ENVIRONMENTAL ENGINEERING

Different effect of nitrogen sources in autotrophic and mixotrophic culture of *Scenedesmus* sp for biomass and carotenoids production using acidic coal mine drainage effluents

INGENIERÍA AMBIENTAL

Efecto de diferentes fuentes de nitrógeno en el cultivo autotrófico y mixotrófico de *Scenedesmus* sp para la producción de biomasa y carotenoides utilizando efluentes ácidos de drenaje de minas de carbón

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Abstract

In the present study, the conditions for an autotrophic and mixotrophic culture of *Scenedesmus* sp were established. Effluents from acidic coal mine drains were used to study biomass and carotenoid production, both under autotrophic and mixotrophic conditions in relation to the effect of different sources of nitrogen: sodium nitrate, urea and ammonium phosphate. Pollutant removal was determined, such as COD, TOC, heavy metals, ions and total suspended solids. Batch cultures lasted nine days and biomass production, carotenoids and pollutant removal were compared in each condition. The highest biomass concentration reached was 1.2 g/L in the mixotrophic culture with urea, followed by the culture with sodium nitrate of 0.9 g/L in a C/N ratio of 6:1. The highest carotenoid specific concentration was reached in the mixotrophic culture with sodium nitrate, 19.43 mg carotenoids /g biomass. The culture with ammonium phosphate was found to be inhibitory for the growth of the microalgae with the lowest results of all the parameters except for the autotrophic culture where the concentration of carotenoids was very similar to those reached with sodium nitrate and urea. Finally, the mixotrophic culture with urea presented the highest removal percentages, being 86.97% for total iron, 58.29% for chlorides and 92.5% for COD.

Keywords: carotenoids, coal mine acid (MAD), mixotrophy, Scenedesmus sp., wastewater.

Resumen

En el presente estudio se estableció las condiciones para el cultivo autotrófico y mixotrófico de *Scenedesmus* sp, utilizando efluentes de drenajes ácidos de mina de carbón con el objeto de estudiar la producción de biomasa y de carotenoides tanto en condiciones autótrofas y mixotróficas en relación con el efecto de diferentes fuentes de nitrógeno: nitrato de sodio, la urea y el fosfato de amonio. Se determinó la remoción de contaminantes como DQO, COT, Metales pesados, iones y Sólidos Suspendidos totales. Los cultivos fueron por lote durante nueve días y se comparó la producción de biomasa, carotenoides y remoción de contaminantes en cada condición. La mayor concentración de biomasa alcanzada fue de 1,2 g/L se alcanzó en el cultivo mixotrófico para el medio con urea, seguido por del medio con nitrato de sodio de 0,9 g/L en una relación C/N de 6:1. La mayor concentración específica de carotenoides se alcanzó en el cultivo mixotrófico para el medio con nitrato de sodio y fue de 19.43 mg carotenoides / g biomasa. El medio con fosfato de amonio resultó ser inhibitorio para el crecimiento de la microalga con los resultados más bajos de todos los parámetros con excepción del cultivo autotrófico donde la concentración de carotenoides fue muy similar que las alcanzadas con el medio con nitrato de sodio y urea. Finalmente, el cultivo mixótrofico en el medio con urea presentó los mayores porcentajes de remoción siendo 86,97% para hierro total, 58,29% para cloruros y 92,5% para DQO.

Palabras clave: aguas residuales, carotenoides, drenaje ácido de minas (MAD), mixotrofía, Scenedesmus sp.

1. Introduction

Microalgae constitute a very diverse group that are primary producers in aquatic environments, and these photosynthetic microorganisms are useful in applications such as the production of metabolites, in bioremediation [1,2] and as biofertilizers [3]. Algae, depending on the species, are capable of growing under different types of trophy (nutrition), autotrophy, heterotrophy and mixotrophy [4-6]. Autotrophy is the type of nutrition given in nature through photosynthesis, but its bitechnological development presents technical and physiological difficulties associated with the supply and distribution of light and CO_2 [7]. Certain microalgae such as those of the genus *Chlorella* and *Scenedesmus* can develop heterotrophically, using an organic carbon source [8,9]. Heterotrophy allows an increased cell concentration and productivity, but not all the products of interest occur under this nutritional condition. Mixotrophy represents the use of light as an energy source coupled with a simultaneous assimilation of CO_2 and organic carbon, therefore, this process can be more efficient to produce biomass as well as some metabolites of interest [10].

The cultivation of microalgae, three key elements are required: 1. a source of carbon (inorganic or organic), 2. a culture medium with sufficient nutrient concentration (nitrogen, phosphorus), and 3. a source of energy, in this case light referred as photosynthetically active radiation [11,12]. One drawback of microalgae cultures is the high cost due to the energy required for growth and production of different metabolites of interest [13]. In the last years, the technical and economic feasibility has been explored to use sources with a high content of nitrogen (N) and phosphorus (P) such as domestic and industrial wastewater [14,15] as indicated on Table 1.

Coal mine acid drain water (MAD) are characterized by low pH values and contain dissolved heavy metals such as Fe, Mn, Zn, Cu, Pb, Cd, among others [16], generate serious negative impacts on the different resources that receive their discharges without prior treatment, mainly on water resources, causing loss of the fauna present, acidification of the resource and decrease in the concentration of oxygen. This problem is caused by mines that do not treat MAD, given the high cost of treating these effluents, as well as the lack of technologies that take advantage of these effluents so they could be reincorporated into the process. Some authors have reported the potential of microalgae to grow using MAD, although there is still not much work in this area [22] Therefore, the objective of this

Table 1. *Production of biomass and carotenoids using wastewater.*

Strain	Type of wastewater	Biomass content	Carotenoids	Reference
Chlorella pyrenoidosa	Olive oil	2400 mg/L	6.1 µg/mg	[16]
Dunaliella salina	Dairy industry	440 mg/L	16.8 mg/L	[17]
Spirulina sp.	Domestic	1080 mg/L	3.4 µg/g.	[18]
Scenedesmus sp.	Domestic	6.7 g/L/d	4.06 mg/g	[19]
Botryococcus braunii	Domestic	2.687 g/L	442 mg/L	[18]
Chlamydomonas bioconvexa	Vinasse	222.22 mg/ L	805.26 µg/ g	[20]
Micractinium sp.	Vinasse	177.78 mg/ L	669.85 µg/ g	[18]
Scenedesmus sp.	Olive oil mill	1.8 g/L	92.75 %	[21]
Consortium: *Scenedesmus* sp, *Chlorella* sp and *Ankistrodesmus* s.p	DAM	0.7 g/L	N.R	[22]

Source: own elaboration

work is to evaluate the effect of three different nitrogen sources under autotrophic and mixotrophic conditions using DAM, generated in a mine in northeastern Colombia, to determine if there is an effect on the production of biomass and carotenoids using *Scenedesmus sp*.

2. Methodology

2.1 Study organism

The green algae *Scenedesmus* sp of the Chlorophyceae class and Chlorococcales order was used, obtained from the INNOVALGAE Laboratory of the Universidad Francisco de Paula Santander (UFPS), Colombia. The inoculum was kept in basal BOLD medium [23] 1 g/L of sucrose was added for the inoculum under mixotrophic condition. The inocula of the autotrophic and mixotrophic conditions were cultured for 20 days in 1 L photobioreactors (0.6 L working volume) under a light intensity of 200 µmol/m^{2}s^1, a photoperiod of 12 h light/12h dark (12/12 h) and an air flow of 0.6 vvm.

2.2 Culture media and operating conditions

Undiluted autoclaved MAD was used as medium, which was characterized physiochemically in the Water Laboratory of the UFPS, as established by the method's standard edition 23 (Table 2). Nitrogen sources: sodium nitrate, urea, and ammonium phosphate were evaluated. The nitrate concentration provided by the BOLD medium was calculated and the necessary sucrose concentration was determined for the mixotrophic condition, to maintain a carbon/nitrogen (C/N) ratio of 6: 1 for each nitrogen source to be evaluated. The BOLD medium was used as a control for each condition.

The cultures were carried out in 1 L photobioreactors stirred at 125 rpm, an air flow rate of 1 vvm, a temperature of 30 ± 2ºC and a pH of 7 ± 0.3. For the autotrophy and mixotrophy conditions, the light was 200 µmol/m^{2}s^1 provided by fluorescent lamps. An inoculum in exponential phase was used to inoculate the reactor (10% of the total operating volume). The photoperiod used was 12/12 h. To monitor the different parameters, samples were taken every 24 hours for the 10 days of the process.

Table 2. *Physicochemical parameters measured*

Parameter	Method	Parameter	Method
COD (mg/L)	Standard Methods 5220C	pH	Standard Methods 4500B
BOD (mg/L)	Standard Methods 5210B-4500-OG	Conductivity (µS/cm)	2510B
Nitrates (mg/L)	Colorimetric	Total Suspended Solids (mg /L)	Standard Methods 2540D
Nitrites (mg/L)	Colorimetric	Heavy Metals (Pb, Mn, Zn, Ca, Fe, Ni, Cd, Cr, Mg, Cu y K) (mg/L)	Standard Methods 3111D
Ammonia nitrogen (mg/L)	Colorimetric	Sulfides (mg/L)	Standard Methods 4500-
Phosphates (mg/L)	Colorimetric	Chlorides (mg/L)	Standard Methods 4500-ClB

Source: own elaboration

2.3 Analysis Methods

2.3.1 Biomass

For dry weight (DW) biomass quantification, 5 mL of sample were taken, vacuum filtered on nitrocellulose membranes of 0.22 µm pore diameter (Millipore®) and dried at 80 °C for 72 h. Previously, these membranes were brought to constant weight by keeping them at 80 °C for 24 h.

2.3.2 Pigments and carotenoids

The pigment specific concentration (mg of pigments/g dry biomass) was quantified using a sample volume of 2 ml. The samples were centrifuged at 3500 rpm for 10 min at room temperature. The pellet from each tube was resuspended in 2 ml of acetone 100% pure. The mixture was vortexed at maximum speed for 10 min. Subsequently, it was centrifuged again at 3500 rpm for 10 min at room temperature. The supernatant from each tube was removed and added 5 ml with acetone 100% pure, then read the absorbance at wavelengths: 662, 645 and 470 nm, in a GENESYS 10S UV-Vis spectrophotometer

from Thermo brand. The concentration of chlorophyll *a*, *b*, and total carotenoids was determined according to the Eq.1, 2 y 3 reported by Wellbur [24] :

$$Chlorophyll\, a = (15.65 \times A_{662}) - (7.34 \times A_{645}) \quad (1)$$

$$Chlorophyll\, b = (27.05 \times A_{645}) - (11.21 \times A_{662}) \quad (2)$$

$$Carotenoids = \frac{[(1000 \times A_{470}) - (2.86 \times Chlorophyll\, a) - (129.2 \times Chlorophyll\, b)]}{221} \quad (3)$$

2.3.3 Nitrogen quantification

Nitrogen quantification was performed by the brucine method for nitrates [1], the potassium persulfate method and the phenol method for ammonium according to methods standard edition 23. Sucrose concentration was measured by the DNS method. Data were analyzed with NSCC data and SigmaPlot 11 ed. software.

2.4 Data analysis

The nitrogen source influence on biomass and carotenoids production was evaluated by an analysis of variance (one-way ANOVA). Post hoc

tests (LSD test) were used to determine the statistical differences (P <0.05), R software 4.0 version for windows was used. The analyzes were carried out in triplicate. Calculations and graphs were performed using the SigmaPlot 11 ed.

3. Results

3.1 MAD physicochemical characterization and biomass production under each condition

Table 3 shows the MAD physicochemical characterization, these are average values obtained during the monitoring time. MAD is known very its acidic pH values, as evidenced in this work. The average pH values obtained were 3.59 ± 0.1, this can be attributed to the oxidation reactions of Fe^{2+} to Fe^{3+} that obtain a higher acid intensity [18, 25]. The pH affects metabolism associated with growth, including ionization of biochemical metabolites, solubility, as well as CO_2 bioavailability and assimilation of nutrients, mainly nitrogen. Very acidic pH can affect this process, so optimum pH can be in the range of 6 to 8 [26]. The concentrations of COD and BOD show the low concentration of organic matter in the MAD, these effluents contain inorganic compounds, mainly iron salts, sulfur, chlorides, and some heavy metals, which generate extreme environments where only some microorganisms can exist with the ability to assimilate these compounds [19]. Also, a low concentration of nitrogen compounds was detected. It is possible that the nitrogen present is rapidly assimilated by the microorganisms present to carry out various metabolic activities, the above evidenced the need to add nitrogen for the biomass and carotenoids production stages.

Figure 1 shows the concentration of ions and heavy metals. The presence of Ni, Cd and Cr was not detected. It was found that the concentrations of Fe and Zn are above the permissible discharge value in Colombia, mainly Fe concentration is considerably high. This can be explained by the chemical characteristics of the soil in the study area, which is clayey and has iron. High iron concentrations can alter the natural balance of ecosystems, it can cause loss of vegetation, desertification, and can impact strongly on the biological component in water sources [20]. The remaining parameters are within the permissible limits established, although the high calcium and magnesium concentrations can increase the hardness of the receiving water resource.

Figure 2 shows the biomass production in autotrophy and mixotrophy conditions with each nitrogen source. The highest biomass concentration occurred in autotrophy, it was reached with sodium nitrate (1.06 g/L $\pm$ 0.06), followed by the culture with urea (0.76 g/L $\pm$ 0.04). In the mixotrophic condition, the highest concentration was reached the culture with urea (1.18 g/L $\pm$ 0.0321), followed by the culture with sodium nitrate (1.1 g/L $\pm$ 0.023). For the culture with ammonium phosphate, it was found that growth was low in both conditions, and it was evidenced by the low nitrogen consumption by the microalgae. The assimilation of nitrogen by the culture with sodium nitrate was 100% in both conditions, the highest assimilation occurs in the first 60 hours. Finally, the cultivation with ammonium phosphate showed low growth in terms of biomass and is corroborated by the low consumption of this nitrogen source.

Various studies have been carried out in order to increase the productivity of microalgae and it has been found that different aspects can limit their growth such as light availability, and the availability and assimilation of nutrients, mainly nitrogen [27, 28]. This nutrient is what limits growth and carotenoid production. Based on the results, the nitrogen source seems to exert a growth limitation, particularly in the case of ammonium

Table 3. MAD physicochemical characterization.

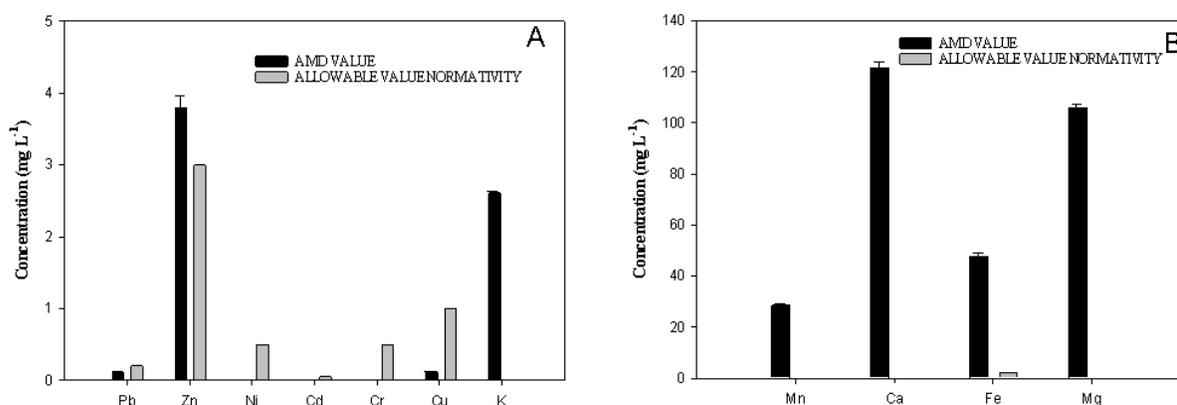| Parameter	Average value	Parameter	Average value
COD (mg/L)	185 ± 0.865	Chlorides (mg/L)	160.37 ± 1.4
BOD (mg/L)	128.53 ± 1.2	pH	3.59 ± 0.1
Nitrates (mg/L)	10.23 ± 0.46	Conductivity (μS/cm)	893.2 ± 2.5
Nitrites (mg/L)	0.142 ± 0.04	Total Suspended Solids (mg/L)	3128.4 ± 3.2
Ammonia nitrogen (mg/L)	2.5 ± 0.6	Sulfides (mg/L)	0.52 ± 0.02
Phosphates (mg/L)	2.81 ± 0.25		

Source: own elaboration

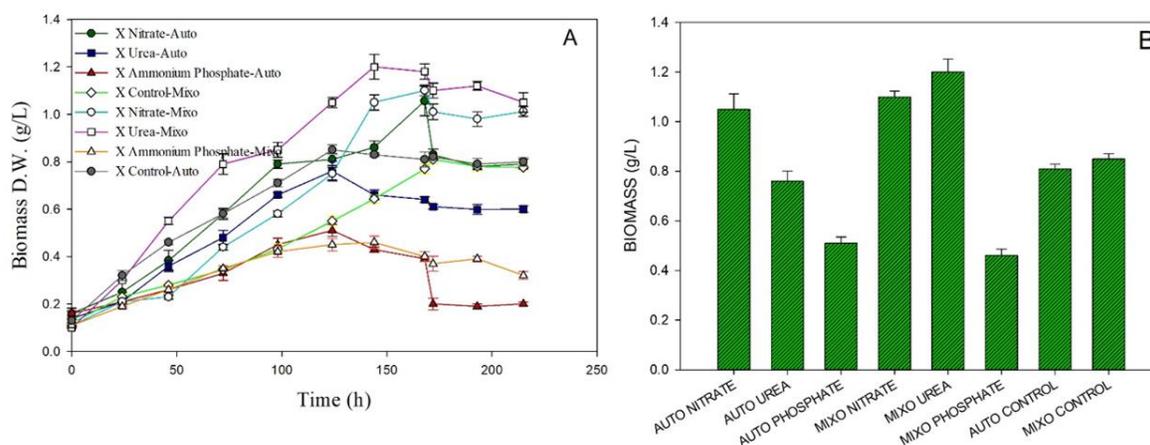*Figure 1. Ions and heavy metals concentrations (A-B) in MAD. Source: own elaboration*

Figure 2. Biomass production on different nitrogen sources using MAD under autotrophic and mixotrophic conditions. A. Growth kinetics and B. Maximum biomass concentration reached. Source: own elaboration

phosphate. It is possible that it exerts toxicity since it is a neutral molecule and easily crosses the membrane, upsetting the balance in the oxidation-reduction reactions for electron transference, which triggers a mismatch in cellular metabolism [29, 30].

3.2 Carotenoid content in each condition

Figure 3 shows carotenoid production under autotrophy and mixotrophy conditions with each nitrogen source.

The highest total carotenoid concentration was obtained in the culture with sodium nitrate in mixotrophy (19.53 mg carotenoids/g biomass). The analysis shows that this concentration has significant differences ($P<0.05$) when compared to the control in both autotrophic and mixotrophic conditions. In the culture with urea, the carotenoid concentration did not show significant differences when compared the control and the mixotrophic culture ($P>0.05$). In the culture with ammonium phosphate, there was a lower carotenoid concentration in both autotrophic and mixotrophic cultures. In the mixotrophic culture, the specific carotenoid concentration was 60% lower when compared to the autotrophic culture. High nitrogen concentration in the culture medium inhibits the growth and carotenoid production [18], because the enzyme nitrate reductase, which introduces nitrogen to the cell, acts at a higher speed when the concentration of this element are low [31]. In the case of ammonium phosphate, the limitation it exerted on the pigment production was 70%. Which can be explained because ammonium unbalances the electrochemical gradient in the thylakoid membrane, which is the organelle where the electron transfer reactions. When this equilibrium is broken, the ATP synthesis in the light phase is affected and, therefore, the reactions of the dark phase are not efficient, thus there is not enough energy for growth and pigment production [32]. As observed in the culture with ammonium

phosphate and the culture with urea for the mixotrophic condition.

3.3 Kinetic parameters estimation and nutrient removal

The specific growth rate of the different cultures was calculated when the cultures were in exponential phase. The pigment concentration shown relates to the maximum pigment concentration reached in each condition. The data obtained are shown in Table 4.

The culture with sodium nitrate had the highest growth rate for the mixotrophic cultures (0.0081 h^{-1}). The lowest growth rates were in the cultures with ammonium phosphate. Regarding the autotrophic culture, the observed rates for the cultures with sodium nitrate and urea were similar. Wang and collaborators [25] reported growth rates in a range of 0.1 - 0.2 d^{-1} using DAM under autotrophic conditions and sodium nitrate as nitrogen source, like those achieved in this work, it should be noted that for the other sources and under mixotrophic conditions no data have been reported yet [26]. According to the statistical analysis, it was found that the cultures with sodium nitrate and urea do not have significant differences when using DAM, and there are significant differences when compared to the culture with ammonium phosphate, as shown in Table 5. Regarding the control culture (BOLD medium), the cultures with sodium nitrate and urea do present significant differences, evidencing that there is a greater growth rate and carotenoid production using the DAM, when compared to the control culture.

The pollutant removal is shown in Figure 4. It is evidenced that the treatments with the best pollutant removal percentages were the cultures with sodium nitrate and urea. Iron was removed by 98% under mixotrophic conditions, while under autotrophic conditions, the removal was 92%. Regarding COD and BOD, the removal percentages were 94% and 98%, respectively, for
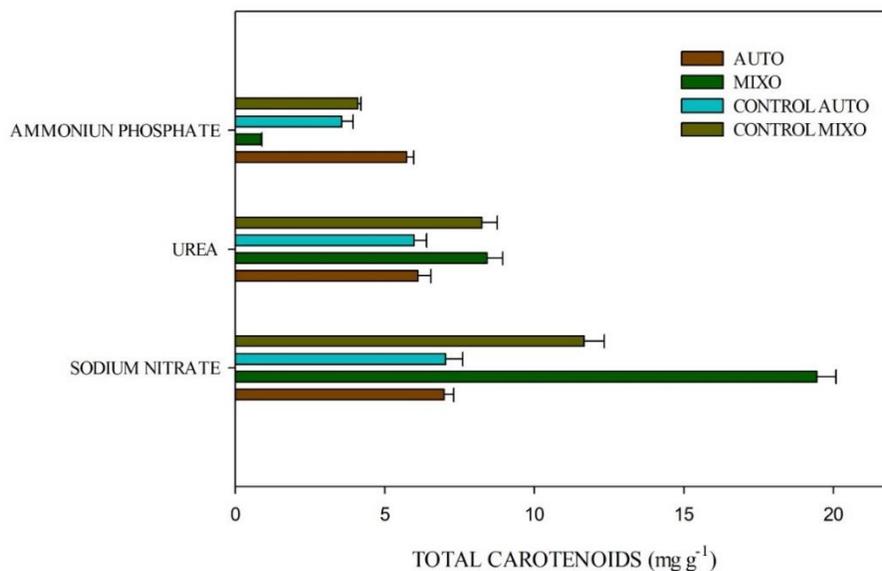
Figure 3. *Total carotenoid concentration under autotrophic and mixotrophic conditions with each nitrogen source.*
Source: own elaboration

Table 4. *Kinetic parameters of the Scenedesmus culture in MAD.*

Culture	Nitrogen source	Biomass productivity (g DW / L·d)	Growth rate (h^{-1})	Carotenoid productivity (mg/g· biomass)
Mixotrophy	Sodium nitrate	$0.183\pm1.9e^{-4}$	0.0081	$19.45\pm6.5e^{-2}$
	Urea	$0.2\pm1e^{-4}$	0.0076	$8.42\pm7.2e^{-2}$
	Ammonium phosphate	$0.077\pm3.2e^{-4}$	0.0025	$0.87\pm6e^{-3}$
Autotrophy	Sodium nitrate	$0.151\pm4.8e^{-5}$	0.0054	$6.98\pm3.5e^{-4}$
	Urea	$0.148\pm5.3e^{-5}$	0.0052	$6.11\pm5.4e^{-4}$
	Ammonium phosphate	$0.099\pm6.1e^{-5}$	0.0025	$5.73\pm8.6e^{-4}$

Source: own elaboration

Table 5. *Growth rate statistical analysis of cultures under mixotrophic and autotrophic conditions.*

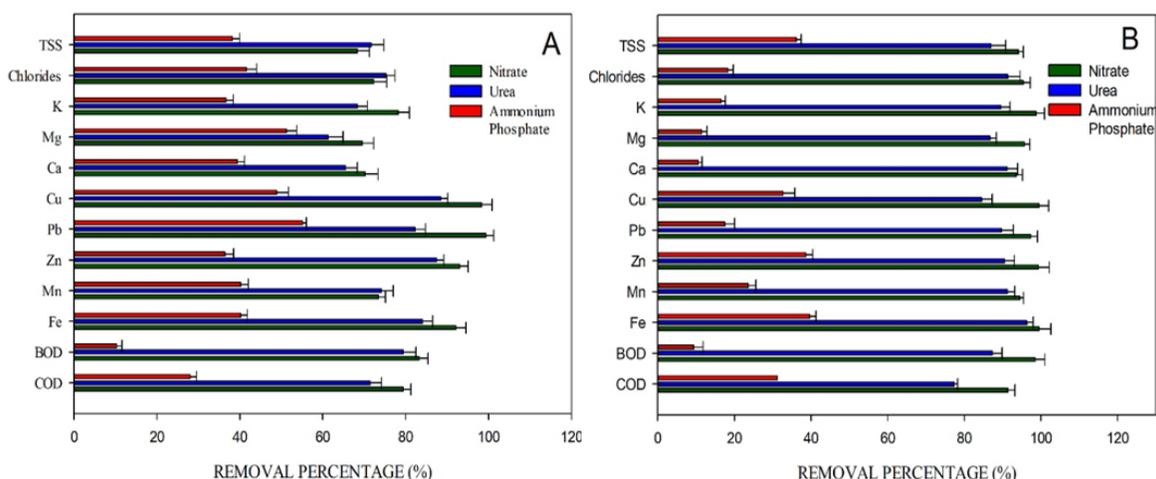| Comparison	Mean Diff	p	Q	P	P<0.05
NO$_3$ vs. A.P.	0.00510	4	38.052	<0.001	Yes
NO$_3$ vs. CONTROL	0.00230	3	17.161	<0.001	Yes
NO$_3$ vs. UREA	0.000400	2	2.984	0.057	No
UREA vs. A.P.	0.00470	3	35.068	<0.001	Yes
UREA vs. CONTROL	0.00190	2	14.176	<0.001	Yes
CONTROL vs. A.P.	0.00280	2	20.891	<0.001	Yes

A.P.: Ammonium Phosphate. Source: own elaboration

Figure 4. Contaminant Removal A. Autotrophy. B Mixotrophy. Source: own elaboration

sodium nitrate under mixotrophic conditions. For the culture with urea, the removal percentages were between 70 and 80%. For the culture with ammonium phosphate, the removal percentages were low when compared to the other cultures. The values of TSS, chlorides, zinc, iron, BOD, and COD removal percentages measured at the end of the process do not comply with the Colombian regulations for these parameters.

Microalgae have the metabolic capacity to remove iron and heavy metal compounds [22]. It has been reported that for microalgae to carry out this process, they follow two mechanisms: absorption (active/passive) and adsorption, mainly through physical adsorption, chemical adsorption and chelation [33]. The acidic pH characteristic of MAD causes the metal ion displacement and complicates absorption and adsorption in the case of microalgae. This phenomenon is solved by the ability of these organisms to alkalize the solution pH, which facilitates ion precipitation. In addition to this, microalgae can act as a selective and absorbent agent for pollutant treatment, managing to remove metals such as Fe, As, Zn, Mn, Cd and Cu and reduce COD and BOD concentrations [34]. In this work, similar results were found for the *Scendesmus* sp strain, the microalgae managed to alkalize the medium (6.9 for the culture with nitrate and 6.6 for the culture with urea), and, therefore, remove the contaminants. Although in the case of the culture with ammonium phosphate, the pH only reached values of 5.5 which may explain the low removal and growth in this culture.

4. Conclusions

Based on the results, the *Scenedesmus* sp strain proved to be an attractive alternative for biomass and carotenoid production using DAM in its culture medium. It can grow under the different trophy conditions and the nitrogen sources proposed. Sodium nitrate was the most suitable nitrogen source for biomass production and for carotenoid accumulation under the mixotrophic condition. In the present study, the operating culture conditions under mixotrophy and autotrophy conditions were determined, and obtained the kinetic parameters for each condition, as well as the pollutant removal percentages achieved in the evaluated treatments. This will allow different works in this area, since there are few works reported for this microalga using MAD.

5. Acknowledgements and Funding Statement

6. References

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