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# Growth and nitrogen uptake by *Arthrospira platensis* cultured in aquaculture wastewater from Nile tilapia reared in biofloc system

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**ABSTRACT.** This study aimed to evaluate the growth of *Arthrospira* (*Spirulina*) *platensis* cultivated in Zarrouk culture medium and effluent from Nile tilapia (*Oreochromis niloticus*) reared in biofloc system. Four treatments were used: Control (100% Zarrouk), E50 (50% Zarrouk + 50% Tilapia effluent), E75 (25% Zarrouk + 75% Tilapia effluent), and E100 (100% Tilapia effluent), and the experiment lasted 10 days. Growth parameters such as maximum cell density (MCD), doubling time (DT), and growth rate (K) were daily evaluated, as well as pH and water temperature. In addition, the concentrations of total ammonia nitrogen (TAN), nitrite-N ($NO_2$-N), and nitrate-N ($NO_3$-N) were analyzed in order to compare nitrogen absorption. Among treatments, E50 and E75 obtained higher maximum cell densities and presented an exponential growth rate similar to the control treatment. Regarding the concentrations of nitrogen compounds, a significant reduction was observed in all treatments, with an $NO_3$-N uptake of 99%, followed by 80% of TAN and 90% of $NO_2$-N. Thus, giving the results obtained, besides being able to grow in wastewater, *A. platensis* can also be used in bioremediation processes, confirming the potential of this species.

**Keywords:** bioremediation; BFT; nitrogen compounds; growth rate; mixotrophic; spirulina.

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## Introduction

Aquaculture activities generate significant quantities of nutrient-rich effluents containing organic and inorganic compounds such as nitrogen, phosphorus, and carbon, which may cause eutrophication of surrounding water bodies. Moreover, these effluents can increase the occurrence of pathogenic microorganisms and the introduction of invasive species (Crab, Defoirdt, Bossier, & Verstraete, 2012). New aquaculture systems, such as biofloc (BFT), have been developed to enhance water quality by balancing nitrogen and carbon in the system (Crab et al., 2012).

The biofloc contain organisms such as microalgae, and autotrophic and heterotrophic bacteria, which are able to convert nitrogen to nitrate, and the nitrogenous waste into bacterial biomass through the system. Protozoa, fungi, nematodes, rotifers, copepods, feed leftovers, and feces are also present in this kind of system (Crab et al., 2012).

This microbial community allows the nitrogen from the feed to be converted into protein biomass, that will be available again for the cultivated animals, improving their zootechnical performance (Zapata-Lovera et al., 2017). Also, reduced water exchange, typical of biofloc, allows the maintenance of water quality, in addition to generating less wastewater to be discarded in adjacent water bodies (Lezama-Cervantes & Paniagua-Michel., 2010). Besides having these benefits, the concentrations of nitrogenous and phosphorus compounds (Zapata-Lovera et al., 2017) in cultures of tilapia in BFT make these effluents suitable for use as a culture medium for microalgae and cyanobacteria. Although there are some studies that report the use of seaweed (Brito et al., 2018), mollusks (Brito et al., 2018), zooplankton (Campos et al., 2020) and microalgae (Magnotti, Lopes, Derner, & Vinatea, 2015) for biological removal of dissolved nutrients in effluent from shrimp and tilapia reared in BFT, there is still no report about the use of *Arthrospira* (*Spirulina*) *platensis* in wastewater from tilapia reared in biofloc system.

The cyanobacteria *Arthrospira* spp. is produced worldwide on a commercial scale since the 20th century in countries such as Australia, India, Israel, Japan, Malaysia, and Myanmar, reaching a production of 89 thousand tonnes (live weight) in 2016 (Food And Agriculture Organization [FAO], 2018). *Arthrospira platensis* has a high commercial and economic potential due to its high content of proteins, vitamins, essential amino acids, gamma-linolenic acid (omega-6) and other polyunsaturated fatty acids, minerals and pigments like beta-carotene (Vonshak, 2014). Due to its biochemical composition, this cyanobacteria is widely used in the pharmaceutical and chemical industries, human and animal nutrition, and production of biofuels (Vonshak, 2014).

Despite the many benefits and biotechnological applications of *A. platensis*, its commercial-scale production is still a challenge due to the high production costs, including those related to the culture medium oftenly used (Vonshak, 2014). Most microalgae can be cultivated autotrophically and some microalgal species can also grow under heterotrophic or mixotrophic modes. In the photoautotrophic mode, microalgae use light energy and $CO_2$ as the main sources of inorganic carbon to produce energy (Huang, Chen, Wei, Zhang, & Chen, 2010). On the other hand, the heterotrophic mode is based on the use of an organic carbon source, and light energy is not required (Perez-Garcia, Escalante, de Bashan, & Bashan, 2011). A combination of the photoautotrophic and heterotrophic mode, the so-called mixotrophic mode, is based on the use of light energy, as well as organic and inorganic sources of carbon for the growth of microalgae (Perez-Garcia et al., 2011).

Therefore, as few studies have investigated the potential use of aquaculture wastewater, which in addition to containing the basic nutrients necessary for the growth of microalgae, still reduce production costs (Abdel-Raouf, Al-Homaidan, & Ibraheem, 2012), the aim of this study was to evaluate the growth and nitrogen uptake by *A. platensis* cultured in Nile tilapia biofloc wastewater.

## Material and methods

### Experimental conditions

An indoor trial was conducted for 10 days in the *Laboratório de Produção de Alimento Vivo* of the *Departamento de Pesca e Aquicultura* at the *Universidade Federal Rural de Pernambuco*, Recife, Brazil. The experimental design was completely randomized with four treatments: Control (100% Zarrouk), E50 (50% Zarrouk + 50% Tilapia effluent), E75 (25% Zarrouk + 75% Tilapia effluent), E100 (100% Tilapia effluent), with three replicates each.

The experimental cultures were developed in 1L Erlenmeyer flasks, maintained at a temperature of 28 ± 1ºC, with constant agitation by bubbling atmospheric air, light intensity of 40 µmol photons $m^{-2} s^{-1}$, and continuous photoperiod.

### Wastewater from tilapia in biofloc

The wastewater which was used as medium culture came from a culture tank of *Oreochromis niloticus* (Nile tilapia) in a biofloc system, where fingerlings of about 9.6 cm and 15.4 g, in the 30th day of culture were being cultivated. Molasses was used as a source of carbon, as well as 36% of crude protein and a C:N ratio of 15:1. This effluent was subjected to sedimentation of the solids, supernatant double filtration (40 µm), chlorination with sodium hypochlorite at 3 ppm for 24 hours (with aeration) and application of sodium thiosulfate. After these procedures, the effluent was autoclaved at 120°C for 15 minutes, and the chemical analysis of the dry effluent presented 132.9 mg $L^{-1}$ of carbon and 8.86 mg $L^{-1}$ of nitrogen.

### Culture medium from BFT and Zarrouk

*Arthrospira platensis* was inoculated in each experimental unit, Zarrouk culture medium and BFT culture medium from waste tilapia, at a density of 10,000 cells $mL^{-1}$. Zarrouk culture medium was composed of 1.948 mg $L^{-1}$ of carbon and 0.41 mg $L^{-1}$ of nitrogen under autotrophic conditions, with a C:N ratio at 4.75:1, while the BFT culture medium was made for maintaining the C:N ratio at 15:1, being considered mixotrophic by the inclusion of organic carbon, as shown in Table 1.

**Table 1.** Concentrations of carbon and nitrogen in different treatments and their respective C:N ratio.

| Treatments | System | Carbon (mg $L^{-1}$) | Nitrogen (mg $L^{-1}$) | C:N ratio |
|---|---|---|---|---|
| Control | Photoautotrophic | 1.948 | 0.41 | 4.75:1 |
| E50 | Mixotrophic | 41.1 | 5.48 | 7.5:1 |
| E75 | Mixotrophic | 64.2 | 5.71 | 11.2:1 |
| E100 | Mixotrophic | 132.9 | 8.86 | 15:1 |

### Growth of *Arthrospira platensis*

Microalgae samples were counted daily using a Sedgewick-Rafter chamber in an optical microscope (the samples were diluted in distilled water to allow counting). The maximum cell density (MCD), that corresponds to the maximum average value of cell density obtained between the first and last days of culture, the doubling time (DT), which represents the time spent for the division of a cell, and the growth rate (K) were calculated. K value was obtained through Equation (1), as described by Stein (1973):

$$K= [3.322\ (T_f-T_i)^{-1}\times(\text{Log}\ N_f\times N_i)^{-1}] \quad (1)$$

Where: 3.322 = conversion factor of logarithm base 2 to base 10;
$(T_f-T_i)$ = time interval in days;
$N_i$ = initial cell density;
and $N_f$ = final cell density.
To calculate DT, Equation (2) was used:

$$DT = \frac{1}{K} \quad (2)$$

Exponential growth rate (K*) was also considered, varying the time interval of the exponential phase for each treatment, according to the Equation (1).

### Water quality

The pH and water temperature were measured daily in the morning, using a multi-parameter probe (YSI 55, Yellow Springs, Ohio, EUA). For the determination of total ammonia nitrogen (TAN), nitrite-N ($NO_2$-N) and nitrate-N ($NO_3$-N), samples were collected at the beginning and at the end of the experiment for analysis in the *Laboratório de Produção Aquícola* of the *Universidade Federal Rural de Pernambuco*. For these analyses, 50 mL samples were centrifuged (5,000 x g; 10 min.), filtered through a 0.22 µm membrane and the nitrogen compounds were measured using the HACH TNT method: 830 (salicylate), 8507 (diazotization) and 8539 (cadmium reduction) for TAN, $NO_2$-N and $NO_3$-N, respectively. The samples were analyzed using a HACH DR 2800 digital spectrophotometer (Hach Company, Colorado, USA).

### Nitrogen uptake

The determination of the percentages of removal and accumulation of nutrients from the culture media were made in triplicate at the end of the experiment and calculated by the equation below, according to Henry-Silva and Camargo (2008) (Equation 3):

$$R(100\%) = \frac{[100-(100\ \text{x final nutriente concetration})]}{\textit{i}\text{nitial nutrient concetration}} \quad (3)$$

### Statistical analysis

Data were submitted to Analysis of Variance (ANOVA) after confirmation of homogeneity (Cochran test) and normality (Shapiro-Wilk test). Some data (MCD and K), after the normality test, required transformation by means of the square root. Tukey test was performed to compare the means among the four treatments, with a confidence interval of 95% ($p < 0.05$). Data analyses were performed using ASSISTAT 7.7 software (Assistat Analytical Software, Campina Grande, Paraíba, Brazil).

Curve Expert 1.4 was used to plot growth curves of each treatment adjusted by the approximation to the logistic curve according to Pindich and Rubenfel (1981), applying the following formula (Equation 4):

$$Y = P1/1 + (P2 - N0/N0.e\text{-}kt) \quad (4)$$

Where:
Y = cell density (cell $mL^{-1}$);
$P_1$ and $P_2$ = first and second parameters of the logistic curve, respectively;
$N_0$ = initial cell density (cell $mL^{-1}$);
k = growth rate ($day^{-1}$);
t = time (days).

Data from obtained curves and adjusted curves were considered corresponding when the coefficient of determination ($r^2$) was equal to or greater than 0.80.

## Results and discussion

### Growth of *Arthrospira platensis*

The growth curves of *A. platensis*, which show the daily cell densities of each treatment, are presented in Figure 1. For each treatment, the curves corresponded to the proposed model and the coefficients showed a correlation above 0.90. As it is shown in the control curve (100% Zarrouk), *A. platensis* entered the exponential phase of growth on the second day, and remained in that phase until the fourth day, showing faster growth from the first day onwards. In the E50 treatment (50% Zarrouk + 50% Tilapia effluent), the microalga initiated the exponential growth phase on the third day of cultivation and remained in this phase until the fifth day, with a longer adaptation phase than the control, as expected. In the E75 (50% Zarrouk + 25% Tilapia effluent), the exponential phase lasted only one day, from the fourth to the fifth day. However, in E100 (100% Tilapia effluent), growth was much lower, with no defined growth phases.

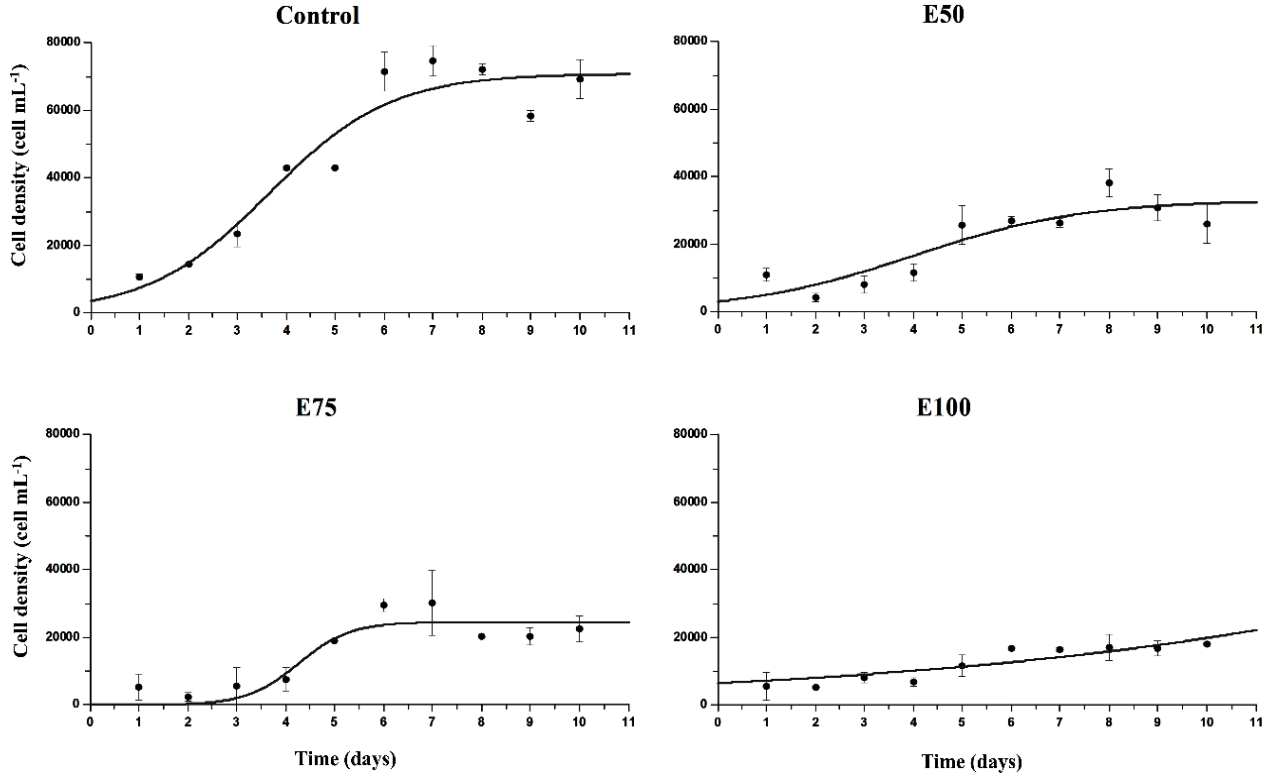


**Figure 1.** Logistic growth curve of the cyanobacteria Arthrospira platensis in 10 days under different proportions of Zarrouk culture medium and effluent from tilapia culture in a BFT system.

Significant differences were observed ($p < 0.05$) for maximum cell density (MCD), growth rate (K) and doubling time (DT) (Table 2). The control treatment took longer than the others to reach the maximum cell density, that was on the seventh day of cultivation. Among treatments, E50 and E75 reached similar values ($p > 0.05$), which were significantly higher than the E100. However, comparing E50 and E75 with the control treatment, cell density reduced by approximately 50% (Table 2).

**Table 2**. Growth parameters of *Arthrospira platensis* cultured in the wastewater from Nile tilapia cultivation in biofloc.

| Treatments | MCD (cell $mL^{-1}$) | K ($day^{-1}$) | K* ($day^{-1}$) | DT (day) |
|---|---|---|---|---|
| Control | 74,800 ± 4,667$^a$ | 0.28 ± 0.01$^a$ | 0.78 ± 0.05$^a$ | 1.81 ± 0.23$^b$ |
| E50 | 38,133 ± 4,162$^b$ | 0.14 ± 0.03$^b$ | 0.82 ± 0.17$^a$ | 1.85 ± 0.23$^b$ |
| E75 | 30,300 ± 5,048$^b$ | 0.12 ± 0,02$^{bc}$ | 1.32 ± 0.4$^a$ | 1.4 ± 0.07$^b$ |
| E100 | 18,200 ± 435$^c$ | 0.09 ± 0.03$^c$ | - | 5.92 ± 1.93$^a$ |

Different letters in the same column indicate significant differences ($p < 0.05$) after one-way ANOVA and Tukey's test. MCD: Maximum Cell Density; K: Growth rate; K*: Exponential growth rate; DT: Doubling Time.

Regarding growth rate (K), the control treatment showed higher values when compared to the other ones (Table 2), on the other hand, exponential growth rate (K*) values of this treatment were similar to those of E50 and E75 ($p > 0.05$), with values ranging between 0.78 and 1.32 day-1. Thus, it is possible to state that *A. platensis* has a high potential to be cultivated in wastewater coming from Nile tilapia reared in biofloc. Likewise, when evaluating the doubling time (DT), control, E50, and E75 treatments presented similar results. The culture medium with 100% Tilapia effluent (E100) showed unsatisfactory growth, with low values for MCD, K, and DT (Table 2).

Several studies have already demonstrated the influence of nutritional metabolism on the growth of microalgae. Yu, Jia, and Daí (2009), when cultivating *Nostoc flagelliforme* under photoautotrophic, heterotrophic, and mixotrophic conditions, found higher biomass concentrations when this microalga was growing mixotrophically (1.67 g L-1), having 4.98 and 2.28 times higher values than the photoautotrophic and heterotrophic modes, respectively. Similarly, Kim, Park, Cho, and Hwang (2013) found higher growth rates of *Chlorella sorokiniana* when grown under a mixotrophic nutritional mode (0.44 day-1), when compared to the growth of this microalga in autotrophic mode (0.24 day-1). The green microalgae *Scenedesmus obliquus* also showed the same behavior in a study conducted by Girard et al. (2014), with exponential growth rates of 1.08 day-1 in the mixotrophic mode, and 0.70 day-1 and 0.26 day-1 in the heterotrophic and autotrophic modes, respectively. Li, Li, Zhai, & Wei (2018) also observed higher biomass and growth rates by *A. plantensis* under a mixotrophic metabolism (5.294 mg L-1; 1.20 day-1) when compared to autotrophic conditions (2.940 mg L-1; 0.88 day-1). Yet, differently from the results presented above, the data obtained in this study showed higher concentrations of biomass, as well as higher growth rates, when *A. platensis* was being grown under autotrophic conditions.

The lower production of cells in mixotrophic systems can be explained by the higher turbidity, which is typical of aquaculture effluents. This high turbidity is commonly caused by suspended organic and inorganic matter, soluble organic compounds, and the presence of plankton and other microorganisms, which impede the passage of light and thus reduce photosynthetic activity (Cai, Park, & Li, 2013). Also, the competition for nutrients between microalgae and the bacterial communities present in the biofloc, and the bioavailability of macro and micronutrients may also explain the results obtained. It is worth mentioning that, even after the sterilization of the effluent, there is still the possibility of the permanence of these microorganisms, which can then influence the growth of the microalgae (Çelekli, Topyürek, Markou, & Bozkurt, 2016). Kuo et al. (2016), when adding a solution of micronutrients (trace metals) in aquaculture effluents for the cultivation of *Chlorella* sp., obtained maximum specific growth rates 1.3 times higher than in effluents without extra addition of this solution, as well as productivity of biomass 2.5 times higher. Furthermore, Abreu et al. (2016) also observed greater growth of *Navicula* sp. that was cultivated with biofloc residues with the addition of trace metals in the culture medium.

The treatments E50 and E75 obtained higher cell densities than the treatment E100, and, although the values were lower than the control, they prove that it is possible to use residues in the production of cyanobacteria. Even though other studies have also observed a growth improvement of microalgae with the addition of more nutrients (nitrogen sources) in aquaculture wastewater (Guldhe, Ansari, Singh, & Bux, 2017), more researches are needed to determine the optimum concentrations of these specific elements in effluents.

Mean temperature values (27-28ºC) did not differ significantly ($p > 0.05$) throughout the experiment among treatments, and remained close to that considered optimal (Vonshak, 2014). Temperature is a very important restrictive factor, and it exerts great influence on the metabolic reactions, affecting the growth and the chemical composition of the cells, as well as the pH. The E100 treatment ($p < 0.05$) was the only treatment with an increase in the pH values between the beginning (8.8) and the end (9.3) of cultivation, and it is worth mentioning that the pH values were different among treatments, being the control with the one with higher values (10.2). For the absorption of chemical compounds present in the culture medium and the satisfactory growth of cyanobacteria, the pH values must be controlled, and according to Pelizer, Pontieri, & Moraes (2007), the optimal range of this parameter for *A. platensis* is between 9.5 and 10.5. In the present study, the pH varied between 9.4 and 10.6, however, as the proportion of effluent in the system increased, the pH started to decrease due to bacterial respiration.

## Nitrogen uptake

Regarding nitrogen sources, ammonia is the nitrogen compound most easily incorporated by this microalgae and also the most energy efficient. This species only uses other sources of nitrogen, such as nitrite

and nitrate, when ammonia is no longer available in the environment. Still, all of these compounds are reduced to ammonia before being incorporated (Markou & Georgakakis, 2011). It is worth noting that the amount of nitrogen inserted into the culture medium should be controlled, as very high levels may inhibit cyanobacteria growth due to the toxic effects (Jha, Ali, & Raghuram, 2007). In the present study, total ammonia nitrogen (TAN) was more abundant in the E50 treatment, while nitrite-N ($NO_2$-N) was higher in the E100 treatment (Table 3), which may have contributed to the lowest rates of growth and density of the cyanobacteria (Table 2).

**Table 3.** Concentrations of nitrogen compounds in different proportions of Zarrouk culture medium and effluent from tilapia culture in a BFT system.

| | TAN (mg $L^{-1}$) | | $NO_2$-N (mg $L^{-1}$) | | $NO_3$-N (mg $L^{-1}$) | |
|---|---|---|---|---|---|---|
| | Initial | Final | Initial | Final | Initial | Final |
| Control | $0^{cA}$ | $1.29 \pm 0.48^{aB}$ | $0^{cA}$ | $0.035 \pm 0.01^{aB}$ | $27.4 \pm 0.04^{aA}$ | $0.17 \pm 0.06^{aB}$ |
| E50 | $4.26 \pm 0.12^{aA}$ | $1.18 \pm 0.59^{aB}$ | $0.26 \pm 0.05^{bA}$ | $0.048 \pm 0.01^{aB}$ | $8.38 \pm 0.23^{bA}$ | $0.078 \pm 0.02^{aB}$ |
| E75 | $3.47 \pm 0.29^{bA}$ | $1.33 \pm 0.50^{aB}$ | $0.31 \pm 0.01^{bA}$ | $0.047 \pm 0.01^{aB}$ | $12.22 \pm 0.48^{bA}$ | $0.102 \pm 0.05^{aB}$ |
| E100 | $3.27 \pm 0.12^{bA}$ | $0.67 \pm 0.28^{aB}$ | $0.41 \pm 0.02^{aA}$ | $0.039 \pm 0.01^{aB}$ | $26.76 \pm 0.22^{aA}$ | $0.256 \pm 0.02^{aB}$ |

Different superscript small letters in the same column indicate significant differences between treatments ($p < 0.05$), different superscript capital letters in the same row indicate significant differences between initial and final values for the same treatment ($p < 0.05$), after ANOVA and Tukey's test.

The nitrogen uptake by *A. platensis* in the wastewater was more than 80% for TAN, 90% for $NO_2^-$-N and 99% for $NO_3$-N. It was observed a decrease in TAN by 84.74, 90.45, and 79.66% in E50, E75, and E100, respectively, but they did not differ significantly ($p > 0.05$). Nitrite-N reduced in E75 (97.22%), was similar to the E50 (93%) and significantly different from E100 (90.67%). The cyanobacteria *A. platensis* removed 99% of nitrate-N in all treatments in 10 days of cultivation (Figure 2).

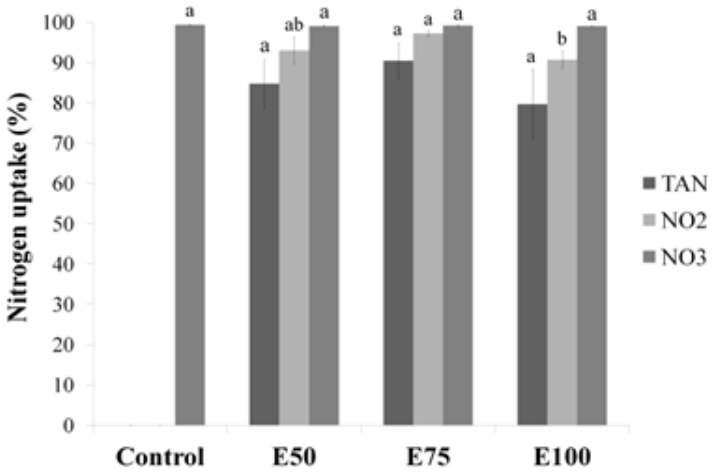


**Figure 2.** Nitrogen uptake (TAN, $NO_2$-N and $NO_3$-N) by *Arthrospira platensis* in 10 days under different proportions of Zarrouk culture medium and effluent from tilapia culture in a BFT system.

Nogueira, Souza Junior, Maia, Saboya, and Farias (2018), in a study that evaluated the reduction of nitrogen compounds by *A. platensis* in wastewater from tilapia reared in a traditional system, found a reduction of 100% for nitrite, 98.7% for nitrate and 19.8% for ammonia, in nine days of cultivation. In this study, nitrate-N was the nitrogen source with more uptake (99%). Wuang, Khin, Chua, and Luo (2016) obtained 100% ammonia and 50% nitrate uptake during three days with *A. platensis* cultivated with wastewater from *Pangasius hypophthalmus*, however, nitrite increased during this time, contrary to this study, in which effluent treatments removed more than 90% of this compound. On the other hand, in the control treatment, nitrite and total ammonia nitrogen were produced throughout the cultivation (Table 3).

According to Markou, Depraetere, Vandamme, and Muylaert (2015), besides absorbing more than 95% of ammonia, *A. platensis* and *C. vulgaris* cultivated in effluents are able to produce biomass with good levels of carbohydrates and lipids. For instance, *A. platensis* is able to accumulate 40% of carbohydrates and 24% of lipids, while *C. vulgaris* 25% and 50% of these same molecules, which make them potential species for the production of biofuels using effluents. Li et al. (2018) observed, in the mixotrophic cultivation of *A. platensis*, that while there was a decrease in nitrogen sources in the cultures, there was an increase in the production of carbohydrates. The authors explained that the supply of organic carbon produced sufficient energy for the

synthesis of carbohydrates, which serve as a source of carbon for the synthesis of lipids in cells. Thus, the biomass produced can be supplied as a source of lipids, carbohydrates, and proteins for other organisms or applied in the production of biofuels.

## Conclusion

Although cell density was lower in the mixotrophic condition than in the control, *A. platensis* had great values regarding exponential growth rate and doubling time in E50 and E75 treatments, being even similar to the control. Moreover, this species also showed efficient uptake of TAN (~ 85%), $NO_2$-N (~ 93%) and $NO_3$-N (~ 99%), which makes *A. platensis* a potential species to be cultivated in wastewater and also to act in the bioremediation of effluents.

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