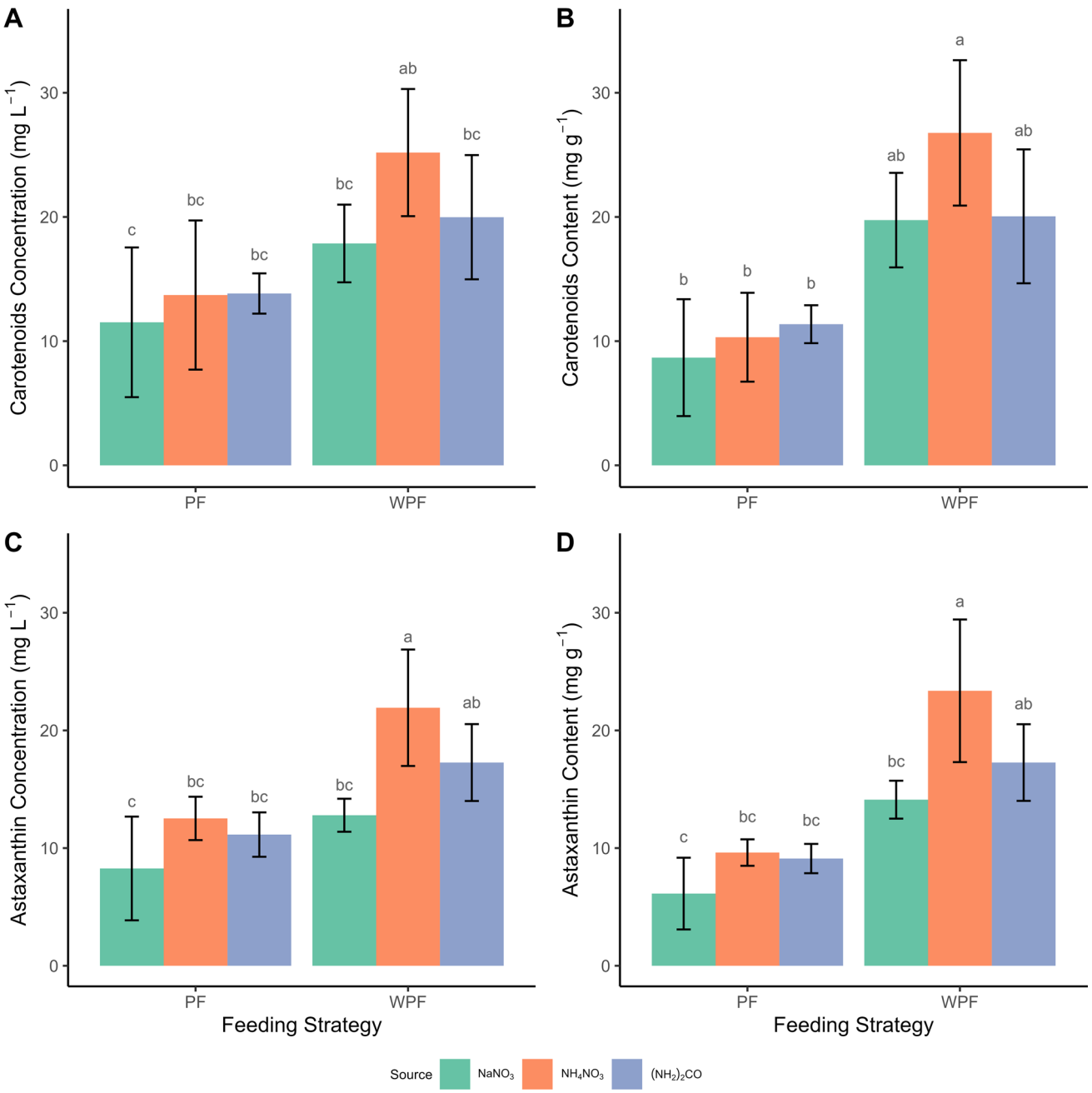

Fig. 5 Concentrations (mg L^{-1}) and contents (mg g^{-1}) of total carotenoids (**A** and **B**) and astaxanthin (**C** and **D**) produced by *Haematococcus pluvialis* cultivated in different nitrogen sources and feeding strategies (different letters indicate significant differences between the factors by Tukey's test)

combinations in WPF, with a significant difference being observed only between NH_4NO_3-WPF (25.18 ± 5.12 mg L^{-1}) and $NaNO_3$-PF (11.52 ± 6.03 mg L^{-1}) (Fig. 5A). When the carotenoid content was evaluated, there was also an effect of the feeding strategy factor ($p = 7.88e\text{-}05$), with a significant difference between NH_4NO_3-WPF (26.77 ± 5.85 mg g^{-1}) and all sources in PF ($NaNO_3$-PF = 8.67 ± 4.71 mg g^{-1}; NH_4NO_3-PF = 10.31 ± 3.58 mg g^{-1}; $(NH_2)_2CO$-PF = 11.36 ± 1.53 mg g^{-1}) (Fig. 5B). Therefore, the highest production of carotenoids occurred in WPF, which showed a shortage of nitrogen from the 14th day of cultivation (Fig. 2). The carotenoid content showed an opposite trend to the biomass, with higher content for the groups with low nitrogen concentration (WPF). This fact can be explained by nitrogen starvation and light supersaturation, which both induce the degradation of chlorophylls and

primary carotenoids (present in the vegetative phase) and consequent accumulation of secondary carotenoids, among which is astaxanthin [43].

Astaxanthin is the most abundant carotenoid in cystic *H. pluvialis*, representing about 80–99% of the total carotenoids [44]. In the present study, the concentration of astaxanthin was influenced both by the nitrogen source ($p = 0.013009$) and by the feeding strategy ($p = 0.000932$), with the highest value for NH_4NO_3-WPF (21.93 ± 4.95 mg L^{-1}), that significantly differed from all sources in PF and $NaNO_3$-WPF (Fig. 5C). Similarly, a significant effect of the two factors on the astaxanthin content was observed, with the highest value for NH_4NO_3-WPF (23.37 ± 6.06 mg g^{-1}), which was similar only to $(NH_2)_2CO$-WPF (17.27 ± 3.26 mg g^{-1}) (Fig. 5D). As with carotenoids, astaxanthin production was higher in WPF cultures, because under nitrogen depletion, there is an increase in the expression of genes linked to astaxanthin biosynthesis to act in the defense metabolism to this stress condition [45].

Furthermore, under nitrogen deprivation, the carbon flux is directed towards the synthesis of astaxanthin; i.e., the exogenous addition of organic carbon (sodium acetate) stimulates the utilization of carbon more quickly and efficiently, mainly through the tricarboxylic acid cycle, for the biosynthesis of this carotenoid [45, 46]. In this perspective, a high C/N ratio was promoted in the cystic stage, through the insertion of sodium acetate at beginning of the stationary phase of growth. The accumulation of astaxanthin was detected through macroscopic—change in color of cultures and microscopic—pigmentation in the cell observations from the 14th day in WPF and 20th day in PF, corresponding to the times of N depletion (Fig. 2). Therefore, the greater accumulation of astaxanthin under high carbon concentration during the cystic stage is due to the change in the C/N ratio and the formation of a relative nitrogen deficiency [47].

The lower yield of carotenoids and astaxanthin by microalgae under pulse feeding strategy can be explained by the excess of nitrogen in the medium after the exponential growth phase, when there should be nitrogen limitation to promote carotenogenesis. In Fig. 2, it is possible to observe excess of N under PF in $NaNO_3$ (Fig. 5A), NH_4NO_3 (Fig. 5B), and $(NH_2)_2CO$ (Fig. 5C) on days 14 and 24, a period in which carotenogenesis occurred in WPF cultures. Considering the greater uptake of N in PF than in WPF, the N inputs were important for the greatest increase in cells and biomass; however, when reaching the limit of cell growth (day 20, approximately), the excess of N in the medium prevented further production of carotenoids. These results indicate that a second N input with a lower concentration could be better used by the microalgae, with less N residue in the medium, without negatively affecting growth or carotenogenesis. The longer time for carotenogenesis in PF was also confirmed by the significant difference in productivity (mg L^{-1} day^{-1}) of carotenoids and astxanthin. Carotenoid productivity was 1.24 ± 0.22 and 0.65 ± 0.06 mg L^{-1} day^{-1}, and astaxanthin productivity was 1.0 ± 0.27 and 0.53 ± 0.08 mg L^{-1} day^{-1} in WPF and PF, respectively.

The production of astaxanthin is also stimulated by other stress factors, such as the presence or absence of certain nutrients, changes in salinity, and variations in temperature and pH. The temperature in the present study remained constant during the cultivation period, varying on average between 21.98 ± 0.09 and 22.34 ± 0.10 °C, with no significant difference between the factors (Table 4). The average pH values ranged from 6.86 to 8.39, with a significant effect of the source and the interaction between the factors (Table 4). Among the nitrogen sources, NH_4NO_3 had the lowest average pH value, both in PF and WPF. Among the WPF cultures, the use of NH_4NO_3 resulted in a greater variation between the initial and final pH of the medium (Fig. 3), so the stress caused by this variation may have contributed to the greater carotenogenesis in NH_4NO_3-WPF.

Stress caused by high pH variation stimulates carotenogenesis, since astaxanthin is a secondary metabolite produced in response to cellular stress [8]. Under conditions of environmental stress, cells intensify the production of reactive oxygen species (e.g., O_2^-, H_2O_2, OH^-); on the other hand, the synthesis of astaxanthin is promoted in order to eliminate reactive oxygen species and avoid oxidative stress [45, 48]. In this sense, the combination of stress-inducing factors, such as pH variation, nitrogen depletion, high luminosity, and insertion of organic carbon, can be used to increase the production of astaxanthin [48]. As a matter of fact, prolonged duration of stress may result in higher astaxanthin production and lower biomass production.

Another challenge in the production of astaxanthin is the high cost of cultivation systems related to low microalgae productivity [8]. In this scenario, the culture medium is one of the main costs, with nitrogen as the macronutrient with the highest proportion [49]. Among the sources of N, NH_4NO_3 is the fertilizer with the lowest cost [50], so, combined with the higher production of astaxanthin obtained with NH_4NO_3, its use would be the most recommended. Therefore, the cultivation of *H. pluvialis* using NH_4NO_3 as a source of N, with the natural depletion of nutrients in the stationary phase and alteration of the C/N balance by the introduction of organic carbon, can be an efficient approach to induce the accumulation of astaxanthin in systems of cultivation of *H. pluvialis*.

Depending on the final application of the biomass, it is important to analyze which is more advantageous, has higher astaxanthin content (under WPF), or has higher biomass (under PF). The biomass of *H. pluvialis* is very valuable; in addition to astaxanthin, it contains a high lipid content and

a suitable fatty acid profile, as well as other metabolites, for applications in food, nutraceutical, pharmaceutical, and biofuel industries [51]. Thus, the evaluation of the influence of the supply of different nitrogen sources on the growth and astaxanthin biosynthesis of *H. pluvialis* has important biotechnological implications.

4 Conclusions

The nitrogen pulse feeding strategy provided greater growth of *Haematococcus pluvialis*, obtaining higher yields and productivity in vegetative and cystic biomass for all nitrogen sources. The average yield of vegetative and cystic biomass was 0.8 and 1.3 g L^{-1}, respectively, for cultures under pulse feeding. On the other hand, higher concentrations and contents of total carotenoids (27 mg g^{-1}) and astaxanthin (23 mg g^{-1}), in general, were observed for the NH_4NO_3 source without pulse feeding strategy, being influenced by nitrogen depletion and pH variation. Future research can be carried out by decreasing the concentration of N pulses, so that there is less N residue in the medium in the carotenogenesis phase, in order to increase the synthesis of astaxanthin. Therefore, the proposal of the present study using the pulse nitrogen supply mode promotes greater production of *H. pluvialis* biomass and a new perspective to increase the production of astaxanthin with NH_4NO_3.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval The experiment was in accordance with Brazilian Law no. 11.794/2008.

Competing interests The authors declare no competing interests.

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